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Immune determinants of COVID-19 protection and disease: A focus on asymptomatic COVID and long COVID

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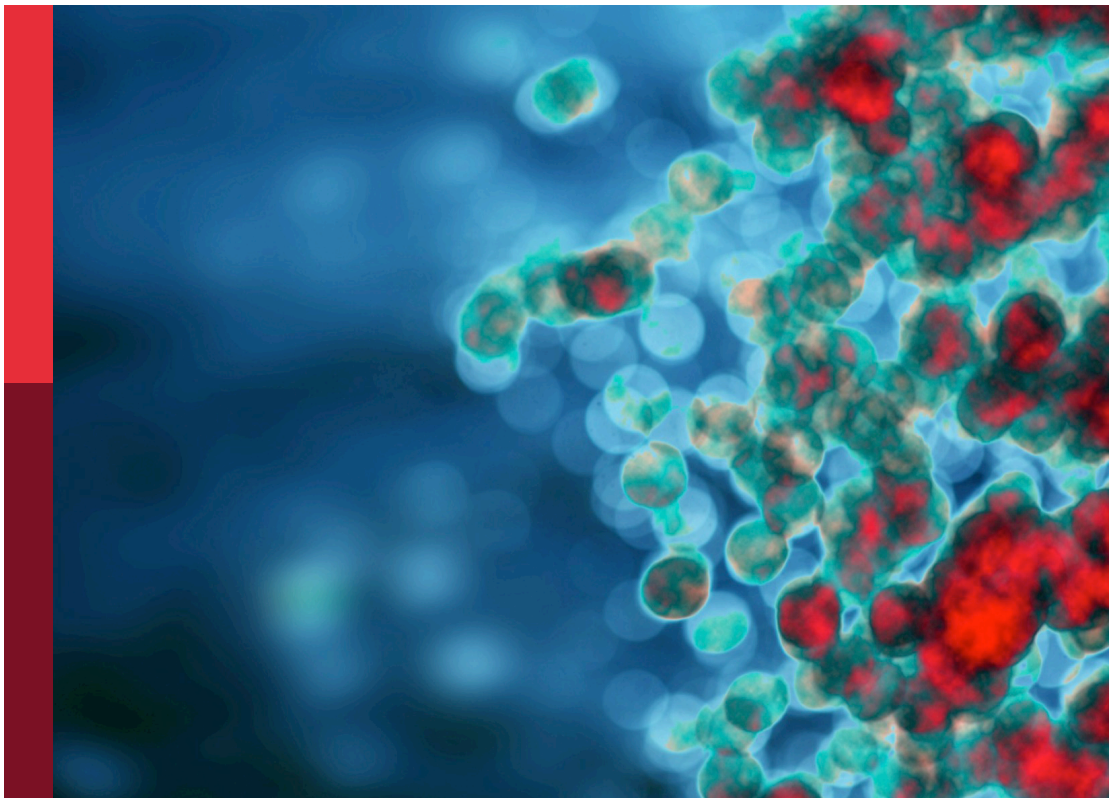

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Immune determinants of COVID-19 protection and disease: A focus on asymptomatic COVID and long COVID

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Editorial: Immune determinants of COVID-19 protection and disease: A focus on asymptomatic COVID and long COVID

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Editorial on the Research Topic
Immune determinants of COVID-19 protection and disease: A focus on asymptomatic COVID and long COVID

SARS-CoV-2 infection manifests as variety of disease presentations, from asymptomatic to mild-moderate COVID-19 symptoms, life-threatening disease, or even persistent debilitating symptoms in some cases (1). Asymptomatic infection occurs in a significant fraction of individuals, and as many as half of all transmission events were reported to occur from pre-symptomatic and asymptomatic individuals (2). One of the most important determinants of disease severity is age as individuals over 65 years have the greatest risk of requiring intensive care, while young children seem to be less severely affected (3, 4). The role of imbalanced immune responses in the overall severity of acute COVID-19 is still not clear. Within this context, we launched our Research Topic "*Immune determinants of COVID-19 protection and disease: a Focus on Asymptomatic COVID and Long COVID*" on May 31st 2021 and invited researchers to contribute towards increasing the understanding of the immunological determinants of COVID-19 disease presentation and severity. We received diverse and insightful manuscript applications, of which Frontiers in Immunology published 15 articles from 232 authors of 12 countries. Despite the diversity of this collective venture, the contributions fall into two main areas of research: serological and cellular responses to SARS-CoV-2 infections.

A first line of research includes contributions examining serological correlates of immune protection. In their perspective commentary, Narasimhan et al. highlighted the importance of following serological responses in asymptomatic individuals, as they could be silent reservoirs to propagate the infection. After evaluating the antibody profiles in 272 plasma samples collected from 59 COVID-19 patients (18 asymptomatic patients, 33 mildly ill patients and 8 severely ill), measuring the IgG against five viral structural proteins, different isotypes of immunoglobulins against the Receptor Binding Domain (RBD) protein, and neutralizing antibodies, Liao et al. concluded that the overall antibody response was lower in asymptomatic infections than in symptomatic infections throughout the disease course. Their

data suggests that asymptomatic infection elicit weaker antibody responses, and primarily induce IgG antibody responses rather than IgA or IgM antibody responses. Similarly, Wu et al. studied antibody profiles from 25,091 individuals enrolled in a surveillance program in Wuhan, China, and compared 405 asymptomatic individuals who mounted a detectable antibody response with 459 symptomatic COVID-19 patients. The authors observed that, while IgM responses rapidly declined in both groups, the prevalence and durability of IgG responses and neutralizing capacities correlated positively with symptoms. Furthermore, Castillo-Olivares et al. noted statistically significantly higher levels of SARS-CoV-2-specific neutralizing antibodies in severe COVID-19 patients than people with mild or asymptomatic infections in a cohort of patients and health-care workers from the Royal Papworth Hospital in Cambridge, UK. They also showed a positive correlation between severity, anti-nucleocapsid assays and intracellular virus neutralization. Collectively, these findings shed important lights on the specific character of immune response and highlight the importance of immunization of individuals after asymptomatic infections. In terms of evaluating the post vaccination anti-SARS-CoV-2 serological response, Xu et al. followed 61 volunteers after receiving the inactivated CoronaVac vaccine over 160 days. They observed an intense antibody response for the vaccine with over 95% neutralizing seropositivity rate, reaching a peak two weeks post second dose, however, a decline of this response has been observed a week after.

Several studies examined whether previous exposure to unrelated coronaviruses could modulate SARS-CoV-2 infection. An interesting study from Khan et al. followed two groups of individuals who tested negative for SARS-CoV-2 infection at the start of the study: individuals with a previously confirmed Middle East respiratory syndrome (MERS)-CoV infection and a control MERS-negative group. Within these groups, 24% of the previously MERS-positive (82 individuals) and 31% of the MERS-negative group (260 individuals) eventually contracted SARS-CoV-2 infection. Thus, previous MERS infection did not correlate with higher probability of SARS-CoV-2 infection (symptomatic or not), but the risk of COVID-19-related hospitalization in the MERS-CoV-positive group was significantly higher. Of note, there could be an age-bias in the analysis of this cohort, and, as it was previously established, older adults have been disproportionately affected during the SARS-CoV-2 pandemic, including higher risk of hospitalization (4). Tanunliong, et al. analyzed presence of anti-human coronaviruses (HCoV) antibodies in a Canadian cohort comprised of over 900 samples (half of them predating 2020) expanding from children under 5 years of age to older adults of >80 years of age. They quantified IgG antibody against the Spike proteins of seasonal HCoV, including alpha (HCoV-229E, HCoV-NL63) and beta (HCoV-HKU1, HCoV-OC43) viruses, the 2003 epidemic beta coronavirus, SARS-CoV-1 as well as Spike, Nucleocapsid, and the Receptor Binding Domain (RBD) of SARS-CoV-2. They concluded that most people have an HCoV priming exposure by 10 years of age with stable IgG levels thereafter, and that some of these anti-HCoV antibodies can cross-react with SARS-CoV-2 epitopes. Finally, Sim et al. investigated potential functions for these cross-reactive antibodies found in the blood of pre-pandemic elderly people and hypothesize that they likely could have two opposing functions: protecting against and enhancing viral infection.

The second line of research contributions focused on the cellular immune response to infection as the serological correlates mentioned above are a direct consequence of the immune cells' activation. Cui et al. report on a critical aspect of this response, reviewing the follicular CD4 T cell (Tfh) subsets, their participation in the humoral immune response, and the important role they play in response to SARS- CoV-2. A review of the reported frequencies of the Tfh subsets in SARS-CoV-2 infected individuals indicate that these cells are expanded in individuals with mild/asymptomatic symptoms, while their numbers are reduced, and germinal centers lost in severe patients. The authors speculate that targeting Tfh cells could serve as therapeutic strategy against SARS-CoV-2 infection.

Several research teams have aimed to understand the immune responses leading to better control of SARS-CoV2 infection using discovery-based methods, comparing individuals spanning the entire spectrum of disease severity. Although SARS-CoV-2 has been demonstrated to be highly transmissible, there are individuals who are resistant to infection despite being exposed to the virus. Castelli et al. explored the possibility that genetic factors led to this resistance by genotyping 83 discordant couples, where one member was COVID-19 symptomatic while the other did not get infected for over six months. Whole-exome sequencing revealed a dominance of several HLA-DRB1 variants in symptomatic individuals while HLA-A alleles encoding 144Q/151R were associated with seronegative women. Interestingly, the highest hits were for the genes MICA and MICB involved in immune modulation of natural killer (NK) cells. The authors speculated that the modified expression of these proteins would likely act to downmodulate NK cell cytotoxic activity and increase susceptibility to SARS-CoV-2 infection. Various immune cell subsets and their abundance have also been correlated to disease severity. Wang et al. performed scRNA sequencing on PBMCs from a cohort of individuals varying from healthy to symptomatic patients with severe disease. The results highlighted the importance of innate immunity in antiviral response as there was an increase in mucosa-associated innate T cells and specific NK cell and classical monocyte subsets in asymptomatic individuals. NKT, Treg and myeloid subsets including monocytes and neutrophils were enhanced in symptomatic patients suggesting they contribute to severe outcome. These results were consistent with previous studies (5), which have shown that the severity of disease caused by SARS-CoV2 infection is correlated with an expansion of myeloid derived suppressor cells (MDSC), particularly the immunosuppressive low-density neutrophil (LDN) subset of these cells. Sieminska et al. were curious if this or other MDSC subsets would result in immunosuppression or long COVID in convalescent individuals who had been infected with SARS-CoV2 but were either asymptomatic or had mild symptoms. They showed that LDN/MDSCs continued to be transiently elevated 35 days after infection, and that the low levels of CD8+ T cells had an exhausted phenotype. The LDN/MDSCs as well as normal density neutrophil subsets expressed PD-L1 and not only affected the production of neutralizing antibodies but also inhibited proliferation of T cells. Together, these results suggest that neutrophil dysfunction is responsible for long-term immunosuppression.

Several research groups have studied the possibility that SARS-CoV2 infection could lead to different outcomes in patients having suppressed or deficient immune systems, e.g., in HIV-1-positive individuals, or those with other types of immunodeficiencies (primary and secondary) (6). This investigation could be relevant in areas with an extremely high prevalence of HIV-1 (e.g., Sub-Saharan Africa) and low level of vaccinations against SARS-CoV-2 – a problem reviewed by Mandala and Liu.

The study of asymptomatic or mild disease has increased our understanding of the immune responses important for protection from SARS-CoV2, since the individuals who are asymptomatic or have mild disease are less likely to be monitored. Soares-Schanoski et al. followed a cohort of United States marine recruits who were initially seronegative, with most seroconverting over time. Although similar dynamics in viral load and generation of specific antibody responses were observed, proteomic analysis revealed a difference in asymptomatic individuals vs those having mild symptoms. For instance, chemokines and cytokines associated with the inflammatory response or immune activation were up-regulated in individuals displaying mild symptoms, while asymptomatic individuals had increased levels of analytes such as IL-17C, MMP-10 and Fibroblast Growth Factors, known to be involved in tissue repair and, in some way, in protection against disease.

Viral infection modulates the intracellular environment to escape host response and create favorable conditions for virus production and spread. Thus, it is plausible that even in the case of asymptomatic infections, SARS-CoV-2 will modify the expression of host genes, albeit differently than during a symptomatic infection. Sfikakis et al. performed a genome-wide transcriptional RNA sequencing of whole blood samples obtained from SARS-CoV-2 seropositive individuals, comparing the differential immune responses relative to symptom presentation. The expression of 15 genes was significantly different, eight of which were associated with interferon related signalling pathways. This led the authors to propose that slight differences in the baseline expression of innate immunity-related genes may be associated with an asymptomatic outcome of SARS-CoV-2 infection.

As seen in this collection, the strength and quality of the host immune response plays important roles in COVID-19 presentation and outcome. March 2023 marked the 3-year anniversary since WHO declared COVID-19 to be a pandemic and since then, collectively and cooperatively worldwide, we have gathered an unprecedented number of critical insights into the roles of the immune system in protection and pathogenesis of COVID-19. Despite how far we have come, the COVID-19 pandemic is not over, it remains a major health concern and there are still many unknowns regarding immune determinants of COVID-19 protection.

Author contributions

All authors contributed to the outline, compiled/wrote the different sections. All authors reviewed and edited for final revisions. All authors approved the final version for publication.

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Conflict of interest

Author MB is employed at Pfizer Inc.

The remaining authors 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.

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Occurrence of COVID-19 Symptoms During SARS-CoV-2 Infection Defines Waning of Humoral Immunity

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Approximately half of the SARS-CoV-2 infections occur without apparent symptoms, raising questions regarding long-term humoral immunity in asymptomatic individuals. Plasma levels of immunoglobulin G (IgG) and M (IgM) against the viral spike or nucleoprotein were determined for 25,091 individuals enrolled in a surveillance program in Wuhan, China. We compared 405 asymptomatic individuals who mounted a detectable antibody response with 459 symptomatic COVID-19 patients. The well-defined duration of the SARS-CoV-2 endemic in Wuhan allowed a side-by-side comparison of antibody responses following symptomatic and asymptomatic infections without subsequent antigen re-exposure. IgM responses rapidly declined in both groups. However, both the prevalence and durability of IgG responses and neutralizing capacities correlated positively with symptoms. Regardless of sex, age, and body weight, asymptomatic individuals lost their SARS-CoV-2-specific IgG antibodies more often and rapidly than symptomatic patients did. These findings have important implications for immunity and favour immunization programs including individuals after asymptomatic infections.

Keywords: COVID-19, humoral immunity, asymptomatic, symptomatic, collective/herd immunity

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INTRODUCTION

Currently, the world faces a global COVID-19 pandemic. As of August 9, 2021, more than 202.7 million people had a laboratory-confirmed SARS-CoV-2 infection and nearly 4.2 million people died during or in the direct aftermath of COVID-19 (1). Calculations of the excess mortality and sero-prevalence surveillance programs indicate that the actual numbers of infections and fatalities are far higher. An important determinant for the number of unrecorded cases is the occurrence of very mild and/or asymptomatic infections, which are the focus of this study. The scarcity of

secondary SARS-CoV-2 infections (2, 3) indicates that adaptive immune responses prevent re-infections in the vast majority of cases - at least during the approximately one-year period during which SARS-CoV-2 has been studied to date. Others and we have shown that binding and neutralizing antibodies develop rapidly after infection and are maintained in the majority of symptomatic COVID-19 patients for a period of 6-10 months after disease onset (4–6). It is important to emphasize that this observation period was defined by the end of the studies rather than the decline of detectable antibodies. However, recent reports suggest that binding antibodies and the neutralizing activity against SARS-CoV-2 is either not similarly strong and/or long-lasting in individuals who had only mild or no symptoms (7–10). Most important landmark studies either included relatively few patients (e.g., 37 per arm) or only examined a relatively short period (e.g., 8 weeks). Additionally, different studies came to contradicting conclusion concerning waning neutralizing antibody responses in asymptomatic individuals (10–12). Therefore, we felt that the duration of protective immunity in asymptomatic individuals should be elucidated in larger cohorts and with a more informative study design.

There is a controversial debate concerning the question with which frequencies bona fide asymptomatic SARS-CoV-2 infections occur. Another important matter of debate is the question if and how they contribute to the spread of the virus (13). A large study from Wuhan suggests that asymptomatic individuals seem not to be very infectious for their contact persons (14). Obviously, unspecific symptoms such as headache, myalgia, and fatigue are not always linked to COVID-19, because they are rather common in the general population and may have various reasons. Thus, the incidence of asymptomatic SARS-CoV-2 infections appears to vary considerably in different studies and/or populations. Descriptions range from 17.8% in Diamond Princess Cruise ship tourists (15) to 21.9-35.8% in a nationwide sero-prevalence study in Spain (16). Factors influencing this wide range appear to be related to the study design (e.g., retrospective questionnaires), personal expectations of being infected, and maybe the patience and perseverance during interviews and interrogations. Regardless of the actual percentage, two points are beyond doubt: (I) a highly relevant proportion of persons acquires a SARS-CoV-2 infection (as indicated by diagnostic antibody testing) without seeking medical help and without recognizing and/or remembering unusual symptoms, and (II) such asymptomatic individuals have no or far milder symptoms as compared to individuals who actively seek medical help due to the occurrence of symptoms. Thus, asymptomatically SARS-CoV-2-infected individuals are often hard to find for larger immunological studies.

Usually, the timing of asymptomatic infections is uncertain given that the virus itself has never been detected by nucleic acid or antigen testing. In such cases, the retrospective diagnosis is exclusively based on the presence of specific antibodies. Since immunity wanes over time, it is very difficult to accurately determine the prevalence and kinetics of binding and neutralizing antibodies in asymptomatic individuals. In the absence of virus detection and/or symptomatic disease episodes, it is nearly impossible to distinguish recent infection events associated with low IgG titers from past infections that had initially elicited strong immune responses that declined afterwards. This level of uncertainty increases even further when re-exposures are taken into account that are almost impossible to detect *in natura* but will almost certainly booster immunity. While the above applies to phases of on-going public virus spread, a clearly defined end of local virus transmission chains may be applied as 'synchronization element' since it excludes infections and the associated antigen re-exposure beyond a defined time point. Since April 2020 and despite large-scale public surveillance programs (17), no autochthonous virus transmissions have been detected in Wuhan strongly suggesting that the stringent non-pharmacologic interventions virtually terminated local virus spread. Given that this end excludes infections, re-infection, antigen re-exposures, and immunological boostering, we inferred that this serendipitous situation would enable an - at least to our knowledge - unprecedented study design dealing with the aftermath of a COVID-19 endemic. We screened 25,091 outpatients in April 2020 and surveyed antibody responses in more than 987 sero-positive persons during a six-month period after the epidemic in Wuhan had ended. Immunoglobulin M (IgM) and G (IgG) responses recognizing the receptor binding domain (RBD) of the spike (S) or the nucleocapsid (N) protein as well as neutralizing activities of clinical specimens derived from 405 asymptomatically infected individuals who mounted a detectable antibody response, and 459 symptomatic COVID-19 patients were determined in a comprehensive and comparative study design. The results provide novel insights into the long-term immune status of asymptomatic individuals and have important implications for the understanding of collective immunity as well as the design of global vaccination programs.

METHODS

Patients and Sample Collection

In total, 29,177 clinical specimens obtained from 25,091 outpatients of the clinic of Wuhan Union Hospital during the period between April 2020 and October 2020 were included in this study. The levels of IgM and IgG antibodies recognizing the RBD of the S protein and the N protein (IgG-S, IgG-N, IgM-S, and IgM-N) were determined. A total of 987 individuals who have not been vaccinated against SARS-CoV-2 tested positive for at least one SARS-CoV-2-specific antibody. Focusing on the antibody-positive patients, we conducted interviews to assess whether the persons experienced symptoms such as fever, sore throat, cough, loss of taste or smell, and chest tightness during the epidemic. Of the 987 SARS-CoV-2-specific antibody-positive persons, 123 had to be excluded from further analyses for one or more of the following reasons: refusal to provide medical information, ambiguity of medical information or sole IgM positivity. The latter were excluded because of the limited specificity of IgM responses. Clinical specimens derived from

repetitive testing of the same individual during an one-month interval were also not taken into account. Individuals co-infected with human influenza A virus, influenza B virus or other viruses associated with respiratory infections were excluded. In the end, data of 405 asymptomatic persons and 459 symptomatic patients, found by screening 25,091 outpatients, were included in this study (**Figure 1**). The age range of asymptomatic and symptomatic individuals are 18-84 and 18-87, respectively. We retrospectively collected patients' medical information including demographic factors (**Supplementary Table 1**). Plasma samples were separated by centrifugation at 3000g for 15 min after 30 min-inactivation at 56°C (complement inactivation) and tested concerning the presence of SARS-CoV-2-specific antibodies. All patients signed a general written consent that residual blood samples can be applied for scientific research. All procedures were approved by the Ethics Commission of Union Hospital of Huazhong University of Science and Technology in Wuhan.

Detection of IgG and IgM Against Spike Protein and Nucleocapsid Protein of SARS-CoV-2

IgG-S, IgG-N, IgM-S, and IgM-N levels were quantified by capture chemi-luminescence immunoassays (CLIA) Kit (Snibe, Shenzhen, China, Lot#: 130219015M/130219016M) using the MAGLUMI^{TM} 4000 Plus as described previously (6). The cut-off value for IgM-S was 0.7 AU/mL and 1.0 AU/mL for IgM-N, IgG-S, and IgG-N.

Virus Neutralization Test Assay

The SARS-CoV-2-neutralizing activity of patient plasma was tested against SARS-CoV-2 (Strain BetaCoV/Wuhan/WIV04/2019, National Virus Resource Center number: IVCAS 6.7512) in highly permissive Vero E6 cells using the previously described co-incubation methodology (6). Virus-specific cytopathic effects (CPE) were visualized and judged by microscopic inspection. The neutralizing antibody titers were expressed as reciprocal value of the highest actual dilution that significantly prevented CPE formation.

Statistics and Reproducibility

The mean and standard deviation were applied for describing continuous variables with a normal distribution. The median and the interquartile range (IQR) were used to describe continuous variables with a skewed distribution. For categorical variables, the number (n) and the percentage (%) were applied for description. We used the Mann-Whitney U test, $\chi 2$ test, or Fisher's exact test as appropriate. A non-parametric Spearman's correlation test was applied for the correlation analyses. Longitudinal changes in antibody titers during April 2020 and October 2020 were depicted using the locally weighted regression and smoothing scatterplots (Lowess) model (ggplot2 package in R). All reported p values were two-sided, and a p value below 0.05 was regarded as hallmark for statistical significance. Levels of statistical significance were depicted as follows: ns, not significance; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. All statistical analyses were conducted using R (The R Foundation, http://www.r-project.org, version 4.0.0).

RESULTS

The local COVID-19 epidemic in Wuhan was discovered in late 2019 (18) and lasted until the end of March 2020. During April and May, only seven new cases were identified among more than 11.2 million inhabitants of Wuhan, China. Despite enormous testing efforts (approximately 9.89 million tests were conducted), no autochthonous infections have been identified since June 2020 (**Supplementary Figure 1**). We figured that this temporarily well-defined epidemic might provide an opportunity to determine humoral immune responses elicited by a novel virus infection that necessarily must have occurred during a very precisely defined and narrow timeframe and in absence of subsequent antigen exposures during the post-epidemic period.

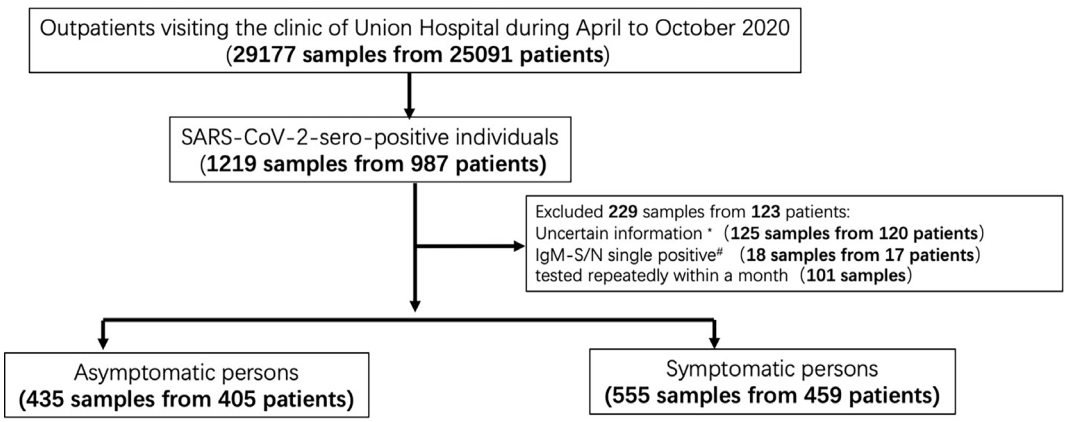

FIGURE 1 | Study population, cohort enrolment process, exclusion criteria, and workflow of surveillance and analysis. Please note that there was an overlap between exclusion criterion 1* ('uncertain information') and 2# ('IgM single positivity').

Previous virus proteome-wide analyses showed that asymptomatic infections mainly produce IgM and IgG antibodies recognizing the S1 or the N protein of SARS-CoV-2 (8). Therefore, we focussed on these antibody responses. We determined specific IgG and IgM responses recognizing the N or RBD-S protein, applying capture chemi-luminescence immunoassays (CLIA). More than 29,177 clinical specimens were analysed from 25,091 outpatients who visited the clinic of Union Hospital during the period from April to October 2020. A total of 1,219 plasma specimens obtained from 987 individuals showed at least one type of SARS-CoV-2-specific antibodies, corresponding to an overall sero-prevalence of 3.93% among the participants. This prevalence is highly consistent with two previous studies conducted among Wuhan residents which described sero-positivity rates of 2.39% and 3.9% (19, 20). After applying exclusion criteria such as uncertainty of medical information, repetitive testing during a one-month period, and IgM positivity only (see the *M&M* section for details), 864 subjects represented by 990 plasma samples were included in this study (**Figure 1** and **Supplementary Table 1**). Interestingly, nearly half of all subjects (n=405; ~46.9%) had no symptoms, whereas 459 (~53.1%) suffered from symptomatic COVID-19. The demographic characteristics of those asymptomatic individuals and symptomatic COVID-19 patients were compared. There were no significant differences concerning age, sex or body weight between individuals who experienced symptomatic and asymptomatic infections (**Supplementary Table 1**).

In the Absence of Antigen Re-Exposure, Asymptomatic Individuals Lose SARS-CoV-2-Specific IgG-S Responses More Rapidly Than Symptomatic Patients Do

As expected, based on the short lifespan of IgM responses and in agreement with the literature (21), most plasma-positive individuals, regardless of the presence or absence of symptomatic episodes, did not show IgM responses recognizing SARS-CoV-2-N and RBD-S two months after the end of the epidemic (**Figure 2A**). Only, in the second month after the epidemic had ended, the levels of anti-RBD IgM-S and IgM-N in asymptomatic patients were significantly higher than that of symptomatic patients (**Figure 2B**). In contrast

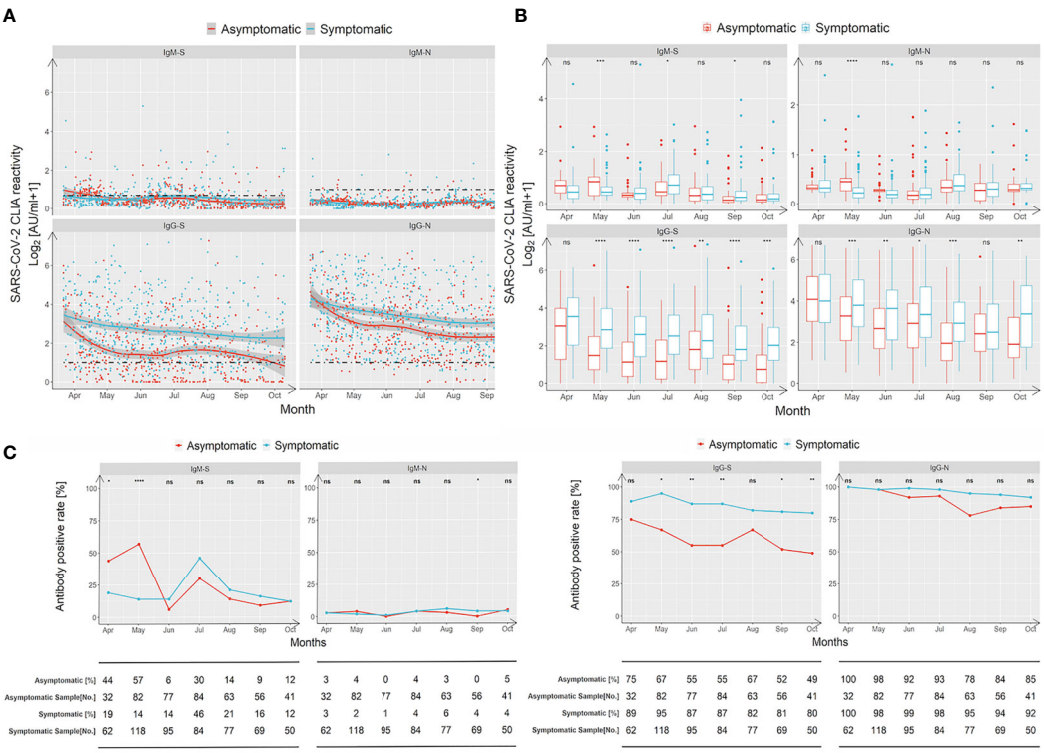

FIGURE 2 | In absence of antigen re-exposure, asymptomatic individuals lose SARS-CoV-2-specific IgG-S responses more rapidly than symptomatic patients do. IgM and IgG recognizing the RBD of the spike protein ('S') and the nucleoprotein ('N') of SARS-CoV-2 were quantified by capture chemi-luminescence immunoassays (CLIA) for 29,177 samples obtained from 25,091 patients. **(A, B)** Plasma antibody levels of IgM-S, IgM-N, IgG-S, and IgG-N in samples obtained from asymptomatic (red) and symptomatic (blue) patients obtained during April 2020 and October 2020 are presented. The line shows the mean value calculated using a Lowess regression model and the shaded area represents the 95% confidence interval. The boxes in **(B)** show medians (middle line), 75% quartiles (upper bound) and 25% quartiles (lower bound), and the whiskers show 1.5-fold the IQR above and below the box. Repeated measures (mixed model) ANOVA was used for statistical analysis. ns, no significance, *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001, two-sided. **(C)** Antibody positivity rates of asymptomatic (red) and symptomatic (blue) groups tested at indicated months after the epidemic ended are shown. The table below the figure depicts the numbers of assessed patients at indicated time points. Chi-square test was used for statistical analysis. ns, no significance, *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001, two-sided.

to the IgM responses, most individuals initially showed IgG responses recognizing N and RBD-S. While both the IgG-N and anti-RBD IgG-S levels remained rather stable during the observation period following symptomatic COVID-19, there was an obvious difference concerning the strength and the sustainability of individual IgG responses compared to asymptomatic individuals, who showed lower and less stable IgG responses (**Figures 2A, B**). Symptomatic patients exhibited an overall anti-RBD IgG-S positivity rate of 89% in April and remained at a prevalence of 80% in October (**Figure 2C**). The positivity rate for IgG-N started at 100% in April and remained at 92% during the six-month observation period. The positivity rate for IgG-N in asymptomatically infected individuals also started at 100% and decreased slightly faster to 85% during the observation period (**Figure 2C**). However, given the importance of RBD-S-specific IgG for protection (e.g., through neutralizing antibodies), we were intrigued by the sharp decline in anti-RBD IgG-S responses (**Figure 2A**, lower left panel) and overall positivity rates that dropped from 75% in April to only 49% six months later (**Figure 2C**).

Symptom Occurrence Is the Dominant Factor Determining the Strength and Stability of SARS-CoV-2-Specific IgG Responses

Given these differences in humoral immunity associated with the symptom occurrence, we stratified asymptomatically infected individuals and symptomatic patients according to sex, age, and body mass index (BMI). We then compared the IgG-N and anti-RBD IgG-S antibody levels to examine the influence of symptomatic disease episodes. As shown in **Figure 3**, IgG-N as well as the anti-RBD IgG-S titers of asymptomatically infected individuals were significantly lower compared to symptomatic patients across most subgroups defined by sex (**Figure 3A**), age (**Figure 3B**), and BMI (**Figure 3C**), except for IgG-N responses in 30-39-year-old and low-weight subjects. We do not think that the lack of significance in the latter two groups indicates a meaningful immunological feature, especially since the trend pointed in the same direction. Taken together, across various groups and biological characteristics of individuals, symptom occurrence during the early phase was the dominant factor defining the strength and sustainability of IgG responses.

Neutralization Activity Is Defined by the Occurrence of Symptoms

Consistent with the essential role of the spike protein for SARS-CoV-2 entry, it represents the main target of neutralizing antibodies. Accordingly, numerous studies including our own documented a strong correlation between IgG-S titers, particularly those antibodies recognizing the receptor-binding domain (RBD) of S, and neutralizing activity (21, 22). The same was observed here: anti-RBD IgG-S titers demonstrated a significant positive correlation with neutralizing activity (Spearman r=0.5795, p<0.0001). A less stringent correlation was found for IgG-N titers (Spearman r=0.1620, p=0.0007) (**Supplementary Figure 2**, left and central panel). Accordingly, high levels of neutralizing activity (1:160 or 1:320) were found in association with high anti-RBD IgG-S levels (**Supplementary Figure 2**, right panel).

Since the IgG-S levels were lower in asymptomatic individuals compared to symptomatic patients, we wondered whether this difference also applies to neutralizing antibodies that are highly relevant for protection from re-infection. We compared neutralizing activities of the two groups at three time points:

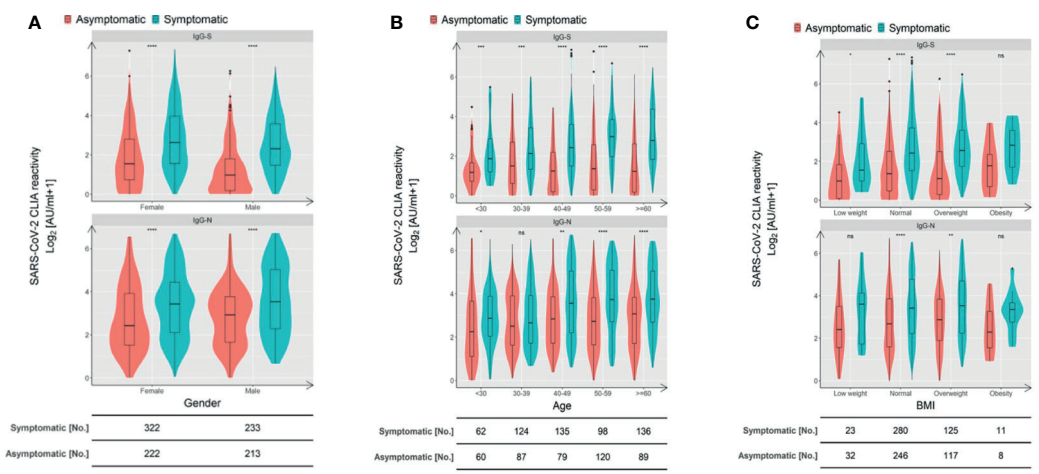

FIGURE 3 | Symptom occurrence is the dominant factor defining the strength of SARS-CoV-2-specific IgG responses. Comparison of RBD S- and N-specific CLIA-reactive IgG titers stratified according to sex **(A)**, age **(B)**, and BMI **(C)**. Violin plots show the distribution of each antibody feature derived from asymptomatic individuals (red) and symptomatic patients (blue). Boxes depict medians (middle line), 75% quartiles (upper bound), and 25% quartiles (lower bound) with whiskers showing a 1.5-fold interquartile ranges above and below boxes. The table below the figure indicates the number of samples obtained from asymptomatic individuals and symptomatic COVID-19 patients. Statistical analysis was performed by a two-tailed Mann-Whitney U test. Asterisks depict the levels of significance as follows: ns, not significant ($p \geq 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

April, July, and October 2020. As shown in **Figures 4A, B**, the neutralization titers and positive rate of sera obtained from asymptomatic individuals were significantly lower than those of symptomatic patients. The frequency of individuals showing neutralizing activity in the asymptomatic group showed a downward trend with 59.3%, 51.2%, and 46.3% in April, July, and October, respectively (**Figure 4B**). In contrast, the frequency of symptomatic patients with neutralizing activity was stable at a far higher level based on prevalence rates of 77.4%, 86.9%, and 86.0% at the indicated time points (**Figure 4B**). From April to July, there was even an increase in the percentage of clinical specimens showing neutralizing antibodies. This may reflect a long-term maturation of antibodies that has been reported after SARS-CoV-2 infection.

In order to investigate the neutralizing capacities longitudinally, serum titers of 17 asymptomatically infected individuals and 54 symptomatic patients with repetitive sampling were analysed. Interestingly, the similar proportion of the two groups, 29.4% in symptomatic individuals and 27.7% in symptomatic individuals, showed increasing neutralizing titers over time (**Figure 4C**). However, the proportion of individuals with decreasing titers of neutralizing antibodies was 52.9% in asymptomatically infected individuals, but only 40.7% in symptomatic individuals. We found the opposite for individuals with no change neutralization titers over time. Here, the proportion of asymptomatic individuals was only 17.6% in contrast to 31.4% in symptomatic patients. This indicates that, on the individual level, more patients in the asymptomatic group compared to symptomatic patients show a decrease in their neutralizing capacity over time.

Taken together our data reveal that symptom occurrence during the primary SARS-CoV-2 infection is the dominant factor defining the strength and sustainability of binding and neutralizing IgG antibodies.

DISCUSSION

We present here, at least to our knowledge, the first comprehensive side-by-side comparison of asymptomatically infected individuals and symptomatic COVID-19 patients in the long-term aftermath of a SARS-CoV-2 endemic. We found striking differences concerning the strength and persistence of SARS-CoV-2-specific IgG responses, in particular in those antibodies recognizing the RBD of S, which mainly comprise neutralizing IgG molecules. Irrespective of sex, age, and body mass index, the symptom occurrence during the early SARS-CoV-2 infection phase was significantly positively correlated

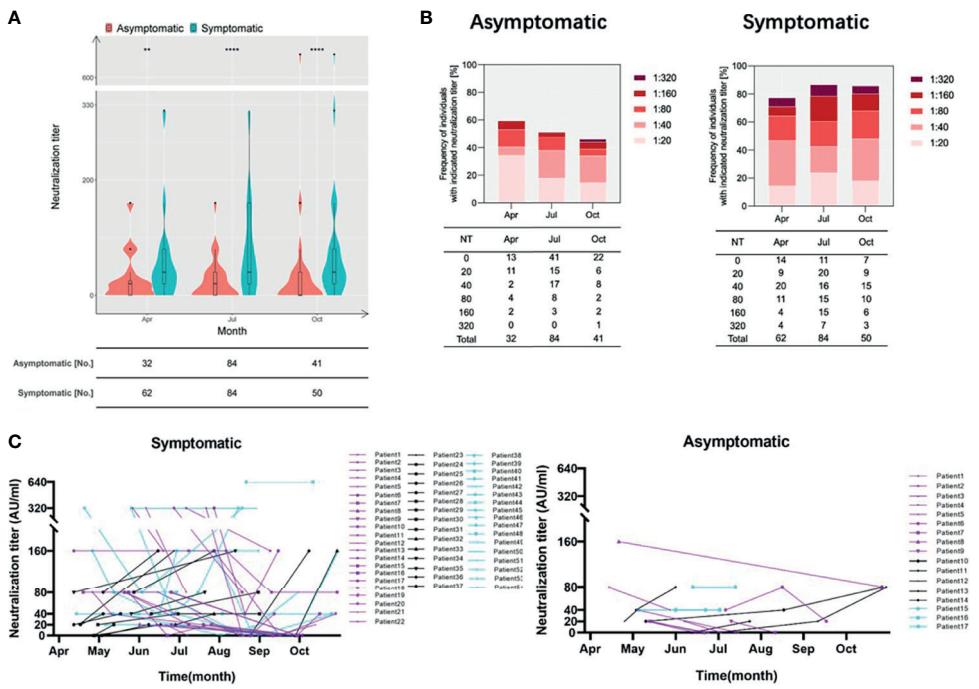

FIGURE 4 | Neutralization activity is defined by the occurrence of symptoms. **(A)** Violin plots show the distribution of neutralization titers of sera derived from derived from asymptomatic individuals (red) and symptomatic patients (blue) during April and October 2020. Boxes depict medians (middle line), 75% quartiles (upper bound), and 25% quartiles (lower bound). Whiskers indicate 1.5-times interquartile ranges above and below boxes. The table below the figure highlights the number of clinical specimens that have been assessed. Statistical analysis was performed by two-tailed Mann-Whitney U test. Asterisks depict levels of significance as follows: **p < 0.01; ****p < 0.0001. **(B)** Like in **(A)** but the frequencies of individuals exhibiting indicated neutralizations titers are depicted. **(C)** Sequential sampling and analyses of neutralization activity in 17 asymptomatic individuals and 54 symptomatic COVID-19 patients. Different colours highlight different trends as follows: purple: declining trend; black: increasing trend; light blue: unchanged neutralization.

with stronger and more sustained anti-RBD IgG-S responses. The same difference was evident concerning the level of neutralizing antibodies.

To enable an investigation such as the present one, several highly unusual circumstances must 'perfectly' align: (I) the beginning and the end of the endemic must be well defined, (II) the duration of the endemic needs to be rather short, (III) public surveillance efforts are needed to trace the spread of the virus in the local community, (IV) sufficient numbers of individuals in general and infected subjects in particular need to be present and willing to share their information, and (V) immunologically related viruses must be negligible during the observational period in the studied area. All of these hold true for Wuhan, leading to an unprecedented situation: SARS-CoV-2 was identified here (18). The endemic started and ended between late 2019 and end of March 2020 (see **Supplementary Figure 1**). Other seasonal coronaviruses such as HCoV-229E did not circulate extensively during the observational period (23) and the antibody responses recognizing the N and S protein of SARS-CoV-2 (if at all) only minimally overlap with responses induced by other seasonal coronaviruses (24). In conjunction, these factors allowed us to probe into the strength and sustainability of humoral immunity in the aftermath of a COVID-19 epidemic and in absence of subsequent antigen re-exposure. Importantly, the most decisive factor across different sex, age, and body mass index groups was the occurrence of symptoms during the early SARS-CoV-2 infection.

Since we separately quantified levels of anti-RBD IgG-S and IgG-N antibodies, we were able to show that neutralizing antibody responses positively correlate best with anti-RBD IgG-S levels in asymptomatic individuals, similar to what we had shown for symptomatic patients (6).

Our results complement other studies based on the Wuhan population (10, 12, 25) collectively showing that the positivity rate and the actual titers of neutralizing antibodies in symptomatic patients are significantly higher and more sustained compared to those found in the asymptomatic group. We also observed that serum neutralizing antibody levels in asymptomatic patients decreased over time, while another study conducted in Wuhan did not find this (25). The reason may be that these authors applied another method to recruit their participants. They used a cluster random sampling method based on households to recruit participants. Accordingly, the study found a positivity rate of antibodies of 6.92% that was far higher than the one defined by us and by other studies based on the Wuhan population (19, 20).

Albeit in far smaller collectives and in shorter analyses, other authors also observed difference between patients who experienced severe symptoms and asymptomatic and/or mildly symptomatic groups (10, 26). We can only speculate why symptoms correlate with the strength and sustainability of IgG responses. Patients with severe infections may produce higher levels of antibodies during the early disease stage because of the stimulation of a large number of antigens and B cell responses outside germinal centres (27). However, damaging effects of SARS-CoV-2 on lymphoid organs affecting the durability of antibody responses and the antibody affinity have also been described (28, 29). Although several studies reported an association between higher viral loads with more severe symptoms, they found little to no difference in respect to virus loads between pre-symptomatic, asymptomatic, and symptomatic patients (30). However, the duration of viral RNA shedding seems to be shorter in people who remain asymptomatic (13, 30). Thus, a shorter virus replication phase may be associated with an antigen availability that is simply not long enough to prime optimal B cell and/or antibody responses. Another explanation may rely on the association between innate immune responses and symptoms on the one hand and innate immune responses and antibody responses on the other hand. It is well known that interferons, besides their important antiviral activity, by themselves cause flu-like symptoms such as fatigue, fever, and myalgia (31, 32). Additionally, interferons enhance antibody responses and induce class switching (33). Thus, a simple and parsimonious explanation for the association between the occurrence of symptoms with strong and long-lasting IgG responses may simply be the overlapping dependence on interferons. Since interferon induction is stimulated by viruses, the first explanation (prolonged virus replication) and the second explanation (increased interferon induction) are by no means mutual exclusive.

Some recent studies claimed that neutralizing activities in clinical serum specimens obtained from patients with mild symptoms and/or asymptomatic infections disappears 2 months after infection (9). However, other studies (34) and our results presented above clearly oppose this view. Despite the apparent decline in antibody responses, nearly half of all asymptomatic individuals exhibited detectable neutralizing activity half a year after the end of the epidemic and in absence of additional antigen exposure. These dynamics are consistent with the change of antibody response during other acute virus infections such as influenza, MERS-CoV, SARS-CoV-1, and the seasonal human coronavirus 229E. Early after such infections, neutralizing antibody titers rise rapidly followed by an obvious contraction phase. However, after this intermediate decline, a stable plateau is established, which can be maintained for several years through the activity of long-lived plasma and memory B cells (5, 29).

Like all observational analyses, our study has certain limitations. Firstly, after half a year, a potential recall bias of asymptomatic carriers may affect the results of this study. Secondly, a fraction of asymptomatic patients may have been missed by our surveillance as consequence of anti-RBD IgG-S levels below the level of detection during the recovery period (10, 18). This study only enrolled asymptomatic individuals, who mounted a detectable antibody response.

In conclusion, half a year after the Wuhan COVID-19 epidemic ended, although asymptomatic individuals had lower anti-RBD IgG-S antibody titers, positivity rates, and neutralizing activities compared to symptomatic patients, nearly half of asymptomatic subjects had sufficient neutralization activity. These results suggest that a considerable fraction of asymptomatic natural infections stimulate a humoral immune response conferring the ability to resist reinfections. Despite this

good news, aforementioned disparity in the strength and duration of anti-RBD IgG-S responses raised by symptomatic and asymptomatic infections, strongly argue in favour of vaccine programmes including individuals who underwent asymptomatic SARS-CoV-2 infections, ideally with an intermediate prioritization adjusted between vulnerable uninfected individuals and symptomatic COVID-19 patients.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Commission of Union Hospital of Huazhong University of Science and Technology in Wuhan. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: JW, BY-L, JL, FD, UD, MT, and XZ. Methodology: BY-L, YH-F, HW, XL-Y, SS, LK-C, SM-L, SH-L, and TD-X. Investigation: YH-F, HW, XL-Y, SS, SM-L, SH-L, and TD-X. Visualization: JW, BY-L, HW, XL-Y, LK-C, and VTKL-T. Funding acquisition: JL, DL-Y, MT, and XZ. Project administration: SS, JL, MJ-L, DL-Y, FD, and UD. Supervision: JW, MJ-L, DL-Y, UD, MT, and XZ. Writing – original draft: JW, BY-L, YH-F, HW, XL-Y, VTKL-T, MT, and XZ. Writing – review and editing: JW, MJ-L, FD, UD, MT, and XZ. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.722027/full#supplementary-material

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Detection of Anti-SARS-CoV-2-S2 IgG Is More Sensitive Than Anti-RBD IgG in Identifying Asymptomatic COVID-19 Patients

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Characterizing the serologic features of asymptomatic SARS-CoV-2 infection is imperative to improve diagnostics and control of SARS-CoV-2 transmission. In this study, we evaluated the antibody profiles in 272 plasma samples collected from 59 COVID-19 patients, consisting of 18 asymptomatic patients, 33 mildly ill patients and 8 severely ill patients. We measured the IgG against five viral structural proteins, different isotypes of immunoglobulins against the Receptor Binding Domain (RBD) protein, and neutralizing antibodies. The results showed that the overall antibody response was lower in asymptomatic infections than in symptomatic infections throughout the disease course. In contrast to symptomatic patients, asymptomatic patients showed a dominant IgG-response towards the RBD protein, but not IgM and IgA. Neutralizing antibody titers had linear correlations with IgA/IgM/IgG levels against SARS-CoV-2-RBD, as well as with IgG levels against multiple SARS-CoV-2 structural proteins, especially with anti-RBD or anti-S2 IgG. In addition, the sensitivity of anti-S2-IgG is better in identifying asymptomatic infections at early time post infection compared to anti-RBD-IgG. These data suggest that asymptomatic infections elicit weaker antibody responses, and primarily induce IgG antibody responses rather than IgA or IgM antibody responses. Detection of IgG against the S2 protein could supplement nucleic acid testing to identify asymptomatic patients. This study provides an antibody detection scheme for asymptomatic infections, which may contribute to epidemic prevention and control.

Keywords: SARS-CoV-2, COVID-19, asymptomatic infections, IgG, antibody response, neutralizing antibody

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INTRODUCTION

COVID-19, caused by SARS-CoV-2 infection, has caused more than 100 million laboratory-confirmed infections and more than 2 million deaths so far. The number of patients is increasing at an alarming rate of hundreds of thousands every day, resulting in a heavy medical and economic burden to the world (1–3).

Since the outbreak of COVID-19, asymptomatic infection has been reported (4). Asymptomatic individuals do not show clinical symptoms, and are usually identified through mass-community screening or contact tracing (5), making them a likely population that contributes to the continuous community spread of SARS-CoV-2 virus. Some studies even believe that asymptomatic individuals are more infectious than those with symptoms (6). Recently, a systematic review showed that asymptomatic infections accounted for at least one-third of SARS-CoV-2 infections (7), further indicating that asymptomatic infections may play a pivotal role in the COVID-19 pandemic (8). Thereby, management of asymptomatic infections has become one of the key measures to control the COVID-19 pandemic (9). Detection of viral nucleic acid by RT-qPCR is considered the gold standard to diagnose SARS-CoV-2 infections (10). However, this approach is limited by the influence of sampling time, types of clinical specimens and method of inactivation, which can yield false-negative results and lead to misdiagnosis, especially in asymptomatic infections (11–13). Detection of SARS-CoV-2-specific antibodies is an important complementary method for the diagnosis of COVID-19 (14). There is an urgent need for a more sensitive antibody detection scheme of asymptomatic infections.

Here, we conducted a study to compare the antibody profile of asymptomatic infections to patients with different disease severity. We also compared the pros and cons of different antibody detection schemes for the diagnosis of asymptomatic infections. We found that asymptomatic infections elicit weaker antibody responses than symptomatic infections, and primarily induced IgG-based antibody responses throughout the disease course. Remarkably, detection of anti-SARS-CoV-2-S2 IgG is more sensitive than anti-RBD IgG in identifying asymptomatic COVID-19 patients.

MATERIALS AND METHODS

Patient Enrollment and Sample Collection

From January to April, 2020, 59 SARS-CoV-2 infected patients, confirmed by real-time PCR and hospitalized in the Guangzhou Eighth People's Hospital, were enrolled in this study. The patients were categorized into 3 groups based on their disease severity, including 18 asymptomatic patients, 33 mild-ill patients and 8 severe patients. Plasma samples were collected from each patient at multiple time points. Clinical data including patient's demographic information and clinical outcome was retrieved from the medical records. Plasma samples from eight healthy donors collected in 2017-2018 were used as controls in this study.

Detection of Anti-SARS-CoV-2-RBD IgA, IgM and IgG by Electrochemiluminescence

The Kaeser 6600 automatic chemiluminescence immunoanalyzer and the matching reagents kit (Guangzhou Kangrun Biotech Co. Ltd, Guangzhou, China) was used to detect the SARS-CoV-2-specific IgM, IgA and IgG levels using a two-step indirect detection method as described previously (15). Briefly, the amino group on the RBD protein was coupled with the carboxyl group of the magnetic beads, and the antigen was fixed to form RBD-coating magnetic beads. The SARS-CoV-2 RBD-specific antibodies in the testing serum could bind to the RBD-coating magnetic beads, and acridine ester derivatives-coupled anti-human IgA, IgM and IgG antibodies were added. After the unbound substances were removed, a photomultiplier was used to detect light signals from acridine ester that were converted to obtain the corresponding signal value. The relative light signal values, expressed in relative light units (RLU), indicated the specific IgM, IgA and IgG levels. The relative light signal value is equivalent to the original signal value over the specific antibody cut-off value. The cut-off values of IgM, IgA and IgG were 11 300, 56 492 and 42 213, respectively. A relative luminescence value (RLV) greater than or equal to 1.0 was considered positive for SARS-CoV-2 RBD specific IgM, IgA and IgG.

Comparison of Antibody Response Against Different SARS-CoV-2 Proteins

To assess the antibody response against the different SARS-CoV-2 proteins or different fragments of the spike protein, SARS-CoV-2 S (spike protein, 1203 aa), S1 (675 aa), S2 (533 aa), RBD (228 aa) and N (424 aa) proteins were obtained from Sino Biological, Inc (Beijing) and used as coating antigens in our in-house ELISA to detect the antigen-specific IgG. Briefly, 96-well plates (Jet, Biofil Co., Ltd, Guangzhou) were coated with 100 μl/well (0.5 μg/ml) of SARS-CoV-2 S, S1, S2, RBD or N protein in DPBS buffer (Thermo Fisher Scientific (China), Shanghai) overnight at 4°C. After blocking (DPBS, 10%FBS), 100 μl of diluted plasma (1:100) were added and plates were incubated at 37°C for one hour. After washing, plates were incubated with 100 μl of HRP-conjugated mouse anti-human IgG (H+L) antibody (Catalog No: 109-035-088, Jackson ImmunoResearch, West Grove, PA) at 37°C for one hour. Reactions were visualized by adding 50 μl of TMB (3,3',5,5'-Tetramethylbenzidine) substrate solution (Biohao Biotechnology Co., Ltd., Beijing). Each plate was monitored to have the same reaction time and the optical densities at 450 nm were then read. The mean value of the healthy donor plasma (named HD group) collected in 2017-2018 plus 3 standard deviations was used as the detection threshold.

Focus Reduction Neutralization Test

SARS-CoV-2 focus reduction neutralization test (FRNT) was performed in a certified Biosafety Level 3 lab. Fifty microliters of plasma samples were serially diluted, mixed with 50 μl of SARS-

Abbreviations: AP, asymptomatic patients; MP, mildly ill patients; SP, severely ill patients; HD, healthy donor; ECL, electrochemiluminescence; TPR, true positive rate; FPR, false positive rate; TNR, true negative rate; FNR, false negative rate; PPV, positive predictive value; NPV, negative predictive value; FDR, false discovery rate; OA, overall accuracy.

CoV-2 virus (100 focus forming unit, FFU) in 96-well plates and incubated for 1 hour at 37°C. The mixtures were then transferred to 96-well plates seeded with Vero E6 cells (ATCC, Manassas, VA) and incubated for 1 hour at 37°C to allow virus entry. Inoculums were then removed before adding the overlay media (100 μl MEM containing 1.2% Carboxymethylcellulose, CMC). The plates were then incubated at 37°C for 24 hours. Overlays were removed and cells were fixed with 4% paraformaldehyde solution for 30 min. Cells were permeabilized with 0.2% Triton X-100 and incubated with cross-reactive rabbit anti-SARS-CoV-N IgG (Cat: 40143-R001, Sino Biological, Inc, Beijing) for 1 hour at 37°C before adding HRP-conjugated goat anti-rabbit IgG(H+L) antibody (1:4000 dilution) (Catalog Number: 111-035-144, Jackson ImmunoResearch, West Grove, PA). Cells were further incubated at 37°C. The reactions were developed with KPL TrueBlue Peroxidase substrates (Seracare Life Sciences Inc, Milford, MA). The numbers of SARS-CoV-2 foci were calculated using an Elispot reader (Cellular Technology Ltd, Shaker Heights, OH).

The Coincidence Between Neutralizing Antibodies and Binding Antibodies

In order to evaluate the predictive value of the binding antibodies for the neutralizing antibodies, we calculated the true positive rate (TPR), false positive rate (FPR), true negative rate (TNR), false negative rate (FNR), positive predictive value (PPV), negative predictive value (NPV), false discovery rate (FDR), and overall accuracy (OA) during the evaluation. The calculation formula is as follows:

$$TPR = TP/(TP + FP)$$

$$FPR = FP/(FP + TN)$$

$$TNR = TN/(TN + FP)$$

$$FNR = FN/(TP + FN)$$

$$PPV = TP/(TP + FP)$$

$$NPV = TN/(TN + FN)$$

$$FDR = FP/(TP + FP)$$

$$OA = (TP + TN)/(TP + FP + FN + TN)$$

Statistical Analysis

Statistical analysis was performed using Graphpad Prism software, version 7.00. Shapiro-Wilk normality test was used to evaluate the type of data distribution. One-way ANOVA with Holm-Sidak's multiple comparisons test or Friedman test with Dunn's multiple comparisons test was used for comparing the IgA/IgM/IgG concentration-time curve of the three groups. Nonlinear regression was used to map the trend of antibodies or positive detection sensitivities of antibodies over time. Linear regression and Spearman correlation coefficient were used to assess the relationship between binding antibodies and neutralizing antibody. A p-value ≤0.05 was considered statistically significant (*p-values of ≤0.05. **p-values of ≤0.01. ***p- values of ≤0.001. ****p-values of ≤0.0001). All values are depicted as mean ± standard error of measurement (SEM).

Ethics Statement

This study was conducted according to the ethical principles of the Declaration of Helsinki. Ethical approval was obtained from the Ethics Committee of Guangzhou Eighth People's Hospital (202002135). Written informed consent was obtained from all participants.

RESULTS

Patients and Clinical Information

Fifty-nine SARS-CoV-2-infected patients, confirmed by real-time qPCR and hospitalized in the Guangzhou Eighth People's Hospital, were enrolled in this study. Plasma samples were collected from each patient at multiple time points to analyze the kinetics of antibody responses, and a total of 272 plasma samples were finally collected. As shown in **Table 1**, 18 asymptomatic patients (AP), 33 mildly ill patients (MP) and 8 severely ill patients (SP) were enrolled in this study. The mean age was 31.8 ± 15.5 years for AP, 47.3 ± 14.6 years for MP, 58.5 ± 9.62 years for SP. All patients were eventually cured and discharged from the hospital. Because asymptomatic infections do not develop symptoms, the day that the nucleic acid test was first positive was defined as day 0 post-onset.

The Kinetics of Anti-SARS-CoV-2-RBD IgA/IgM/IgG Were Different Among AP, MP and SP

To understand the kinetics of antibody responses against SARS-CoV-2 in patients, plasma IgA, IgM and IgG against the SARS-CoV-2 receptor binding domain (RBD) were detected by electrochemiluminescence (ECL).

As shown in **Figure 1A**, the average IgA, IgM and IgG responses in MP and SP were much more robust than in AP. The kinetics of IgA antibodies in the three groups of patients showed a similar trend, beginning to rise in the first week after disease onset, reaching a peak in the third week, and starting to decline from the fourth week. In the seventh week, reduced but still detectable IgA against SARS-CoV-2 RBD was observed in each group. The concentrations of IgA reached similar peak values in MP and SP, but decreased faster in MP. Unlike IgA, the IgM response peaked in the third week for AP, and in the fourth to fifth week for MP and SP, with a very close dynamics between MP and SP. As for the IgG response, the average anti-RBD IgG concentrations of AP and SP peaked in the fourth week and plateaued till the end point of this study. However, the IgG level in MP did not reach its peak until the end of the fifth week, which was about 2 weeks later than the other two groups, and no

TABLE 1 | Patients involved in this study.

Group		Asymptomatic Patients	Mildly ill Patients	Severely ill Patients
Numbers		18	33	8
Age (years old)	$\bar{X} \pm SD$	31.8 ± 15.5	47.3 ± 14.6*	58.5 ± 9.62*
Sex				
Male		7	17	7
Female		11	16	1
Outcome				
Discharge		18	33	8
Death		0	0	0
Virus shedding time (days)	*median ± IQR*	3.0 ± 4.5	19.0 ± 15.5*	18.0 ± 27.5
Duration of hospitalization (days)	*median ± IQR*	8.0 ± 5.0	21.0 ± 10.0*	26.5 ± 14.3*

$\bar{X}$ mean; SD, standard deviation; IQR, interquartile range; *P < 0.05 (*vs.* asymptomatic patients, Mann-Whitney test).

plateau was observed. These results suggest that SARS-CoV-2 can only elicit a limited anti-RBD antibody response in asymptomatic infections.

Further analysis of the antibody isotypes against RBD in the plasma of patients revealed that anti-RBD IgG was always the dominant type of antibody in AP. The levels of IgA and IgM were similar by the fourth week of admission, but IgM declined faster than IgA and became undetectable within week 7 (**Figure 1B**). In MP, anti- SARS-CoV-2-RBD antibodies were dominated by IgM in the first week, IgA in the second and third weeks, and IgG after the third week. From the fourth week, the IgA level fell below the IgM level. In SP, the antibodies were dominated by IgM in

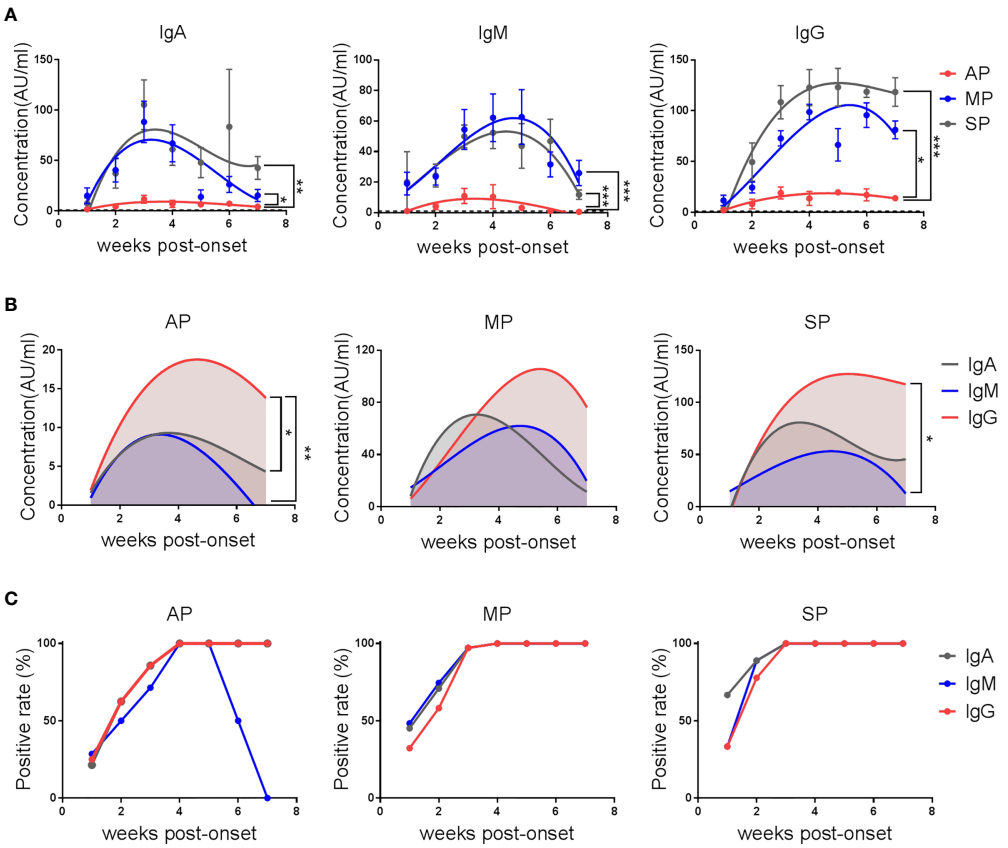

FIGURE 1 | The kinetics of anti-SARS-CoV-2-RBD IgA/IgM/IgG in COVID-19 patients. Serum IgA, IgM and IgG against the RBD protein of SARS-CoV-2 were detected by electrochemiluminescence. **(A)** The kinetics of anti-SARS-CoV-2-RBD IgA/IgM/IgG antibodies in AP, MP and SP groups. The line is a trend line fitted by Nonlinear Regression method. Antibody concentrations were expressed as mean ± SEM. **(B)** The kinetics of different anti-SARS-CoV-2-RBD antibody isotypes in each group. The line is a trend line fitted using the nonlinear regression method. **(C)** Positive rates of different anti-SARS-CoV-2-RBD isotypes over time in each group. One-way ANOVA with Holm-Sidak's multiple comparisons test was used. A p-value of ≤ 0.05 was considered statistically significant (*p-values of ≤ 0.05; **p-values of ≤ 0.01; ***p-values of ≤ 0.001).

the first week, IgA and IgG in the second week, and IgG from the third week on.

Consistent with the much weaker antibody responses in AP, the percent positive rates of SARS-CoV-2 RBD-specific IgA, IgM and IgG were lower in AP, with the positive rates of all these three isotypes reaching 100% by the end of the third week in MP and SP but not in AP. Specifically, the positive rate of IgM dropped rapidly from the sixth week and became 0 by the end of the 7th week in AP (**Figure 1C**).

These results demonstrate that the kinetics and magnitude of the antibody responses in patients with different disease severity are different. More importantly, detecting anti-RBD IgG rather than IgM and IgA antibodies, even at early stage after infection, can be helpful for identifying AP.

The Kinetics of IgG Response Against SARS-CoV-2-RBD/S1/S2/S/N Varied Among AP, MP and SP

In order to further dissect which structural proteins of SARS-CoV-2 are mainly targeted by the IgG antibodies and to understand their kinetics, we used ELISA to detect the antigen-specific IgG recognizing the different SARS-CoV-2 proteins. We included RBD (aa 319-514 of the spike protein), S1 (aa 1-685 of the spike protein), S2 (aa 686-1213 of the spike protein), S (aa 1-1213 of the spike protein), and N (aa 1-419 of the nucleocapsid protein) (**Figure 2**). The kinetics of anti-RBD IgG detected by ELISA (**Figure 2**) was consistent with that detected by the ECL assay (**Figure 1A**). Surprisingly, although the S1 protein contains the RBD fragment, the anti-S1 IgG kinetics were quite different from those of anti-RBD IgG, with a concordant upward trend in all the three groups. While the anti-S2 IgG response peaked in the third week post-onset in both MP and SP, it peaked in the fifth week in AP.

Overall, assays against the RBD and S2 proteins gave better signal and higher positive rates for all the three groups, and the IgG responses against all the indicated SARS-CoV-2 structural proteins were much weaker in AP than in MP or SP. Taken together, RBD and S2, but not other proteins (S1, S and N) are suitable targets for detecting SARS-CoV-2-reactive IgG antibodies in AP.

The Kinetics of Neutralizing Antibodies and Their Correlation With Binding Antibodies

In the previous assays, we have evaluated the SARS-CoV-2-reactive IgG antibodies that possess binding activity, but not their neutralization capacity. Here, neutralizing antibodies in the different groups of patients were assessed using authentic SARS-CoV-2 virus in a focus reduction neutralization test (FRNT). As shown in **Figure 3A**, the dynamics of neutralizing antibodies in the three groups was similar to those of anti-RBD IgG (**Figure 1A**), which is in line with previous studies demonstrating that neutralizing antibodies are mainly IgG antibodies that bind to the RBD region (16). The magnitude of

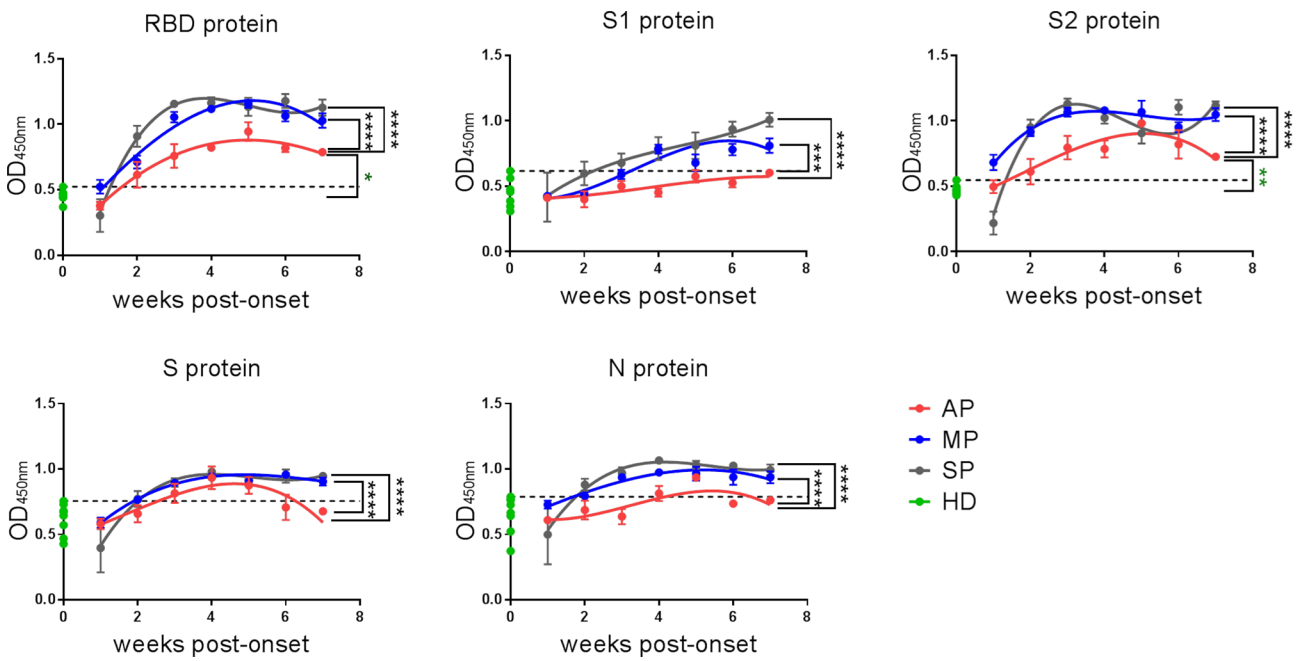

FIGURE 2 | The kinetics of IgG against SARS-CoV-2-RBD/S1/S2/S/N of COVID-19 patients. IgG against RBD/S1/S2/S/N proteins of SARS-CoV-2 in plasma were detected by ELISA. The line is a trend line fitted by Nonlinear Regression method. OD values were expressed as mean ± SEM. Eight healthy donor plasma samples collected in 2017-2018 served as a control group (HD group) in this experiment. Threshold (dashed line) was defined as mean (of HD group) + 3SD. One-way ANOVA with Holm-Sidak's multiple comparisons test was used. A p-value of ≤ 0.05 was considered statistically significant (*p-values of ≤ 0.05; **p-values of ≤ 0.01; ***p-values of ≤ 0.001; ****p-values of ≤ 0.0001).

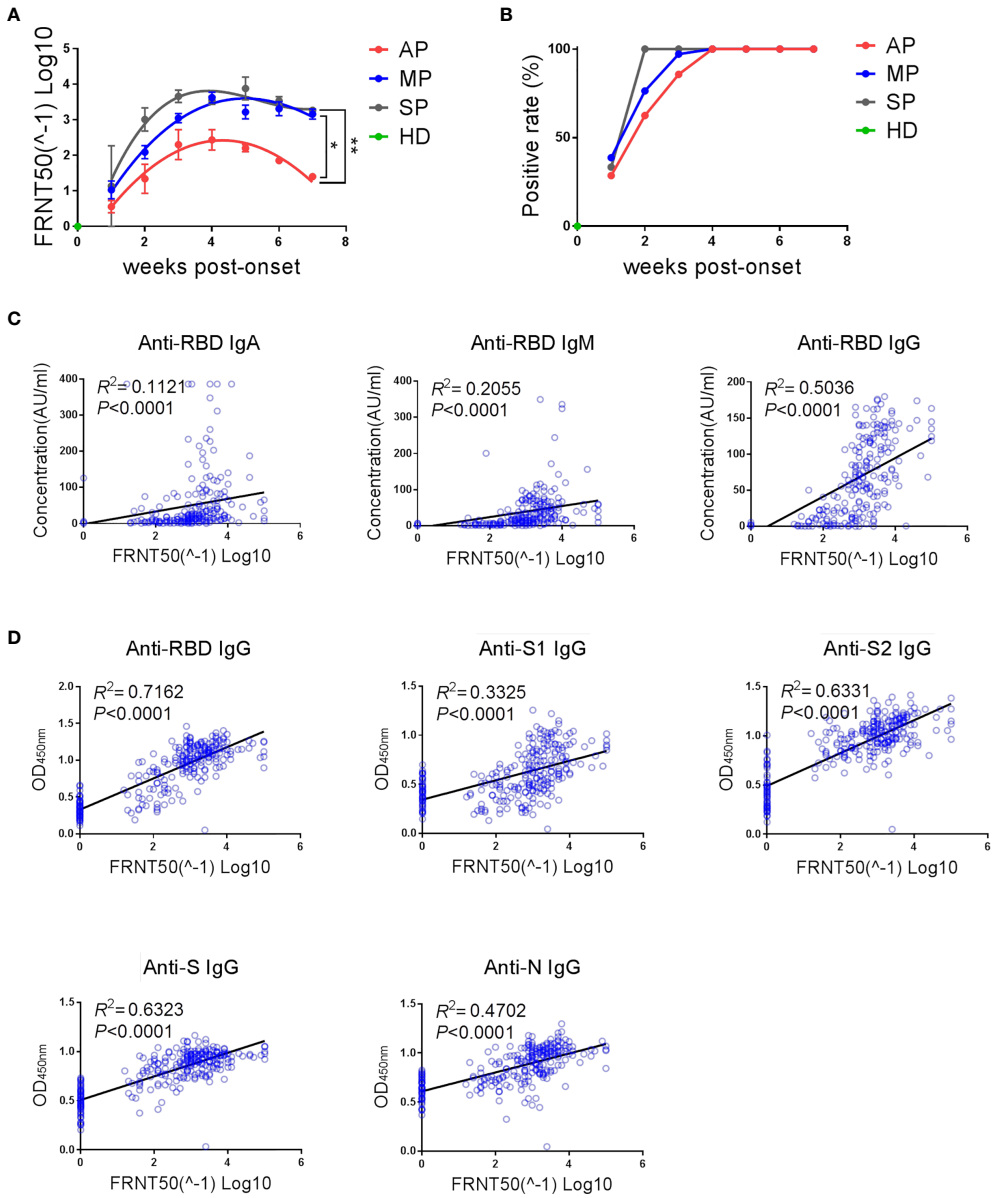

FIGURE 3 | The kinetics of neutralizing antibodies and its strong correlation with the magnitude of anti-SARS-CoV-2 binding antibodies. **(A)** Kinetics of neutralizing antibodies in different groups. The line is a trend line fitted by Nonlinear Regression method. The neutralizing antibody titers were expressed as mean ± SEM. **(B)** Positive rates of neutralizing antibodies over time in different groups. **(A, B)** One-way ANOVA with Holm-Sidak's multiple comparisons test was used. A p-value of ≤ 0.05 was considered statistically significant (*p-values of ≤ 0.05; **p-values of ≤ 0.01). **(C)** Correlation between neutralizing antibody titers and RBD-specific IgA/IgM/IgG concentrations detected by ECL assay was analyzed by linear regression. **(D)** Correlation between neutralizing antibody titers and multiple structural protein (RBD/S1/S2/S/N)-specific IgG levels detected by ELISA assay was analyzed by linear regression.

neutralizing antibody response was high in SP, moderate in MP, and low in AP (**Figure 3A**). Further, the positive rate of neutralizing antibodies reached 100% two weeks faster in SP than in MP or AP (**Figure 3B**).

In order to investigate the association between binding antibodies and neutralizing antibodies, we performed correlation analysis and linear regression analysis on the levels of neutralizing antibodies and binding antibodies (**Figures 3C, D**). As shown in **Figures 3C, D**, the titers of neutralizing antibodies (FRNT 50) had a significant linear correlation with the concentration of IgA/IgM/IgG against SARS-CoV-2-RBD (**Figure 3C**) and with the level of IgG antibodies against multiple SARS-CoV-2 structural proteins (**Figure 3D**). To further explore whether the presence of neutralizing antibodies can be predicted by detection of binding antibodies, the consistency between the detection results of different binding antibodies and the neutralizing antibodies was analyzed (**Table 2**). As expected, there was indeed a certain degree of consistency between the presence of neutralizing antibodies and

TABLE 2 | Overall performance of binding antibodies for predicting neutralizing antibodies.

Method	Binding antibody		Neutralizing antibody		TPR(%)	FPR(%)	FNR(%)	TNR(%)	PPV(%)	NPV(%)	FDR(%)	OA(%)
			Positive	Negative								
ECL	Anti-RBD IgA	Positive	202	7	94.84	11.86	5.16	88.14	96.65	82.54	3.35	93.38
		Negative	11	52								
	Anti-RBD IgM	Positive	203	6	95.31	10.17	4.69	89.83	97.13	84.13	2.87	94.12
		Negative	10	53								
	Anti-RBD IgG	Positive	194	3	91.08	5.08	8.92	94.92	98.48	74.67	1.52	91.91
		Negative	19	56								
ELISA	Anti-RBD IgG	Positive	195	3	91.55	5.08	8.45	94.92	98.48	75.68	1.52	92.28
		Negative	18	56								
	Anti-S1 IgG	Positive	107	4	50.23	6.78	49.77	93.22	96.40	34.16	3.60	59.56
		Negative	106	55								
	Anti-S2 IgG	Positive	212	13	99.53	22.03	0.47	77.97	94.22	97.87	5.78	94.85
		Negative	1	46								
	Anti-S IgG	Positive	177	0	83.10	0.00	16.90	100.00	100.00	62.11	0.00	86.76
		Negative	36	59								
	Anti-N IgG	Positive	155	4	72.77	6.78	27.23	93.22	97.48	48.67	2.52	77.21
		Negative	58	55								

TPR, true positive rate; FPR, false positive rate; TNR, true negative rate; FNR, false negative rate; PPV, positive predictive value; NPV, negative predictive value; FDR, false discovery rate; OA, overall accuracy.

binding antibodies, although this degree depended on the type of binding antibody. Among the binding antibodies, different isotypes of anti-RBD antibodies detected by ECL and anti-S2 or anti-RBD IgG detected by ELISA showed an overall accuracy (OA) of over 90% in predicting the presence of neutralizing antibodies. Specifically, anti-S2 IgG had the highest overall accuracy (94.85%) and highest negative prediction value (97.87%), indicating that when anti-S2-IgG is negative, the neutralizing antibody is most likely to be negative. Whereas anti-RBD-IgG detected by ELISA had the highest positive prediction value (98.48%), indicating that when anti-RBD-IgG is positive, the neutralizing antibody is most likely to be positive. In line with this, the linear regression results showed that the linear fit between RBD-IgG and neutralizing antibody detected by ELISA assay was the best (R^2 = 0.7162, P<0.0001) (**Figure 3D**).

Taken together, asymptomatic infections elicit significantly weaker neutralizing antibody responses and the presence and abundance of neutralizing antibodies show strong correlation with anti-RBD and anti-S2 IgG binding antibodies.

Sensitivity of Various Detection Schemes in Identifying AP

In order to better control the spread of SARS-CoV-2, accurate identification of asymptomatic or pre-symptomatic infections is essential. We compared the overall sensitivity (the overall antibody positive rate in all samples covering all the time points) of different detection schemes in identifying asymptomatic infections (**Figures 4A–C**). By comparing the sensitivity of anti-RBD-IgG detected by different methods (**Figure 4A**), we found that the sensitivity of ECL was higher than that of ELISA (49.00% *vs.* 45.10%). By comparing the sensitivity of IgG against different SARS-CoV-2 proteins detected by ELISA (**Figure 4B**), we found that the detection of anti-S2-IgG can achieve the highest positive detection rate (56.86%). By comparing the sensitivity of different antibody isotypes against RBD detected by ECL (**Figure 4C**), we found that detection of IgG can achieve the highest sensitivity (49.00%).

In order to further explore whether and how the sensitivity of these schemes changes over time, they were plotted against the time post-onset (**Figure 4D**). The sensitivity of all schemes increased steadily in the first five weeks, and most of them declined rapidly afterwards, except anti-S2 IgG, anti-RBD IgG and anti-RBD IgA. Detection of anti-S2 IgG was better than anti-RBD IgG, manifested by a higher sensitivity in the first two weeks post-onset. Comparing the sensitivity-time curves of anti-RBD-IgG detected by ECL and ELISA, ECL showed slightly higher sensitivity than ELISA, mainly in the first 1-2 weeks after admission.

Taken together, detection of IgG against SARS-CoV-2-S2 protein by ECL may improve the sensitivity for early detection of asymptomatic infections.

DISCUSSION

In this study, we tested SARS-CoV-2-specific antibodies in 272 plasma samples from 59 COVID-19 patients. The dynamics of the different binding antibody isotypes against the major SARS-CoV-2 structural proteins, as well as neutralizing antibodies in patients with different disease severity were evaluated and the sensitivity of the different detection schemes were compared.

Since asymptomatic SARS-CoV-2 infection was reported, it has caused widespread concerns for not only the high risk of silent spread of the disease, but also limited understanding of the immune response (6). A previous study showed that asymptomatic individuals develop a weaker immune response to SARS-CoV-2 infection than symptomatic patients, including lower levels of virus-specific IgG (17). Consistent with this, we found that the overall antibody response is lower in asymptomatic infections than in symptomatic infections throughout the disease

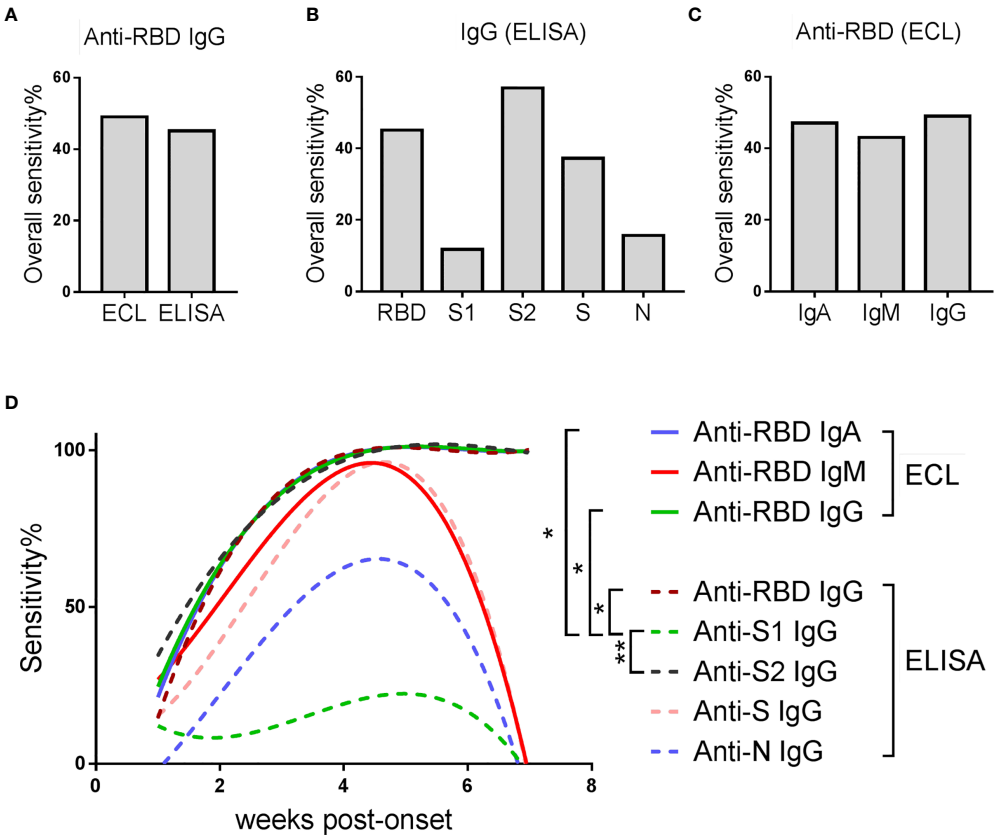

FIGURE 4 | Comparison of sensitivity of various detection schemes in identifying AP. **(A)** Overall sensitivity of anti-RBD-IgG detected by different methods in all samples. **(B)** Overall sensitivity of IgG against different SARS-CoV-2 proteins detected by ELISA assay. **(C)** Overall sensitivity of different antibody isotypes against SARS-CoV-2-RBD detected by ECL assay. **(A–C)** Overall sensitivity = 100% × (the number of antibody positive samples/the number of all samples). **(D)** Sensitivity of various detection schemes over time. The line is a trend line fitted using the nonlinear regression method. Sensitivity = 100% × (the number of antibody positive samples at each time point/the number of total samples at each time point). Friedman test with Dunn's multiple comparisons test was used. A p-value of ≤ 0.05 was considered statistically significant (*p-values of ≤ 0.05; **p-values of ≤ 0.01).

course, based on the response against various viral structural proteins, and the different isotypes of immunoglobulins against the RBD protein, as well as neutralizing antibodies. Besides, asymptomatic infections also show a lower antibody positive rate when compared with symptomatic infections at the same stage after infection, indicating that some asymptomatic infections develop antibody responses later. Taken together, these results demonstrate that asymptomatic infection induces a weaker humoral immune response.

We also found that the characteristics of antibody isotypes are different in patients with different disease severity. First, IgA appears later than IgG in asymptomatic infections, but earlier than IgG in symptomatic infections, especially in severe patients (**Figure 1C**). Our findings are consistent with the previous reports showing that IgA levels in severe cases were higher than those in mild or moderate cases (18, 19). Second, IgG is always dominant in asymptomatic individuals but not in symptomatic patients.

Neutralizing antibodies are important correlates of protection against infection. Detection of neutralizing antibodies in plasma is useful for evaluating the risk of infection in COVID-19 vaccinees and the risk of reinfection in COVID-19 convalescents (20). However, high-throughput detection of neutralizing antibodies is difficult due to the need for authentic SARS-CoV-2 viruses in biosafety level 3 laboratories and the long experimental procedure. Therefore, based on our findings, we propose here a candidate detection scheme to predict the level of neutralizing antibodies. Presence of IgG antibodies against S2 and RBD protein, which has the highest negative predictive value and the highest positive predictive value respectively, may best predict the presence of neutralizing antibodies. Further, the magnitude of neutralizing antibodies can be predicted from the anti-RBD IgG level. This approach may provide a safe alternative to predict the presence of neutralizing antibodies after infection.

Currently, a big challenge to control the pandemic is to rapidly and accurately identify asymptomatic infections at an early stage. As a gold standard, viral nucleic acid detection has high specificity, but its sensitivity is affected by many factors including sample collection. Serological testing can be a good supplement to nucleic acid testing (14). Although anti-RBD or anti-N IgG antibodies are

often the major targets in serological testing, here we find that detection of IgG against the S2 protein by ECL improves the sensitivity for early identification of asymptomatic infections. Although the S2 protein of SARS-CoV-2 is relatively conserved within the beta-coronaviruses, no non-specific reactivity were observed in healthy donors (21), indicating that this test could be a good candidate for auxiliary testing.

In summary, our findings suggest that compared with a symptomatic infection, an asymptomatic infection elicits a weaker humoral immune response, that is dominated by an IgG-based response. Detection of IgG antibodies against S2 and RBD proteins may help to estimate the abundance of neutralizing antibodies. Additionally, the detection of IgG against SARS-CoV-2-S2 protein may be a good supplement to nucleic acid testing. Our findings should be further validated with a larger cohort and could only be applicable within the context of natural infection but not vaccination. In conclusion, this study provides a more sensitive antibody detection scheme for identification of asymptomatic infections that may help mitigate the COVID-19 pandemic.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical approval was obtained from the Ethics Committee of Guangzhou Eighth People's Hospital (202002135). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

XT, YL, AZ, BS, JincZ, and JingZ conceived the study. BL, LL, CW, and WC collected clinical specimen. ZC, AZ, PZ, JianZ, and SW executed the experiments. ZC, JianZ, FL, SL, DC, CW, WC, SW, YT, LD, PW, FC, JHY, JWY, and JF analyzed the data. ZC, JincZ, JingZ, and AZ drafted the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Follicular Helper T Cells in the Immunopathogenesis of SARS-CoV-2 Infection

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Coronavirus disease 2019 (COVID-19), caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a serious infectious disease that has led to a global pandemic with high morbidity and mortality. High-affinity neutralizing antibody is important for controlling infection, which is closely regulated by follicular helper T (Tfh) cells. Tfh cells play a central role in promoting germinal center reactions and driving cognate B cell differentiation for antibody secretion. Available studies indicate a close relationship between virus-specific Tfh cell-mediated immunity and SARS-CoV-2 infection progression. Although several lines of evidence have suggested that Tfh cells contribute to the control of SARS-CoV-2 infection by eliciting neutralizing antibody productions, further studies are needed to elucidate Tfh-mediated effector mechanisms in anti-SARS-CoV-2 immunity. Here, we summarize the functional features and roles of virus-specific Tfh cells in the immunopathogenesis of SARS-CoV-2 infection and in COVID-19 vaccines, and highlight the potential of targeting Tfh cells as therapeutic strategy against SARS-CoV-2 infection.

Keywords: COVID-19, SARS-CoV-2, Tfh cells, B cells, neutralizing antibody

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), an emerging and acute novel coronavirus mainly transmitted *via* the respiratory tract, has rapidly caused pandemic-level cases of coronavirus disease 2019 (COVID-19), which has a high morbidity and mortality worldwide (1–5). Globally, as of 22 June 2021, there have been 178,503,429 confirmed cases of COVID-19, including 3,872,457 deaths from 195 countries and 28 regions according to the World Health Organization (WHO) report (6). SARS-CoV-2 is a serious threat to human health and life worldwide.

Humans who are immune-naive to SARS-CoV-2 are considered to be a major factor for the COVID-19 pandemic worldwide, and high-affinity neutralizing antibodies are especially essential

for the control and clearance of SARS-CoV-2 infection (7–10). Several studies have reported sustained antibody responses in patients with SARS-CoV-2 infection, in which specific antibody titers are increased along with the progression of infection (11–13) (**Figure 1**). Notably, the titers of specific antibodies against SARS-CoV-2 are usually low in the first week. When the high cumulative seroconversion rate occurs between 2 and 3 weeks after symptom onset, the titers of neutralizing antibodies are significantly decreased in the early convalescent phase, with the titers of neutralizing antibodies not detectable in some patients, which indicate that several weeks may be needed to generate antibodies against SARS-CoV-2 (12–17). These findings suggest that further studies are needed to explore the production and function of neutralizing antibody inSARS-CoV-2 infection.

Antibody responses are closely correlated with $CD4^+$T cell subsets that play important roles in the control of viral infections, including T helper (Th) 1 (Th1), Th2, and Th17 cells and follicular helper T (Tfh) cells (18, 19). Among $CD4^+$Th cell subsets, naive $CD4^+$T cells differentiated into Tfh cells can promote humoral immunity by mediating the interaction between T cells and B cells, which are essential for the control of viral infections and vaccine responses (19–21). Tfh cells, as a novel $CD4^+$T cell subset, are characterized by the high expression of CXC chemokine receptor 5 (CXCR5), inducible T cell costimulator (ICOS), programmed cell death protein 1 (PD-1), B-cell lymphoma 6 (Bcl-6), and interleukin-21 (IL-21) in both mice and humans and can usually initiate B cells to differentiate into plasma cells that produce high-affinity antibodies to neutralize the virus, such as lymphocytic choriomeningitis virus (LCMV), influenza virus and hepatitis B virus (22–25). Loss of Tfh cell function can result in primary immunodeficiencies characterized by impaired humoral immunity, including COVID-19 infection, autosomal-dominant hyper IgE caused by *STAT3* deficiency and common variable immunodeficiency (21, 25, 26). However, the roles and function features of Tfh cells in SARS-CoV-2 infection remain largely unclear (19, 20). Here, we will discuss the characteristics and functions of Tfh cells in the immunopathogenesis of SARS-CoV-2 infection and in COVID-19 vaccine responses, as well as their implications in eliciting effective immunity against SARS-CoV-2 infection.

THE PHENOTYPES AND FUNCTIONS OF TFH CELLS

Tfh cells can help B cells generate high-affinity antibodies, long-lived plasma cells, and memory B cells through functional markers (20, 21). The markers of Tfh cells are important to identify Tfh cells and their distinct subsets in the lymphoid tissue and circulation, which commonly include chemokine receptor CXCR5, transcription factor Bcl-6, PD-1, CD40 ligand (CD40L), and ICOS in humans and mice (25, 27–29). Moreover, the phenotypes of Tfh cells are associated with different stages of immune responses (30, 31). In secondary lymphoid organs, naïve $CD4^+$T cells are differentiated into Tfh cells with the upregulation of CXCR5 and downregulation of CC-chemokine receptor 7 (CCR7), which are mediated by antigen-specific conventional dendritic cells (DCs) or monocyte-derived DCs (28, 32, 33). The increased CXCR5 and decreased CCR7contribute to the migration of Tfh cells toward CXC-chemokine ligand 13 (CXCL13)-enriched B lymphoid follicles in the germinal center (GC) (28, 34). The specific transcription factor Bcl-6 is selectively expressed in Tfh cells but is highly expressed in $CXCR5^{hi}CCR7^{low/-}$Tfh cells in human and mouse GCs (34–37). The IL-21 cytokine is highly and specifically secreted by Tfh cells, which promotes the proliferation of Tfh cells and helps B cell differentiation and antibody secretion, which is characteristic of Tfh cells (38–42). ICOS deficiency

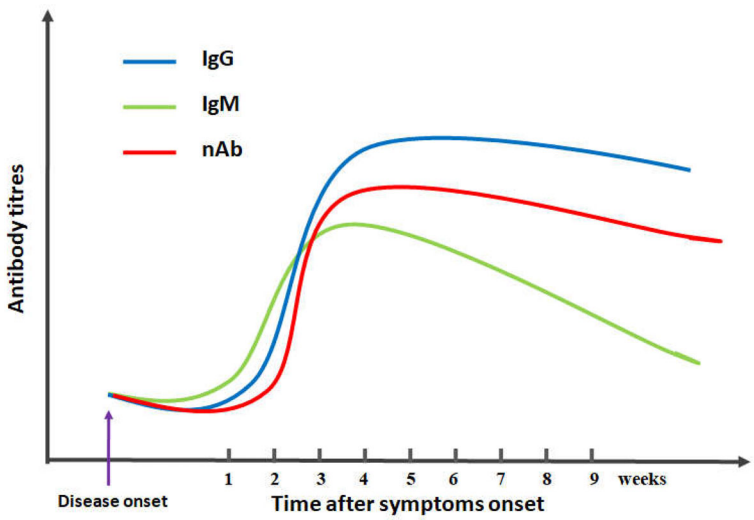

FIGURE 1 | Schematic diagram of antibody kinetics in COVID-19 patients. IgG/IgM/nAb indicates IgG antibodies/IgM antibodies/neutralizing antibodies, respectively.

significantly reduces GC reactions and Tfh cells in mice and humans, which indicates that ICOS expressed in Tfh cells is essential for the differentiation and maintenance of Tfh cells, GC formation, B cell differentiation and antibody responses (43–45). ICOS, as a key costimulatory molecule, can also induce the secretion of IL-21 in Tfh cells (45–47). HighPD-1 expression on Tfh cells can significantly promote the differentiation and activity of Tfh cells (48–50). Collectively, Tfh cells are commonly identified as having three phenotypes: canonical GC Tfh cells with PD-1^{++} and $ICOS^{++}Bcl\text{-}6^{+}CCR7^{-}CXCR5^{++}CD4^{+}$T cells, precursor-Tfh (Pre-Tfh) cells characterized as PD-$1^{+}ICOS^{+}Bcl\text{-}6^{low}CCR7^{low}CXCR5^{+}CD4^{+}$T cells, and memory Tfh cells similar to Pre-Tfh cells in lymphoid tissue (36, 50–52). In GC, Tfh cells are responsible for regulating B cell differentiation into memory B cells and plasma cells, controlling the selection of high-affinity antibody production and the development of long-term humoral immunity (53–56).

Circulating Tfh (cTfh) cells in the peripheral blood are usually composed of two distinctive phenotypes: effector memory Tfh cells (PD-$1^{+}ICOS^{+}CCR7^{low}BCL\text{-}6^{-}CXCR5^{+}CD4^{+}$T cells) and central memory Tfh cells (PD-$1^{-}ICOS^{-}CCR7^{high}BCL\text{-}6^{-}CXCR5^{+}CD4^{+}$T cells) (32, 57, 58). Additionally, based on the expression of CXCR3 and CCR6, cTfh cells are further divided into three subsets: Tfh1 ($CXCR3^{+}CCR6^{-}$), Tfh2 ($CXCR3^{-}CCR6^{-}$), Tfh17 ($CXCR3^{-}CCR6^{+}$), and Tfh1/17 ($CXCR3^{+}CCR6^{+}$) cells, which share the signature transcription factors and cytokines of Th1 (T-bet and IFN-γ), Th2 (GATA3, IL-4, IL-5 and IL-13), Th17 (RORγt, IL-17 and IL-22) cells, respectively (32, 58, 59). cTfh2 and cTfh17 cells can induce B cell differentiation and antibody secretion and regulate immunoglobulin (Ig) isotype switching. cTfh1 cells are commonly considered not to be a helper for B cells, but $ICOS^{+}PD\text{-}1^{high}CCR7^{low}$cTfh1 cells effectively regulate B cell differentiation and induce antibody responses (59–65). These studies display functionally distinct cTfh cell subsets based on ICOS, PD-1, and CCR7 expression, as well as CXCR3 and CCR6. Moreover, these novel subsets are different from Th1, Th2 and Th17 cells but share some of their characteristics. Additionally, Tfh-like cells have also been identified in non-lymphoid tissues, including the synovium of arthritis, skin and salivary glands of patients, which commonly express low or undetectable CXCR5 and Bcl-6 and high PD-1, ICOS, OX40 and IL-21 compared to Tfh cells in secondary lymphoid organs, which also express tissue-specific chemokine receptors, including CCR2, CCR5, CX3C-chemokinereceptor 1 (CX3CR1) and CXCR4 (52, 66–71). Recently, Tfh13 cells, a novel Tfh cell subset that secretes IL-4 and IL-13, were shown to be responsible for IgE production in human and mouse allergies and to highly express the transcription factors Bcl-6 and GATA3 (72–74). Current studies indicate that distinct phenotypes of Tfh cells are critical for B cell differentiation and high-affinity antibody production (**Table 1**). Interestingly, follicular regulatory T (Tfr) cells are considered a subset of $Foxp3^{+}$Treg cells in the GC that are initiated from $Foxp3^{+/-}$ precursors but not from Tfh cells (75–78). Tfr cells share canonical Tfh cell molecules, including CXCR5, Bcl-6, PD-1 and ICOS, as well as Treg cell molecules, including CD25, Foxp3, Blimp-1 and CTLA-4 (79–82). Importantly, Tfr cells, similar to Treg cells, play a critical role in immunosuppression, rather than Tfh cells, which can limit GC responses and suppress the activation of Tfh cells and B cells within GCs through inhibitory molecules, including CTLA-4, PD-1, IL-10 and TGF-β secretion. The balance of Tfh/Tfr cells is essential to maintain immune homeostasis and mediate humoral immunity (63, 66, 82–85).

THE DIFFERENTIATION OF TFH CELLS

Tfh cell differentiation is regulated by multiple complex factors and stages. Naïve $CD4^{+}$T cells are primed by binding their T cell receptors with peptide-loaded major histocompatibility complex (MHC) class II (pMHC-II) on professional antigen-presenting cells (APCs), such as DCs and monocytes. Strong TCR signaling and continuous antigenic stimulation play critical roles in favoring Tfh cell differentiation by upregulating BATF to promote Bcl-6 expression (86–90). The early differentiation of

TABLE 1 | Phenotypes of Tfh cell subsets in blood and lymphoid tissues.

Location	Cell subsets	Phenotypic markers	References
Blood			
	Central memory Tfh cells	$PD\text{-}1^{-}ICOS^{-}CCR7^{high}Bcl\text{-}6^{-}Blimp\text{-}1^{-}CXCR5^{+}$	(32, 57, 58)
	Effector memory Tfh cells	$CD40L^{+}/PD\text{-}1^{+}/ICOS^{+}CCR7^{low}Bcl\text{-}6^{-}Blimp\text{-}1^{-}CXCR5^{+}$	
	cTfh1 cells	$IFN\text{-}\gamma^{+}Bcl\text{-}6^{-}Blimp\text{-}1^{-}CXCR5^{+}$or $PD\text{-}1^{+}ICOS^{+}CCR7^{low}CXCR3^{+}CCR6^{-}Bcl\text{-}6^{-}Blimp\text{-}1^{-}CXCR5^{+}$	(32, 57–59)
	cTfh2 cells	$IL\text{-}4^{+}Bcl\text{-}6^{-}Blimp\text{-}1^{-}CXCR5^{+}$ or $CXCR3^{-}CCR6^{-}Bcl\text{-}6^{-}Blimp\text{-}1^{-}CXCR5^{+}$	
	cTfh17 cells	$IL\text{-}17A^{+}Bcl\text{-}6^{-}Blimp\text{-}1^{-}CXCR5^{+}$or $CXCR3^{-}CCR6^{+}Bcl\text{-}6^{-}Blimp\text{-}1^{-}CXCR5^{+}$	
	cTfh1/17 cells	$IFN\text{-}\gamma^{+}$ $IL\text{-}17A^{+}Bcl\text{-}6^{-}Blimp\text{-}1^{-}CXCR5^{+}$or $CXCR3^{+}CCR6^{+}Bcl\text{-}6^{-}Blimp\text{-}1^{-}CXCR5^{+}$	
	cTfh13 cells	$IL\text{-}13^{hi}IL\text{-}4^{hi}IL\text{-}5^{hi}IL\text{-}21^{low}Bcl\text{-}6^{+}GATA3^{+}CXCR5^{+}$	(72–74)
Lymphoid tissues			
	Pre-Tfh cells	$PD\text{-}1^{+}ICOS^{+}CCR7^{low}Bcl\text{-}6^{low}Blimp\text{-}1^{-}CXCR5^{+}$	(32, 57, 58)
	GC Tfh cells	$PD\text{-}1^{++}ICOS^{++}CCR7^{-}Bcl\text{-}6^{+}Blimp\text{-}1^{-}CXCR5^{++}$	
	Memory Tfh cells	$PD\text{-}1^{+}ICOS^{+}CCR7^{low}Bcl\text{-}6^{low}Blimp\text{-}1^{-}CXCR5^{+}$	

PD-1, programmed cell death protein-1; CCR7, CC-chemokine receptor 7; CXCR3, CXC-chemokine receptor 3; CCR6, CXC-chemokine receptor 6; CXCR5, CXC-chemokine receptor 5; ICOS, inducible T cell co-stimulator.

Tfh cells is sufficiently initiated by DCs predominantly localized to T cell zones of lymphoid organs, which are considered Pre-Tfh cells that upregulate Bcl-6 and CXCR5 and repress CCR7 expression, and $Bcl6^{+}CXCR5^{+}$Pre-Tfh cells are attracted by the chemokine CXCL13 (CXCR5 ligand) produced within the B cell follicle zones toward the T-B border (36, 64, 91–94). Pre-Tfh cells migrate to the T-B cell border and interact with cognate B cells to further upregulate Bcl-6, CXCR5, ICOS, PD-1 and IL-21 and downregulate CCR7 expression, which further drives GC-Tfh differentiation and maturation and GC formation. These processes also require available costimulatory molecules and cytokines, including ICOS-ICOSL, OX40-OX40L, PD-1-PD-Ll/2, CD40-CD40L, IL-21, IL-6 and IL-12 cytokines (25, 32, 36, 95–102).

The transcription factor Bcl-6 in $CD4^{+}$T cells is mostly essential for Tfh differentiation and function, and loss of Bcl-6 represses Tfh differentiation, GC formation, B cell differentiation and antibody responses (34, 35, 43). Bcl6-expressing Tfh cells are also regulated by multiple transcription factors, including positive inductors such as TCF-1 and LEF-1, BATF, NOTCH1/2, and IRF4 and negative regulators such as Blimp-1, FOXO1 and STAT5 (22, 25, 32, 103–110). Some costimulatory molecules expressed on Tfh cells are considered markers of Tfh cells, including ICOS, OX40, PD-1 and CD40L, which can also induce Tfh cell differentiation and maintenance (32, 111, 112). In GC, B cells highly express costimulatory ligands, including ICOSL, CD80, CD86, PD-L1, and PD-L2, which contribute to the maintenance of Tfh cells, and then Tfh cells also mutually promote B cells to differentiate into plasma cells to further produce specific antibodies that mediate humoral immune responses (113, 114). Bcl-6 induces secretion of the cytokine IL-21, which can promote Tfh cell differentiation by upregulating STAT-1 and STAT-3 signals to further induce Bcl-6 expression, and similarly, the cytokine IL-6 plays a critical role in Tfh cell differentiation by upregulating the STAT1/3-Bcl-6 signal axis (56, 85, 115, 116). In addition, Tfh1 cells are characterized by IL-21 and IFN-γ production, and Tfh1 cell differentiation characterized by increased T-bet and Bcl-6 expression is initiated by phosphorylation of STAT1 and STAT4 in $CD4^{+}$T cells that are induced through IL-12, which is partially inhibited by a high concentration of IL-2 that reduces Bcl-6 expression (85, 115–120). Tfh2 cells are characterized by IL-4 and IL-21 production; Tfh2 cell differentiation is driven by IL-4 but suppressed by IL-6 *via* STAT3 signaling, and IL-4-secreted Tfh2 cells contribute to humoral immunity (85, 121–123). Tfh17 cells are characterized by IL-21 and IL-17 production; Tfh17 cell differentiation is primed by IL-23, IL-21, ICOS, TGF-β and IL-6, which upregulate Bcl-6 and RORγt expression. Consistent with its well established role in driving B cell response during infection, IL-17 secreted by Tfh17 cells can promote interactions of cognate T-B cells in the GC, inducing the formation of spontaneous GC and Ig isotype class-switching (124–127). However, low doses of IL-2, TGF-β and CTLA-4 promote the development of Tfr cells that play critical roles in inhibiting Tfh cell differentiation and GC responses by activating STAT5, Blimp-1, and Bach2 transcription factors in Tfr cells characterized by $CXCR5^{+}Foxp3^{+}CD4^{+}$T cells (128–133). Tfr cells can inhibit Tfh cell and plasma cell differentiation by inhibitory molecules, including CTLA-4, IL-10 and TGF-β; conversely, Tfh cells also inhibit the expansion of Tfr cells by the IL-21 cytokine (27, 131–139). This suggests that the balance of Tfh and Tfr cells plays a critical role in regulating B cell differentiation and specific antibody production (140).

TFH CELLS IN SARS-COV-2 INFECTION AND VACCINE

Currently, the SARS-CoV-2 infection pandemic has led to a serious threat to human health worldwide. Neutralizing antibodies of humoral immunity play a critical role in vaccine responses and battles against infectious viruses, including SARS-CoV-2, which is closely associated with Tfh cells differentiation and function (18, 19, 21, 141–144) (**Figure 2**). The role and function of Tfh cells in the control and clearance of SARS-CoV-2 infection and in the development of new vaccines have been investigated.

Previous reports showed that the frequencies of cTfh cells characterized by$CXCR5^{+}ICOS^{+}PD\text{-}1^{+}$progressively increased up to 20 days from the onset of infection in a case with non-severe convalescent COVID-19, in addition to elevated specific plasma SARS-CoV-2-binding IgM and IgG antibodies (145). Single-cell analysis revealed that expanded frequencies of cTfh cells were found in patients with active COVID-19 disease, as well as a high percentage of specific anti-SARS-CoV-2 antibodies, including IgA and IgG (146). The frequencies of spike (S)-specific cTfh cells ($CD3^{+}CD4^{+}CD45RA^{-}CXCR5^{+}$) are consistently elicited after S peptide stimulation in convalescent COVID-19 cases and exhibit a clear phenotypic bias to a$CCR6^{+}CXCR3^{-}$cTfh17 cell phenotype; however, neutralizing activity is inversely correlated with S-specific cTfh17 cell frequencies but positively correlated with S-specific cTfh, cTfh1 ($CCR6^{-}CXCR3^{+}$) and cTfh2 ($CCR6^{-}CXCR3^{-}$) cell frequencies (147). Previous reports suggested that expanded $CXCR3^{+}$cTfh1 cells positively correlated with the neutralizing antibody response against influenza vaccination and live-attenuated yellow fever vaccination (148, 149). A recent study showed that increased frequencies of $CCR7^{low}PD\text{-}1^{+}$cTfh-effectormemory (em), cTfh1 and cTfh2 cells in $CXCR5^{+}CD45RA^{-}CD25^{-}CD4^{+}$T cells are significantly increased, as well as high IL-1β and TNF-α, and that the frequencies of cTfh1 cells are associated with SARS-CoV-2-specific IgG/IgM antibodies, although $CCR7^{high}PD\text{-}1^{-}$cTfh-central memory (cm) and cTfh17 cells in $CXCR5^{+}CD45RA^{-}CD25^{-}CD4^{+}$T cells are decreased, as well as cTfr cells in Treg cells in convalescent patients compared to healthy subjects. Moreover, the frequencies of high cTfh-em, low cTfh-cm and cTfr cells are positively correlated with disease severity (150). These observations indicated that cTfh cell phenotypes can induce potent neutralizing responses against SARS-CoV-2 in COVID-19convalescent patients, which will contribute to antibody-based therapeutics and vaccination design for COVID-19.

Additionally, increased frequencies of virus-specific cTfh cells ($CD4^{+}CXCR5^{+}OX40^{+}CD40L^{+}$) were observed in acute and convalescent COVID-19 cases, and the frequencies of both

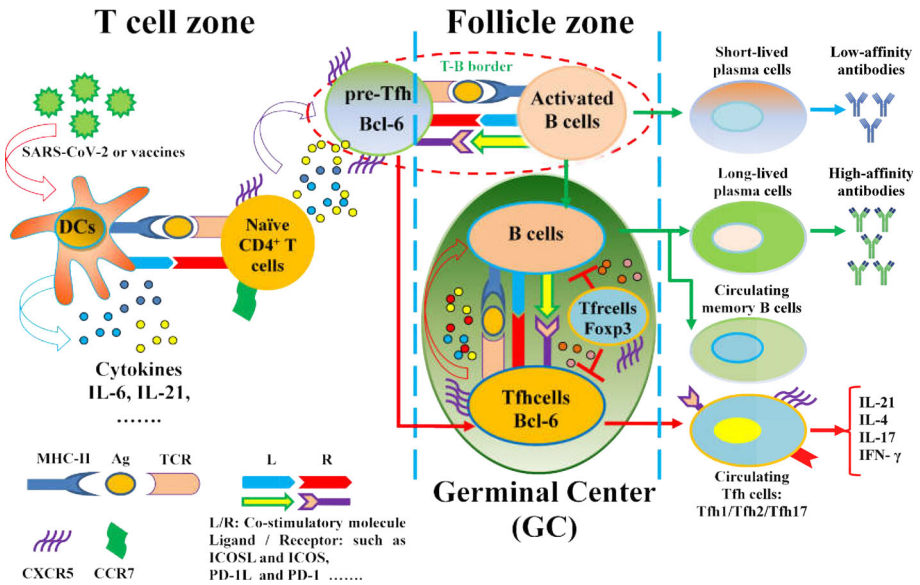

FIGURE 2 | The regulation of Tfh cell differentiation and function in SARS-CoV-2 infection and vaccines. Naïve $CD4^+$ T cells are driven by APCs (DCs) upon exposure to SARS-CoV-2 or virus antigens, which are activated toward antigen-specific Pre-Tfh cells with upregulation of CXCR5 and Bcl-6 and downregulation of CCR7 under the interaction of MHC-II molecules on DCs and cognate TCR on $CD4^+$T cells, as well as the expression of costimulatory molecules and cytokine production. Pre-Tfh cells interact with activated B cells at the T-B border in the follicle zone, which further differentiate into various Tfh cell subsets that migrate to the GC, where Tfh cells promote B cell differentiation and specific antibody production. However, the loss of GC structures reduces Bcl-6^+Tfh cells in severe COVID-19 patients. Notably, SARS-CoV-2-specific Tfh cells are expanded in mild and asymptomatic patients with COVID-19.Moreover, vaccines can efficiently induce Tfh cell differentiation, GC formation, and protective antibody responses.

SARS-CoV-2-specific cTfh cells and S-specific $CCR6^+CXCR3^-$ cTfh17 cells were closely associated with low disease severity (151). Longitudinal studies on COVID-19 infection and convalescent subjects indicate that the levels of SARS-CoV-2 antibodies are low and insufficient in humoral immunity response, although the underlying mechanism is poorly understood (11–14). The numbers of $CD4^+CXCR5^+$Tfh, $ICOS^+$Tfh, Bcl-6^+Tfh and Bcl-6^+B cells are decreased in lymph nodes and spleens, which are possibly associated with exclusively abundantTh1 cells, increased Treg cells (but not Tfr cells) and aberrant TNF-α production in COVID-19 lymph nodes in COVID-19 patients, as well as loss of GCs in lymph nodes and spleens from acute and dead COVID-19 patients (26, 152, 153). These data indicated that defective Tfh cell generation and dysregulated humoral immunity provide a possible mechanistic explanation for the limited durability of antibody responses in COVID-19 disease. Furthermore, low frequencies of $CD45RA^-$ $PD\text{-}1^+CXCR5^+$cTfh cells were also observed, but elevated frequencies of activated cTfh ($CD38^+ICOS^+$) cells were positively correlated with anti-SARS-CoV-2 IgM and IgG titers in hospitalized COVID-19 patients (154). These findings indicated that activated cTfh cells may be more reflective of recent antigen encounter and emigration from the GCs. Additionally, a single-cell transcriptomic analysis revealed that increased proportions of cytotoxic cTfh cells in hospitalized COVID-19 patients early in the illness are negatively correlated with the IgG levels of anti-spike protein antibodies to SARS-CoV-2, although the total SARS-reactive cTfh cells show a positive correlation with anti-spike antibody levels in hospitalized COVID-19 patients but not in non-hospitalized COVID-19 patients, which provided insights into cytotoxic cTfh cells in the distinct disease severities of COVID-19 patients (155). Moreover, reduced cTfh and $PD\text{-}1^+$cTfh and increased exhausted $TIM\text{-}3^+$cTfh cell frequencies are significantly observed, but the correlations between cTfh cells and anti-SARS-CoV-2IgM and IgG titers were not analyzed in hospitalized COVID-19 patients (156). These results indicated that cytotoxic cTfh and exhausted cTfh cells may inhibit specific anti-SARS-CoV-2 antibody production, which plays a critical role in severe SARS-CoV-2 infection (157). In a recent cohort study of COVID-19 patients within six months of recovery, the $CXCR5^+CD4^+$cTfh cell frequencies were significantly higher in COVID-19 patients in the long-term clinically recovered (20∼26 weeks) cohort (LCR) than in those in the short-time clinically recovered (4∼9 weeks) cohort (SCR). However, the frequencies of cTfh cells in both the LCR and SCR cohorts were lower than those in the healthy donor cohort (HD). Moreover, three cTfh subsets were similar between the LCR and HD cohorts; cTfh1 cell frequencies in the SCR cohort were shown to be significantly low, but cTfh2 and cTfh17 subsets were found to be high compared with the LCR and HD cohorts (158). Virus-specific Tfh cell frequencies, memory B cell responses, and serum CXCL13 levels were not different between asymptomatic or mild symptomatic COVID-19 patients. In contrast, COVID-19 patients with moderate or severe disease exhibited vigorous virus-specific GC B cell responses and Tfh cell responses. Moreover, potent virus-specific Th1 and $CD8^+$T cell responses

were observed in asymptomatic or mildly symptomatic patients but not in severely symptomatic patients. These data suggest that asymptomatic and mild patients have weak and transient SARS-CoV-2 antibody responses (159).

During acute COVID-19 infection, expanded activated $CD38^+HLA\text{-}DR^+PD\text{-}1^+ICOS^+CXCR5^+CD4^+$cTfh cells, $CD38^+HLA\text{-}DR^+CXCR3^+$cTfh1 cells, and activated $CD38^+HLA\text{-}DR^+$Th1 cells emerged, together with cytotoxic $CD8^+$T cells. The number of activated cTfh1 cells positively correlated with the levels of RBD- and spike-specific antibodies, including IgG, IgA and IgM isotypes (160). These data indicated that activated cTfh cell responses were associated with robust antibody responses elicited during SARS-CoV-2 infection, which may be valuable as potential biomarkers in vaccine clinical trials. Similarly, $CD38^+HLA\text{-}DR^+$cTfh cells, activated $CD4^+$T cells and cytotoxic $CD8^+$T cells were expanded in COVID-19 patients, and increased $CD38^+HLA\text{-}DR^+$cTfh cells indicated a recent antigen encounter and emigration from the GC of the patients (161). The frequencies of $PD\text{-}1^+ICOS^+$cTfh, cytotoxic $CD4^+$T and exhausted T cells were strongly expanded in COVID-19 patients, particularly in severe patients compared to healthy individuals, which suggested that extensive T cell dysfunction was associated with COVID-19 severity (162). In severe COVID-19 patients, the frequencies of $CCR6^+$cTfh cells and $CCR4^+$cTfh cells were expanded, but $CCR3^+$cTfh cells and Th1 cells were low in severe COVID-19 patients compared to healthy individuals (163). The frequencies of $PD\text{-}1^+ICOS^+$cTfh cells, activated cTfh cells and cytotoxic $CD8^+$T cells were strongly upregulated in COVID-19 patients, particularly in severe patients compared to healthy donors. Moreover, an increase in $CD4^+CD127^-CD25^+$Treg cells was found in mild patients, and upregulation of CCR4 in activated $CD8^+$T cells indicated enhanced lung homing in severe COVID-19 patients (164). Additionally, in rhesus macaques, SARS-CoV-2 infection induces predominantly GC $CXCR3^+$Tfh cells (but not a $PD\text{-}1^+Foxp3^+$Tfr cell subset) specific for the SARS-CoV-2 spike and nucleocapsid proteins and produce high titers of antiviral serum IgG and IgM antibodies againstSARS-CoV-2 (165) (**Table 2**). These data indicated that variable Tfh cell subsets dysregulated the humoral immune responses in COVID-19 patients caused by SARS-CoV-2 infection.

The COVID-19 pandemic continues to spread worldwide, and a safe and protective vaccine is urgently needed to effectuate herd protection and control of SARS-CoV-2. Currently, rapid advances have been made in the design and development of SARS-CoV-2 vaccines, such as inactivated vaccines, DNA vaccines, mRNA vaccines and specific SARS-CoV-2 proteins (166). mRNA-1273 vaccine could significantly induce Th1 and interleukin-21-producing $CXCR5^+PD\text{-}1^+ICOS^+$Tfh cell responses, and elicit robust SARS-CoV-2 neutralizing activity, which provided rapid protection in the upper and lower airways from SARS-CoV-2 infection in Rhesus Macaques (167). When compared to SARS-CoV-2 with recombinant SARS-CoV-2 receptor-binding domain (rRBD) formulated with AddaVax (rRBD-AddaVax) protein vaccine, the SARS-CoV-2 mRNA vaccines encoding RBD and full-length spike protein efficiently induce SARS-CoV-2-specific GC B cell and Tfh cell responses, which promoted specific neutralizing antibody production in vaccinated mice. Interestingly, the rRBD-AddaVax vaccine could elicit high frequencies of $IL\text{-}4^+$ Tfh cells (168). In human vaccination, the BNT162b2 mRNA vaccine for SARS-CoV-2 had significantly elicited $AIM^+CXCR5^+CD45RA^-CD3^+$cTfh cell responses, AIM (activation induced marker) cells include $CD69^+OX40^+$ or $CD69^+CD40L^+$ or$CD69^+4\text{-}1BB^+$ or $OX40^+4\text{-}1BB^+$ or $CD40L^+4\text{-}1BB^+$ or $CD40L^+OX40^+$cells, and the frequency of AIM^+cTfh cells is positively correlated with anti-Spike-specific IgA and IgG antibody titers (169). These findings have indicated that SARS-CoV-2 mRNA vaccines can effectively promote antigen-specific Tfh cell differentiation, B cell responses and the generation of protective antibodies, which are considered as promising candidates for eliciting high-quality adaptive

TABLE 2 | Characteristics and function of Tfh cells in COVID-19 patients.

Severity of disease	Characteristics	Function	Isotype of antibodies	References
Convalescent	$CD3^+CD4^+CD45RA^-CXCR5^+$Tfh cells expansion; bias to a $CCR6^+CXCR3^-$cTfh17 cells.	Positively associate with plasma neutralizing activity.	—	(146)
	cTfh-em and cTfh1 cells expansion.	Positively associate with the SARS-CoV-2-specific antibody titers.	IgM	(149)
Mild	$CXCR5^+ICOS^+PD\text{-}1^+$cTfh cells expansion,	Correlate with better clinical outcomes.	IgM, IgG	(144)
	$CD45RA^-PD\text{-}1^+CXCR5^+$cTfh cells reduction, activated cTfh ($CD38^+ICOS^+$) cells expansion.	Positively correlate with anti-SARS-CoV-2IgM and IgG titers.	IgM, IgG	(153)
Moderate	$TIM\text{-}3^+$Tfh-like cells expansion, $CD226^+$Tfh-like cells reduction.	Benefit the maintenance of balanced cellular and humoral immune responses.	—	(155)
Severe	Tem and Tfh-em cells expansion, Tcm, Tfh-cm, and Tfr cells reduction.	cTfh-em cells negatively correlate with recorded PaO_2/FiO_2.	IgG, IgA	(149)
	Cytotoxic cTfh cells and cytotoxic T helper cells expansion, Treg cells reduction.	Negatively correlate with antibody levels to SARS-CoV-2spike protein.	—	(154)
	$PD\text{-}1^+ICOS^+CXCR5^+CD4^+$cTfh cells expansion.	Correlate with robust humoral immunity.	IgG, IgM, and IgA	(159)
	$CCR6^+$cTfh cells and $CCR4^+$cTfh cells expansion, $CCR3^+$cTfh cells and Th1 cells reduction.	Favor the development of the antibody response.	—	(162)

"—" indicates not mentioned; Tfh, follicular helper T cell; cTfh, circulating Tfh cell; cTfh-em, effector-memory-like circulating Tfh cell; Tfh-cm, central-memory-like circulating Tfh cell; Tfr, follicular T regulatory cell; PaO_2, arterial oxygen tension; FiO_2, inspiratory oxygen fraction;Treg, regulatory T cells.

TABLE 3 | Tfh cell responses in various vaccine candidates of SARS-CoV-2.

Vaccine candidates	Phenotypes	Function	Antibody isotypes	References
mRNA vaccines				
mRNA-1273	IL-21$^+$CXCR5$^+$PD–1$^+$ICOS$^+$Tfh cells expansion.	Induce robust and specific antibody responses including neutralizing antibody.	IgA, IgG	(166)
full SΔ furin mRNA	B220$^-$CD4$^+$CD44^{hi}CD62L$^-$CXCR5$^+$Bcl-6$^+$ Tfh cells, B220$^-$CD4$^+$CD44^{hi}CXCR5$^+$PD-1^{hi} IL-21$^+$Tfh cells, B220$^-$CD4$^+$CD44^{hi}CXCR5$^+$Bcl-6$^+$ ICOS$^+$Tfh cells B220$^-$CD4$^+$CD44^{hi}CXCR5$^+$PD-1^{hi} IFN-γ$^+$Tfh cells notable expansion.	Elicit potent SARS-CoV-2-specific GC B responses, induce robust and specific antibody responses including neutralizing antibody.	IgG1, IgG2a, IgG2b,	(167) (167)
RBD mRNA (receptor binding domain, RBD)	B220$^-$CD4$^+$CD44^{hi}CD62L$^-$CXCR5$^+$Bcl-6$^+$ Tfh cells, B220$^-$CD4$^+$CD44^{hi}CXCR5$^+$PD-1^{hi} IL-21$^+$Tfh cells, B220$^-$CD4$^+$CD44^{hi}CXCR5$^+$Bcl-6$^+$ ICOS$^+$Tfh cells, B220$^-$CD4$^+$CD44^{hi}CXCR5$^+$PD-1^{hi} IFN-γ$^+$Tfh cells notable expansion	Elicit potent SARS-CoV-2-specific GC B responses, induce robust and specific antibody responses including neutralizing antibody.	IgG1, IgG2a, IgG2b,	
BNT162b2 mRNA vaccine	AIM$^+$CXCR5$^+$CD45RA$^-$CD3$^+$cTfh cells expansion, AIM cells include CD69$^+$OX40$^+$ or CD69$^+$CD40L$^+$ orCD69$^+$4-1BB$^+$ or OX40$^+$4-1BB$^+$ or CD40L$^+$4-1BB$^+$ or CD40L$^+$OX40$^+$	Positively correlate with anti-spike-specific IgA and IgG titers.	IgA, IgG	(168)
Protein vaccines				
rRBD-AddaVax	B220$^-$CD4$^+$CD44^{hi}CD62L$^-$CXCR5$^+$Bcl-6$^+$ Tfh cells, B220$^-$CD4$^+$CD44^{hi}CXCR5$^+$PD-1^{hi} IL-21$^+$Tfh cells B220$^-$CD4$^+$CD44^{hi}CXCR5$^+$PD-1^{hi} IL-4$^+$Tfh cells slight expansion	Delay to elicit potent SARS-CoV-2-specific GC B responses, induce robust and specific antibody responses including neutralizing antibody.	IgG1,	(167)
NVX-CoV2373	CXCR5$^+$PD-1$^+$CD4$^+$Tfh cells expansion	Induce specific antibody responses including neutralizing antibody.	IgG	(169)
Spike (S) and receptor binding domain (RBD) protein subunit vaccine	CXCR5^{++}BCL-6$^+$CD4$^+$CD3$^+$B220$^-$Tfh cells expansion	Induce specific antibody responses including neutralizing antibody.	IgG	(170)
StriFK-FH002C	PD-1$^+$CXCR5$^+$CD4$^+$Tfh cells expansion	Induce specific antibody responses including neutralizing antibody.	IgG, IgG1, IgG2a, IgG2b	(171)

Tfh, follicular helper T cell; cTfh, circulating Tfh cell.

immune responses to control and clear SARS-CoV-2 infection. Additionally, the specific protein vaccines including SARS-CoV-2 subunit vaccine (NVX-CoV2373) with the full-length spike (S) protein, StriFK-FH002C and Spike (S)/receptor binding domain (RBD) protein subunit vaccine significantly induce specific cTfh cell and GC B cell responses, resulting in high neutralizing antibody titers of SARS-CoV-2 (170–172) (**Table 3**). Various clinical trials in humans indicate that inactivated SARS-CoV-2 vaccines can induce satisfactory high neutralizing antibody titers that notably reduce the number of patients with severe COVID-19 (173–176). These data suggested that SARS-CoV-2 vaccines can safely and effectively promote humoral immune responses, enhance neutralizing antibody titers, and reduce the incidence and mortality of critically ill patients.

CONCLUSIONS

Tfh cells and associated molecules play a critical role in the development of viral infection, and Tfh cell subsets are required for high-quality neutralizing antibodies from B cells to control and clear viruses including SARS-CoV-2, which can effectively promote humoral immune responses. Emerging evidence indicates that functional characterization of Tfh cells and their subsets will provide novel insights into improved vaccine design and therapeutic strategies to prevent and control various viral infections including SARS-CoV-2 infection.

AUTHOR CONTRIBUTIONS

DC drafted the manuscript and designed the figures and tables. FX, QJ, DJ, YL, DX, and JW revised the manuscript. DC, JX, CW, and LL conceived the topic and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Potential Cross-Reactive Immunity to COVID-19 Infection in Individuals With Laboratory-Confirmed MERS-CoV Infection: A National Retrospective Cohort Study From Saudi Arabia

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Background: A growing number of experiments have suggested potential cross-reactive immunity between severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) and previous human coronaviruses. We conducted the present retrospective cohort study to investigate the relationship between previous Middle East respiratory syndrome-coronavirus (MERS-CoV) infection and the risk of SARS-CoV-2 infection as well as the relationship between previous MERS-CoV and COVID-19-related hospitalization and mortality.

Methods: Starting in March 2020, we prospectively followed two groups of individuals who tested negative for COVID-19 infection. The first group had a previously confirmed MERS-CoV infection, which was compared to a control group of MERS-negative individuals. The studied cohort was then followed until November 2020 to track evidence of contracting COVID-19 infection.

Findings: A total of 82 (24%) MERS-positive and 260 (31%) MERS-negative individuals had COVID-19 infection. Patients in the MERS-positive group had a lower risk of COVID-19 infection than those in the MERS-negative group (Risk ratio [RR] 0.696, 95% confidence interval [CI] 0.522-0.929; p =0.014). The risk of COVID-19-related hospitalization in the MERS-positive group was significantly higher (RR 4.036, 95% CI 1.705-9.555; p =0.002). The case fatality rate (CFR) from COVID-19 was 4.9% in the MERS-positive group and 1.2% in the MERS-negative group (p =0.038). The MERS-positive group had a higher risk

of death than the MERS-negative group (RR 6.222, 95% CI 1.342-28.839; p =0.019). However, the risk of mortality was similar between the two groups when death was adjusted for age (p =0.068) and age and sex (p =0.057). After controlling for all the independent variables, only healthcare worker occupation and >1 comorbidity were independent predictors of SARS-CoV-2 infection.

Interpretation: Individuals with previous MERS-CoV infection can exhibit a cross-reactive immune response to SARS-CoV-2 infection. Our study demonstrated that patients with MERS-CoV infection had higher risks of COVID-19-related hospitalization and death than MERS-negative individuals.

Keywords: coronavirus disease 2019, Middle East respiratory syndrome coronavirus, Saudi Arabia, cross-immunity, mortality

INTRODUCTION

Coronaviruses (CoVs) within the *Coronaviridae* family are a group of enveloped, positive-strand RNA viruses that infect numerous animal species (1). However, since the severe acute respiratory syndrome (SARS) outbreak in 2003, it was realized that CoVs could cause devastating zoonotic diseases that raise concerns regarding the significant health threats of these viral strains in humans (2). Six species of human CoV are known, of which three, SARS-CoV, Middle East respiratory syndrome-coronavirus (MERS-CoV), and SARS-CoV-2, are highly pathogenic and cause pneumonia and systemic symptoms in humans (3, 4). These strains share many similarities, such as genomic structure, route of transmission, and clinical manifestations (5). Furthermore, they have similar sequence homology and antigenic epitopes that can induce an adaptive immune response (6). However, unlike SARS-CoV-2, SARS-CoV and MERS-CoV displayed only limited person-to-person spread, leading to fewer confirmed cases (7).

The MERS outbreak originated in Saudi Arabia in 2012, where the virus was mainly transmitted through dromedary camels (8). As of December 2020, a total of 2566 laboratory-confirmed cases and 882 deaths due to MERS were reported globally, leading to a high fatality rate of 34% (9). The clinical features of MERS-CoV infection vary substantially and can range from asymptomatic or flu-like symptoms to severe pneumonia, acute respiratory distress, multiorgan failure, and death (10). To date, there are no approved vaccines or specific therapies for MERS-CoV infection (8).

On the other hand, coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2 which was first reported in China in December 2019, is an ongoing global pandemic that has affected more than 110 million persons to date (11). The global COVID-19 fatality rate is approaching 2.2%, and it notably rises to 49% in critically ill patients (11, 12). The COVID-19 pandemic poses a significant challenge to healthcare services and societies globally. The pandemic has also exerted a tremendous socioeconomic toll, the consequences of which are yet to be recognized. A significant contributor to the difficulty of adequately managing the outbreak is the sheer volume of cases, which threatens overwhelming the available resources (such as ventilators and ICU beds) of healthcare facilities (13). Thus, a global effort was cumulated to develop a number of effective vaccines. Nonetheless, several unresolved issues remain concerning the longevity of these vaccines and their efficacy against emerging viral variants (14).

A growing number of research studies have suggested potential cross-reactive immunity between SARS-CoV-2 and previous human CoVs, which is thought to stem from the high sequence similarity between these viruses, leading to reactive CD4+ T cells (6, 15–17). A case series by Barry et al. (18), assessed the clinical characteristics of 99 hospitalized patients with COVID-19 in a MERS-CoV center in the Kingdom of Saudi Arabia (KSA) and reported no co-occurrence of MERS-CoV among SARS-CoV-2-infected persons. Immunity resulting from previous MERS-CoV infection has been suggested to underlie the lower mortality rates of COVID-19 (16, 19). These results accord with Kim et al. (20), who found persistence of antibodies against spike protein in 70 patients with previous MERS-CoV for three years after the infection (21). A more recent report detected MERS-CoV–specific neutralizing antibodies for six years post-infection in 48 patients with previous MERS-CoV infection. A case report of a 31-year-old physician with COVID-19 and previous MERS-CoV infection suggested that prior MERS-CoV infection provided partial immunity leading to mild disease (22).

The current statistics reveal that nearly 85% of the total MERS-CoV cases worldwide occurred in the KSA. As of December 2020, there were 2167 laboratory-confirmed MERS cases in the KSA and 804 related deaths (9). On the other hand, a total of 362,979 COVID-19 cases were reported in the KSA by the end of December 2020 (23). Thus, the KSA provides a unique setting where both MERS-CoV and SARS-CoV-2 are circulating. Hence, we conducted the present retrospective cohort study to investigate the relationship between previous MERS-CoV infection and the risk of SARS-CoV-2 infection, as well as the relationship between previous MERS-CoV and COVID-19-related hospitalization and mortality.

MATERIALS AND METHODS

The Institutional Review Board of the Ministry of Health (MoH) in Saudi Arabia approved the study protocol (Central/RB log

No: 21 -28M). All study procedures were compliant with the principles of the Declaration of Helsinki (24) and local regulatory laws. As the present study was a retrospective chart review, the need for informed consent was waived by the IRB committee. The present manuscript was prepared in compliance with the recommendations of the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement (25).

Study Design and Population

The present retrospective cohort study retrieved the data of all individuals who were screened for COVID-19 infection in March 2020 in Saudi Arabia. A total of six million individuals were screened from March to September 2020. We included all cases with a previously confirmed MERS-CoV infection who tested negative for SARS-CoV-2 infection during the screening phase and had no previous history of SARS-CoV-2 infection (n =342). In addition, we utilized a random sampling technique with a ratio of 1:3 to include MERS-CoV-negative individuals from the screened population who were negative for SARS-CoV-2 infection at that time. The studied cohort was then followed until December 2020 to track the evidence of contracting SARS-CoV-2 infection. The diagnosis of COVID-19 infection was confirmed only by reverse transcription-polymerase chain reaction (RT-PCR) laboratory tests (26). The data were collected from the Health Electronic Surveillance Network (HESN) database of the Saudi MoH.

Sample Size Calculation

The calculated sample size for this study was based on the hypothesis that participants with previous MERS-CoV infection might be at least two times less at risk of SARS-CoV-2 infection *versus* the general population. This assumption was based on a pilot test that randomly compared the risk of SARS-CoV-2 infection between 20 cases with previous MERS-CoV infection and a similar number of MERS-CoV-negative individuals. The latest data from Saudi MoH's official website suggest that the general population has a positivity rate of approximately 10%. Therefore, the minimum sample size given by Fleiss without continuity correction would be approximately 1380 subjects, including 345 previously infected with MERS and 1035 from the general population. This hypothesis assumes a two-sided type 1 error of 5% and a power of 80% (27).

Data Collection

The data of eligible participants were obtained from the HSEN. Extracted data included demographic characteristics (age, gender, nationality, region, occupation), smoking status, comorbidities that were defined according to the International Statistical Classification of Diseases and Related Health Problems-10 (ICD-10) (28), history of flu vaccine, presence of laboratory-confirmed MERS-CoV infection, the occurrence of COVID-19 infection, COVID-19 symptoms, the severity of symptoms, need for hospitalization, ICU admission, and death. The data underwent thorough data management steps to ensure accuracy and validity. Each variable was checked for any typing or entry mistakes and bizarre or irrelevant observations.

Study's Outcomes

The main parameter of the present study was to compare the incidence of SARS-CoV-2 infection between previously confirmed MERS-CoV cases and MERS-CoV-negative individuals. The secondary parameters included the risk of COVID-19-related hospitalization and mortality among previously confirmed MERS-CoV cases.

Statistical Analysis

Continuous variables are expressed as medians with interquartile ranges (IQRs), and categorical variables are expressed as percentages (%). Categorical variables were analyzed using the chi-square test or Fisher's exact test. The relative risk and its associated 95% confidence interval were calculated based on MERS-CoV exposure (Yes/No) and the risk of different outcomes, such as SARS-CoV-2 infection, hospitalization, and death. Multivariable logistic regression was used to test the association of important baseline characteristics with the binary outcome of SARS-CoV-2 infection. The following variables were included in the multivariate analysis: age, previous MERS-CoV infection, flu vaccination during the past year, being a healthcare worker, and > 1 comorbidity. Graphical presentations of some important variables were generated using Microsoft Office Excel 2013 for Windows (Microsoft Corporation, USA). All mean and median values, as well as their measures of variability, were formatted to one decimal place. All percentages were rounded to one decimal place. The significance level was two-sided with a type 1 error of 5%. The analysis was performed using Statistical Package for Social Sciences version 24 (IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp).

RESULTS

The MERS-infected population was part of the general Saudi population screened for recent SARS-CoV-2 infection. All MERS-positive cases were selected (n=342) who were negative for SARS-CoV-2 infection at the time of initial screening in March 2020. In addition, a random sample of MERS-negative cases (n=1035) was selected from the screened population; out of them, the complete dataset was retrieved for only 834 individuals. All selected individuals were negative for SARS-CoV-2 infection at that time. The cohort was followed up to November 2020 for evidence of COVID-19 infection. A total of 82 (24%) MERS-CoV-positive and 260 (31%) MERS-CoV-negative individuals had SARS-CoV-2 infection (**Figure 1**).

Baseline Characteristics of the Study Population

The median age of the studied cohort was 36 (IQR =21) years. The MERS-CoV-positive patients were significantly older than the MERS-CoV-negative patients (p <0.001). The majority of cases in both groups were males (64.4% and 63.7%, respectively; p =0.773). The median duration from MERS-CoV infection and SARS-CoV-2 infection was 3.4 (IQR =3.6) years. Most of the

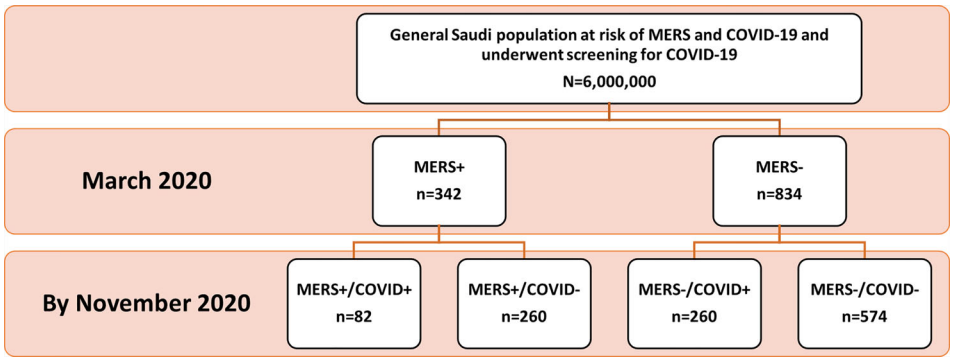

FIGURE 1 | Patient Disposition Flowchart. Out of 6,000,000 people who underwent the national screening on March 2020, 342 patients with a previously confirmed MERS-CoV infection and did not test positive for SARS-CoV-2 infection were identified. The random sample of the control group was taken approximately to be 3 times that of MERS exposed group based on the sample size. Patients were followed-up retrospectively until November 2020 to identify patients who captured SARS-CoV-2 infection.

identified cases were of Saudi nationality, with no significant difference between MERS-CoV-positive and MERS-CoV-negative cases (p =0.834). Notably, the number of healthcare workers was significantly higher in the MERS-CoV-positive group than in the MERS-CoV-negative group (34.3% *versus* 12.6%, respectively; p <0.001). MERS-CoV-positive patients were more likely to have one or more comorbidities than MERS-CoV-negative patients (p <0.001). The prevalence of hypertension (p <0.001), diabetes (p <0.001), cardiac diseases (p =0.006), and end-stage renal diseases (p <0.001) was significantly higher in the MERS-CoV-positive patients. Additionally, the flu vaccine was taken in a higher percentage in the MERS-CoV-positive group (41.3%) than in the MERS-CoV-negative group (27%; p =0.012) (**Table 1**).

The COVID-19 subpopulation had a high prevalence of comorbidities (27.3%). Those having more than one

TABLE 1 | Baseline characteristics of the cohort screened for COVID-19.

Characteristic	Total N=1176	MERS+N=342	MERS-N=834	P-value
Age				
Median (IQR)	36(21)	47(25)	32(17)	<0.001*
0-9	58(4.9%)	3(0.9%)	55(6.6%)	<0.001*
10-19	63(5.4%)	4(1.2%)	59(7.1%)	
20-29	227(19.3%)	24(7.0%)	203(24.3%)	
30-39	339(28.8%)	82(24.0%)	257(30.8%)	
40-49	198(16.8%)	70(20.5%)	128(15.3%)	
50-59	146(12.4%)	73(21.3%)	73(8.8%)	
60-69	89(7.6%)	56(16.4%)	33(4.0%)	
70-79	27(2.3%)	14(4.1%)	13(1.6%)	
≥80	29(2.5%)	16(4.7%)	13(1.6%)	
Male	757(64.4%)	218(63.7%)	539(64.6%)	0.773
Duration since MERS-CoV infection, Median (IQR)	—	3.4 (3.6)	—	—
Nationality (n =1162)				
Saudi	822(70.6%)	243(71.1%)	579(70.4%)	0.834
Non-Saudi	342(29.4%)	99(28.9%)	243(29.6%)	
Healthcare Worker (n =523)	88(16.8%)	35(34.3%)	53(12.6%)	<0.001*
Smoking Status (n =477)	91(19.1%)	14(19.4%)	77(19.0%)	0.931
Comorbidities (n =484)	**484**	**76**	**408**	
One comorbidity	132(27.3%)	47(61.8%)	85(20.8%)	<0.001*
>1 comorbidity	46(9.5%)	22(28.9%)	24(5.9%)	<0.001*
Diabetes	60(12.4%)	28(36.8%)	32(7.8%)	<0.001*
Hypertension	52(10.7%)	27(35.5%)	25(6.1%)	<0.001*
Cardiac	11(2.3%)	5(6.6%)	6(1.5%)	0.006*
Asthma & COPD	19(3.9%)	3(3.9%)	16(3.9%)	0.992
ESRD	9(1.9%)	6(7.9%)	3(0.7%)	<0.001*
Cancer	6(1.2%)	2(2.6%)	4(1.0%)	0.232
Flu vaccine (n =475)	139(29.3%)	31(41.3%)	108(27.0%)	0.012*

Bold values represent the total number of patients with co-morbidities.
**Statistically significant at p-value <0.05.*

comorbidity were (9.5%). Symptoms occurred in 86.5% of the cases, with fever reported by 75.5%, cough by 60.4%, and sore throat by 46% of cases. Regarding the clinical outcomes, hospitalization and ICU admission occurred in 36.1% and 9% of the cases, respectively. The total percentage of cases requiring mechanical or assisted ventilation was 4.5%. The median and IQR of the duration of hospitalization was 7 (16) days (**Figure 2**).

Risk of SARS-CoV-2 Infection

A total of 342 (29.1%) included cases had SARS-CoV-2 infection by November 2020. The incidence of SARS-CoV-2 infection was significantly higher in the MERS-CoV-negative group (n=260, 31.2%) than in the MERS-CoV-positive group (n=82, 24%; p=0.014) (**Table 2**). Patients in the MERS-CoV-positive group had a significantly lower risk of SARS-CoV-2 infection than those in the MERS-CoV-negative group (RR 0.696, 95% CI 0.522-0.929; p =0.014) (**Table 3**).

Risk of COVID-19-Related Hospitalization and Death

The risk of having symptoms of COVID-19 was similar between the MERS-CoV-positive and MERS-CoV-negative groups (RR 0.945, 95% CI 0.404-2.211; p =0.89). On the other hand, the risk of COVID-19-related hospitalization in the MERS-CoV-positive group was significantly higher (RR 4.036, 95% CI 1.705-9.555; p =0.002) (**Table 3**).

The number of deaths from COVID-19 was numerically higher in the MERS-CoV-positive group than in the MERS-CoV-negative group (1.2% *versus* 0.4%, respectively; p =0.114). However, the CFR from COVID-19 was 4.9% in the MERS-CoV-positive group and 1.2% in the MERS-CoV-negative group (p =0.038) (**Table 2**). The MERS-CoV-positive group had a higher risk of death than the MERS-CoV-negative group (RR 6.222, 95% CI 1.342-28.839; p =0.019). However, the risk of mortality was similar between the two groups when death was adjusted for age (RR 4.29, 95% CI 0.897-20.511; p =0.068) and age and sex (RR 4.65, 95% CI 0.956-22.62; p =0.057) (**Table 3**). Further stratification of the groups based simultaneously on the presence of the exposure (MERS infection) and presence of the outcome (COVID-19 infection) showed that those who did not contract SARS-CoV-2 infection had approximately the same mortality rate of 0.77% for MERS+/COVID- and 0.87% for MERS-/COVID- (**Figure 3**).

Independent Predictors of COVID-19 Infection

Multivariable logistic regression was performed to test the association between SARS-CoV-2 infection as a dependent variable and selected independent variables based on their significance in a univariable model or their clinical importance. The model included age in years, previous MERS-CoV infection, flu vaccination, occupation, and >1 comorbidity. After controlling for all the independent variables, only healthcare worker occupation and >1 comorbidity were independently significant, with RRs (95% CI) of 2.336 (1.199-4.550) and 0.389 (0.168-0.898), respectively (**Table 4**).

DISCUSSION

Since 2002, the world has witnessed the emergence of three human CoV outbreaks. Nonetheless, there is much ambiguity concerning the human immune response to CoVs and whether

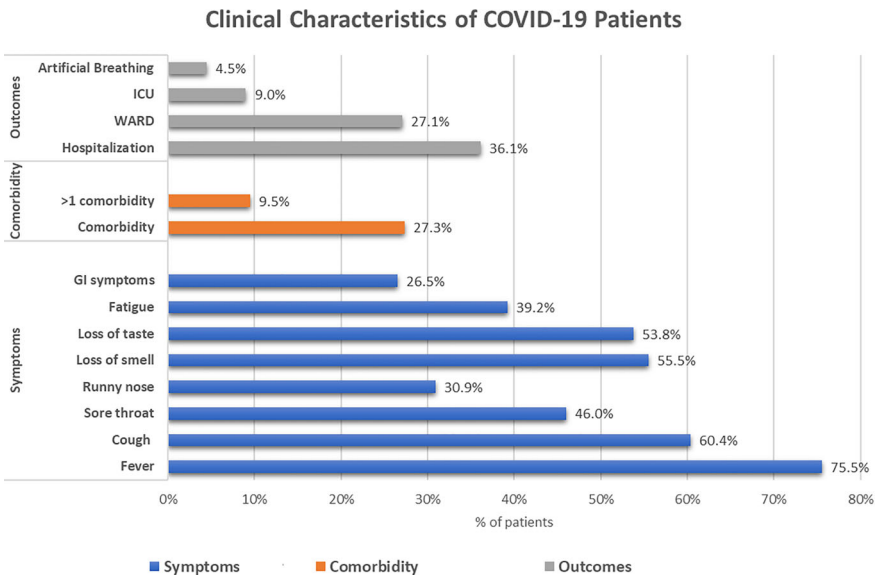

FIGURE 2 | Clinical characteristics of the COVID-19-infected subgroup within the cohort. The blue bars represent the symptomatic presentations of the patients, the orange bars represent the presence of comorbidities, and the grey bars represent the outcomes of the patients.

TABLE 2 | SARS-CoV-2 infection and mortality measures within the cohort subjects.

	Total N= 1176	MERS+N =342	MERS-N=834	P-value
Number of COVID-19 cases	342(29.1%)	82(24.0%)	260(31.2%)	0.014*
Death from COVID-19	7(0.6%)	4(1.2%)	3(0.4%)	0.114
Death from all Causes	14(1.2%)	6(1.8%)	8(1.0%)	0.254
COVID-19 Case Fatality Rate	2.0%	4.9%	1.2%	0.038*

**Statistically significant at p-value <0.05.*

TABLE 3 | Risk of the MERS-exposed group relative to SARS-CoV-2 infection, symptom presence, hospitalization and death.

	N	RR	95% Confidence Interval	P-value
SARS-CoV-2 infection	342	0.696	0.522-0.929	0.014*
Presence of COVID-19 symptoms	259	0.945	0.404-2.211	0.896
Hospitalization	133	4.036	1.705-9.555	0.002*
Death	245	6.222	1.342-28.839	0.019*
Adjusted Death[#]	245	4.290	0.897-20.511	0.068
Adjusted Death*	245	4.651	0.956-22.618	0.057

*[#]adjusted for age; *adjusted for age and gender.*

TABLE 4 | Multivariable logistic regression for independent predictors of SARS-CoV-2 infection in the study cohort.

	RR	95% Confidence Interval	P-value
Age in years	1.004	0.991-1.017	0.531
MERS infection	1.181	0.621-2.245	0.612
Flu-vaccine	0.640	0.391-1.049	0.077
Healthcare worker	2.336	1.199-4.550	0.013*
>1 comorbidity	0.389	0.168-0.898	0.027*

**Statistically significant at p-value <0.05.*

there is cross-reactive immunity between different human CoVs. To our knowledge, this is the first clinical study that assessed the risk of SARS-CoV-2 infection among individuals with a history of laboratory-confirmed MERS-CoV infection as well as the impact of previous MERS-CoV infection on the clinical course of COVID-19. The outcomes of this study shape the currently ongoing hypotheses regarding the presence of cross-reactive immunity between MERS-CoV and SARS-CoV-2 infection (5).

Our results indicated that individuals with MERS-CoV infection had a lower risk of SARS-CoV-2 infection than MERS-negative individuals. Interestingly, patients with MERS-CoV infection had higher risks of COVID-19-related hospitalization and death than MERS-negative individuals. However, the mortality risk of death was similar between MERS-CoV-positive and MERS-CoV-negative cases when death was adjusted for age and sex. In the multivariate analysis, only being a healthcare worker and having >1 comorbidity were independent predictors of COVID-19 infection.

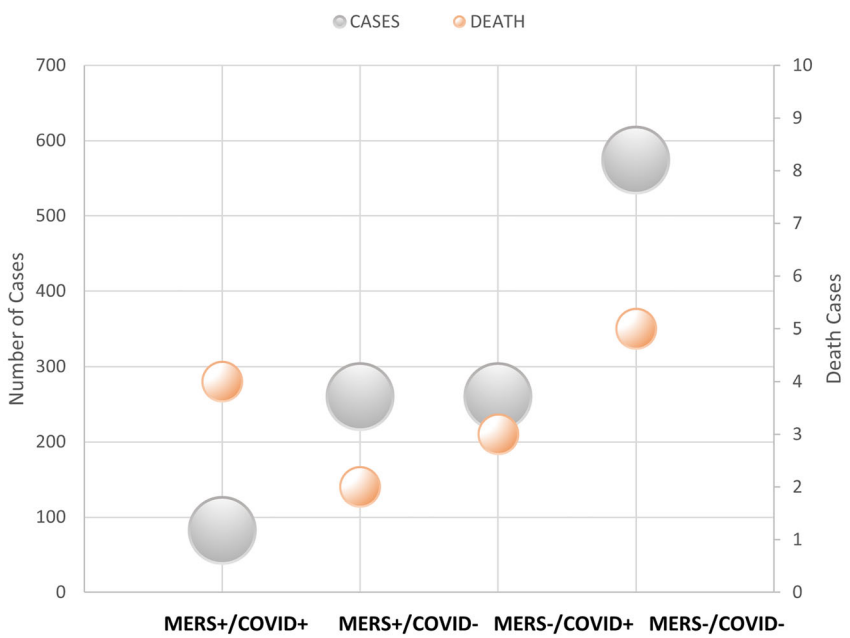

FIGURE 3 | Number of cases and deaths based on MERS and COVID-19 infection.

The immune system remains the ideal defense supporting the human's natural ability to defend itself against foreign pathogens. Innate immunity is the first line of defense against viral infections, including human CoVs. In the context of shared sequence homology of human CoVs (29), it is expected that the innate immune response against SARS-CoV-2 is similar to that of other human CoVs and involves signaling through the Toll-like receptor (TLR) pathway to induce type I and type III interferons (IFNs). Other pro-inflammatory cytokines, such as tumor necrosis factor-β (TNF-β), interleukin-1 (IL-1), and IL-6, are also released (30). This nonspecific antiviral defense potentiates a more specific adaptive immune response through activation of T lymphocytes when the viral antigen is expressed by antigen-presenting cells such as macrophages and dendritic cells (31, 32). T lymphocyte activation produces inflammatory mediators such as interferon-I (INF-I), tumor necrosis factor-β (TNF-β), interleukin-1 (IL-1), IL-6, and monocyte chemoattractant protein-1 known as CCL2. In addition to perforin and granzyme B, a process also occurs in other respiratory infections (33, 34). Animal models for SARS-CoV-1 have suggested that T cells are protective. In mouse models, depletion of $CD4^+$ delayed the clearance of the virus and worsened the disease; similarly, T cell augmentation led to rapid clearance of the virus and ameliorated the disease (35, 36). T cell memory is long-lived, and SARS-CoV-1 T cell specificity was identified four years after infection (37, 38). For SARS-CoV-2, T cells have been identified in asymptomatic cases or with mild COVID19 symptoms (39); moreover, the specific T cells of SARS-CoV-2 were detected in contacts of infected cases (40). T cells were fewer in patients with SARS-CoV-2 than in healthy individuals (41). Humoral immunity is essential in later phases of infection and has a role in reinfection suppression. Coronavirus-specific antibodies were identified in 80 to 100% of patients with SARS-CoV-1 and MERS two weeks after the disease onset, with delayed immune response in severe infection (42–44). A systematic review in 2020 reported that antibodies to coronavirus were infrequently seen in the first seven days after infection but increased in the second and third weeks of infection (45). ADE (antibody-dependent enhancement) was reported to play a role in previous SARS and MERS infections (6). It is unknown whether antibodies are associated with disease severity.

Due to the similarities in the immune responses between MERS and SARS-CoV-2 infections, particularly the T cell response, several authors hypothesized potential cross-reactive immunity between the two pathogens (46). Yaqinuddin (16) hypothesized that T cells contain specific receptors (TCRs) that may recognize the SARS-CoV-2 peptides in individuals with previous MERS-CoV infection due to sequence homology between the two viruses. In experimental models, variable degrees of cross-reactivity between T- and B-cell antibodies against SARS-CoV-2 and MERS-CoV were detected (19, 47). In the present report, we found that patients with previously confirmed MERS-CoV infection had a lower risk of SARS-CoV-2 infection than the MERS-CoV-negative group. In Barry et al. (18), there was no co-occurrence of MERS-CoV among SARS-CoV-2-infected persons in the KSA. Such findings are crucial in vaccine development against the ongoing COVID-19 pandemic, as understanding the immune response against human CoVs could help in the vaccine development process. According to a large-scale Korean study, serum antibodies against the spike antigen of MERS-CoV persist for three years after infection (48). A more recent report detected MERS-CoV–specific neutralizing antibodies for six years post-infection in 48 patients with previous MERS-CoV infection (20), which is significantly longer than the reported duration for serum antibodies against the spike antigen of SARS-COV-2 (nearly eight months) (49). In the present study, the median duration from MERS-CoV infection and SARS-CoV-2 infection was 3.4 years. We argue for future multicenter immunological studies from MERS-CoV endemic areas to characterize the potential cross-reactivity between MERS-CoV and SARS-COV-2 infections, aiming to guide ongoing research on vaccine development.

The virulence mechanism of SARS-COV-2 is thought to depend on its nonstructural and structural proteins. ACE2 is the receptor of SARS-COV-2 within the body and is predominately expressed in type II alveolar cells (50). Once the virus binds with its receptor, ACE2 expression is markedly elevated, leading to alveolar cell damage and subsequent immune and inflammatory reactions within the body. Previous reports showed that patients with COVID-19 showed pulmonary edema and important proteinaceous exudates, such as large protein globules (51). In severe form, a 'cytokine storm' can be initiated by the release of interleukin-6 (IL-6) and other pro-inflammatory cytokines, leading to wide disturbance in thermoregulation, lymphocytes, central nervous system functions, and eventually death (52). Cardiac involvement, such as acute myopericarditis and microthrombi of coronary arteries, was reported in patients with COVID-19 (53). Similar to SARS-CoV-2, MERS-CoV infection is characterized by excessive inflammatory reactions and cytokine release (54). Yaqinuddin (16) hypothesized that cross-reactive immunity can potentially reduce the risk of severe COVID-19 infection in individuals with previous MERS-CoV infection. In contrast, our observations showed that patients with MERS-CoV infection had higher risks of COVID-19-related hospitalization and death than MERS-negative individuals, though the significance was lost when adjusting for age and gender. While the exact mechanisms of severe COVID-19 infection in patients with MERS-CoV infection are unclear, such observations may be explained by the results of previous experiments in MERS-CoV models. Human CoVs can develop immune-escape mechanisms; it was previously noted that MERS-CoV reduces memory T-cell activation through decreased antigen presentation to major histocompatibility complex (MHC) class I and II (55). In addition, MERS-CoV was found to induce T cell apoptosis and severe immunosuppression (56). Thus, patients with previous MERS-CoV may be more susceptible to acquired immune escape mechanisms and more severe immunosuppression. Mutations in the receptor-binding domain (RBD) of the S protein of human CoV were reported to account for the impaired humoral immune response to COVID-19 infection (57, 58). A notable fraction of

COVID-19 patients were found to harbor genetic variants in the immune response to infection by human CoV which render them susceptible to severe diseases, as recently reported for defects in type I IFN immunity and the presence of autoantibodies to type I IFN (59, 60). We hypothesize that patients with previous MERS-CoV may carry a higher risk of developing mutations than unexposed individuals. However, our findings should be interpreted cautiously, as MERS-CoV cases in our study were significantly older and had a higher prevalence of comorbidities, which might have represented potential confounders to the study findings. In addition, a previous case report showed a mild COVID-19 infection in a healthcare worker with co-occurrence of MERS-CoV infection (22). As stated above, future multicenter immunological studies from MERS-CoV endemic areas should be conducted to explore the relationship between COVID-19 severity and previous MERS-CoV infection.

The current body of evidence highlights that the risk of SARS-CoV-2 infection and severe disease is closely related to a wide range of patient-specific factors, including age and the presence of comorbidities (61, 62). It was noted that healthcare workers had a higher risk of SARS-CoV-2 infection, and they roughly account for one-quarter of the global COVID-19 cases (63). Healthcare workers had a higher chance of being exposed to patients with SARS-CoV-2 infection; besides, insufficient information about COVID-19 transmission and clinical symptoms can contribute to the higher risk of infection among healthcare workers (64). The present study found that being a healthcare worker and having >1 comorbidity were independent predictors of COVID-19 infection.

The published literature is scarce concerning the potential cross-reactive immunity between SARS-CoV-2 and previous CoVs. To our knowledge, this is the first comparative study that assessed the relationship between previous MERS-CoV and the risk of COVID-19 infection in a MERS-CoV endemic area. However, we acknowledge that the present study had some limitations. The present study data were collected retrospectively with many potential biases, such as case ascertainment bias. Most of the patient variables were self-reported and could not be verified. Besides, the patients' medical records did not contain information about the MERS-CoV and SARS-CoV2 specific IgG antibodies levels; the measurement of MERS-CoV and SARS-CoV2 specific IgG antibodies are required to elucidate the mechanism underlying the protection. Another limitation is that the sample size calculation of the present study was based on a small pilot study that included 40 participants only. The MERS-CoV cases in our study were significantly older and had a higher prevalence of comorbidities, which might have represented potential confounders to the study findings. In addition, the MERS-CoV positive individuals had a higher percentage of influenza vaccination than the MERS negative group; according to previous reports, influenza vaccination provides bystander immunity to a wide range of viral infections (65). Thus, the history of influenza vaccination might have represented a potential confounder to the study findings.

CONCLUSION

In conclusion, individuals with previous MERS-CoV infection can exhibit a cross-reactive immune response to SARS-CoV-2 infection. In the present clinical study, the incidence of SARS-CoV-2 infection was lower in individuals with MERS-CoV infection than in MERS-CoV-negative individuals. This potential cross-reactive immunity can guide ongoing global efforts to develop effective vaccines against the COVID-19 pandemic, particularly with the reported "relatively" long duration of serum neutralizing antibodies against MERS-CoV compared to neutralizing antibodies against SARS-CoV-2. Nonetheless, many gray areas need to be addressed before translating our findings to clinical application. First, the adequacy of the T-cell response elicited by MERS-CoV against SARS-CoV-2 clinical disease should be widely characterized by future research. In addition, long-term studies are recommended to study the longevity of this cross-reactive immunity.

On the other hand, our study demonstrated that patients with MERS-CoV infection had higher risks of COVID-19-related hospitalization and death than MERS-CoV-negative individuals. Experimental research should identify factors that contribute to the dysregulated immune response and immunopathology in patients with severe COVID-19 disease and the co-occurrence of MERS-CoV infection.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ministry of Health, Saudi Arabia. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AK contributed to concept and design, definition of intellectual content, literature search, clinical studies, data acquisition, statistical analysis, manuscript preparation, manuscript editing, and manuscript review. AAl contributed to concept and design, literature search, clinical studies, data acquisition, statistical analysis, manuscript preparation, manuscript editing, and manuscript review. YA, FA, YA, and AAl contributed to literature search, clinical studies, statistical analysis, manuscript editing, and manuscript review. SA, MK, SY, GC, AAs, and HJ contributed to literature search, clinical studies, data acquisition,

data analysis, statistical analysis, manuscript preparation, manuscript editing, and manuscript review. All authors contributed to the article and approved the submitted version. The requirements for authorship as stated earlier in this document have been met, and each author believes that the manuscript represents honest work.

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Silent SARS-CoV-2 Infections, Waning Immunity, Serology Testing, and COVID-19 Vaccination: A Perspective

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus causes a spectrum of clinical manifestations, ranging from asymptomatic to mild, moderate, or severe illness with multi-organ failure and death. Using a new machine learning algorithm developed by us, we have reported a significantly higher number of predicted COVID-19 cases than the documented counts across the world. The sole reliance on confirmed symptomatic cases overlooking the symptomless COVID-19 infections and the dynamics of waning immunity may not provide 'true' spectrum of infection proportion, a key element for an effective planning and implementation of protection and prevention strategies. We and others have previously shown that strategic orthogonal testing and leveraging systematic data-driven modeling approach to account for asymptomatics and waning cases may situationally have a compelling role in informing efficient vaccination strategies beyond prevalence reporting. However, currently Centers for Disease Control and Prevention (CDC) does not recommend serological testing either before or after vaccination to assess immune status. Given the 27% occurrence of breakthrough infections in fully vaccinated (FV) group with many being asymptomatics and still a larger fraction of the general mass remaining unvaccinated, the relaxed mask mandate and distancing by CDC can drive resurgence. Thus, we believe it is a key time to focus on asymptomatics (no symptoms) and oligosymptomatics (so mild that the symptoms remain unrecognized) as they can be silent reservoirs to propagate the infection. This perspective thus highlights the need for proactive efforts to reevaluate the current variables/strategies in accounting for symptomless and waning fractions.

Keywords: asymptomatic, waning, serology testing, COVID-19, vaccination

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INTRODUCTION

This is a perspective chiefly based on the reports that have suggested antibody quantitation could be a prevaccination screening strategy and specifically, a single dose of COVID-19 vaccine may likely suffice for the already SARS-CoV-2 infected cohort (1–5). These studies along with other findings similar to ours, have shown that the serological assessment of nucleocapsid (N)- and spike (S)-specific

IgG antibody levels could differentiate vaccine-induced responses from those acquired following SARS-CoV-2 infection (1, 6, 7). In addition, these interesting and elegant studies demonstrated that the first dose (1D) of mRNA vaccine generated similar protective antibody responses in previously SARS-CoV-2 infected healthcare workers to that of a second dose (2D) seen in immunologically naïve patients (1, 2, 4, 5). Further, the modest ACE2 binding inhibition responses of 2D *versus* 1D vaccine doses among COVID-19 recovered individuals reassures that a single dose could govern a protective antibody response in this population (1). This emphasis concerning the potential for single dose vaccination in prior COVID-19 individuals is relevant and timely, given the drastic decrease in new cases reported with that approach in many countries alongside a prolonged antigenic stimulation that has the likelihood of dampening the immune response *via* effector T-cells exhaustion, as has been observed with several other viruses (8). Among the vaccinated, there appears to be some difference in the extent and duration of immune response depending on the number of doses taken, time gap given between the first and second dose, prior infection/disease burden besides the physical, environmental, and general health status' influences (9). Based on some select studies, **Table 1** enlists some key responses and differences seen after administration of 1D and 2D of COVID-19 vaccines.

Along these lines, the potential biases around presumed high proportion of 'silent' asymptomatic patients must be duly acknowledged. Also concerning is the nebulously defined asymptomatic testing by Centers for Disease Control and Prevention (CDC) guidelines on one hand (14–17), and its relaxed guidelines on masking and distancing for FV population that can still contract breakthrough infections. Together, these clearly portend the need for careful surveillance/assessment mechanism(s) for the symptomless and distinguishing them from presymptomatic cases. Notably, as of May 25, 2021 CDC's report, about 27% (2725/10262) vaccine breakthrough infections were asymptomatic, and in those, 29% (289/995) of hospitalizations were related to asymptomatic or unrelated to COVID-19 (18). While some anecdotal evidence raises optimism that asymptomatic-driven transmission of the infection can subtly result in comprehensive immunization of the population towards herd immunity. There are other studies that report only one in five asymptomatic carriers possesses the capacity to seroconvert compared with severe and mild COVID-19 cases during or after hospitalization (19). Thus, it is tenuous whether asymptomatic infections can allow protective immunity. Hence, we believe it is the right time to proactively characterize asymptomatics and oligosymptomatics from such studies that assess and deduce prevalence-based protection and prevention measures. Only then, the challenges surrounding vaccine redirection to hotspots/appropriate groups, mitigation of vaccination inequities, and efforts to enhance the speed, coverage, and impact of vaccination across the globe will be tackled adeptly.

ASYMPTOMATIC INFECTIONS AND WANING IMMUNITY REMAIN AS AN UNCHARTED TERRITORY IN THE UNDERSTANDING OF SARS-COV-2 INFECTION

A vast majority of the studies alluding to emphasize the potential of a single dose vaccination strategy in prior infected cohorts has included only the confirmed cases (symptomatic) and assessed the effectiveness of vaccination in terms of quantifying the antibody levels (1–7). However, it is to be noted that a significant percentage of COVID-19 infections were silent/asymptomatic causing many infections to go unreported (14–17). Using a new machine learning algorithm developed by our team (accounting for undocumented infections), we have reported that the total numbers of predicted COVID-19 cases to be significantly higher than reported across the nation and

TABLE 1 | Some key responses and differences seen after 1D and 2D of COVID-19 vaccines.

S.No.	Particulars	1D	2D	Comments	Reference
1.	IgM	Low	High	Low and high was relative to the unvaccinated controls. IgM levels were found to be *increased* by 1.7-fold in the 2D-received naïve group (seronegative) with no appreciable change in the prior infected group. This was, however, only transient and during the initial period following vaccination.	(7)
2.	IgG	Low	High	Low and high was assigned based on the relative levels with the pre-vaccine status. Median IgG levels was *increased* by 7.0-fold in sero+ 2D group; 8.6-fold in sero- 2D group; no change in the prior COVID-19+ group; with ~1.8-fold in the overall 2D population that includes sero+, sero-, and prior COVID-19+ subgroups.	(10)
3.	Virus neutralizing potency	Low	High	Low and high was based on the neutralization antibody titers relative to pre-vaccine (1D). The median potency of 2D when adjusted and compared to 1D was *increased* in the range between 2.6 and 26-fold in sero- group; between 1.3 and 1.7-fold in 2D sero+ group.	(10, 11)
4.	Vaccine Efficacy-(VE)			❖ VE* was assessed in terms of onset of COVID infection and a low VE indicates a high infection. VE was found to be 52.4% in the period between 1D and 2D and was *increased* to 92.7% at 2 or more days after 2D. ❖ In a multicenter SIREN study including 23,324 participants from 104 sites (*all in England*), the VE# assessed in terms of new infections observed at ≥21 days after 1D was reduced by 50% at 7 days after 2D. ❖ In a nationwide historical cohort study from *Israel* with 6286 subjects, between ≥14 days after 1D until the receipt of 2D, VE@ was found to be 61%, which was increased to 82% from 1 to 6 days after the 2D.	(12) (4) (13)

**VE was derived according to the Clopper–Pearson method using the formula 100× (1–IRR), where IRR is the calculated ratio of confirmed COVID-19 cases per 1000 person-years of follow-up in the active vaccine group to the corresponding illness rate in the placebo group.*

#VE was deduced from the incidence density of new infections/10,000 person-days (8 following 1D vs 4 after 2D).

@VE was deduced from the effectiveness of vaccine against PCR positive SARS-CoV-2 (with or without symptoms).

worldwide (14). Represented in the **Figures 1A, B** is an estimated cumulative incidence (~27%) and estimated total current infections (90 million) across the U.S, respectively as of April 16, 2021. This is very critical given the relevant published study's (1) central theme is that a single dose vaccination could be sufficient for prior infected, for which an accurate estimation of the true size of infected population is pivotal. Note that stringent reliance on confirmed cases only can lead to under-ascertainment of COVID-19 infections (14). Not accounting for the asymptomatic and oligosymptomatic population in the context can mislead the experts in gauging the vulnerability of a community to the virus and confound the subsequent decisions on mitigation strategies. Could strategic surveillance testing using adequate follow-up (serial PCR) and orthogonal immunological assessments (antigen- and antibody- based tests) of people that are likely exposed to confirmed cases (e.g., contact tracing) or are high-risk spreaders (e.g., front-line and congregate facility workforce) be leveraged along with systematic data-driven modeling approach (integrating age, sex, chronic conditions and COVID-19 risk factors, etc.) allow better characterization of infection dynamics (proliferation, clearance, and persistence) in asymptomatic pool and ably guide the planning and optimization of specific actions?

Compounding the asymptomatics, waning immunity, *per se*, may challenge the testing and interpretation with false negative immunoassay results because of decreasing levels of antibodies and higher positive cut-off thresholds set by vendors that were mainly derived from active infection cases. Importantly more the 'interacting asymptomatics and oligosymptomatics (e.g.: working age)' socially mix with the non-immunized or immunized (under waning immunity), greater and sustained will be the spread of infection. Thus, a disruption in the timing and intensity of interventional strategies and/or efforts is imminent when the asymptomatic and waning immunity considerations are discounted. While the effectiveness of authorized COVID-19 vaccines is apparent from real-world scenarios and a spate of clinical studies, the emergence of new variants and reports of a worldwide surge of recent vaccine breakthrough infections with the Delta variant (B.1.617.2) of coronavirus in the FV have raised alarms about the waning vaccine immunity (23). In an unreviewed study that evaluated the mRNA vaccines' longitudinal effectiveness across different states in the U.S. including Minnesota, Wisconsin, Arizona, Florida, and Iowa between January and July 2021, the efficacy of Pfizer vaccine was found to have dropped by nearly two-fold than Moderna (24). An Israel study has also underscored the concerns of rising breakthrough infections with an ebb in vaccine's efficacy by reporting 2.26 times greater risk of infection in the early Pfizer vaccinees (Jan-Feb, 2021) compared to those vaccinated later (Mar-Apr, 2021) (25). However, so far, the rate of breakthrough infections reported in vaccinated population is modest compared to the soaring new infections in unvaccinated populations. Notably, prior vaccination appears to be strongly reducing the risk of hospitalization and developing severe COVID-19 in non-immunocompromised individuals. Yet unbeknownst, whether the diverging effectiveness of COVID-19 vaccines is owing to inherent differences in potency of vaccines against the Delta variant or their varying durability characteristics, the need and value of an additional dose to refresh the fading immunity in the general population has become a subject of intense scientific debate in the COVID community. However, to translate this idea of additional doses as 'a' key to stop the pandemic into reality, a speedy coverage of remaining worldwide population that is yet to receive either 1D or 2D of vaccines coupled with efforts to properly track and understand breakthrough infections in real-time is equally important.

From the laboratory-based analysis, to monitor such circumstances, binding immunoassay format such as anti-nucleocapsid-pan-Immunoglobulin (anti-N-pan-Ig) electro-chemiluminescence assay (ECLIA) that detects late, mature, high affinity antibodies regardless of the subclass with high sensitivity

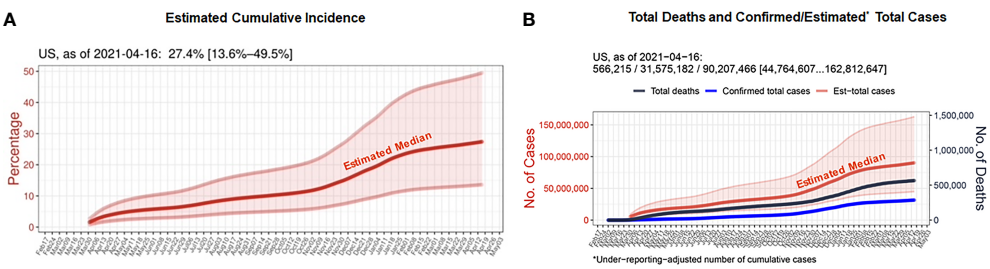

FIGURE 1 | **(A)** Estimates of cumulative incidence rates for the period until April 16, 2021, for the 50 U.S. states. **(B)** Seven-day rolling-averaged counts of daily confirmed total cases and deaths until April 16, 2021, for the U.S. For this computational study, the COVID Tracking project provided the U.S. dataset (20) and the Center for Systems Science and Engineering (CSSE) repository at Johns Hopkins University remained as a source of confirmed cases and deaths for countries (21). The actual number of infections across countries and regions were inferred in terms of the Infection-Fatality-Rate (IFR), since it is one of the key epidemiological parameters that afforded us a clue to fill the gap between confirmed and actual infections, under the assumption that the number of undocumented deaths is negligible (14). While the IFR is subjected to fluctuations depending on age structure of population, timeline, the current estimation uses a consensus and previously established estimate of 0.66% IFR that encompass a wide band of uncertainty (0.39%–1.33%, 95%-confidence interval) among all the PCR-confirmed infections including asymptomatic cases (22). It is worth noting that considering the estimate's large estimation uncertainty, the confidence interval is expected to cover the true IFRs of most countries and U.S. states and our machine-learning-based IFR estimates and current framework of daily counts of 'actual' COVID-19 infections were in line with the existing seroprevalence rates in 46 U.S. states (14).

and specificity from <5 days (proportion of infection detected only by PCR) until >15–22 days samples post-symptom when used in serial measurements could come in handy (26). It is notable that CDC recommends serial serological screening and surveillance testing to identify carriers with asymptomatic infections and waning conditions (17). However, feasibility of screening chiefly used in the setting of outbreaks or in high prevalent areas and how it must fit in this context must also be considered along with other potential alternatives. Yet, amidst the ambiguity, very recently (July 27, 2021), the CDC has made an encouraging recommendation by reversing the previous testing exemption granted for FV with no COVID-19-like symptoms even after a close contact with confirmed COVID-19 patient(s) to mandating the testing for FV who still don't show symptoms after an exposure (27).

IS THERE A CASE TO CONSIDER AN 'EDITABLE THRESHOLD' OF SEROLOGY ASSAYS TO REVEAL PREVIOUSLY UNDIAGNOSED INFECTIONS?

The bigger purpose of studies focused on understanding if a single dose vaccination is sufficient for prior-infected subjects (1–5) must primarily involve identification and clustering of the truly infected (prior) from uninfected subjects. Serological evaluation of SARS-CoV-2 antibody profiles is an important tool to assess prior SARS-CoV-2 infection and infection prevention strategies. However, the SARS-CoV-2 antibody levels become low as in mild infections or decline over time owing to waning immunity and thus, using manufacturer-established PC thresholds of N-IgG can underestimate actual case numbers, yielding an incomplete number of true past infections (8). Relevantly, most informative data to improve the identification of individuals either prior-infected and resolved or under waning immunity came from the idea of orthogonal testing and an in-depth optimization of the manufacturer-established positive cut-off (PC) of N-IgG assay without compromising assay specificity (28–30). Under the 'editable gray-zone' threshold, the European Union recently also approved refinement of Abbott's IgG SARS-CoV-2 assay, allowing laboratories to adapt PC carefully and achieve a 'near-perfect' quantification of infected subjects (*Personal communication with Abbott*). Notably, a misclassification of prior-infected individual as uninfected can severely impact the health, economic, and social picture, which can complicate actual intent of effective COVID-19 single-dose vaccination strategy. While the study uses antibody testing results as one criterion, broaching on the concept of an in-depth serology testing was circumvented in these studies. If a reliable solution is of ultimate interest, it is critical to ensure an optimal clustering of the 'target' population (prior-infected) for 1D vaccination. Pertinently, a careful reflection of data collection on orthogonal testing and alternative data analysis approaches like editable serology cut-off could thus be more germane.

Many reports, including Ebinger's study, identified a fraction of the naïve individuals following 1D reached the neutralizing threshold titer or beyond (for instance, nearly 8% in the 1D category; Figure 1 from Ref #1). This raises important questions whether these naïve individuals that exhibited a hyper-IgG response following 1D (compared to the prior-infected individuals) were truly naïve, or perhaps asymptomatically infected, or their samples collected at a later period within the 7-21 days (≥14 days, where typical IgG response is highly likely). This can be addressed (i) if those naïve group subjects who had all 3 data points (baseline, 1D and 2D) are plotted longitudinally per individual basis and (ii) by providing distribution of antibody response over time for the 1D and 2D separately. These data are critical in obtaining a clearer picture when defining a cohort for a single dose vaccination. Moreover, vaccine-triggered protective immunity is also known to decay progressively and wane over time, requiring revaccinations. In such situations, studies reflecting the accurate prevalence and persistence of infection (e.g.: accounting for asymptomatics) and immune status/sustainability (e.g.: waning) could be valuable to illuminate the impending patterns of oscillating infection(s)/outbreaks. This, in turn, can help guide and drive an effective periodic immunization program (e.g.: extending immunity duration *via* administering another booster).

IS IT WORTH ESTABLISHING A PAN IG: NEUTRALIZATION TITER?

In the course of COVID-19 disease, the kinetics of generation and persistence of IgM and IgG antibodies are typically asynchronous and vary with time. In particular, IgM emerges early during primary and secondary immune responses, while IgG typically appears later, but remains in circulation for a longer duration. Akin to IgG, IgM also functions in toxic neutralization, agglutination, complement activation, and acts as a mediator of inflammation. But, since IgM class of antibodies has a shorter persistence in relation to IgG, its detection may be used to indicate a recent event (infection or immunization). In the context of SARS-CoV-2 infection or COVID-19 immunization, determining IgM antibodies early in the event (within 2 weeks that may persist up to nearly 3 weeks following disease onset or immunization) and IgG antibodies (beyond 2 weeks until several months after infection or immunization), have been evidently recognized to be more informative for evaluating antibody-based immunological responses with sufficient sensitivity and specificity (31–33). Interestingly, the beneficial role of IgA, a potent and early SARS-CoV-2–neutralizing agent (34) has been recently documented for intranasal immunization with a Middle East respiratory syndrome coronavirus (MERS)-derived vaccine (35). Viewed in light of these facts, nearly all reports disregard the relevance of IgM despite presenting the data and there is no mention of IgA. Accordingly, future research studies involving a meticulous analysis of the results reflecting on both S-IgM and S-IgA values and establishing a neutralizing titer like that of the conservative IgG (S-RBD) could be helpful to obtain critical complimentary information. In this connection, it could be worth to consider a combined pan-Ig serological tests

hatsimultaneously measures reactive S-IgM (early antibodies), S-IgA (often detectable early antibodies before IgG), and IgG, like these newly developed assays (33, 36). In the hindsight, the bigger picture of 'S-pan Ig:neutralization titer' correlation will perhaps broaden the dataset of serological diagnosis and vaccine assessments regarding our understanding of the early phase of immunological response.

WHAT IS THE IMPACT OF TIME INTERVAL BETWEEN PRIOR INFECTION AND VACCINATION ON VACCINE-ELICITED ANTIBODY RESPONSES?

While a slew of studies claim that a single dose of vaccine is sufficient to protect the prior infected individuals as they mounted robust immune response following 1D (1–5), they left a gap in understanding whether the duration of time since resolution of infection had any impact on the level of antibody response following 1D. If ever there is an influence of the former on the latter, would it be different between an asymptomatic cluster and symptomatic subgroup? We have recently passed the anniversary of the first cases of COVID-19 appearing in the U.S., illustrating the point that there is a broad range of time since recovery in the U.S. population. Since protective immune response depends on the level of immunity (immunocompetence), which is a function of time since infection or vaccination, it is also possible that a single dose vaccine may elicit a more robust immune response in a recently recovered individual than in a person recovered more than a year ago or vice versa. In other words, more the period after vaccination (immunity wanes), the greater the susceptibility level to the illness, as reported for other infection scenarios (37, 38). After all, minimizing the susceptible group (waning) is a/the best way to eliminate the infection or epidemic (39). Thus, for a single dose vaccination strategy to be successful in prior-infected group, the feasibility of defining the optimal time interval allowed between prior infection and vaccination, and also determining the clinically reliable threshold of 'prime-boost' mechanism (functional immunity/inherent memory of the immune system) must be further explored.

CONCLUSIONS

While clearly endorsing the compelling findings on the adequacy of single-dose vaccination to prior infected cohorts (1–5), we feel detailed analysis and considerations as described here about asymptomatics and waning pools would be an inclusive approach to help define a population (prior-infected) that would benefit from single dose COVID-19 vaccination critically and confidently. Further, as countries prepare to implement novel and customized vaccination programs, addressing these questions in the context of newly emerging variants and breakthrough infections could certainly be impactful, and allowing experts to build upon this idea to enact practices and policies to combat COVID-19. It is appropriate to recall a thorough analysis-based personal view in a recent issue of '*The Lancet-Infectious Diseases*', emphasizing a sustained role for asymptomatic SARS-CoV-2 subset unless the scientific approaches are systematically and accurately approached (40).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author. The code, latest updated estimates, and their visualizations presented in Figure 1 are freely available at a GitHub repository (https://github.com/JungsikNoh/COVID19_Estimated-Size-of-Infectious-Population).

AUTHOR CONTRIBUTIONS

NM, LM, and AM conceived the idea, wrote, and proofread the manuscript. JN provided the data. All authors contributed to the article and approved the submitted version.

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MHC Variants Associated With Symptomatic *Versus* Asymptomatic SARS-CoV-2 Infection in Highly Exposed Individuals

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Despite the high number of individuals infected by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) who develop coronavirus disease 2019 (COVID-19) symptoms worldwide, many exposed individuals remain asymptomatic and/or uninfected and seronegative. This could be explained by a combination of environmental (exposure), immunological (previous infection), epigenetic, and genetic factors. Aiming to identify genetic factors involved in immune response in symptomatic COVID-19 as compared to asymptomatic exposed individuals, we analyzed 83 Brazilian couples where one individual was infected and symptomatic while the partner remained asymptomatic and serum-negative for at least 6 months despite sharing the same bedroom during the infection. We refer to these as "discordant couples". We performed whole-exome sequencing followed by a state-of-the-art method to call genotypes and haplotypes across the highly polymorphic major histocompatibility complex (MHC) region. The discordant partners had comparable ages and genetic ancestry, but women were overrepresented (65%) in the asymptomatic group. In the antigen-presentation pathway, we observed an association between *HLA-DRB1* alleles encoding Lys at residue 71 (mostly DRB1*03:01 and DRB1*04:01) and DOB*01:02 with symptomatic infections and *HLA-A* alleles encoding 144Q/151R with asymptomatic seronegative women. Among the genes related to immune modulation, we detected

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variants in *MICA* and *MICB* associated with symptomatic infections. These variants are related to higher expression of soluble MICA and low expression of MICB. Thus, quantitative differences in these molecules that modulate natural killer (NK) activity could contribute to susceptibility to COVID-19 by downregulating NK cell cytotoxic activity in infected individuals but not in the asymptomatic partners.

Keywords: SARS-CoV-2, COVID-19, MHC, HLA, resistance, asymptomatic, MICA, MICB

INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, became a worldwide pandemic affecting millions of people and the leading cause of death in Brazil in 2020 and 2021. Clinical manifestations range from severe cases with a lethal outcome to mild forms or asymptomatic cases. About 35%–50% of infected individuals are asymptomatic (1, 2) and are believed to be responsible for about 60% of transmissions (3).

Investigations on COVID-19 in thousands of samples from worldwide populations demonstrated the role of host genetics in disease susceptibility. Some variants and specific genome regions are related to disease severity and hospitalization (a proxy for disease severity), with different genome-wide association studies (GWASs) pointing to similar results. Among these associated regions and variants, we may cite rs11385942 at 3p21.31, in the region encompassing genes *SLC6A20*, *LZTFL1*, *CCR9*, *FYCO1*, *CXCR6*, and *XCR1*, rs657152 at 9q34.2 (the ABO blood group), rs10735079 at 12q24.13, in a gene cluster that encodes antiviral restriction enzyme activators (*OAS1*, *OAS2*, and *OAS3*), and rs74956615 at 19p13.2 (gene *TYK2*). There is also rs2109069 at 19p13.3 within the gene that encodes dipeptidyl peptidase 9 (*DPP9*) and rs2236757 at 21q22.1 in the interferon receptor gene *IFNAR2 (*4–7). One GWAS detected hits within the major histocompatibility complex (MHC), rs9380142 (*HLA-G*) and rs143334147 (*CCHCR1*) (5). However, while all these reported associations present p-values below the GWAS threshold ($p < 10^{-8}$), the odds ratios (ORs) are very low (usually less than 1.5), and they cannot be considered predictive genomic markers of disease severity. Since many genes influence COVID-19 severity, polygenic risk must be considered. Major efforts have been made to evaluate polymorphism and disease severity, usually by comparing hospitalized patients with a population-based sample (the normal control), but they do not evaluate COVID-19 resistance in exposed individuals.

Identifying asymptomatic individuals or those resistant to the infection who are seronegative even after high exposure is challenging, since controlling for and measuring different degrees of exposure are complex. Asymptomatic or resistant individuals, however, may provide clues on the mechanisms of resistance and infection itself.

Genes modulating immune responses are natural candidates in studying resistance to infectious agents and disease outcomes. Together with other genomic regions, as the ones listed earlier, they may contribute to the "resistant' phenotype. It has been shown that both innate and adaptive immune responses are crucial in the fight against COVID-19 (8, 9). In this context, the human MHC, harboring genes related to antigen processing, presentation, and immune modulation (10), is critical for both adaptive and innate immune responses. The human leukocyte antigen (HLA) genes within the MHC are among the most polymorphic genes in the human genome, and they are important targets for natural selection (11). The HLA class I molecules present antigens to CD8+ T lymphocytes (e.g., *HLA-A*, *HLA-B*, *and HLA-C*, usually expressed in all somatic cells) and HLA-class II to CD4+ T lymphocytes (e.g., *HLA-DRB1*, *HLA-DQA1* and *-DQB1*, *HLA-DPB1*, and others, usually expressed on professional antigen presentation cells, but also in activated T lymphocytes). In addition, HLA molecules play a critical role in the modulation of NK cell activity (e.g., *HLA-C*, *HLA-G*, *HLA-E*, and *HLA-F*, with a more restricted expression profile) (12). The MHC also harbors genes involved in antigen processing and loading (*HLA-DOA*, *HLA-DOB*, *HLA-DMA*, *HLA-DMB*, *TAP1*, and *TAP2*), cytokines such as tumor necrosis factor (TNF) and complement components, and genes that modulate the activity of NK cells, as *MICA* and *MICB.* The expression of *MICA and MICB* is low in normal tissues but is induced in tumors or during infections, upregulating (when expressed in the membrane) or downregulating (when expressed as soluble isoforms) NK cell cytotoxic activity (13).

Because of their unusually high polymorphism and extensive paralogy, GWAS findings for genes from the MHC are often ignored or treated with caution, as a consequence of HLA allele frequencies varied markedly across the world, and there may be different associations for different populations with diverse ethnicities. Because of that, the few studies on the frequency and distribution of HLA alleles and their clinical relevance for the SARS-CoV-2 infection (14–17) have shown conflicting results. However, the HLA locus is among the top hits in one GWAS from the COVID-19 Host Genetics Initiative (6).

To identify genetic factors involved as key players in the immune response of symptomatic SARS-CoV-2 infection and resistance, we have analyzed a cohort of couples discordant for the infection. While one was infected with symptomatic disease, the household-sharing partner, despite being closely exposed during the infection period, remained asymptomatic and seronegative for SARS-CoV-2 up to 6 months after the putative exposure. We whole-exome sequenced these couples, applied a bioinformatics pipeline to properly analyze variants within the MHC, and tested for genetic associations with disease/resistance phenotypes.

SUBJECTS AND METHODS

Volunteers' Recruitment and Datasets

Initial recruitment for screening involved broad media advertising based on the main inclusion criteria: couples discordant for SARS-CoV-2 symptomatic infection. From more than 2,000 received emails, we recruited 100 couples, all from São Paulo city (Brazil's most populated metropolis). All couples filled out an online questionnaire, which included basic personal information (age, sex, blood type, comorbidities), and clinical progression of COVID-19 as well as diagnostic tests. The asymptomatic and seronegative member remained in close contact with his/her symptomatic partner throughout the SARS-CoV-2 infection, sharing the same bed (except when the symptomatic one needed to be hospitalized). Confirmatory tests (RT-PCR for symptomatic and RT-PCR or serology for asymptomatic) endorsed that just one of the pair had symptomatic viral infection at the time and that all asymptomatic are seronegative. The collection of biological samples occurred at intervals from 30 to 180 days after the reported viral infection. Serological testing was repeated in the blood plasma with two different techniques (electrochemiluminescence and ELISA-SARS-CoV-2 RBD/NP IgA and IgG). This excluded seven couples where the asymptomatic partner was found to have IgA or IgG antibodies against SARS-CoV-2. After exome sequencing quality control, we obtained data on 83 couples. To provide a baseline of allelic diversity and frequency, we also compared the exposed asymptomatic seronegative (the COVID-19[-] group) and the symptomatic group (the COVID-19[+] group) with MHC data from a previously whole-genome sequenced census-based sample of elderly individuals from the same city, pairing samples by gender and genetic ancestry (18). The characteristics of each group are available in **Supplementary Table S1**. Notably, the samples were collected between June and October 2020, before new SARS-CoV-2 variants were reported in Brazil (especially Gamma).

Definition of Groups

The dataset is composed of couples sharing the same household and bedroom during the infection of one individual (**Supplementary Figure S1**). Three comparisons were conducted. Comparison 1 was made between COVID-19[+] *vs.* COVID-19[-] (n = 83 per group), with sex, age, and genetic ancestry as covariates. Due to the recurrent COVID-19 host hypothesis raised by our own analyses and literature about sex-specific factors driving infection risk, Comparison 2 subdivided the cohort into two sex-specific directions of resistance/susceptibility: COVID-19[+] males (n = 50) *vs.* COVID-19[-] females (n = 50) or COVID19[-] males (n = 28) *vs.* COVID-19 [+] females (n = 28). In Comparison 2, homosexual couples were excluded of the analyses; age and ancestry were covariates. Lastly, Comparison 3 subdivided the cohort into two sex-specific groups: COVID-19[+] males (n = 51) *vs.* COVID-19[-] males (n = 29) or COVID-19[+] females (n = 32) *vs.* COVID-19[-] females (n = 54) using age and genetic ancestry as covariates.

Exome Sequencing and Variant Calling

We used the Nextera Rapid Capture Custom Enrichment Kit or the Nextera Flex Kit (Illumina, San Diego, CA, USA) for library preparation and the IDT xgen-V1 kit for capture following manufacturer protocols. Whole-exome sequencing was performed on the NovaSeq 6000 equipment (Illumina, USA) with a 150-base paired-end dual index read format. Reads were aligned to the human reference GRCh38 using Burrow–Wheeler Aligner (BWA) (https://github.com/lh3/bwa/tree/master/bwakit). We also called genotypes using GATK HaplotypeCaller (version 4.0.9). The pipeline used for alignment, variant calling, variant refinement, and genetic ancestry assessment is detailed in the supplementary methods.

Genotype and Haplotype Calls for the Major Histocompatibility Complex

Genes from the MHC are prone to alignment bias and genotyping errors because of the significant similarity and high polymorphism. To circumvent this issue, we used hla-mapper version 4 (19) to optimize read alignment for specific genes from the MHC (**Figure 1**), as described elsewhere (18, 19). After applying hla-mapper, we called genotypes using GATK HaplotypeCaller with a further refinement step using vcfx.

To obtain phased variants for each gene, we first phased close variants using GATK ReadBackedPhasing and then analyzed phase sets using the phasex program (https://github.com/erickcastelli/phasex), as described previously for a large Brazilian sample (18).

After the hla-mapper optimization and phasing, we obtained the complete exonic sequences for each individual and the translation of these sequences (**Supplementary Figure S2**). These methods are detailed in the supplementary methods, including the procedure to call HLA alleles.

Statistical Analyses

Associations of phenotypic status with biallelic and multiallelic variants, allotypes, and specific amino acids were tested by fitting a logistic regression that considers each allele of a variant as an independent marker, controlling for age, genetic ancestry (all comparisons), and sex (when not stratifying by sex] using a local Perl script to convert the Variant Call Format (VCF) data into a plink-like table and R to fit the logistic regression. Due to sample size limitations, likely multifactorial inheritance, and the high number of variable sites (many multiallelic) in the MHC, we did not expect large effect sizes to be detected. Therefore, we used the threshold ($p < 0.005$) to detect candidates that may influence symptomatic infection susceptibility. To test if rare variants of larger effects contribute to the outcome, we also performed gene-based variant collapsing tests using SKAT-O within the rvtest program (20), enabling the analysis of multiallelic variants. SKAT-O is an optimized method for rare variants that combines and compares burden and SKAT tests, resulting in an optimal p-value for a given variant set (gene or gene set), controlling for the same covariates as individual variant associations.

RESULTS

We first compared the COVID-19[-] and COVID-19[+] groups, controlling for age, sex, and genetic ancestry (Comparison 1,

Supplementary Figure S2). These groups had comparable ages, socioeconomic status, and genetic ancestry proportions (**Supplementary Table S1**). We observed a large sex difference between the two groups; 51 men and 32 women among symptomatic individuals compared to 29 males and 54 females among COVID-19[-]. Because some variants might be linked to symptomatic infection only in men or women, we also stratified the groups by sex (21) in comparisons 2 and 3 (**Supplementary Figure S2**). In all cases, for each group, we selected a population-based sample paired by sex and genetic ancestry to compare the frequencies with the expected in the general population.

Among 1,723 queried variants within the genes illustrated in **Figure 1**, 13 attained significance at the $p < 0.005$ threshold (**Figure 2**). When controlling for sex, we found candidate variants for *MICA*, *MICB*, *HLA-DRB1*, *HLA-DOB*, and *HLA-DPB1* genes (**Figure 2** upper panel), including three missense variants in *MICB*, *HLA-DOB*, and *HLA-DRB1*. All variants from *MICB* and *HLA-DPB1* are in absolute Linkage Disequilibrium (LD). MICA promoter allele rs2596541/C is underrepresented in COVID-19[-] individuals when compared to symptomatic patients ($p = 0.0034$, OR = 1.9) and to the paired population sample ($p = 0.0168$) and overrepresented in the COVID-19[+] when compared to its paired population sample ($p = 0.0331$). Likewise, variants from *MICB* listed in **Figure 2** are overrepresented in COVID-19[+] compared with COVID-19[-] subjects ($p < 0.005$, OR = 2.6) and with the paired population sample ($p < 0.05$). The *HLA-DOB* missense variant rs2071554/T is underrepresented in the COVID-19[-] compared to COVID-19[+] ($p = 0.0039$, OR = 7.3) and the general population ($p = 0.0037$).

We detected different associations when stratifying the groups by sex. When evaluating sex-specific direction of infection within couples (comparison 2, **Figure 2** middle and lower panels), COVID-19[+] men and COVID-19[-] women, the strongest association is for two missense mutations in *HLA-A*, and this association is not detected when controlling for sex (**Figure 2** upper panel) or when testing for other directions (**Figure 2** lower panel). We also observed little to no effect of other MHC genes, except for a synonymous mutation in *HLA-DRB1* (rs3167799). *HLA-A* variants rs1059536/G and rs1059517/C, which are in high LD, are overrepresented in COVID-19[-] women compared to COVID-19[+] men ($p < 0.005$ and OR < 0.44 for both) and to the general population ($p < 0.05$). For COVID-19[+] women and COVID-19[-] men, the strongest association is for a missense mutation in *HLA-DRB1* (rs767010367), which is not detected in other comparisons. *HLA-DRB1* variant rs767010367/AG (the absence of a guanine deletion at chr6:32584370) is underrepresented in symptomatic women ($p < 0.005$, OR = 0.25) and in the general population ($p < 0.05$). When evaluating COVID-19[+] and COVID-19[-] individuals from the same sex (**Supplementary Figure S3**), we observed that the susceptibility *MICB* variants are overrepresented in symptomatic men and women, but significant only among men ($p = 0.0128$). The same can be observed for variants regarding HLA-DPB1. The associations regarding *HLA-A* are only observed when we compare COVID-19[+] men and COVID-19[-] women, but not when comparing individuals of the same sex. Finally, variant rs2071554 from HLA-DOB seems to be overrepresented only among COVID-19[+] women.

We applied SKAT-O in each candidate gene to evaluate the contribution of rare and common variants collapsed into gene-wide sets. Among MHC genes, we identified a significant association between *MICB* and COVID-19[+] ($p = 0.017$). We verified that the association signal was driven by the same common variants identified by the single variant association test described in **Figure 2**.

Most of the candidate variants in **Figure 2** presented intermediate frequencies in the general population samples when compared to the COVID-19[-] and COVID-19[+] groups, despite their similar genetic ancestry backgrounds. This finding may be explained by the mix of individuals prone to both phenotypes in the general population.

To investigate how specific protein sequences or amino acid residues may influence susceptibility to SARS-CoV-2 symptomatic infection, we translated all the exonic sequences to proteins, *in silico*, comparing the frequency of every full-length protein (the allotypes) and every amino acid residue between groups (**Figure 3**). Considering all samples (**Figure 3**, upper panel), the allotype MICB*004 and the amino acid residue that defines it, 75-N, both related to the missense mutation rs3131639/A, and the presence of K at residue 101 from HLA-DRB1 (related to rs9296942/T), are overrepresented among COVID-19[+] when compared to COVID-19[-] ($p < 0.005$) and the general population ($p < 0.05$). Conversely, allotype HLA-DOB*01:02 and the residue that defines it, 18-Q, both related to the missense mutation rs2071554/T, are underrepresented in the COVID-19[-] when compared to their paired COVID-19[+] and the general population ($p < 0.005$).

When stratifying the groups by sex, the HLA-A residues 168Q and 175R, both corresponding to *HLA-A* missense mutations listed in **Figure 2**, are underrepresented in symptomatic men ($p < 0.005$) compared to COVID-19[-] women and in the general

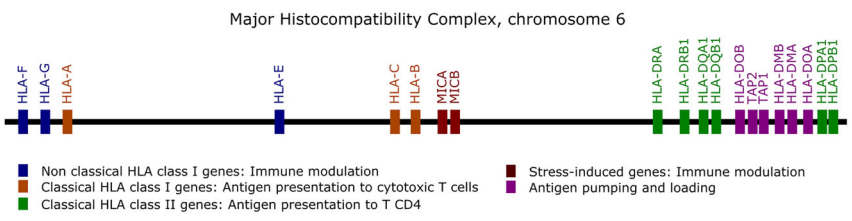

FIGURE 1 | List of the genes optimized by the hla-mapper program in the Major Histocompatibility Complex (MHC).

	Variants, alleles, and effects						Couples cohort				Population sample cohort			
	Position	Allele	SNPid	Gene	Effect	Association	Covid-19[-] n=83	Covid-19[+] n=83	P-value (1)	Odds Ratio	Paired to COVID-19[-] n=415	P-value (3)	Paired to COVID-19[+] n=415	P-value (3)
Comparison 1 (all samples)	29943426	C	rs1059517	HLA-A	missense	Protective	0.3434	0.2470	0.0974	0.63	0.3060	0.3594	0.3169	0.0796
	29943448	G	rs1059536	HLA-A	missense	Protective	0.2952	0.1928	0.0411	0.57	0.2385	0.1395	0.2518	0.1127
	31400061	C	rs2596541	MICA	promoter	Susceptibility	0.4518	0.6145	0.0034	1.93	0.5554	0.0168	0.5229	0.0331
	31505769	A	rs3131639	MICB	missense	Susceptibility	0.0964	0.2169	0.0029	2.60	0.1494	0.0861	0.1482	0.0360
	31506067	T	rs2286713	MICB	intronic	Susceptibility	0.0964	0.2134	0.0042	2.54	0.1496	0.0861	0.1484	0.0481
	31507350	A	rs3131638	MICB	intronic	Susceptibility	0.0904	0.2134	0.0028	2.73	0.1460	0.0630	0.1451	0.0351
	31509684	C	rs3132464	MICB	intronic	Susceptibility	0.0915	0.2125	0.0036	2.68	0.1458	0.0630	0.1446	0.0351
	32584180	T	rs9269942	HLA-DRB1	missense	Susceptibility	0.0060	0.0488	0.0041	8.50	0.0219	0.3448	0.0207	0.0525
	32584218	C	rs3167799	HLA-DRB1	synonymous	Protective	0.2289	0.1506	0.3218	0.60	0.1679	0.0745	0.1848	0.3202
	32584369	AG	rs767010367	HLA-DRB1	missense	Protective	0.3735	0.3375	0.0804	0.85	0.3768	1.0000	0.3631	0.5949
	32816899	T	rs2071554	HLA-DOB	missense	Susceptibility	0.0060	0.0427	0.0039	7.39	0.0530	0.0037	0.0506	0.8440
	33085249	G	rs9277457	HLA-DPB1	intronic	Protective	0.2831	0.1688	0.0017	0.51	0.2783	0.9246	0.2711	0.0061
	33085959	G	rs879317257	HLA-DPB1	intronic	Protective	0.2840	0.1603	0.0012	0.48	0.2783	0.9246	0.2711	0.0032
Comparison 2 (men symptomatic)	Position	Allele	SNPid	Gene	Effect	Association	COVID-19[-] women, n=50	COVID-19[+] men, n=51	P-value (2)	Odds Ratio	Paired to COVID-19[-] women, n=250	P-value (3)	Paired to COVID-19[+] men, n=255	P-value (3)
	29943426	C	rs1059517	HLA-A	missense	Protective	0.3700	0.2000	0.0007	0.43	0.3060	0.2389	0.3118	0.0230
	29943448	G	rs1059536	HLA-A	missense	Protective	0.3000	0.1600	0.0019	0.44	0.2300	0.1588	0.2529	0.0410
	31400061	C	rs2596541	MICA	promoter	Susceptibility	0.4300	0.5900	0.0268	1.91	0.5400	0.0487	0.5176	0.2319
	31505769	A	rs3131639	MICB	missense	Susceptibility	0.1100	0.2100	0.0875	2.15	0.1500	0.3501	0.1588	0.2462
	31506067	T	rs2286713	MICB	intronic	Susceptibility	0.1100	0.2100	0.0875	2.15	0.1500	0.3501	0.1591	0.2462
	31507350	A	rs3131638	MICB	intronic	Susceptibility	0.1000	0.2100	0.0527	2.39	0.1440	0.2686	0.1578	0.2424
	31509684	C	rs3132464	MICB	intronic	Susceptibility	0.1020	0.2083	0.0676	2.32	0.1440	0.2686	0.1569	0.2424
	32584180	T	rs9269942	HLA-DRB1	missense	Susceptibility	0.0000	0.0510	0.0147	10.31	0.0161	0.3639	0.0219	0.1625
	32584218	C	rs3167799	HLA-DRB1	synonymous	Protective	0.2800	0.1100	0.0048	0.32	0.1680	0.0113	0.2028	0.0257
	32584369	AG	rs767010367	HLA-DRB1	missense	Susceptibility	0.3300	0.4388	0.1150	1.59	0.3768	0.4274	0.3674	0.1797
	32816899	T	rs2071554	HLA-DOB	missense	Susceptibility	0.0100	0.0204	0.5510	2.06	0.0460	0.1559	0.0529	0.2023
	33085249	G	rs9277457	HLA-DPB1	intronic	Protective	0.2600	0.1875	0.2210	0.66	0.2900	0.6277	0.2529	0.1650
	33085959	G	rs879317257	HLA-DPB1	intronic	Protective	0.2653	0.1809	0.1730	0.61	0.2900	0.7181	0.2529	0.1270
Comparison 2 (women symptomatic)	Position	Allele	SNPid	Gene	Effect	Association	COVID-19[-] men, n=29	COVID-19[+] women, n=28	P-value (2)	Odds Ratio	Paired to COVID-19[-] men, n=145	P-value (3)	Paired to COVID-19[+] women, n=140	P-value (3)
	29943426	C	rs1059517	HLA-A	missense	Susceptibility	0.2857	0.3214	0.7631	1.18	0.3172	0.7589	0.3429	0.8773
	29943448	G	rs1059536	HLA-A	missense	Protective	0.2679	0.2500	0.7700	0.91	0.2886	0.8752	0.2643	0.8696
	31400061	C	rs2596541	MICA	promoter	Susceptibility	0.4821	0.6607	0.0213	2.09	0.5759	0.1962	0.5071	0.0402
	31505769	A	rs3131639	MICB	missense	Susceptibility	0.0714	0.2143	0.0475	3.55	0.1552	0.0990	0.1214	0.0861
	31506067	T	rs2286713	MICB	intronic	Susceptibility	0.0714	0.2037	0.0742	3.33	0.1557	0.0990	0.1214	0.1363
	31507350	A	rs3131638	MICB	intronic	Susceptibility	0.0714	0.2037	0.0742	3.33	0.1557	0.0990	0.1143	0.1222
	31509684	C	rs3132464	MICB	intronic	Susceptibility	0.0714	0.2037	0.0742	3.33	0.1552	0.0990	0.1143	0.1222
	32584180	T	rs9269942	HLA-DRB1	missense	Susceptibility	0.0179	0.0357	0.1864	2.03	0.0316	1.0000	0.0216	0.6248
	32584218	C	rs3167799	HLA-DRB1	synonymous	Susceptibility	0.1607	0.1964	0.7308	1.28	0.1563	1.0000	0.1607	0.5557
	32584369	AG	rs767010367	HLA-DRB1	missense	Protective	0.4464	0.1731	0.0049	0.26	0.3668	0.2397	0.3357	0.0258
	32816899	T	rs2071554	HLA-DOB	missense	Susceptibility	0.0000	0.0714	0.0142	8.92	0.0690	0.0324	0.0429	0.3182
	33085249	G	rs9277457	HLA-DPB1	intronic	Protective	0.3393	0.1481	0.0148	0.34	0.2552	0.1944	0.3000	0.0206
	33085959	G	rs879317257	HLA-DPB1	intronic	Protective	0.3393	0.1346	0.0143	0.30	0.2552	0.1944	0.3000	0.0206

FIGURE 2 | The frequency of each candidate variant at the Major Histocompatibility Complex (MHC) associated with susceptibility to SARS-CoV-2 symptomatic infection or with asymptomatic and seronegative after exposure. COVID-19[+]: Patients with symptomatic COVID-19. COVID-19[-]: Individuals sharing the same bed with symptomatic patients (exposed individuals) and are asymptomatic and seronegative. P-value (1): Logistic regression comparing all COVID-19[+] and all COVID-19[-] individuals, controlling for sex, age, and genetic ancestry; P-value (2): Logistic regression comparing COVID-19[+] and COVID-19[-] individuals, controlling for age, and genetic ancestry; P-value (3): Fisher exact test, comparing COVID-19[+] or COVID-19[-] individuals with a population sample paired by gender and genetic ancestry. In green, P-values < 0.005; In yellow, P-value between 0.005 and 0.05.

population ($p < 0.05$). However, we do not observe these differences when comparing COVID-19[+] and COVID-19[-] with the same sex (**Supplementary Figure S4**). HLA-DOB*01:02 is overrepresented mostly in COVID-19[+] women ($p = 0.0044$, OR = 9.0678). Overall, the protein and amino acid analyses corroborate what was observed in the single-nucleotide polymorphism (SNP) analysis for missense mutations.

We also tested *HLA-A* and *HLA-B* alleles grouped as supertypes (22). There is a trend to lower frequency of supertype A03 among exposed seronegative ($p = 0.0378$) when controlling for genetic ancestry, sex, and age, but this association was not significant after correction for multiple tests (six different HLA-A supertypes) or when stratifying samples by sex (**Supplementary Table S2**).

Finally, we discussed potential mechanisms underlying the associations presented in **Figures 2**, **3** (detailed at the **Supplementary Results**). In brief, the promoter variant from *MICA* is associated with higher mRNA expression levels (**Supplementary Figure S5**). In comparison, the susceptibility variants for *MICB* are associated with lower mRNA expression (**Supplementary Figure S6**). The susceptibility variant from *HLA-DOB* modifies the signal peptide and possibly the cellular localization and trafficking of the protein. The female *HLA-A* protective variants do not influence *HLA-A* expression levels.

DISCUSSION

Environmental factors such as protective measures, socioeconomic status, and access to healthcare may explain in part the high variability in COVID-19 disease incidence and

	Allotypes and associated SNPs				Couples cohort				Population sample cohort			
Comparison 1 (all samples)	Gene	Polymorphism	Associated SNP	Notes	COVID-19[-] n = 83	COVID-19[+] n=83	P-value (1)	Odds Ratio	Paired to COVID-19[-] n=415	P-value (3)	Paired to COVID-19[+] n=415	P-value (3)
	HLA-DOB	DOB*01:02	rs2071554/T	A	0.0060	0.04217	0.0039	7.26	0.0530	0.0037	0.0506	0.8440
	MICB	MICB*004	rs3131639/A	B	0.0843	0.20482	0.0021	2.80	0.1410	0.0583	0.1349	0.0298
	MICB	residue 75N	rs3131639/A	B	0.0964	0.21687	0.0029	2.60	0.1494	0.0861	0.1482	0.0360
	HLA-A	residue 168Q	rs1059517/C	C	0.3434	0.24699	0.0974	0.63	0.3060	0.3594	0.3169	0.0796
	HLA-A	residue 175R	rs1059536/G	D	0.2952	0.19277	0.0411	0.57	0.2385	0.1395	0.2518	0.1127
	HLA-DRB1	residue 101K	rs9269942/T	E	0.1084	0.21084	0.0044	2.20	0.1386	0.3204	0.1253	0.0066
Comparison 2 (men symptomatic)	Gene	Polymorphism	Associated SNP	Notes	COVID-19[-] women, n=50	COVID-19[+] men, n=51	P-value (2)	Odds Ratio	Paired to COVID-19[-] women, n=250	P-value (3)	Paired to COVID-19[+] men, n=255	P-value (3)
	HLA-DOB	DOB*01:02	rs2071554/T	A	0.0100	0.0200	0.5480	2.02	0.0460	0.1559	0.0529	0.2023
	MICB	MICB*004	rs3131639/A	B	0.1000	0.2000	0.0752	2.25	0.1420	0.3359	0.1412	0.1718
	MICB	residue 75N	rs3131639/A	B	0.1100	0.2100	0.0875	2.15	0.1500	0.3501	0.1588	0.2462
	HLA-A	residue 168Q	rs1059517/C	C	0.3700	0.2000	0.0007	0.43	0.3060	0.2389	0.3117	0.0230
	HLA-A	residue 175R	rs1059536/G	D	0.3000	0.1600	0.0019	0.44	0.2300	0.1588	0.2529	0.0410
	HLA-DRB1	residue 101K	rs9269942/T	E	0.1200	0.1800	0.2420	1.61	0.1360	0.7491	0.1254	0.2013
Comparison 2 (women symptomatic)	Gene	Polymorphism	Associated SNP	Notes	COVID-19[-] men, n=29	COVID-19[+] women, n=28	P-value (2)	Odds Ratio	Paired to COVID-19[-] men, n=145	P-value (3)	Paired to COVID-19[+] women, n=140	P-value (3)
	HLA-DOB	DOB*01:02	rs2071554/T	A	0.0000	0.0714	0.0142	8.92	0.0690	0.0324	0.0429	0.3182
	MICB	MICB*004	rs3131639/A	B	0.0536	0.1964	0.0351	4.32	0.1448	0.0552	0.1143	0.1222
	MICB	residue 75N	rs3131639/A	B	0.0714	0.2143	0.0475	3.55	0.1552	0.0990	0.1214	0.0861
	HLA-A	residue 168Q	rs1059517/C	C	0.2857	0.3214	0.7631	1.18	0.3172	0.7589	0.3429	0.8773
	HLA-A	residue 175R	rs1059536/G	D	0.2679	0.2500	0.7700	0.91	0.2586	0.8701	0.2643	0.8696
	HLA-DRB1	residue 101K	rs9269942/T	E	0.1071	0.2500	0.0462	2.78	0.1552	0.4161	0.1393	0.0453

FIGURE 3 | The frequency of each candidate allotype and amino acid residue encoded in the Major Histocompatibility Complex (MHC) associated with susceptibility to SARS-CoV-2 symptomatic infection or with asymptomatic and seronegative after exposure. COVID-19[+]: Patients with symptomatic COVID-19. COVID-19[-]: Individuals sharing the same bed with symptomatic patients (exposed individuals) and are asymptomatic and seronegative. P-value (1): Logistic regression comparing all COVID-19[+] and all COVID-19[-] individuals, controlling for sex, age, and genetic ancestry; P-value (2): Logistic regression comparing COVID-19[+] and COVID-19 [-] individuals, controlling for age, and genetic ancestry; P-value (3): Fisher exact test, comparing COVID-19[+] or COVID-19[-] individuals with a population sample paired by gender and genetic ancestry. In green, P-values < 0.005; In yellow, P-value between 0.005 and 0.05. Notes: (A) DOB*01:02 is the only HLA-DOB allotype carrying residue 18Q; (B) MICB*004, *024, *028 carry residue 75N (residue 75 in the full-length protein); (C) This residue is common to many HLA-A alleles, including A*23:01, A*25:01, A*26:01, *29:02, *30:01, *30:02, *31:01, *32:01, *33:01; (D) This residue is common to many HLA-A allotypes, including A*23:01, *29:02, *30:01, *30:02, *31:01, *32:01, *33:01; (E) This residue occurs in DRB1*03:01, *03:02, *04:01,*04:09, *13:03. rs9269942/T captures only a fraction of the sequences encoding K at position 101 from HLA-DRB1, since the composition of other surrounding variants, including indels, can produce the codon for K.

mortality among individuals. However, few studies have focused on the genetics of resistance against SARS-CoV-2 infection due to limitations in controlling for exposure. Previous reports on host genetic factors with resistance to COVID-19 have investigated SARS-CoV-2 receptors such as the angiotensin-converting enzyme 2 (ACE2), the transmembrane protease serine-type 2 (TMPRSS2), glucose-regulated protein 78 kDa (GRP78), and the extracellular matrix metalloproteinase inducer or cluster of differentiation 147 (CD147) [reviewed by (23)].

GWASs addressing COVID-19 severity detected many different genomic regions and specific variants influencing the disease outcome (4–7, 24). However, it became clear that polygenic risk must be considered, since the ORs of these associations are relatively underpowered, usually lower than 1.5. The Brazilian media reported that both sibs from seven pairs of monozygotic adult twins died from SARS-CoV-2 infection within a few days of difference. Furthermore, recent observations showed that secondary transmission among close household contacts was 53% (25). These observations support the influence of host genetics in COVID-19 severity and resistance (6), particularly in a polygenic fashion.

An efficient response in the early course of SARS-CoV-2 infection may strongly influence infection outcome, differentiating CODIV-19-unaffected resistant individuals or asymptomatic after exposure and symptomatic ones. Several studies aimed to understand the human genetics of protective immunity to SARS-CoV-2 (24, 26, 27). Innate immune genes and genes involved in antigen presentation seem to be strong candidates in this matter, contributing with an additional layer to other known genomic regions that influence COVID-19 outcome (4–7, 24).

Spouses of infected and symptomatic COVID-19 patients (COVID-19[+]) sharing the same bedroom without protective measures represent an efficient approach to identify and ascertain resistant individuals highly exposed to the same viral strain of SARS-CoV-2. Here, we investigated 83 discordant couples, in which one was symptomatic and the partner remained asymptomatic and seronegative for at least 6 months. Since we collected all samples in the first semester of 2020, all couples were likely exposed to the same or closely related viral strains (28). Our study suggests that genes of innate and adaptive immune responses may play a vital role in susceptibility/protection to symptomatic SARS-CoV-2 infection. The innate response is key for controlling primary encounters with a pathogen (29). In the case of COVID-19, although SARS-CoV-2 is a novel pathogen, it shares extensive CD4+ and CD8+ T-cell cross reactivity with human endemic coronaviruses (30–32) and

would therefore elicit a secondary T-cell response to the cross-reactive epitopes. This cross-reactive secondary T-cell response could eradicate SARS-CoV-2 infection in a proportion of individuals, even before a specific SARS-CoV-2 antibody response is made, as demonstrated in family contacts of COVID-19 patients (33, 34).

Associations of the Antigen-Presentation Pathway

MHC variants showed no important associations between class I antigen-presentation genes (*HLA-A*, *HLA-B*, and *HLA-C*) and nonclassical HLA class I genes (*HLA-G*, *HLA-E*, and *HLA-F*) in symptomatic infection susceptibility. The only exception is two missense mutations of *HLA-A* protecting mainly women when the male partner is symptomatic (rs1059517/C and rs1059536/G), both related to some common allotypes such as HLA-A*23:01, *29:02, *30:01, *30:02, *31:01, *32:01, and A*33:01 (**Figure 3**). None of these alleles present relevant frequency differences when considered alone, but most of them are overrepresented in COVID-19[-] women, particularly HLA-A*23:01 and *29:02. These SNPs lead to two amino acid exchanges in the HLA-A molecule, encoding Q and R at residues 168 and 175 of the full protein (or 144 and 151 in the mature molecule), respectively. These residues belong to the alpha-2 domain, but they seem to not influence peptide binding (35), although residue 175 can interact with the T-cell receptor. The binding prediction of SARS-CoV-2 peptides to HLA class I genes indicated that alleles A*23:01 and A*29:02 are intermediate binders, thus enhanced antigen presentation should not be the case (36). Moreover, these variants seem to not influence *HLA-A* mRNA expression levels in men, women, or the combined sample. HLA-A*23:01 was detected as risk markers for severe COVID-19 among Spanish patients (37), but not in Russia (38). A GWAS detected a trend to a higher frequency of A*23:01 among the general population than in severe COVID-19 Italian patients, but no differences for A*29:02 (4). A study from China comparing COVID-19 patients and the general population did not detect any *HLA-A* association (39). Moreover, in our study, polymorphisms of nonclassical HLA genes involved in immune modulation (e.g., *HLA-E*) were not associated with symptomatic infection susceptibility in discordant couples, but a higher frequency of HLA-E*01:01 has been reported to be associated with hospitalized patients (9). In addition, we cannot rule out the influence of *HLA-G*, particularly variant rs9380142 as described in a recent GWAS (5), because this is a 3'-untranslated region (UTR) variant not captured by our exome analysis. Previous studies focusing on HLA genes and SARS-CoV-2 infection, comparing infection outcome (mild *vs.* severe COVID-19) or infection susceptibility in infected individuals against population control samples, show different associated alleles for each population and sample (4, 9, 14, 17, 38, 40). This lack of consistency among studies possibly reflects the wide frequency differences of HLA alleles among populations and how each study characterized its samples.

For HLA class II genes, we found an association of K at residue 101 of HLA-DRB1 when considering the full-length molecule (or residue 71 in the mature protein) with symptomatic infection susceptibility, mostly related to increased frequencies of DRB1*03:01 and DRB1*04:01 among symptomatic patients (**Figure 3**). HLA-DR molecules are important antigen-presenting molecules, essentially for CD4+ T cells, and lower HLA-DRB1 expression has been associated with the severity of the SARS-CoV-2 infection (41). Thus, inappropriate antigen-presentation by some HLA-DR molecules might facilitate infection susceptibility. Accordingly, DRB1*04:01 has been associated with lower HLA-DRB1 mRNA expression than many other alleles (42). Furthermore, these results conflict with previous data from other populations. For instance, in North East of England (43) and Saudi Arabia (44), HLA-DRB1*04:01 was 2–3 times more frequent in the asymptomatic group. Likewise, DRB1*03:01 had a protective effect against SARS-CoV-2 infection in the Sardinian population (40). In our survey, HLA-DRB1*04:01 and DRB1*13:03 were six times and three times more frequent among symptomatic individuals ($p = 0.0187$ and $p = 0.0229$, respectively), and DRB1*03:01 was also more frequent among symptomatic individuals (individually not significant). Taken together, they contribute to the much higher frequency of residue 71K among symptomatic individuals than exposed seronegative ones ($p = 0.0044$), which was also confirmed by the comparison with the general population ($p = 0.0066$). DRB1*04:01 is common in European populations (France, Denmark, England, Germany) and relatively rare in Asian and African populations (www.allele-frequencies.net). Conversely, DRB1*03:01 is common in Europe and Africa but uncommon in East Asia. In our sample, Brazilians carrying DRB1*04:01 present a much higher East Asian genetic ancestry (10.7%) than observed in the entire dataset (around 1.5%), while Brazilians carrying DRB1*03:01 present a similar ancestry background than the entire dataset. This lack of consistency for HLA-DRB1 associations among populations is another example of population-specific associations.

The DOB*01:02 (rs2071554/T, a missense variation in the HLA-DOB signal peptide) may have a potential effect in protein function, since *in silico* analysis of functional effects of this variation predicted a possible damage to the protein function (45). HLA-DO molecule is a modulator of HLA-DM, a peptide exchange factor required for efficient loading of endosomal peptides onto MHC-II molecules (46). This may lead to inadequate antigen presentation failure to recognize important epitopes.

Natural Killer Cell Activity Pathway

Interestingly, most of the MHC class I region hits coincide with two genes, *MICA* and *MICB*. *MICA* and *MICB* are constitutively expressed in a few cell types, such as fibroblasts and epithelial cells but are markedly upregulated in stress conditions like cancer and infections (47). Here, the regulatory *MICA* variant associated with symptomatic infection is related to higher mRNA expression, possibly higher soluble MICA (sMICA), while the opposite is observed for all *MICB* variants (supplementary results). MICA and MICB interact with activating receptor C-type lectin-like receptor NKG2D and play an important role in mediating NK and TCD8+ cytotoxic activity (48).

Previous studies suggest the participation of the unconventional T (uT) cells, gamma-delta T (γδ T), NKT, and NK cells in the immune response against several infections such as tuberculosis, HIV, Influenza A, Influenza A (H1N1) [reviewed by (49)], as well as SARS-CoV-1 (50) and SARS-CoV-2 infections (51). These cells recognize non-peptide antigens in an MHC-independent way and produce mostly inflammatory cytokines such as interferon (IFN)-γ and eliminate target cells by perforin/granzyme-mediated cytotoxicity *in vivo* (52). Thus, these cells could be a key for a rapid defense against bacterial and viral infections (53) and contribute to control viral load through a higher MICB expression (from the exposed seronegative individuals), activating an effective natural response *via* NKG2D.

The missense *MICB* variant associated with SARS-CoV-2 symptomatic infection, MICB*004 and rs3131639/A, is clearly overrepresented in symptomatic patients compared to both exposed seronegative and the general population. Interestingly, its frequency is also increased in patients with secondary dengue hemorrhagic fever (54). Thus, higher MICB expression may positively influence DENV infection control by activating early NKG2D-mediated immune responses. A similar mechanism may be important for SARS-CoV-2 infection, and higher MICB expression may play a role in the innate immune defense against SARS-CoV-2. Conversely, variants associated with lower MICB expression, possibly implicating a diminution of NK cytotoxicity, may facilitate infection. Besides, the amino acid exchange (75-N, **Figure 3**) may also be related to ligand/receptor binding impairment or lower protein stability. In our study, rs3131639/A and MICB*004 are related to lower MICB expression (**Supplementary Figure S3**), which is confirmed by the GTEx portal (https://www.gtexportal.org). The mechanism underlying this differential expression or whether other polymorphisms in LD with rs3131639, particularly in the *MICB* promoter region, may also play a role in the expression regulation is not clear.

MICA alleles are also associated with susceptibility to infectious diseases (55). Here, the *MICA* variant associated with symptomatic infection is linked to higher MICA mRNA expression (**Supplementary Figure S2**). This variant is also in LD with MICA allotypes that associated with high levels of sMICA (56, 57), MICA*008 and MICA*019, both overrepresented among symptomatic individuals. High levels of soluble sMICA would have an inverse effect on NK cell activation. Other viruses are known to stimulate the release of sMICA to escape NKG2D recognition by activating endocytosis and degradation of the NKG2D receptor (58). A recent study suggested that dysregulation of the NKG2D-MICA axis could be a possible mechanism of NK cell exhaustion in SARS-CoV-2 infection, resulting in suppressive effects by excessive cytokines in the disease course. SARS-CoV-2 might escape from NKG2D recognition using a similar mechanism of elevated plasma levels of sMICA. For instance, the disintegrin and metalloproteinase17 (ADAM17) activity is upregulated upon binding of SARS-CoV to ACE2, facilitating viral entry. This might be responsible for the higher shedding of MICA after spike-ACE2 interaction during SARS-CoV-2 infection [reviewed by (59)]. Although not confirmed by protein expression analysis, we can hypothesize that the variants associated with higher MICA expression are also associated with higher sMICA levels and/or NK exhaustion, resulting in NK dysfunction in the early stages of SARS-CoV-2 infection. Indeed, decreased NK cell numbers, impaired cytotoxic activity, and a biased inflammatory phenotype have been reported in SARS-CoV-2 infection, indicating that NK cells likely integrate into the underlying immune dysregulation in COVID-19. In addition, sMICA levels were considerably higher in COVID-19 patients with severe disease (60). In this context, our findings bring additional potential mechanisms involving NK dysfunctions [reviewed by (61)], which could be confirmed by functional assays.

Innate immunity efficiency may be a critical feature between symptomatic and asymptomatic infections, and it also may facilitate the prompt elimination of the virus after exposure. Host genetics influence this feature. For instance, inborn errors of TLR3- and IRF7-dependent type I IFN immunity are associated with life-threatening COVID-19 pneumonia (24), and neutralizing autoantibodies against IFN type I were detected in 10% of patients with life-threatening COVID-19 pneumonia (62). These findings highlight the importance of the innate immunity against SARS-CoV-2. Here, we add another potential layer to this complexity—host genetics influencing NK cell activation efficiency due to differential expression capabilities of MICA and MICB.

The susceptibility haplotype formed by the *MICB* variants in **Figure 2** is more frequent in Europe (about 23%) and Africa (15%) and less frequent in Asia (9.4%). Conversely, MICA allele rs2596541/C is more frequent in East Asia (about 72%), South Asia (about 63%), and Europe (about 60%) and less frequent in Africa (about 46%). Interestingly, the *MICB* susceptibility haplotype can be detected in archaic humans, such as the Neanderthal from the Vindija Cave, and the *MICA* susceptibility allele in the Neanderthal from the Altai Mountains and one Denisovan.

Concluding Remarks

In short, here we performed a candidate region approach to compare polymorphisms in the MHC region in symptomatic COVID-19-infected individuals and in highly exposed partners who were seronegative. We used a state-of-the-art method to call genotypes and haplotypes in the MHC. We observed little to no impact of polymorphisms in class I antigen-presentation genes, except for HLA-A allotypes carrying 144Q and 151R among asymptomatic women when the male partner is symptomatic, increasing susceptibility to symptomatic infection. We also observed an association of HLA-DRB1 alleles encoding K at residue 71 (DRB1*03:01, DRB1*04:01, and others) with susceptibility to symptomatic infections. Allele HLA-DOB*01:02 is associated with symptomatic infection mostly among women. Moreover, our results suggest that genes related to immune modulation, mainly involved in NK cell killing activation/inhibition, harbor variants potentially contributing to infection resistance. We hypothesize that

individuals prone to produce higher amounts of sMICA, and low amounts of MICB, would be more susceptible to symptomatic infections. Accordingly, quantitative differences in these NK activity-related molecules could contribute to susceptibility to COVID-19, likely downregulating NK cell cytotoxic activity in infected individuals but not in resistant partners. Functional assays will provide the means to test the hypothesis of differential NK cell activity between COVID-19 symptomatic and asymptomatic exposed individuals, involving *MICA* and *MICB*.

Host genetics influencing NK cell activation due to differential expression of MICA and MICB is another layer in the complex interaction between the human genome and COVID-19 outcome. The current knowledge regarding this matter supports the link between polymorphisms and susceptibility to life-threatening COVID-19, the majority promoting moderate–higher risk. The associations described here follow the same path but with slightly higher ORs than those described in previous GWASs (4–7). For instance, the OR for MICB*004 and HLA-DOB*01:02 for symptomatic infection is 2.8 and 7.39, respectively.

Due to the likely multifactorial nature of resistance itself, the putative resistant individuals in our study could be protected by NK cell response or cytotoxic effects present due to previous endemic coronavirus with common antigenic exposure to SARS-CoV-2 (cross reaction).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

This study was approved by the Committee for Ethics in Research of the Institute of Biosciences at the University of São Paulo (CAAE 34786620.2.0000.5464). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EC, MC, MN, DM, and MZ contributed to the conceptualization. MC, LM, and MVRS contributed to data curation. EC, MN, MOS, NS, HA, AS, RP, CC, and KN contributed to the formal analysis. MZ contributed to funding acquisition. MC, MN, MOS, EC-N, and KS contributed to the investigation. EC, MN, MOS, CM-J, DM, and KN contributed to the methodology. MZ contributed to the project administration. EC, MN, and MOS contributed the software. EC, MN, MOS, CM-J, and KN contributed to visualization. EC, MC, MN, and MOS contributed to writing–original draft. NS, HA, AS, RP, CC, CM-J, DM, KN, LM, MVRS, JE, VC, RB, MH, JM, EC-N, VC, KS, MM, JK, MN, RM, MP-B, and MZ contributed to writing–review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.742881/full#supplementary-material

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Mild and Asymptomatic COVID-19 Convalescents Present Long-Term Endotype of Immunosuppression Associated With Neutrophil Subsets Possessing Regulatory Functions

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The SARS-CoV-2 infection [coronavirus disease 2019 (COVID-19)] is associated with severe lymphopenia and impaired immune response, including expansion of myeloid cells with regulatory functions, e.g., so-called low-density neutrophils, containing granulocytic myeloid-derived suppressor cells (LDNs/PMN-MDSCs). These cells have been described in both infections and cancer and are known for their immunosuppressive activity. In the case of COVID-19, long-term complications have been frequently observed (long-COVID). In this context, we aimed to investigate the immune response of COVID-19 convalescents after a mild or asymptomatic course of disease. We enrolled 13 convalescents who underwent a mild or asymptomatic infection with SARS-CoV-2, confirmed by a positive result of the PCR test, and 13 healthy donors without SARS-CoV-2 infection in the past. Whole blood was used for T-cell subpopulation and LDNs/PMN-MDSCs analysis. LDNs/PMN-MDSCs and normal density neutrophils (NDNs) were sorted out by FACS and used for T-cell proliferation assay with autologous T cells activated with anti-CD3 mAb. Serum samples were used for the detection of anti-SARS-CoV-2 neutralizing IgG and GM-CSF concentration. Our results showed that in convalescents, even 3 months after infection, an elevated level of LDNs/PMN-MDSCs is still maintained in the blood, which correlates negatively with the level of $CD8^+$ and double-negative T cells. Moreover, LDNs/PMN-MDSCs and NDNs showed a tendency for affecting the production of anti-SARS-CoV-2 S1 neutralizing antibodies. Surprisingly, our data showed that in addition to LDNs/PMN-MDSCs, NDNs from convalescents also inhibit proliferation of autologous T cells. Additionally, in the convalescent sera, we detected significantly higher concentrations of GM-CSF, indicating the role of emergency granulopoiesis. We conclude that in mild or

asymptomatic COVID-19 convalescents, the neutrophil dysfunction, including propagation of PD-L1-positive LDNs/PMN-MDSCs and NDNs, is responsible for long-term endotype of immunosuppression.

Keywords: COVID-19 convalescents, normal density neutrophils, immunosuppression, granulocyte-macrophage colony stimulating factor (GM-CSF), low-density neutrophils (LDNs), granulocytic myeloid-derived suppressor cells (PMN-MDSCs)

INTRODUCTION

Since the beginning of 2020, the COVID-19 pandemic has affected more than 200 million people worldwide, causing over 4.5 million deaths so far. The causative agent of COVID-19 is severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), airborne transmitted between the humans (1). Many comorbidities, such as hypertension, obesity, diabetes, and other pathologies affecting the immune system are the risk factors of the severe course of COVID-19 (2). The clinical manifestations of COVID-19 are diverse and range from asymptomatic, through mild to severe disease with lung injury and respiratory distress, often followed by multiorgan failure and death (3, 4). Blood lymphopenia is one of the hallmarks of COVID-19 and its severity correlates with worse prognosis (5, 6). However, the mechanisms underlying lymphopenia, and particularly reduction of T-cell number during COVID-19, remain unclear. The lymphocytes due to a relatively low surface expression of angiotensin converting enzyme 2 (ACE2), the entry receptor for the virus (7), seem not to be its direct target (8). Lymphopenia is not subset-specific within T cells and the numbers of both $CD4^+$ and $CD8^+$ T cells are rapidly reduced during the virus infection. This may be caused by the cytokine storm and rapid release of IL-6, TNF-α, and IL-1 (9), subsequent thymic involution, and/or T-cell sequestration in the specific organs due to the hyperinflammation (9, 10). However, lymphopenia has been reported concurrently with onset of the clinical symptoms (6). In this context, an alternative hypothesis claims the collapse of the host protective immunity ("immunologic collapse"), leading to failure in control of viral replication and dissemination (11–14). In this scenario, an increased production of prostaglandin D_2 by the respiratory epithelium (15) causes inhibition of the dendritic cell response *via* DP_1 receptor signaling and/or upregulation of myeloid cells with regulatory functions, including myeloid-derived suppressor cells (MDSCs), which may be one of the mechanisms attenuating inflammatory response (16, 17). From the other side, the early expansion of MDSCs may inhibit SARS-CoV-2 antigen-specific T-cell response and predict fatal outcome (18), suggesting these cells as important players during COVID-19.

The population of MDSCs has been defined as innate bone-marrow-derived myeloid cells suppressing effector T-cell response (19), and are considered as key cellular components connecting innate and adaptive T-cell response. They are detected mainly in cancer, where their blood level correlates with disease progression (20). Increased MDSC level was also shown in viral infections (21, 22), including COVID–19 (18, 23). By phenotype and morphology assessment, three populations of MDSCs, differing in their origin, have been distinguished so far: granulocytic (PMN–MDSCs)—$Lin^-HLA–DR^{low/-}CD11b^+CD14^-CD15^+$, monocytic (Mo–MDSCs)—$Lin^-HLA–DR^{low/-}CD11b^+CD14^+CD15^-$, and early stage (e–MDSCs)—$Lin^-HLA–DR^{low/-}CD11b^+CD14^-CD15^-$, all classified as immature myeloid cells with strong immunosuppressive properties (24). The MDSC subsets differ in the mechanism of action—PMN–MDSCs are mainly responsible for reactive oxygen species (ROS) production, while Mo–MDSCs possess higher expression of inducible nitric oxide synthase (iNOS), and capacity to release large amounts of nitric oxide (NO), although some common pathways, including arginase–1 (Arg1) activity and PD–L1 expression, are also relevant (25). Their function relies mainly on suppression of T–cell response, including T–cell proliferation, IFNγ production (26), and/or induction of regulatory T cells (27). The PMN–MDSCs have lower density in contrast to normal granulocytes, which typically sediment on top of erythrocytes after density gradient centrifugation, hence, they are also called immunosuppressive low–density neutrophils (LDNs) (28) or LDNs/PMN–MDSCs, due to the lack of specific markers distinguishing neutrophil subsets within LDNs (29).

The major differences between MDSC populations and corresponding mature neutrophils and monocytes had been described (30), however, recently due to the progress in resolution techniques, including high–dimensional single–cell assays and reporter–fate mapping, concerns regarding the development and activation state of MDSCs were raised, questioning previously accepted nomenclature (31).

The role of myeloid cells with regulatory activity has been indicated in SARS–CoV–2 infection, discriminating between patients with mild and severe disease (23, 32–34). In particular, LDNs were shown to emerge in severe COVID–19 patients (32), and expansion of PMN–MDSCs with Arg1 activity was associated with an increase of the disease severity (33). The role of Mo–MDSCs, although less frequent, was also documented as related to the course of SARS–CoV–2 infection (34). All of them correlated with poor T–cell response in severe COVID–19 patients (35–37). Here, we asked whether a mild or asymptomatic course of COVID–19 may also lead to consequences in the form of systemic immunosuppression and long–COVID.

MATERIALS AND METHODS

Study Group

The study group consisted of convalescents who recovered from a mild or asymptomatic infection with SARS–CoV–2, confirmed by the positive PCR test for SARS–CoV–2 mRNA. There were 13

individuals (7 women and 6 men) in this group aged from 29 to 58 years, with no persistent symptoms or post–COVID complications, who at the time of COVID–19 diagnosis had no symptoms (n = 3) or had symptoms of fever, chills, fatigue, new loss of taste or smell, cough, congestion or runny nose, headache, muscle or body aches, especially orbital region pain, and sore throat (n = 10), related to SARS–CoV–2 infection (convalescent characterization is presented in **Supplementary Table S1**). On the day of blood sampling, all convalescents were approximately 35 (20–60) days after the first manifestations of the disease or positive result of the RT–PCR test. The control group consisted of 13 healthy subjects without SARS–CoV–2 infection in the past, with ages from 18 to 65 years. All participants were non–vaccinated against SARS–CoV–2 before blood donation. The Bioethical Committee of the Jagiellonian University approved the study (Approval no. 1072.6120.83.2020), and all subjects gave written informed consent to participate in the study.

Peripheral blood was drawn to citrate–containing tubes (10 ml) and tubes with clot activator (3 ml). The blood count was assessed by routine procedure using a hematology analyzer (Sysmex XN–350, Sysmex, Norderstedt, Germany).

Flow Cytometry Analysis

Whole blood (100 µl) was used for T–cell subset analysis after the staining with cocktail of the following monoclonal antibodies (mAbs): anti–CD3–FITC + anti–CD8–PE + anti–CD45–PerCP + anti–CD4–APC (BD Multitest™, BD Biosciences, Franklin Lakes, NJ, USA) and anti–PD–1–BV605 mAb (BD Biosciences). The subsets of T cells were identified after the lysis of red blood cells (RBC Lysis Buffer, eBioscience™, Invitrogen, Carlsbad, CA, USA) on FACS CantoII flow cytometer (BD Biosciences, Immunocytometry Systems, San Jose, CA, USA). Populations of $CD45^+CD3^+CD4^+$, $CD45^+CD3^+CD8^+$, and $CD45^+CD3^+CD4^-CD8^-$, corresponding to $CD4^+$, $CD8^+$, and double–negative T cells (DNTs), respectively, were identified using FACS Diva v. 8.0.1 software (BD Biosciences).

From the remaining blood volume, mononuclear cells (PBMC) were isolated by standard Pancoll (PAN BIOTECH, Aidenbach, Germany) density gradient centrifugation. For analysis of MDSCs, PBMCs (approximately 1×10^6 cells) were stained with the following mAbs: anti–CD33–PE (clone P67.6), anti–LIN–AF700 (CD3, CD19, CD56, clones UCHT1, HIB19, and B159), anti–HLA–DR–PerCP (clone L243), anti–CD11b–BV510 (clone ICR F44), anti–CD14–FITC (clone MφP9), anti–CD15–PE–Cy7 (clone HI98), anti–PD–L1–APC (clone 10F.9G2), anti–CD64–AF700 (clone 10.1), anti–CD16–PE (clone 3G8), and anti–CD66b–FITC (clone G10F5) (all from BioLegend, San Diego, CA, USA) for 20 min at 4°C. After incubation, cells were washed twice in PBS and suspended in 0.2 ml of PBS. To determine the level of non–specific staining and cell autofluorescence, the respective isotype controls and fluorescence minus one (FMO) control samples were incubated in parallel. The samples were analyzed on FACS CantoII flow cytometer (BD Biosciences) using FACS Diva v. 8.0.1 software (BD Biosciences) and FlowJo v.10 software (BD Biosciences). The Mo–MDSCs were characterized as $LIN^-HLA–DR^{low/-}CD11b^+CD14^+CD15^-$ cells, whereas LDNs/PMN–MDSCs were characterized as $LIN^-HLA–DR^{low/-}CD11b^+CD14^-CD15^+$ cells. The e–MDSCs were gated as $LIN^-HLA–DR^-CD11b^+CD14–CD15^-$ cells. The level of MDSC subsets was presented as the percentage of nucleated cells (NC) (positive for SYTO™ 9, Invitrogen, Eugene, OR, USA). Detailed gating strategy is presented in **Supplementary Figure S1**.

Assessment of Cell Suppressive Activity

Suppressive activity of the myeloid cell subsets was analyzed in cultures with autologous T cells activated with anti–CD3 mAb (clone HIT3, 1 µg/µl) by H^3–thymidine incorporation assay. Briefly, the FACS–purified T cells ($CD3^+$) with 10% of autologous monocytes (FACS sorted $CD14^+HLA–DR^+$) were co–cultured with LDNs/PMN–MDSCs (FACS sorted $HLA–DR^{low/-}CD33^+CD66b^+CD14^-$) or NDNs, used as control (FACS–sorted $CD66b^+$ from the bottom fraction after Pancoll separation and RBC lysis) at the T cells to MDSCs/NDNs ratio 2:1 (established experimentally), and activated with *anti–CD3* mAb (clone HIT3, 1µg/µl, BD Pharmingen™, Franklin Lakes, NJ, USA). After 3 days of culture, T cells were pulsed with H^3–thymidine (1 µCi/well, GE Healthcare, Marlborough, CT, USA) for an additional 6 h and thymidine uptake was measured as counts per minute (cpm) in a liquid scintillation counter LS1801 (Beckman Coulter, Indianapolis, IN, USA). The results are presented as mitotic index:

$$\text{mitotic index} = \frac{\text{anti} - \text{CD3 stimulated test culture [cpm]}}{\text{non} - \text{stimulated culture [cpm]}}$$

Testing for Anti–SARS–CoV–2 S1 IgG

Anti–SARS–CoV–2 S1 IgG antibodies were quantified in fresh serum samples. The level of antibodies was determined by SARS–CoV–2 S1 IgG II Quant chemiluminescent microparticle immunoassay (Abbott Laboratories, Lake Bluff, IL, USA), using "Aliniti i" immune analyzer (Abbott), following the manufacturer's instructions.

Evaluation of GM–CSF Concentration

Concentration of GM–CSF was assessed in serum samples stored at −20°C in one batch measurement. All samples were thawed, and the concentration of GM–CSF was evaluated by ELISA immunoassay (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's recommendations.

Statistical Analysis

Statistical analysis was performed using the PRISM GraphPad 8 package (GraphPad Software Inc., San Diego, CA, USA). Data were analyzed using t–test or one–way analysis of variance (ANOVA) with Tukey Multiple Comparison Test, as a *post–hoc* test. The magnitude of the relationship between two quantitative features was evaluated using Pearson's correlation coefficient. All data are expressed as median with interquartile range. $p < 0.05$ was considered statistically significant.

RESULTS

PBMCs From COVID–19 Convalescents Contain High Frequency of LDNs/PMN–MDSCs

The group of convalescents was examined, on average, after 35 days (20–60 days) from the first symptoms or, in the case of asymptomatic course, from the day of positive result of the RT–PCR test for SARS–CoV–2 mRNA. The MDSCs were identified as $Lin^{-}HLA–DR^{low/-}CD11b^{+}$ cells and further divided into Mo–MDSCs, LDNs/PMN–MDSCs, and e–MDSCs, based on the expression of CD14 and CD15. Their frequency was evaluated in PBMCs of convalescents and compared with the age–matched healthy controls (CTR). Level of e–MDSCs was negligible (data not shown), whereas the frequency (percent value of NC) of Mo–MDSCs in the study group did not differ from healthy controls (**Figure 1B**). In contrast, frequency of LDNs/PMN–MDSCs was significantly higher in PBMCs of convalescents in comparison to healthy controls ($p < 0.0001$) (**Figure 1A**).

Therefore, in the follow–up, we focused on LDNs/PMN–MDSCs only. An elevated level of these cells had reduced individually with various rates, in three cases with the highest frequency, it reached the level of healthy donors after ca. 3 months from infection (**Figure 1C**).

Composition of the Main Subsets of T Cells Is Altered in Peripheral Blood of COVID–19 Convalescents and Includes High Level of $CD8^{+}$ T Cells With Phenotype of Exhaustion

At first, we analyzed the count of white blood cells (WBCs) and specific leukocyte populations, including neutrophils, monocytes, lymphocytes, T cells, and their main subsets, namely, $CD8^{+}$, $CD4^{+}$, and DNTs in peripheral blood of COVID–19 convalescents. This analysis revealed that WBC counts already normalized in all individuals at the time of the study. Similarly, blood counts of neutrophils, monocytes (except one case), lymphocytes, T cells, and their subsets, except one case, were in normal ranges. In some individuals the mean value of the $CD4^{+}/CD8^{+}$ ratio significantly differed from the reference interval after recovery from COVID–19 (**Figure 2A**). In the case of six convalescents, this parameter was still less than 1.5, in two cases, it was even less than 1.0, and in two other cases, it was higher than 2.5 (**Figure 2A**). These data indicate that in majority of the convalescents, $CD8^{+}$ T cells were induced by SARS–CoV–2 (**Figure 2B**). However, these $CD8^{+}$ T cells when further analyzed, showed to be positive for PD–1 expression (**Figure 2C**), suggesting their exhaustion due to stimulation by viral antigens. In parallel, we correlated the absolute numbers of circulating $CD8^{+}$, $CD4^{+}$, and DNTs with the level of LDNs/PMN–MDSCs. This analysis showed that the numbers of $CD8^{+}$ T cells and DNTs negatively correlated with the frequency of PMN–MDSCs (**Figures 2D, E**). In respect to $CD4^{+}$ T cells, such an association was not observed (data not shown). Moreover, we have noticed a positive correlation between the $CD4^{+}/CD8^{+}$ ratio and the number of circulating neutrophils (**Figure 2F**).

LDNs/PMN–MDSCs and NDNs May Affect Anti–SARS–CoV–2 Antibody Production

In the next step, we asked if LDNs/PMN–MDSCs may impact on anti–SARS–CoV–2 antibody production. To address this question, we evaluated the serum level of anti–SARS–CoV–2 S1 IgG antibodies and correlated with the frequency of LDNs/PMN–MDSCs in blood of COVID–19 convalescents. The obtained results showed a clear tendency for negative correlation between these two parameters. Additionally, similar dependency was observed in relation to neutrophils. Although these data did not reach a statistical significance (**Figures 2G, H**), the tendency suggests that LDNs/PMN–MDSCs and NDNs interfere with anti–SARS–CoV–2 antibody production in COVID–19 convalescents.

Both LDNs/PMN–MDSCs and NDNs From COVID–19 Convalescents Possess Immunosuppressive Activity

Identification of myeloid cells with regulatory activity requires, in addition to their immunophenotype characterization, also a confirmation of their suppressive nature (30). Following this requirement, the FACS–sorted LDNs/PMN–MDSCs from the blood of COVID–19 convalescents were added to the cultures of autologous anti–CD3–stimulated T cells. Simultaneously, as controls, anti–CD3–stimulated T cells from COVID–19 convalescents and healthy donors were cultured alone or in the presence of autologous NDNs, added at the same ratio. The obtained results showed that T cells from COVID–19 convalescents already have a tendency (n.s.) for diminished proliferation ability in response to stimulation (**Figure 3A**). These data, although not statistically significant, collaborate the results by others (39). Moreover, LDNs/PMN–MDSCs from the COVID–19 convalescents effectively suppressed anti–CD3–induced proliferation of autologous T cells ($p < 0.01$). Surprisingly, NDNs from the convalescents also showed suppressive activity, and this was even more pronounced compared to LDNs/PMN–MDSCs ($p < 0.0001$). On the contrary, NDNs from healthy donors' blood did not have such activity, instead, they slightly enhanced proliferation of autologous T cells stimulated with anti–CD3 mAb (**Figure 3A**).

Searching for the reason of suppression induced by neutrophil subsets from COVID–19 convalescents, we looked at the PD–L1 expression as a key marker related with such an activity and found that both populations of LDNs/PMN–MDSCs and NDNs showed high expression of immunosuppressive PD–L1 (**Figure 3B**). Further, we took an advantage from the study by Khanna et al., who described a similar phenomenon operating in mesothelioma patients, and pointed on GM–CSF as a factor promoting emergency myelopoiesis and granulocyte–related immunosuppression (40). With this in mind, we analyzed the GM–CSF level in the convalescent's sera. Results from the ELISA measurements showed that sera from COVID–19 convalescents contain significantly higher concentration of GM–CSF than sera from healthy donors (**Figure 3C**).

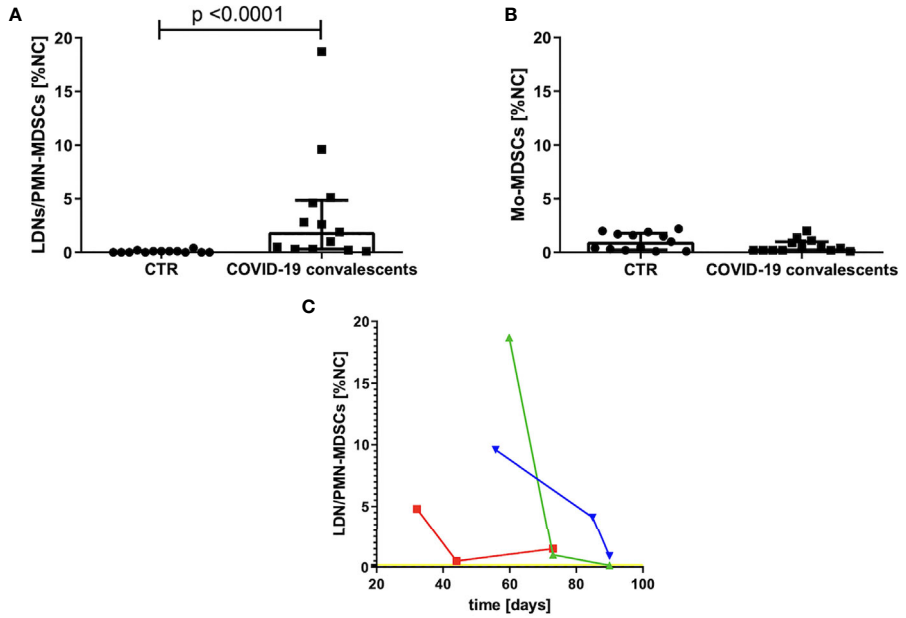

FIGURE 1 | **(A)** Frequency of LDNs/PMN–MDSCs in the blood of convalescents and healthy controls (CTR). MDSCs were identified by flow cytometry after gating according to the cell surface antigens, as described in *Materials and Methods* and presented in **Supplementary Figure S1**. Cell frequency was calculated as percentage of nucleated cells (NC) from PBMCs. **(B)** Frequency of Mo–MDSCs in the blood of convalescents and healthy controls (CTR). MDSCs were identified by flow cytometry after gating according to the cell surface antigens, as described in *Materials and Methods* and presented in **Supplementary Figure S1**. Cell frequency was calculated as percentage of nucleated cells (NC) from PBMCs. **(C)** Changes in LDNs/PMN–MDSCs frequency in peripheral blood as a function of time after COVID–19 recovery. Around day 80 from COVID–19 infection, the level of LDNs/PMN–MDSCs is dropping noticeably in the blood of convalescents. LDNs/PMN–MDSCs level was analyzed in three time points by flow cytometry as $LIN^{-}HLA–DR^{low/-}CD11b^{+}CD14^{-}CD15^{+}$ cells and calculated as percentage of nucleated cells (NC) from PBMC. Data from three patients with initial highest level of LDNs/PMN–MDSCs are presented. The average level of LDNs/PMN–MDSCs in peripheral blood of healthy donors is indicated by a yellow line.

DISCUSSION

SARS–CoV–2 infection is associated with lymphopenia and profound alterations of the myeloid compartment (32, 39). Here we showed dysfunctions of myeloid cells also in convalescents from mild/asymptomatic COVID–19. Specifically, our data suggest that propagation of LDNs/PMN–MDSCs and presence of NDNs with regulatory functions are responsible for long–term endotype of immunosuppression in this group. Recently, in the elegant study, Schulte–Schrepping et al. using scRNA–seq analysis showed in–depth COVID–19–associated alterations in monocyte and neutrophil components, documenting occurrence of immature and dysfunctional neutrophils and $HLA–DR^{low}$ monocytes during the severe course of disease (35). Accumulation of $HLA–DR^{low}$ monocytes, suggesting an impairment of antigen presentation to naive T cells in severe form of infection, has also been detected by others (3, 41–44). HLA–DR downregulation, typical for MDSCs, was also shown to immediately precede progression to severe respiratory failure (45). In opposite to severe COVID–19, in patients with mild course of infection, the $HLA–DR^{high}/CD11c^{high}$ inflammatory monocytes with an interferon–stimulated gene signature were detected (35). In our study, 1 month after infection, we observed no difference in HLA–DR expression level on monocytes, comparing convalescents to healthy donors (**Supplementary Figure S2A**), suggesting that such cells might be only temporarily present in peripheral blood. Also, Mo–MDSCs were found to expand in blood of COVID–19 patients and associate with disease severity (36), however, in the case of convalescents, we did not detect changes in the frequency of this cell subset in PBMCs, when comparing to healthy controls (**Figure 1**).

In respect to neutrophils, they comprise a heterogenous cell population, differing both in their functions and density. In pathological conditions, including infections, the presence of LDNs within the fraction of mononuclear cells in the interphase after density gradient centrifugation has been reported (28, 46), with a substantial composition of immunosuppressive PMN–MDSCs (47). Neutrophils upon activation and degranulation secrete arginase–1 and produce ROS to mediate cell suppression, indicating functional and phenotype overlap with PMN–MDSCs (48–50). In line with this, many studies have described LDNs as being composed of immature neutrophils (51), heterogeneous populations consisting of both immature and mature "neutrophil–like" populations (52, 53), and "activated/degranulated" mature neutrophils (49, 54, 55).

Several markers, including maturation, e.g., CD10, CD11b, and CD16, or activation ones, e.g., CD66b, CD64, PD–L1,

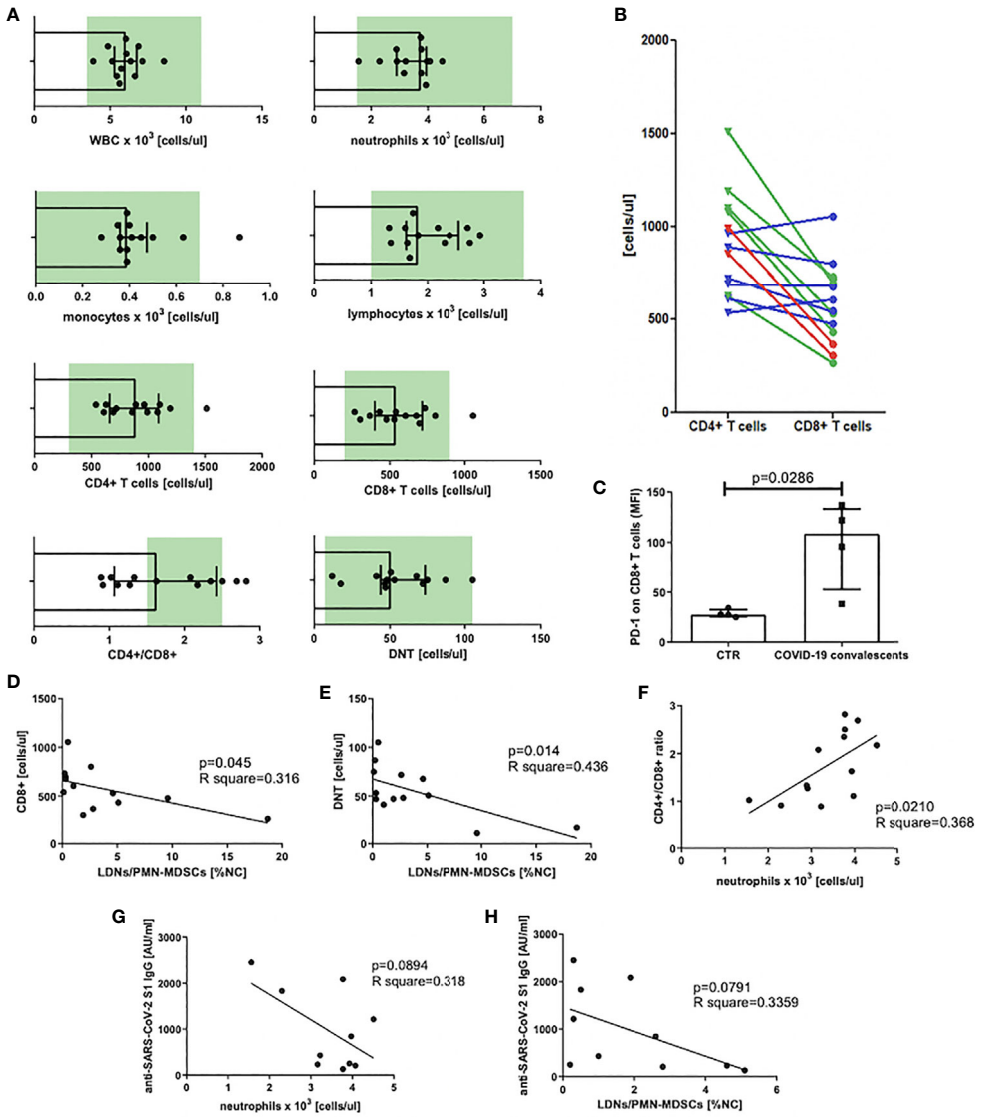

FIGURE 2 | **(A)** Blood WBC, leukocyte populations, and T–cell subset count in peripheral blood of COVID–19 convalescents with normal range marked in green. Normal range for DNTs was calculated from T cells [1%–5% of T cells, (38)] (n = 13). **(B)** $CD4^+$ and $CD8^+$ T–cell distribution in individual patients. Patients with $CD4^+/CD8^+$ ratio above the normal range are marked in red, within the norm in green, and below the norm in blue (n = 13). **(C)** PD–1 expression on $CD8^+$ T cells of COVID–19 convalescents. Expression of PD–1 on $CD8^+$ T cells of COVID–19 convalescent and healthy controls was evaluated by flow cytometry and presented as mean fluorescent intensity (MFI). Data from four subjects in each group are presented (n = 4). **(D)** Correlation of the level of LDNs/PMN–MDSCs and the count of $CD8^+$ in blood of COVID–19 convalescents. $CD8^+$ T cells were identified by flow cytometry as $CD45^+CD3^+CD8^+$ cells and their concentrations were calculated from the total lymphocyte counts (n = 13). **(E)** Correlation of the level of LDNs/PMN–MDSCs and the count of double–negative T cells (DNT) in blood of COVID–19 convalescents. Double–negative T cells (DNTs) were identified by flow cytometry as $CD45^+CD3^+CD4^-CD8^-$ and their concentrations were calculated from the total lymphocyte counts (n = 13). **(F)** Correlation of $CD4^+/CD8^+$ T cell ratio with neutrophil count in blood of COVID–19 convalescents (n = 13). **(G)** Correlation of anti SARS– CoV–2 S1 IgG antibody concentration with the number of neutrophils in COVID–19 convalescents (n = 10). **(H)** Correlation of anti–SARS CoV–2 S1 IgG antibody concentration with the frequency of LDNs/PMN–MDSCs in COVID–19 convalescents (n = 10).

CD11b, and CD16, have been proposed to differentiate neutrophil subsets in LDNs, depending on the study design (56–58). However, recent sc–RNAseq analysis in severe and mild COVID–19 patients revealed the presence of seven phenotypically distinct neutrophil clusters within LDNs (35). With this in mind, to indicate the complexity of LDNs, in the current paper, we have used the previously proposed acronym LDNs/PMN–MDSCs (28).

In the case of SARS–CoV–2 infection, myeloid cells with regulatory functions, including those of neutrophil origin, have been studied so far, mainly in terms of their effect on the duration and course of disease (23, 36, 59, 60). In this context, it has been

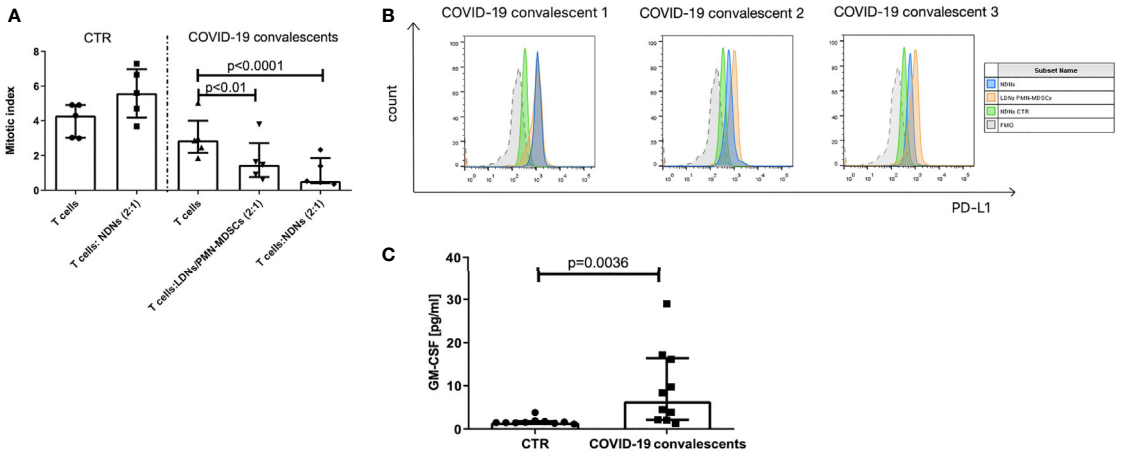

FIGURE 3 | **(A)** LDNs/PMN–MDSCs and NDNs from COVID–19 convalescents inhibit proliferation of autologous T cells. T cells were stimulated with anti–CD3 mAb for 3 days in the presence of LDNs/PMN–MDSCs or NDNs. LDNs/PMN–MDSCs were isolated by FACS as HLA–DR$^{low/-}$CD33^{+}CD66b^{+}CD14^{-} cells from PBMCs, while NDNs were sorted out as CD66b^{+} cells from the pellet obtained by density gradient centrifugation and RBC lysis. LDNs/PMN–MDSCs and NDNs were added to the culture of FACS purified autologous CD3^{+} T cells with 10% of autologous monocytes (sorted CD14^{+} HLA–DR^{+} cells). After 3 days of co–culture, T cells were pulsed with H^3–thymidine for an additional 6 h and β^{-} radiation was measured as cpm in a liquid scintillation counter. The index of proliferation was calculated as a ratio of anti–CD3 stimulated test culture [cpm] and non–stimulated culture [cpm] (n = 5). **(B)** PD–L1 expression on LDNs/PMN–MDSCs (orange) and NDNs (blue) of COVID–19 convalescent and healthy control (green). LDNs/PMN–MDSCs were gated as presented in **Supplementary Figure S1**. Individual data from three convalescents are shown. **(C)** Serum concentration of GM–CSF in COVID–19 convalescents and healthy controls (n = 10).

shown that early expansion of these cells may predict a fatal outcome of COVID–19 (18) and their level is higher in patients with severe course of infection (61). In line with this, MDSCs have been postulated as a potential biomarker and therapeutic target in COVID–19 (23). Here, we have shown that the frequency of LDNs/PMN–MDSCs is increased in the convalescents' blood even 2–3 months from mild or asymptomatic infection (e–MDSCs were not detectable at this time). A higher level of PMN–MDSCs, compared to healthy donors, was already observed in patients with mild course of disease (61), while delayed and transient expansion of this cell subset in the cohort of severely ill Japanese patients was correlated with inhibition of the harmful immune response (34). These authors even proposed the level of PMN–MDSCs as a prognostic factor for severe COVID–19 patients. At the same time, they did not notice any increase in this cell subset level in cases of mild course of COVID–19. This discrepancy may result from the use of frozen PBMCs in the abovementioned study, not recommended for the detection of this type of cells (62). In the case of our study group, decrease in the frequency of LDNs/PMN–MDSCs in PBMCs after infection was slow and was close to the level of healthy donors after ca. 3 months of infection. To the best of our knowledge, this is the first study documenting such an observation in convalescents after mild or asymptomatic infection. In the case of convalescents, we did not observe, typical for acute COVID–19 lymphopenia (63, 64), which is associated with severe disease (6, 65) and usually is reversed when patients recover (6, 66). In some patients, lymphopenia has been reported to affect CD4^{+} and CD8^{+} T cells, and other lymphocytes (12, 45, 66), whereas many data suggest that SARS–CoV–2 infection has a preferential impact on CD8^{+} T cells (63, 67). In this context, CD8^{+} T cells from our convalescents, although in a normal range for their absolute number, were positive for PD–1, known as a T–cell exhaustion marker (68). Functional exhaustion of T cells during COVID–19 has been already documented by many groups (12, 14, 37, 69).

Zheng et al. reported that elevated exhaustion levels and reduced functional diversity of T cells in peripheral blood may predict the disease progression (13). However, expression of the exhaustion markers could also reflect recent activation, and it is not clear whether T cells in patients with COVID–19 are exhausted or just highly activated (69). In keeping, some reports question the exhaustion of PD–1^{+} cells in COVID–19, suggesting that PD–1^{+} T cells are fully functional in these patients (70, 71). In our experimental settings, we did not assess function of CD8^{+} T cells, however, analysis of their concentration in peripheral blood of convalescents clearly showed a negative correlation between the two. This indirectly supports hypothesis on the regulatory effect of LDNs/PMN–MDSCs on CD8^{+} effector T cells in COVID–19 convalescents. Importantly, a drop in CD8^{+} level was associated with the severe course of disease, and posttreatment decrease in CD8^{+} T cells and increase in CD4^{+}/CD8^{+} ratio were indicated as independent predictors of poor effectiveness of therapy (71, 72).

We have noticed the imbalance in the CD4^{+}/CD8^{+} T cell ratio, which normally oscillates between 1.5 and 2.5 (72). In 46% of convalescents, this ratio was below 1.5. Such disturbances were already observed in COVID–19 patients (64). Interestingly, our research showed a strong association between CD4^{+}/CD8^{+} ratio and neutrophil count, suggesting that cells of granulocyte origin may have an impact on this parameter, most likely affecting frequency of CD8^{+} T cells. In the work by Li et al., the neutrophil

count and $CD4^+/CD8^+$ ratio were among the top five variables contributing in mild COVID–19 cases, selected using a machine learning approach (69).

Suppression of $CD8^+$ T cells by MDSCs is well documented and one of the mechanisms involved is the production of immunosuppressive cytokines, mainly TGF–β and IL–10 (73). In the case of SARS–CoV–2 infection, the decrease of blood level of DNTs was also negatively correlated with IL–10 (74). DNTs contribute to inflammation and were found to act as regulatory and/or cytotoxic T cells (75). Given that the level of DNTs decreases in the initial stage of infection and it correlates with fever in COVID–19 (76), these cells can be assigned a cytotoxic role in SARS–CoV–2 infection. Additionally, MDSCs constitute a source of IL–10 (73) and the level of IL–10 is elevated in COVID–19 patients (12). In a current study, we have shown a negative correlation between DNTs count and frequency of LDNs/PMN–MDSCs. This observation indirectly supports previous data on the role of IL–10 in reducing the number of DNTs (74) and suggests such a mechanism operating in COVID–19.

Although humoral immune response may also be hampered by MDSCs (77), there is no direct evidence on the diminished antibody production by B cells during COVID–19 (37). In our study, we have shown a tendency for negative correlation between LDNs/PMN–MDSC level and the concentration of anti–SARS–CoV–2 neutralizing IgG antibody in the convalescents after 20–60 days from infection. In line with this, it was documented that the level of the spike protein–specific memory B cells increases around 30–60 days after infection (78). Although we have no formal proof, it is tempting to speculate that this might be accompanied by normalization of the LDNs/PMN–MDSCs level observed in our study. Tentatively, the phenomenon could be explained by antibody–dependent enhancement if complexes of neutralizing antibodies and viral antigens were bound to FcγRII. This aspect, however, needs to be further investigated.

While the suppressive nature of LDNs/PMN–MDSCs is not surprising, detection of the similar activity of NDNs was unexpected. In our experimental settings, NDNs from COVID–19 convalescents exhibited robust suppressive activity on proliferation of autologous T cells, and this was even stronger than mediated by LDNs/PMN–MDSCs. Such NDNs have been already described in cancer patients (40, 79) and patients with severe COVID–19, where the presence of dysfunctional neutrophils, including LDNs, was linked to emergency myelopoiesis (35). Several studies have identified emergency myelopoiesis as a hallmark of fatal COVID–19 (42, 80) and particularly neutrophil counts were found to be significantly elevated in patients with COVID–19 and correlated with disease severity (81, 82). In this context, it is worth mentioning that in differential analysis, both LDNs/MN–MDSCs and NDNs are counted as peripheral blood neutrophils, affecting the neutrophil–to–lymphocyte ratio in COVID–19 patients. The use of HLA–DR, CD16, CD64, and CD66b markers was able only to indicate the presence of activated neutrophils within the LDNs/PMN–MDSCs (slight increase in HLA–DR and CD66b expression, and presence of $CD64^{high}$ cells) and more mature $CD16^+$ subset within NDNs but did not allow one to precisely distinguish the composition of the two. In respect to HLA–DR, its expression on LDNs/PMN–MDSCs (characteristic feature of these cells) of convalescents seems to further decrease over time (**Supplementary Figure S2**).

Interestingly, neutrophils from patients with severe SARS–CoV–2 infection featured expression of genes related to suppressive functionality, including *ARG1* and *CD274* (PD–L1) (83), while culture supernatants from neutrophils isolated from COVID–19 patients inhibited T–cell proliferation (84). It was also postulated that activated neutrophils may exert myeloid–derived suppressor cell activity (48). In our study, both LDNs/PMN–MDSCs and NDNs were positive for the surface expression of PD–L1, indicating its role in direct cell–to–cell mediated immunosuppression of T–cell response. This, however, does not exclude the involvement of other suppressive–like molecules released by these cell subsets and operating in COVID–19 patients, e.g., arginase–1 or ROS (22, 33, 85). This mechanism could explain a more potent suppressive nature of NDNs despite their generally lower expression of PD–L1, compared to LDNs/PMN–MDSCs. Altogether, our data suggest that in case of COVID–19 convalescents, NDNs and LDNs/PMN–MDSCs may differ in activation/maturation status and mechanism of suppressive activity, with a common pathway involving PD–L1 expression. However, whether and to what degree NDNs display properties like those described for LDNs is still unclear (29).

The mechanism responsible for neutrophil dysfunction further leading to T–cell suppression may be related to GM–CSF activity (40). GM–CSF is an emergency myelopoiesis cytokine and may induce neutrophil (hyper)–activation and degranulation (86) through STAT3 phosphorylation (87). In addition, both GM–CSF and STAT3 are associated with the induction of neutrophils with regulatory functions (88). In this context, an increased GM–CSF concentration in convalescents observed in our study, also shown by others in COVID–19 patients (89), may be responsible for neutrophil (hyper)–activation and induction of their suppressive activity. In this context, we cannot exclude the role of other cytokines, e.g., IL–6, TNF–α, and IL–1, responsible for "cytokine storm" associated with several detrimental clinical features of COVID–19 in patients with severe course of disease. In the case of convalescents from mild/asymptomatic COVID–19, these cytokines (in significantly lower concentrations) could be involved in secondary activation of "immature" myeloid cells, further developing their regulatory functions (90). Recent data by Chu et al. showing no difference in serum cytokine concentrations between the mildly and more severely affected COVID–19 patients 6 weeks after infection seem to support this scenario (91), however, the levels of respective cytokines were not compared to healthy subjects.

In conclusion, although our group of subjects was small, we were able to show that in convalescents from COVID–19 after 2–3 months from infection, both LDNs/PMN–MDSCs and NDNs possess immunosuppressive properties against T cells. Transient expansion of LDNs/PMN–MDSCs and dysfunction of NDNs after asymptomatic and mild course of SARS–CoV–2 infection may be caused by GM–CSF production and upregulation of PD–L1 expression, leading to prolonged immunosuppression in COVID–19 convalescents. However, in the light of the current

controversy in definition of myeloid regulatory cell populations (31), the precise origin of immunosuppressive LDNs, altered granulopoiesis, and/or regulatory properties acquired by NDNs in response to SARS–CoV–2 infection and long–COVID remains to be determined.

DATA AVAILABILITY STATEMENT

Upon reasonable request, the raw data supporting the results of this article will be made available by the authors without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Bioethical Committee of the Jagiellonian University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IS and JB conceived the study. IS performed most of the experiments, analyzed data, and wrote the draft version of the manuscript. KW performed FACS sorting. MSu performed GM–CSF analysis. DK–B performed anti–SARS–CoV–2 S1 IgG testing. MSa and JB contributed to funding acquisition. MSa and MSi revised the manuscript. JB and IS wrote the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.748097/full#supplementary-material

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Blood Transcriptomes of Anti-SARS-CoV-2 Antibody-Positive Healthy Individuals Who Experienced Asymptomatic *Versus* Clinical Infection

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The reasons behind the clinical variability of SARS-CoV-2 infection, ranging from asymptomatic infection to lethal disease, are still unclear. We performed genome-wide transcriptional whole-blood RNA sequencing, bioinformatics analysis and PCR validation to test the hypothesis that immune response-related gene signatures reflecting baseline may differ between healthy individuals, with an equally robust antibody response, who experienced an entirely asymptomatic (n=17) *versus* clinical SARS-CoV-2 infection (n=15) in the past months (mean of 14 weeks). Among 12.789 protein-coding genes analysed, we identified six and nine genes with significantly decreased or increased expression, respectively, in those with prior asymptomatic infection relatively to those with clinical infection. All six genes with decreased expression (*IFIT3, IFI44L, RSAD2, FOLR3, PI3, ALOX15*), are involved in innate immune response while the first two are interferon-induced proteins. Among genes with increased expression six are involved in immune response (*GZMH, CLEC1B, CLEC12A*), viral mRNA translation (*GCAT*), energy metabolism (*CACNA2D2*) and oxidative stress response (*ENC1*). Notably, 8/15 differentially expressed genes are regulated by interferons. Our results suggest that subtle differences at baseline expression of innate immunity-related genes may be associated with an asymptomatic disease course in SARS-CoV-2 infection. Whether a

certain gene signature predicts, or not, those who will develop a more efficient immune response upon exposure to SARS-CoV-2, with implications for prioritization for vaccination, warrant further study.

Keywords: innate immunity, anti-SARS-CoV2 antibody, asympomatic, RNAseq, whole-blood

INTRODUCTION

Since December 2019 the SARS-CoV-2 has spread throughout the world infecting dozens of millions of people and resulting in over 2.8 million deaths, as of April 2021. Although the case fatality rate in hospitalized patients may exceed 10% (1, 2), 35-50% of infected adults do not develop, perceive and report any clinical symptom (3, 4). Asymptomatic infected persons may be responsible for viral transmission for more days than aware self-isolated cases, which may also explain, at least partially, the exponential increase in the number of infections globally (5–7). Notably, we only know in retrospect who was indeed asymptomatic, since individuals without symptoms at the time of a positive molecular test should be followed for 14 days to determine the clinical picture, being "pre-symptomatic" if they develop symptoms later.

The proportion of asymptomatic individuals varies widely among different viral infections, whereas relevant biomarkers do not currently exist due to our limited knowledge of the molecular host-pathogen interactions and immune response to particular infections (8). For example, a significant fraction of cytomegalovirus infections, similarly to SARS-CoV-2, are asymptomatic and unsuspected (9). In contrast, measles infected individuals are very rarely asymptomatic (10). The reasons why certain individuals, including even people living with HIV (11) or other immunodeficiencies (12), do not develop clinical symptoms during SARS-CoV-2 infection are essentially unknown (13, 14). So far, studies assessing the immune response during asymptomatic infection are few. In an elegant study, Long et al. showed that asymptomatic individuals presented with significantly longer duration of viral shedding compared to symptomatic patients, lower levels of IgG antibodies to SARS-CoV-2, and lower serum levels of 18/48 cytokines, including interferon-gamma levels, suggesting that asymptomatic individuals indeed displayed a weaker anti-virus-reactive immune response to SARS-CoV-2 (15). More recently, Chan et al. also showed in a whole blood transcriptomic analyses that asymptomatic patients display a less robust response to type-I interferon than symptomatic patients, whereas differences between asymptomatic and symptomatic patients may be present at the cellular, innate, and adaptive immune response levels (16)

While the role of genetics in determining immune and clinical response to the SARS-CoV-2 virus is currently under investigation, it is well established that individual human immune systems are highly variable (17). Most of this inter-individual immune variation is explained by environmental exposures early in life (18) but genetic factors are clearly also involved. For example, a gene expression signature dominated by interferon-inducible genes in the blood is prominent in systemic lupus erythematosus (19), whereas interferon-α is increased not only in the serum of these patients but also in their healthy first-degree relatives (20) pointing to genetic influences on the interferon-mediated immune interactions.

Clearly, the most successful immune response against SARS-CoV-2 occurs in those individuals who, while remaining asymptomatic, develop a robust adaptive response. This is not always the case since antibodies are not detected in a proportion of asymptomatic infections (21). We have recently examined the humoral immune response to SARS-CoV-2 in members of the National and Kapodistrian University of Athens, Greece (22). Overall, among 4.996 people the unweighted seroprevalence of SARS-CoV-2 antibodies was 1.58%, whereas 49% of the seropositive individuals denied having had any clinical symptom compatible with previous SARS-CoV-2 infection, which was also unsuspected for 33% of them. Interestingly, in our study, the mean levels of antibodies to both the nucleocapsid (N) protein and the receptor-binding-domain (RBD) of the spike (S) protein were comparable between asymptomatic and clinical infection cases and not associated with age or sex (4). Others have also reported that IgG antibodies are commonly observed in both asymptomatic and clinical infections (85% *versus* 94% of patients, respectively) (23), whereas durable B cell-mediated immunity against SARS-CoV-2 after mild or severe disease occurs in most individuals (24).

To further study COVID pathogenesis, herein we aimed to identify mRNA expression patterns that could serve as baseline correlates for development, or not,of clinical symptoms following contact with SARS-CoV-2. Since variations in the strength and/or extent of the immune response may be critical for the clinical picture and progress after infection with SARS-CoV-2, existing inter-individual differences at the transcriptome level may be observed even later, after convalescence. Indeed, gene expression pattern in blood samples collected 21 days after influenza infection are indistinguishable from baseline (25). Therefore, we performed 3' mRNA next generation sequencing-based genome-wide transcriptional whole blood profiling to test the hypothesis that the baseline mRNA expression of theimmune response-related genes are differentially expressed between healthy individuals who developed an equally robust antibody response following either an entirely asymptomatic or clinical SARS-CoV-2 infection.

MATERIALS AND METHODS

Blood Collection and Anti-SARS-CoV-2 Antibody Testing

Blood samples were collected from members of the NKUA, Athens, Greece in June–November 2020. The protocol was

approved by the Ethics and Bioethics Committee of the School of Medicine, NKUA (protocol #312/02-06-2020) and study participants provided written informed consent. All plasma samples were analyzed as previously described (4) using, a) the CE-IVD Roche Elecsys® Anti-SARS-CoV-2 test, an electrochemiluminescence immunoassay (ECLIA) for the detection of total antibodies (IgG, IgM, and IgA; pan-Ig) to SARS-CoV-2 N-protein (Roche Diagnostics GmbH, Mannheim, Germany), and b) the CE-IVD Roche Elecsys® Anti-SARS-CoV-2 S, an ECLIA for the quantitative determination of antibodies (including IgGs) to the SARS-CoV-2 S-protein RBD (Roche Diagnostics).

3' mRNA Sequencing, Mapping, Quality Control, and Quantifications

Total RNA was isolated from whole blood, stored in paxgene, using the ExtractionMonarch® Total RNA Miniprep Kit (NEB #T2010). Upon blood isolation, Monarch DNA/RNA Protection Reagent (supplied as a 2x concentrate) was added undiluted to an equal volume of blood. Addition of the protection reagent and the following RNA isolation was performed as described in the kit's manual for Total RNA Purification from Mammalian Whole Blood Samples.

After quantification on a NanoDrop ND-1000 (Thermofisher) and Bioanalyzer RNA 6000 Nano assay (Agilent), 140-300ng of total RNA from samples passing quality control were processed using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD (Lexogen, 015.96) for library preparation. Libraries were assessed for molarity and median library size using Bioanalyzer High Sensitivity DNA Analysis (Agilent, 5067-4626). After multiplexing and addition of 13% PhiX Control v3 (Illumina, FC-110-3001) as spike in, the NGS was performed on a NextSeq550 with NextSeq 500/550 High Output Kit v2.5 - 75 cycles (Illumina, 20024906).

The quality of FASTQ files was assessed using FastQC (version 0.11.9) (26). The reads were mapped to the GRCh38 reference human genome using STAR, as part of a pipeline provided by Lexogen and BlueBee. After quality control, we obtained quantifications for 16.737 (12.789 protein coding) genes with more than five reads in more than 25% of the 17 asymptomatic and 15 clinical disease samples. Raw bam files, one for each sample, were summarized to a 3'UTR read counts table, using the Bioconductor package GenomicRanges (27), through metaseqR2 (28). The gene counts table was normalized for inherent systematic or experimental biases (e.g., sequencing depth, gene length, GC content bias) using the Bioconductor package EDASeq (29). For the downstream analysis, 12 hemoglobin (*HBQ1, HBG2, HBZ, HBA2, HBA1, HBM, HBZP1, HBE1, HBG1, HBD, HBBP1, HBB*) genes were removed from all samples.

Blood Immune Cell Subsets Deconvolution

CIBERSORTx (30) was used to estimate the proportion of blood immune cell subsets for each individual. As a signature matrix, the LM22 signature matrix for 22 subsets obtained at the single cell level was used. The Mann-Whitney U test was applied in order to calculate the significance of the difference in distributions between the asymptomatic and clinical groups. Statistical significance calculation and plotting were applied with R.

Differential Gene Expression

The resulting gene counts table was subjected to differential expression analysis (DEA) to compare individuals with a history of asymptomatic *versus* clinical ("symptomatic") infection using the Bioconductor packages DESeq (31), edgeR (32), NOISeq (33), limma (34), NBPSeq (35), baySeq (36). In order to combine the statistical significance from multiple algorithms and perform meta-analysis, the PANDORA weighted P-value across results method was applied through metaseqR2. The weighted meta p-value was used as metric for the statistical significance for the differentially expressed genes. Multidimensional scaling was also applied through metaseqR2. DAVID analysis (37) was performed for the increased and decreased genes, both for enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and for biological processes [Gene Ontology (GO)]. For the prediction of enriched regulons in asymptomatic disease we used the TRRUST (v2) reference transcription factor (TF)–target interaction database (38) and enrichR (39) focusing on the ChEA prediction with the increased genes in asymptomatic disease as input. For the identification of interferon-regulated genes the interferome database (v2) (40) was used.

Real Time PCR

Validation of the gene expression signatures was performed not only on the 32 samples for which the RNAseq propocol was followed, but also for 9 additional available samples (5 and 4 from prior asymptomatic or clinical infection, respectively). Briefly, 1000 ng starting material (or 500 ng for the samples with limited available RNA) was reverse transcribed to cDNA using a Takara PrimeScript RT Reagent Kit (Takara RR037A), following the manufacturer's protocol. Real-time quantitative PCR (qPCR) was performed using the SYBR™ Select Master Mix, Applied Biosystems, ThermoFisher Scientific on the LightCycler® 96, Roche. Primers specific for each gene were designed with Primer Blast, while GAPDH served as the housekeeping gene (**Supplementary Table S1**.). All samples were measured in duplicates. Relative expression of each sample was defined as 2^-DCt, where DCt = Ct (gene target) – Ct (housekeeping gene).

RESULTS

Whole Blood Transcriptional Profiling and Determination of Immune Cell Subsets in Healthy Seropositive Individuals With Prior Asymptomatic *Versus* Clinical Infection Time PCR

As shown in **Table 1**, the two groups under study comprised 15 seropositive individuals (9 men, mean age 34 years) who experienced clinical infection 7 to 25 weeks before sampling (mean 13; SD5.3)and 17 seropositive individuals (11 men, mean age 37 years)with entirely asymptomatic infection. Of those with

TABLE 1 | Demographics and antibody measurements.

	Number of Individuals (males)	Age, mean ± SD (range)	Interval (weeks, mean ± SD) between sampling and symptoms or putative SARS-CoV-2 exposure	anti-SARS-CoV-2 N-protein Abs, mean ± SD (range)	anti- SARS-CoV-2 S-protein RBD Abs, mean ± SD (range)
Clinical Disease	15 (9)	34 ± 14 (18-57)	13 ± 5.3	38 ± 39 (5-119)*	179 ± 255 (6-752)
Asymptomatic Disease	17 (11)	37 ± 17 (19-70)	14 ± 6.8	46 ± 45 (1-166)**	122 ± 131 (3-426)***

n = 14, n = 15**, n = 14***.*

Age, sex distribution and levels of antibodies to both SARS-CoV-2 N-protein and the S-protein RBD were comparable between asymptomatic and clinical cases.

clinical infection, 12/15 experienced fever and only one was in need of hospitalization which was uneventful. Individuals were considered asymptomatic in the absence of any symptoms since the onset of the pandemic, according to a detailed history obtained by a physician (absence of fever of any grade, fatigue, conjunctivitis/sweating coughs, headaches, respiratory distress/dyspnea, smell or taste loss, diarrhea). These individuals reported putative exposure to SARS-CoV-2 5 to 21 weeks before sampling (mean 14; SD 6.8).

Whole blood-derived, 3' mRNA next generation sequencing-based, genome-wide transcriptional profiling was performed and, overall, more than 386 million reads were generated. Genes with fewer than five counts in fewer than 25% of the samples were filtered out, resulting to 16.737 profiled genes, of which 12.789 were protein coding. Twelve hemoglobin genes (*HBQ1, HBG2, HBZ, HBA2, HBA1, HBM, HBZP1, HBE1, HBG1, HBD, HBBP1, HBB*) were removed. A multidimensional scaling (MDS) plot generated using all 16.737 expressed genes, in order to avoid gene-type biases, revealed no clear separation of the two sample groups (**Figure 1A**).

The proportions of immune cell populations, namely, naive B cells, memory B cells, plasma cells, CD8+ T cells, naive CD4+ T cells, resting memory CD4+ T cells, activated memory CD4+ T cells, follicular helper T cells, regulatory T cells, gamma delta T cells, resting NK cells, activated NK cells, monocytes, M0 macrophages, M1 macrophages, M2 macrophages, resting dendritic cells, activated dendritic cells, resting mast cells, activated mast cells, eosinophils and neutrophils in the peripheral blood estimated by CIBERSORTx were also comparable between the two groups (**Figure 1B**).

Differentially Expressed Genes Are Associated With Innate Immunity and Interferon Activity

Although the differential expression analysis of 12.777 protein coding genes did not reveal a distinct transcriptional profile between the two groups of healthy individuals, 24 genes were returned as differentially expressed (logFC=|1|, meta p-value<0.05) in a primary analysis (**Supplementary Figure S1**). Because the number of these genes was small, we repetitively applied the DEA pipeline, removing samples that were possible outliers in terms of expression of each differentially expressed gene. Therefore, genes that were repeatedly returned as significantly differentially expressed in those with prior asymptomatic infection relatively to those with clinical SARS-CoV-2 infection were characterized as differently expressed (**Figure 2**). Brief description of the function of six and nine genes that were found significantly decreased (**Supplementary Table S2**) and increased (**Supplementary Table S3**), respectively, in prior asymptomatic *versus* clinical SARS-CoV-2 infection is shown in **Supplementary Tables**.

In order to validate our DGEA results we performed SYBR Green-based qPCR to quantify the mRNA expression of 5 randomly selected differentially expressed genes Indeed, all 5 genes showed the same pattern of expression as in the RNA-sequencing experiment, reaching statistical significance in 4 genes, despite the small sample size (**Supplementary Figure S1**).The statistical significance remained excluding the 9 additional samples that were not included in the RNAseq run from the PCR validation (data not shown).

Notably, all six decreased genes in asymptomatic SARS-CoV-2 infection (*IFIT3, IFI44L, FOLR3, RSAD2, PI3, ALOX15*), are involved in innate immune responses (41–45) while the first two are interferon-inducible genes. Similarly, three increased genes (*GZMH, CLEC1B, CLEC12A*) are involved in innate immunity mechanisms (43, 46, 47), one (*GCAT*) in viral mRNA translation (48), one (*CACNA2D2*) in the integration of energy metabolism (49) and one (*ENC1*) in oxidative stress responses (50). The expression patterns of these 15 genes across all samples are depicted in **Figure 2**. Enrichment analysis returned no statistically significant enriched KEGG or GO terms. Similarly, there were no common upstream transcriptional regulators revealed by transcription factor (TF)–target interaction databases for these genes.

Furthermore, the interferome database which hosts genomic and transcriptomic data generated from cells or tissues treated with interferons was used for the 15 genes that were found to be differently expressed in asymptomatic *versus* clinical SARS-CoV-2 infections. Collectively, 8 out of 15 genes are regulated by interferons (*ENC1, FOLR3, IFIT3, PI3, RSAD2, IFI44L, CLEC12A, ALOX15*). Specifically, six genes are regulated by both type I and type II Interferons (*ENC1, FOLR3, IFIT3, PI3, RSAD2, IFI44L*), whereas the remaining two are targets of interferon type II only (*CLEC12A, ALOX15*) (40) (**Figure 3**).

DISCUSSION

Genome-wide transcriptome analyses studies using next generation sequencing technology in patients infected with

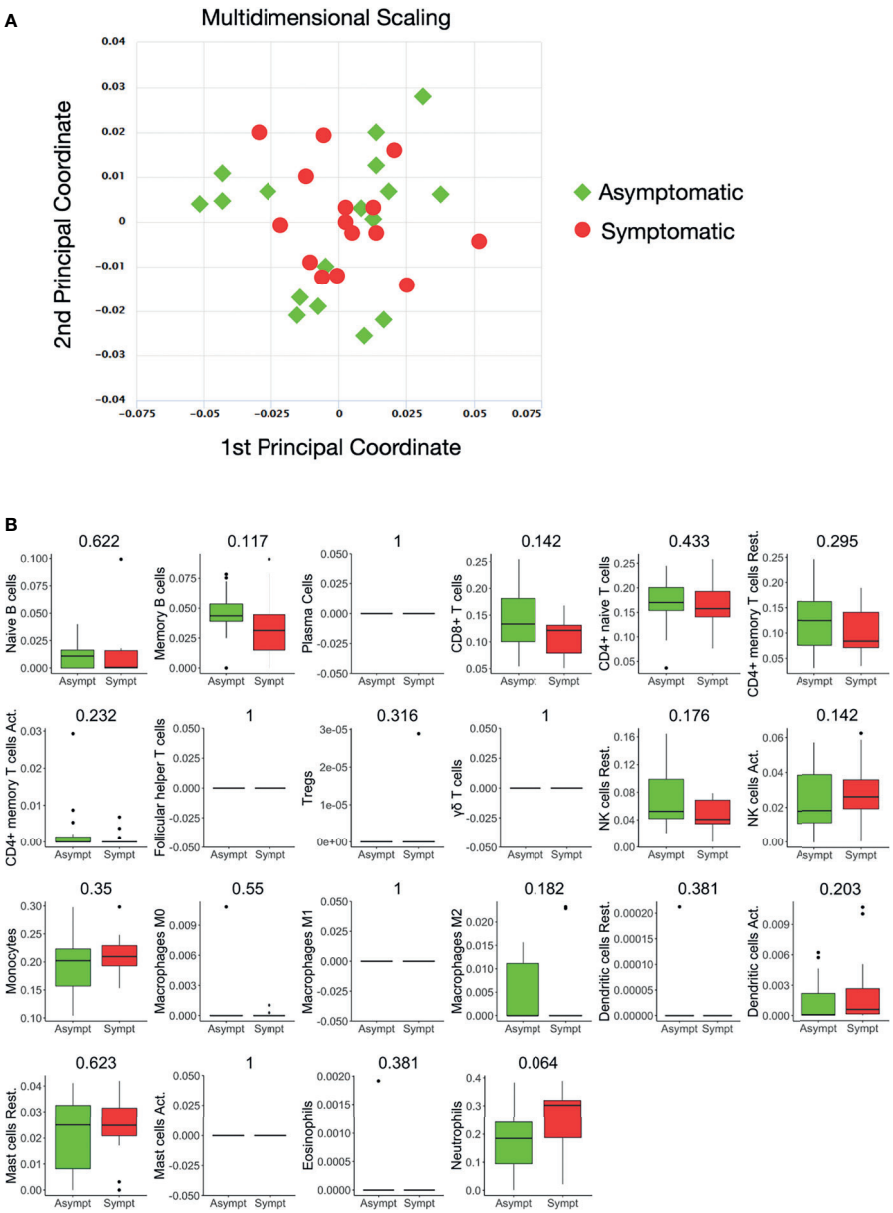

FIGURE 1 | Whole blood transcriptional profiles and immune cell subsets in seropositive healthy individuals with prior asymptomatic or clinical SARS-CoV-2 infection. **(A)** Dimensionality reduction of all samples: Multidimensional scaling of all samples from individuals with prior clinical (n=15) and prior asymptomatic (n=17) infection. Each dot corresponds to the sample of one individual. All expressed elements were used (16.737, out of which 12.789 were non-zero protein-coding genes), in order to avoid gene type biases. The smaller the distance between each sample pair, the greater the similarity of the gene expression profile of the samples. No separation of the two sample groups is revealed, reflecting their similarity. **(B)** Blood transcriptome deconvolution with CYBERSORTx in prior asymptomatic (AS) and prior clinical (CL) disease groups. For every cell type, the Mann-Whitney U test p-value comparing the two groups is displayed on top. No statistically significan differences (meta p-value< 0.05) were detected between the two groups.

SARS-CoV-2 provide evidence that transcriptome-wide changes may serve as predictors of morbidity and possibly of response to specific therapies (51). In addition, transcriptomic analyses may provide mechanistic insights into certain complications associated with SARS-CoV-2 infection (52). To our knowledge, this is the first whole blood genome-wide transcriptomic comparative analysis in seropositive healthy individuals who either experienced a clinical SARS-CoV-2 infection or an entirely asymptomatic infection around 3 months before sampling and developed an equally robust antibody response. In one previous study published so far in asymptomatic seropositive individuals infected in a super spreading event, the transcriptome in peripheral blood mononuclear cells was similar to that of seronegative highly exposed individuals from the same

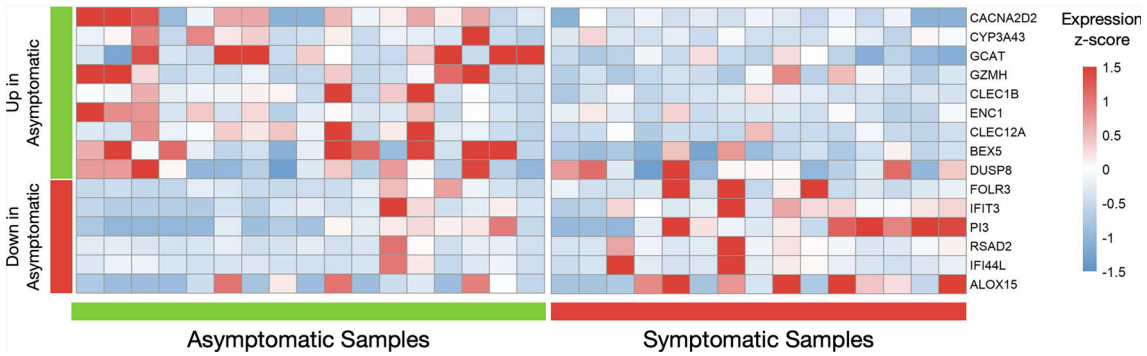

FIGURE 2 | Differential gene expression analysis in seropositive healthy individuals with prior asymptomatic or clinical SARS-CoV-2 infection. Heatmap of robustly differentially expressed genes (genes that were differentially expressed and highly expressed in three or more samples, logFC>|1|, meta p-value<0.05) in individuals with prior asymptomatic infection relatively to those with clinical ("symptomatic") SARS-CoV-2 infection, with raw expression values being scaled. The values for all samples (17 asymptomatic on the left and 15 clinical on the right) is plotted. The first nine genes are increased in the Asymptomatic group, while the next six are decreased.

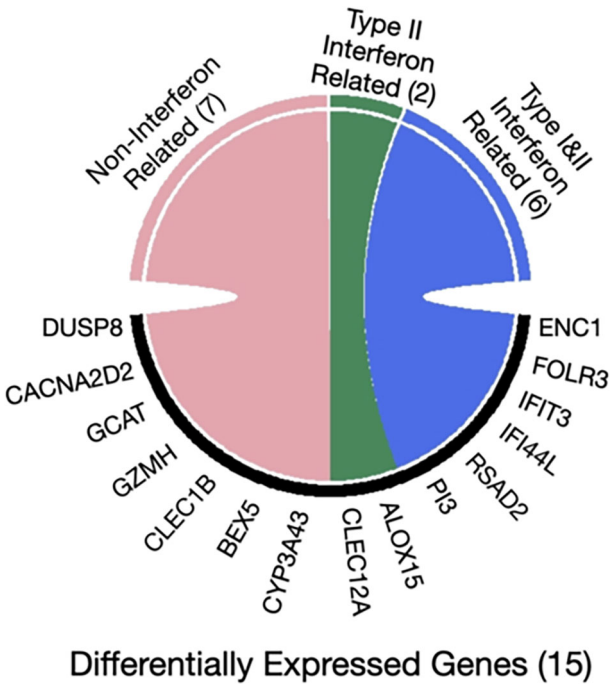

FIGURE 3 | Chord diagram of differentially expressed genes in seropositive healthy individuals with prior asymptomatic or clinical SARS-CoV-2 infection with respect to interferon activity. The 15 genes characterized as differentially expressed in those with prior asymptomatic relatively to those with clinical infection were queried in the Interferome database and 8/15 were found to be associated with interferon activity. Of those, 6 are regulated by both interferon type I and II (blue), while 2 genes only by type II (green) and none by type III; the 7 remaining genes are not regulated by interferons (pink).

community. The putative time of infection of seropositive asymptomatic individuals was 4–6 weeks prior to sample collection, suggesting that the development of antibody response following viral exposure in asymptomatic cases is not necessarily associated with sustained alterations in the immune transcriptome (53).

Variations in innate immune system responses and cytokine networks could explain, at least in part, the wide heterogeneity in clinical presentation of SARS-CoV-2 infection (54). The symptom that best reflects the potency of the immune response, namely fever, has been repeatedly shown to be a poor diagnostic marker in severe disease (55, 56). Along these lines, it has been speculated that asymptomatic infection could be partly explained by the examples of altered innate immunity mechanisms operating in bats and pangolins. Despite carrying an enormous load of viral species, these animals display an apparent genetic resistance to coronavirus pathology (57). For example, decomposition of many type I interferon genes (58) and partial loss of function in stimulator of interferon genes (STING) is observed in bats (59). Regarding pangolins, recent findings suggest that these animals have lost interferon-ϵ (60) as well as interferon-induced with helicase C domain 1 (IFIH1), also known as IFIH1/MDA5 (61).

Our results provide evidence that among 12.777 genes, there were only 15 with significantly different expression when comparing healthy, relatively young individuals after convalescence from a previous entirely asymptomatic SARS-CoV-2 infection to those with a clinical infection history. While there were no apparent differences in cellular components and no specific immune deficiencies or co-morbidities to explain the different clinical presentation, the small, only, number of differentially expressed genes is expected since the cohort comprised healthy individuals at the time of sampling, who experienced in the past a SARS-CoV-2 infection. The small number of differentially expressed genes was also the reason why further bioinformatics analysis, i.e. enrichment and/or functionality analysis could not be applied. It should be highlighted that the transcriptome analysis was performed several weeks after the time of active infection; thus, certain potential differential responses may have been blunted during assessment after infection. This could also explain the

limitation of the absence of differentially expressed genes with >2-fold change in our primary analysis. However, such differential responses should be more robust at the time of infection and more genes and immune networks may be differentially expressed.

Among the six genes that were found with significantly decreased expression in previously asymptomatic cases relatively to clinical cases, and in line with our research hypothesis, all are involved in innate immune responses (**Supplementary Table S2**) and two of these genes (*IFIT3, IFI44L*) belong to the interferon-induced family of genes. Overall, 8 of the 15 differentially expressed genes in those with prior asymptomatic infection relatively to those with clinical SARS-CoV-2 infection can be found in datasets that include genes which have been implicated in interferon related signaling pathways *in vitro* (38). A detailed explanation according the functionality of those genes and the pathways though which they act requires further studies. However, on the basis of these findings some assumptions can be made, since, indeed, the first 6 genes with higher expression in individuals with clinical infection compared to asymptomatic individuals, namely *IFIT3, IFI44L, FOLR3, RSAD3, PI3, and ALOX15*, share some common characteristics that can be relevant. For example, *IFIT3, IFI44L and RSAD3* expression can be induced by viruses which in turn enhances the progress of the viral infection (62–64), whereas higher intrinsic expression of *IFIT3, FOLR3, PI3* and *ALOX-15* have all been associated with immune-mediated chronic diseases (65–68) In contrast, a protective effect of *GZMH, CLEC1B, CLEC12A*, that have a higher expression in asymptomatic individuals, may be associated with the effectiveness of *GZMH* in viral eradication (69) and the ability of *CLEC1B* and *CLEC12A* to enhance neutrophil extracellular trap formation, thus presenting an antiviral effect that helps to control systemic virus levels (70). Despite the fact that our findings have to be validated in a larger independent cohort of prior SARS-CoV-2 infected individuals, taken together with those of the literature support the hypothesis that there are differences in the innate immune responses between clinical and asymptomatic individuals during SARS-CoV-2 infections (15, 16).

As happens in all viral infections, type I interferon response plays a major protective role for the host because not only promotes viral clearance but also triggers a prolonged adaptive immune response (71). Insights into the innate and adaptive immune responses to SARS-CoV-2 have been gained by many research efforts over the past year (52). The innate immune responses that protect against disease and particularly the role of type I and III interferons have been addressed in numerous studies, mainly in patients with severe disease at the time of sampling. Important findings by Casanova and collaborators have shown that either neutralizing autoantibodies to type I interferons (72) or deleterious mutations in components involved in interferon induction or signaling (17) predispose patients to life-threatening infections. Along these lines, a highly impaired type I interferon response has been reported in patients with severe disease (73). However, in contrast to these findings, increased levels of interferons and interferon-stimulated genes have been observed in severe and life-threatening infections in many other studies (74–76). Indeed, increased interferon-alpha levels are a biomarker of mortality (77).

Moreover, the SARS-CoV-2 receptor ACE2, which is expressed in specific cell subsets across tissues is an interferon-stimulated gene in human airway epithelial cells (78), suggesting that a weaker individual interferon response may be protective. The latter may explain the low infection levels and morbidity in children (55, 56) who, relative to adults, display, in general lower interferon responses (79) and lower ACE2 expression (80). Taken together, in individuals infected with SARS-CoV-2, interferon-mediated responses may be protective or detrimental depending on the timing and the stage of infection, in addition to other factors, including viral load, age, and co-morbidities (71, 81, 82).

To conclude, our results suggest that subtle differences in the expression levels of innate immunity-related genes, including lower expression of genes involved in interferon signaling, may be beneficial for the host upon SARS-CoV-2 infection. The current study attempts to fill the existing gap regarding the potential implication of certain pathways in the clinical phenotype of SARS-CoV-2 infection. The described association of a 'weaker' immune response to SARS-CoV-2 with a lack of clinical symptoms needs further investigation, which hopefully will be performed in the near future. Whether a certain innate immunity signature predicts, or not, those who will develop a more successful immune response upon contact with SARS-CoV-2, with possible implications for prioritization of vaccination, warrant further study.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE173317.

ETHICS STATEMENT

The protocol was approved by the Ethics and Bioethics Committee of the School of Medicine, NKUA (protocol #312/02-06-2020). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, PS, and MD. Methodology, PS and KMV. Formal analysis and investigation, PS and KMV. Writing - original draft preparation, PS. Writing - review and editing, PS, KMV, GA-M, OT, DP, EK, EL, ParM, ET, IT, VC, MM, PanM, GP, GK, PH, and MD. Funding acquisition, PS and MD. Resources, OT, EL, ParM, ET, and IT. Supervision: PS and MD. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.746203/full#supplementary-material

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SARS-CoV-2 and HIV-1: Should HIV-1-Infected Individuals in Sub-Saharan Africa Be Considered a Priority Group for the COVID-19 Vaccines?

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Since its emergence in 2019 SARS-CoV-2 has proven to have a higher level of morbidity and mortality compared to the other prevailing coronaviruses. Although initially most African countries were spared from the devastating effect of SARS-CoV-2, at present almost every country has been affected. Although no association has been established between being HIV-1-infected and being more vulnerable to contracting COVID-19, HIV-1-infected individuals have a greater risk of developing severe COVID-19 and of COVID-19 related mortality. The rapid development of the various types of COVID-19 vaccines has gone a long way in mitigating the devastating effects of the virus and has controlled its spread. However, global vaccine deployment has been uneven particularly in Africa. The emergence of SARS-CoV-2 variants, such as Beta and Delta, which seem to show some subtle resistance to the existing vaccines, suggests COVID-19 will still be a high-risk infection for years. In this review we report on the current impact of COVID-19 on HIV-1-infected individuals from an immunological perspective and attempt to make a case for prioritising COVID-19 vaccination for those living with HIV-1 in Sub-Saharan Africa (SSA) countries like Malawi as one way of minimising the impact of COVID-19 in these countries.

Keywords: COVID-19, HIV, immunity, vaccine, Sub-Sahara Africa

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INTRODUCTION

Coronaviruses have been in existence since time immemorial and six coronaviruses are known to cause disease in humans (1, 2). Four of these human coronaviruses (hCoV), 229E, HKU1, NL63 and OC43, cause infections attributed as the common cold and are endemic in different parts of the world (1). However, the other two have been major health concerns with severe acute respiratory syndrome coronavirus (SARS-CoV) and the Middle East respiratory syndrome coronavirus (MERS-CoV) emerging in 2002 and 2012, respectively (3).

The first case of COVID-19 (coronavirus disease) caused by SARS-CoV-2 was reported to the World Health Organisation (WHO) by the Chinese authorities on 31st December 2019 (4, 5). Since its emergence SARS-CoV-2 has proven to be more lethal than the other hCoV with the WHO declaring it a global pandemic in March 2020 (6, 7). Globally, as of October 2021, there have been over 240 million cases and over 4.8 million deaths (8, 9).

SARS-CoV-2 has however not spread evenly throughout the world with countries like Italy, the UK, the USA, Iran, Brazil and India being the most affected countries (9). A report published in November 2020 showed that SARS-CoV-2 incidences appeared low in most African countries recording less than 4% of the global cases and deaths but in a background of limited COVID-19 testing (10) with African countries conducting the least tests (8, 9). Furthermore, as of September 2021, SSA, which has a population of 1.15 billion (about 14% of the global population) (11), had only received 2% of the world's COVID-19 vaccine supply with most of the countries not reaching the target of vaccinating 10% of their population (12). In this review we report on the impact COVID-19 on HIV-1-infected individuals from an immunological perspective. We emphasize the need to prioritise COVID-19 vaccination for HIV-1-infected individuals in order to reduce the burden of COVID-19 upon low-resource countries in SSA such as Malawi.

COVID-19 IN MALAWI AND AFRICA

The first COVID-19 cases in Malawi were confirmed in April 2020 (13) and these were three cases, one index case and two local transmission cases. As of 17th October 2021, there had been 61,716 confirmed cases and 2,292 deaths (14, 15). Of the confirmed cases, the average age was 36 years and 66.9% were male (14). Among the confirmed COVID-19 deaths, the average age was 56.7 years and 82.5% were male (9, 14). While initial COVID-19 cases were primarily imported, the number of local transmissions surpassed imported cases by July 2020 (14). The lower than expected burden of COVID-19 on the African continent massively contradicted various projections which had been calculated at the onset of the pandemic (16, 17). Various theories, ranging from genetics to BCG vaccine administration, lower testing rate compared to other countries, have been proposed to explain the lower than expected burden of the disease on the continent (18–21) but these are yet to be proven.

Although the vast majority of those who get infected with COVID-19 remain asymptomatic or merely manifest mild flu-like symptoms, some individuals develop life-threatening severe disease and require hospitalization or long COVID-19 disease (15, 22, 23). For the original variant first detected in Wuhan, China (4, 5), the main risk factor for developing severe COVID-19 disease and COVID-19-related mortality was age with those aged 65 years or more being at higher risk (24). Other risk factors included being male, having other underlying conditions such as diabetes, severe asthma, smoking, blood group and obesity (24, 25). However, lately some variants, especially the Delta, are causing disease even amongst the young (26).

Although Malawi as a country has lower rates of COVID-19 infection compared to other African countries, HIV-1/AIDS prevalence is still high. With a population of close to 17 million, the HIV-1 prevalence amongst individuals aged between 15 and 64 years is 10.6% (27–29). This rate is quite similar to other African countries like South Africa with the national prevalence rate of 12.2% with approximately 6.4 million living with HIV-1 (30) but lower than that of Eswatini (formerly Swaziland) which is estimated at 26% (31) (**Figure 1**).

Recent studies have not established any association between being HIV-1-infected and being more vulnerable to contracting COVID-19 (32), but have shown that being infected with HIV-1 is a risk factor for developing severe COVID-19 and for COVID-19-related mortality (33–35). It is not known how many of the cases of COVID-19 and COVID-related deaths in Malawi were people living with HIV-1 (15). Since the pandemic started over 90 vaccine candidates have been developed. Malawi, like most other African countries, started receiving the Vaxzevria/Oxford-AstraZeneca vaccine through the COVAX initiative with 360,000 doses delivered in March 2021 with more doses in August 2021 supplemented with doses of the Johnson and Johnson's Jansen vaccine (36). Despite this, by 17th October 2021 only 3.01% Malawians had been fully vaccinated (12).

IMMUNOLOGY OF COVID-19, VIRUS MUTATION, AND VACCINES

SARS-CoV-2 gains entry into human cells by binding to the Angiotensin Converting Enzyme 2 (ACE2) receptor, which is expressed by various cells including lung epithelial cells (37). Entry of virus into the body triggers the host immune system starting with the innate immune cells which recognise the molecular patterns associated with the virus (38). Two recent reviews (39, 40) provide detailed outlines of the different immune components that are involved in the human response against SARS-CoV-2 infection.

At the innate immune response level, a significant increase of monocytes and macrophages has been observed in individuals infected with SARS-CoV-2 with the macrophages infiltrating the lungs and secreting inflammatory cytokines such as IL-6 and IL-1β (41)Infected individuals have also been observed to have a suppressed type 1 IFN response, which is fundamental in the fight against viral infections (41). In contrast, other immune cells such as eosinophils were observed to be much lower than normal in those infected with SARS-CoV-2 (42) whereas mast cells were reported to secrete inflammatory cytokines such as IL-1, IL-6, IL-33 and other mediators such as histamine and protease (43, 44). Decreased cell counts of natural killer (NK) cells were observed in individuals infected with SARS-CoV-2 and these predominantly expressed an exhaustion phenotype (45). Failure of the innate immune response to eliminate the virus will normally lead to the activation of the adaptive immune system with T and B cells involved. The CD4+ and CD8+ T cell responses target all parts of the SARS-CoV-2 proteome with CD4+ T cells dominating the response (46, 47).

The severity of COVID-19 patients has been associated with a skewed CD4+ T cell response to cytotoxic CD4+ T follicular helper (Tfh) cells, reduced regulatory T cells (48) and a less coordinated CD4+ and CD8+ T cell response (47). In infected individuals B cell subsets were observed to be lower than normal but the actual amount of SARS-CoV-2 specific IgG produced was

SE Africa	6.70	87	83	90	2.00
Malawi	10.60	90	86	92	2.89
South Africa	12.20	92	75	92	16.57
Kenya	4.50	90	82	92	4.08
Uganda	5.80	89	94	90	2.97
Lesotho	22.80	93	71	93	8.88
Eswatini	26.00	>95	>95	>95	11.35
Zambia	11.30	87	89	75	2.23
Zimbabwe	12.80	90	94	86	18.85
Botswana	20.70	92	89	>95	16.35
Tanzania	4.80	83	90	92	0.80

FIGURE 1 | Progress made by various countries in the Sub-Saharan Africa towards achieving the 90-90-90 Targets as of 2020 (70) and COVID-19 percentage coverage for individuals who had been fully vaccinated, regardless of HIV status, in each country as of September 2021 (71).

high (49). Antibodies are thought to be protective with convalescent plasma and neutralizing monoclonal antibodies used as treatment for COVID-19 patients (50, 51). However, the antibody titre levels against the receptor binding domain and anti-spike neutralizing antibodies were reportedly higher in more severely affected patients compared to mildly ill patients (52). This may also be a consequence of prolonged infection.

>The efficacy levels reported for each vaccine type are those observed against the original strain of SARS-CoV-2 initially detected in Wuhan, China. However, since then several different variants of the virus have emerged (53). The Alpha variant, first documented in the UK in September 2020, became dominant, but has now been superseded by the Delta variant that emerged from India in October 2020 (53). Other variants of concern (VOC) are the Beta variant and the Gamma variant (53). The emergence of these new variants has questioned whether the various COVID-19 vaccines currently in use would maintain their efficacies.

One recent study reported reduced efficacy of the Pfizer vaccine against the Alpha and Beta variants (54) with others hypothesizing that some of the vaccines will drastically lose their efficacy against the new variants (55). The clinically approved human monoclonal antibody treatments, bamlanivimab and etesevimab, were unable to neutralize the Beta variant (56). Furthermore, both the Beta and Delta variants were more resistant to neutralization to sera from Moderna/Spikevax, Comirnaty/Pfizer-BioNTech and Vaxzevria/Oxford-Astra Zeneca vaccines than the Alpha variant (57).

One of the main concerns with the COVID-19 vaccines has been whether their efficacy could be affected when administered to HIV-1-infected individuals due to their immunocompromised status (58). However, results of two recent studies investigating the efficacy of the AstraZeneca COVID-19 vaccine in the UK and South Africa reported that there were no differences in terms of vaccine-related antibody or T cell-mediated responses between HIV-1-infected participants and HIV-1-negative controls at any stage of the vaccination process (58, 59). The studies also showed that antibody responses were not affected by the CD4 T cell count in the HIV-1-infected individuals (58, 59). Similar results were

reported by a group working on a nanoparticle vaccine, Novavax, although the researchers still propose that more work needs to be done in HIV-1-infected individuals with CD4 T cell counts lower than 350 cells/μl blood and with detectable viral loads (60).

HIV-1 AND IMMUNE DYSFUNCTION

It has been shown that years of untreated HIV-1 infection before commencing combination antiretroviral treatment (cART) can substantially affect the length of time the immune system needs to fully recover (61). Thus, it is beneficial for HIV-1+ patients to start cART as early as possible (62). An environment of activation, dysfunction and inflammation pervades the immune system during untreated HIV-1 infection. Chronic immune activation of T cells occurs through persistent depletion and expansion of T cells accompanied with over-expression of CD38 and HLA-DR (62). In B cells there is an over-production of autoantibodies, increased expression of activation markers but also dysfunctional responses to T cell help and a loss of memory B cells (63). The innate immune system shows raised levels of IL-1, IL-6, TNF and C-reactive protein (CRP) amongst other things (62). Meanwhile CD4+ Tfh cells are expanded in untreated HIV-1, which leads to changes in certain B cell populations, an increase of germinal B cells, fewer memory B cells and more BCL6 transcriptional repressor (64).

Furthermore, B cell vaccine responses to influenza, HPV and yellow fever are attenuated in HIV-1 patients (65–67). During HIV-1 infection, there is also loss of the mucosal barrier leading to microbial translocation demonstrated through the increased levels of soluble CD14 and soluble CD163 (62). This drives activation of the innate immune system, such as macrophages along with abnormally raised levels of pro-inflammatory cytokines such as IFN-α, TNF-α, IL-1, IL-6 and IL-18 (62). One of the roles of plasmacytoid DCs (pDC) is to produce IFN-α and IFN-β during early stages of a viral infection. Unfortunately, HIV-1 infection impairs the function of pDC and reduces their frequency (68).

The introduction of cART in the late nineties has had a major impact on the lives of millions of HIV-1+ patients increasing life expectancy to non-infected levels. However, depending largely on the CD4+ T cell count before cART commencement (61), full immune restoration is not always attained such that as many as 16% may not attain CD4+ T cell counts greater than 200 cells/μl blood after four years of treatment (69).

According to UNAIDS, 83% (with a range of 60-92%) of HIV-1-infected individuals in eastern and southern Africa had access to cART in 2020 (70). For Malawi, it was estimated that 88% had access to cART and 92% had suppressed viral loads. The respective percentages for some selected SSA countries are presented in **Figure 1**. Based on these figures, it is clear that a significant population of HIV-1+ patients in SSA are potentially vulnerable to further HIV-1 associated opportunistic infections and other pathogens such as SARS-CoV-2.

While cART does have an effect on reducing T cell activation, it does not completely abolish all chronic immune activation as there are still elevated levels of IL-6, CRP, D-dimer and sCD14 (72). The frequency of Tfh cells drops to normal levels under cART but there is an over-representation of germinal centre B cells and an under-representation of memory B cells (72). Meanwhile, the innate immune functionality of pDC remains affected in patients on cART, which may compromise the anti-viral response (68). Myeloid dendritic cells frequencies appear normal before and during cART but their ability to skew towards to Th1 responses is impaired (73).

Hearps and colleagues showed that monocytes have impaired phagocytic activity in cART patients and they resemble those of elderly HIV-1-negative subjects (74).

Natural killer (NK) cells play a major role against viral pathogens producing IFN-γ and killing virally-infected cells through antibody-dependent cellular cytotoxicity (ADCC). However, in HIV-1+ patients their ability to performed ADCC is compromised and persists under cART (75). Therefore, all these factors may affect the ability of HIV-1+ patients to respond to further viral infections including SARS-CoV-2 especially if the CD4+ T cell counts of the affected individuals have not yet "normalized" in the course of being on cART.

Specifically, if plasma levels of IL-6 are already elevated in cART patients, some have speculated that such individuals would be more likely to get more severe symptoms of COVID-19 (76). One of the major risk factors of mortality in COVID-19 is age (24) and it has been suggested that many of the immuno-cellular disturbances associated with HIV-1 infection have similarities with immune systems in the elderly (77). The hallmarks of immune systems in the elderly consist of declining frequencies of naive T cells and hematopoietic progenitor cells with heightened levels of inflammation, which all have parallels with HIV-1 affected immune systems (77).

Although, multiple factors contribute to pathology associated with COVID-19, an aged immune system because of a HIV-1 infection may not be beneficial. Currently, several developed countries are already administering COVID-19 vaccine booster shots to individuals aged 65 years and above and those who are immunocompromised (78). Considering the similarities of the immune systems, comparable requirements might also be essential for HIV-1-infected patients.

COVID-19 IN HIV-1-INFECTED INDIVIDUALS

As mentioned earlier, no study so far has established any link between being HIV-1-infected and being more vulnerable to contracting COVID-19 (32). What is known though is that being infected with HIV-1 is a risk factor for developing severe COVID-19 (79) and for COVID-19 related mortality (33–35). Furthermore, a recent case report in South Africa revealed the development of over ten new SARS-CoV-2 variants in one individual who presented with untreated HIV-1 infection and had been co-infected with SARS-CoV-2 (80). The authors proposed that untreated HIV-1 infection might provide a fertile environment that favours intra-host mutation of SARS-CoV-2 (80).

With a seemingly waning efficacy of the current vaccines against some of the SARS-CoV-2 variants (54–57), a scenario whereby the current vaccines become ineffectual with time is a real possibility. The recent reports of individuals who had been infected with two different SARS-CoV-2 variants (81–83) suggest that it is possible for some individuals to be concurrently infected by more than one variant of SARS-CoV-2 and subsequently more virulent variants could emerge through recombination (84). With COVID-19 vaccines proving to be just as effective in HIV-1-infected individuals, prioritising inoculation of this group might be one of the ways of mitigating variant development. The WHO provides guidelines on the prioritization of vaccine administration if supplies are limited (85). The potential risk of intra-host mutation development in HIV-1-infected individuals reported in South Africa (80) may serve as an additional justification for the WHO to move this population group further up the COVID-19 vaccination priority list. If this observation is repeated in other studies involving both untreated and treated HIV-1-infected individuals in countries where HIV/AIDS prevalence is high, countries might wish to prioritise all HIV-1-infected individuals to be vaccinated against SARS-CoV-2 in order to keep the variants development in check.

CHALLENGES AHEAD

As long as there is significant community transmission taking place, the virus will continue to mutate and variants emerge (84). One such variant, C.1.2, was first detected in May 2021 in Mpumalanga South Africa and by August 2021 it had spread to various provinces in the country (84). Although much about the C.1.2 variant in terms of virulence and transmissibility is yet to be fully elucidated, its discovery emphasises the point that in countries where a good proportion of the population is unvaccinated, VOCs will continue to arise posing a threat to the world at large. As it has been shown with the Delta variant, the efficacy of the current COVID-19 vaccines against the new variants tends to be compromised (54, 55). In the event that a new variant emerges on the global scene, which is completely resistant to all current vaccines, it would derail the fight against SARS-CoV-2 and downgrade the gains so far attained. As more countries gradually but cautiously lift travel bans from high-risk countries, the risk of new vaccine-resistant variants spreading to different parts of the world remains high (86).

Of major concern is the recently observed disparity in vaccine coverage between developed countries, which have already attained over 75% vaccine coverage, and most African countries (**Figure 1**) which, on average, have only achieved 2% coverage (87). This so-called "vaccine apartheid" phenomenon could be exacerbated if the current trend of more developed countries proceeding with the implementation of proposed "booster" or third jab (88, 89). These vaccines could have been more beneficial, and more effective against the pandemic, if administered either as first or second jabs in developing countries. The recent report by WHO (90) on some developed countries having even a greater access towards the vaccines originally meant for developing countries under the COVAX initiative makes this scenario even worse. Meanwhile the ensuing low COVID-19 vaccination coverage in Africa (71) still provides a potentially conducive environment for the development of new SARS-CoV-2 variants.

While it is clear that the majority of unvaccinated individuals survive primary infection without the need for hospitalization (91) what remains to be fully elucidated are the specific immune correlates of protection against SARS-CoV-2 (92–94). The pandemic will continue until further studies reveal the veritable correlates of protection against SARS-CoV-2 and/or the whole world is immunized, including those most vulnerable to severe COVID-19 such as those living with HIV-1. Waning vaccine-induced antibodies (95) and emerging new variants (54, 55, 79) will inadvertently prolong the period during which vaccine booster jabs and vaccines against VOC will be required.

The introduction of the Extended Program on Immunization (EPI) by WHO back in 1974 has been one of the success stories in the region (96). Given, the necessary support, SSA countries are capable of contributing substantially to the global fight against COVID-19.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to this work in writing, reviewing, and editing.

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Analysis of Serological Biomarkers of SARS-CoV-2 Infection in Convalescent Samples From Severe, Moderate and Mild COVID-19 Cases

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Precision monitoring of antibody responses during the COVID-19 pandemic is increasingly important during large scale vaccine rollout and rise in prevalence of *Severe Acute Respiratory Syndrome-related Coronavirus-2* (SARS-CoV-2) variants of concern (VOC). Equally important is defining Correlates of Protection (CoP) for SARS-CoV-2 infection and COVID-19 disease. Data from epidemiological studies and vaccine trials identified virus neutralising antibodies (Nab) and SARS-CoV-2 antigen-specific (notably RBD and S) binding antibodies as candidate CoP. In this study, we used the World Health Organisation (WHO) international standard to benchmark neutralising antibody responses and a large panel of binding antibody assays to compare convalescent sera obtained from: a) COVID-19 patients; b) SARS-CoV-2 seropositive healthcare workers (HCW) and c) seronegative HCW. The ultimate aim of this study is to identify biomarkers of humoral immunity that could be used to differentiate severe from mild or asymptomatic SARS-CoV-2 infections. Some of these biomarkers could be used to define CoP in further serological studies using samples from vaccination breakthrough and/or re-infection cases. Whenever suitable, the antibody levels of the samples studied were expressed in International Units (IU) for virus neutralisation assays or in Binding Antibody Units (BAU) for ELISA tests. In this work we used commercial and non-commercial antibody binding assays; a lateral flow test for detection of SARS-CoV-2-specific IgG/IgM; a high throughput

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multiplexed particle flow cytometry assay for SARS-CoV-2 Spike (S), Nucleocapsid (N) and Receptor Binding Domain (RBD) proteins); a multiplex antigen semi-automated immunoblotting assay measuring IgM, IgA and IgG; a pseudotyped microneutralisation test (pMN) and an electroporation-dependent neutralisation assay (EDNA). Our results indicate that overall, severe COVID-19 patients showed statistically significantly higher levels of SARS-CoV-2-specific neutralising antibodies (average 1029 IU/ml) than those observed in seropositive HCW with mild or asymptomatic infections (379 IU/ml) and that clinical severity scoring, based on WHO guidelines was tightly correlated with neutralisation and RBD/S antibodies. In addition, there was a positive correlation between severity, N-antibody assays and intracellular virus neutralisation.

Keywords: COVID-19, SARS-CoV-2, Serological biomarkers, Antibodies, WHO International Standard, Correlates of Protection, COVID-19 immune response

1 INTRODUCTION

From the moment the World Health Organisation (WHO) declared COVID-19 a Public Health Emergency of International Concern (1), SARS-CoV-2 continued its global spread and caused more than 3 million deaths up to April 2021 (2). A total of 184 candidate vaccines are now in pre-clinical development and 92 have entered the clinical phase. Of the latter, seven vaccines have been approved by National Regulatory Authorities in different parts of the world and WHO have issued Emergency Use Listing for four of these (3). All these developments occurred in less than a year, thanks to the unprecedented joint effort made by the scientific community, WHO and other international public-health entities, the pharmaceutical Industry and philanthropic organisations. Because a defined Correlate of Protection (CoP) to COVID-19 did not exist, and still remains elusive, the efficacy of these vaccines was evaluated in large placebo-controlled clinical trials involving large numbers of participants exposed naturally to SARS-CoV-2 in countries that had active COVID-19 epidemics (4). Though successful, this process was very costly and logistically demanding. The last few months of the pandemic are being characterised by the emergence of SARS-CoV-2 variants that carry mutations that resulted in increased transmissibility, increased pathogenicity or increased potential to evade the immune response of the host (5). Determining the vaccine efficacy against these variants of concern (VOC) is of high priority for regulatory bodies and vaccine manufacturers in the coming months or perhaps years.

In the absence of a universally accepted CoP against COVID-19, data from Phase III clinical trials suggested that virus neutralising antibodies (nAb) are a candidate CoP (6). Likewise, observations made on the natural history of COVID-19 indicated the association of nAb and protection (7). However, the protective threshold for nAb has been difficult to establish in different settings. Early studies comparing clinical progression and case fatality rates found that the magnitude of the antibody response correlated with the severity of disease (8–10). Patients with fatal outcomes often had the strongest IgG responses to nucleoprotein (N), and were often accompanied by marked responses to the Spike (S) protein (11). Furthermore, patients with severe disease have also been reported to have high nAb titres (12, 13), with studies showing a strong correlation between live-virus or pseudotype based micro-neutralisation (pMN) and anti-Spike antibody binding assays (10, 14).

Cellular immune responses directed against internal viral antigens often play an important role in the clearance of viral infections. The effector mechanisms of anti-viral immunity of non-neutralising antibodies are becoming better understood (15, 16) and it is often that these are directed against viral internal antigens, such as the N antigen of SARS-CoV-2. One of these mechanisms is mediated by the cytosolic antibody receptor TRIM21 (17), which captures antibody-antigen complexes and accelerates their degradation and processing through the proteasome, facilitating the loading of antigenic peptides in nascent MHC molecules and promoting antigen presentation to T cells (18). While the latter study focused on antibodies against the nucleoprotein of the enveloped positive strand RNA virus LCMV, it is possible that antibodies directed against the N antigen of SARS-CoV-2 function in a similar way. We therefore analysed TRIM21-mediated biomarkers of immunity in our cohorts using established methods (19) to determine the holistic role of antibodies in protection from COVID-19 disease.

One of the factors that have precluded the derivation of well-defined humoral CoP in general, and to COVID-19 disease in particular, is the diverse number of quantitative antibody assays available and the different units used to quantify the antibody levels of clinical samples. Measuring nAb against SARS-CoV-2 is typically done by virus neutralisation tests such as plaque reduction neutralisation test (PRNT), infectious centre assays or micro-neutralisation tests (20–24). These are performed with live SARS-CoV-2 or with pseudotyped viruses (typically lentivirus or vesicular stomatitis virus) displaying SARS-CoV-2 Spike protein in their envelope. The antibody levels of these assays are quantified in serum titres for specific percentages of neutralisation (i.e. $PRNT_{50}$, $PRNT_{80}$), or as IC_{50} or other readouts. The choice of antibody binding assays is also varied, from the traditional ELISA format to more refined commercial assays (ECLIA, multiplexed micro-sphere assays, semi-automated immunoblotting assays). These methods use the

Spike protein or sub-domains thereof or internal nucleoprotein as target antigens. The readouts of these assays are expressed using a diverse suite of units such as antibody titre, OD (optical density) values for specific wave lengths (450 nm, 490 nm) or mean fluorescent intensity (MFI) units or Chemiluminescent units (MCI). In order to harmonise results of quantitative COVID-19 immuno-assays, the WHO has advocated the use of the 'International Standard for SARS-CoV-2 immunoglobulin' (NIBSC code '20/136') as a primary assay calibrant (25, 26). We used this reagent and its assigned unitage (1000 units/ml) as a reference to derive, in International Units (IU), the potency of our 'in-house' internal assay calibrants. In this way, antibody levels of samples tested by either neutralisation of antibody binding assays can be expressed in IU or Binding Antibody Units per ml (BAU/ml) and thus immunogenicity and vaccine efficacy data can be compared between different laboratories.

The Humoral Immune Correlates for COVID-19 Project (HICC) aims to dissect the humoral immune response to SARS-CoV-2 and identify the mechanisms of immunity that protect from COVID-19 and to distinguish them from pro-inflammatory and complement responses leading to severe disease. The specific aim of the present study is to define antibody-based biomarkers that differentiate severe from mild/asymptomatic COVID-19 cases. These antibody based parameters can also be used to define CoP in vaccine breakthrough and/or re-infection studies. Wherever possible, we expressed these antibody measurements in IU or BAU. Towards this objective, we have analysed the antibody levels and antigenic specificity of convalescent antibody samples of HCWs and hospitalised COVID-19 patients exposed and infected during the first pandemic wave (between March 2020 – October 2020) in the UK. This study defines the methods and findings establishing a benchmark for future longitudinal studies to define COVID-19 CoP.

2 MATERIALS AND METHODS

2.1 Selection of Sera and Plasma

Serum and plasma samples were obtained from healthcare workers (HCW) and patients referred to the Royal Papworth Hospital, Cambridge, UK for critical care. COVID-19 patients hospitalised during the first wave and as well as NHS healthcare workers working at the Royal Papworth Hospital in Cambridge, UK, served as the exposed HCW cohort (Study approved by Research Ethics Committee Wales, IRAS: 96194 12/WA/0148. Amendment 5). NHS HCW participants from the Royal Papworth Hospital were recruited through staff email over the course of two months (20th April 2020-10th June 2020) as part of a prospective study to establish seroprevalence and immune correlates of protective immunity to SARS-CoV-2. Patients were recruited in convalescence either pre-discharge or at the first post-discharge clinical review. All participants provided written, informed consent prior before enrolment in the study. Sera from NHS HCW and patients were collected between July and September 2020, 3-5 months after they were enrolled in the study.

Clinical assessment and WHO criteria scoring of severity for both patients and NHS HCW (**Table 1**) was conducted following the 'COVID-19 Clinical Management: living guidance' (27).

For cross-sectional comparison, representative convalescent serum and plasma samples were collected from seronegative HCWs, seropositive HCW and convalescent PCR-positive COVID-19 patients. The serological screening to classify convalescent HCW as positive or negative was done according to the results provided by a UKAS-accredited Luminex assay detecting N-, RBD- and S-specific IgG, a lateral flow diagnostic test (IgG/IgM) and an Electro-chemiluminescence assay (ECLIA) detecting N- and S-specific IgG. Any sample that produced a positive result by any of these assays was classified as positive. The severity score of the individuals from which the sample was obtained ranged from 0 to 7 according to the WHO classification described above. Thus, the panel of convalescent serum samples (3-5 months post-infection) were grouped in three categories: a) Patients (n=38); b) Seropositive HCW (n=24 samples); and c) Seronegative HCW (n=39) (**Table 2**).

2.2 Internal and External Calibration Reagents

The reference reagents used as external, or primary calibrants in our assays included: a) the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (NIBSC 20/136); b) the Anti-SARS-CoV-2 Antibody Diagnostic Calibrant (NIBSC 20/162; and c) the Research Reagent for anti-SARS-CoV-2 Ab (NIBSC 20/130). Details of these are described in the NIBSC catalogue (28).

TABLE 1A | Severity score classification.

Severity Code	Severity Name	Description
1	Asymptomatic	
2	Mild Disease	Case definition without of COVID-19 without pneumonia
3	Moderate pneumonia	Fever, cough, dyspnoea, SpO_2 >90%
4	Severe pneumonia	Fever, dyspnoea, cough plus RR>30, SpO_2 <90%requirement;
5	ARDS (Acute Respiratory Distress Syndrome)	diffuse bilateral infiltrates PaO2/FiO2<300
6	Sepsis	Life-threatening organ disfunction: severe dyspnoea, delirium, low O2 saturation, oliguria, tachycardia, weak pulse, low blood pressure, coagulopathy
7	Septic Shock	As above plus Vasopressor requirement

TABLE 1B | Cohort demographic and severity score classification.

	Symptom Severity Score						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Patients	2	3	0	15	1	2	15
HCW-P	4	12	8	0	0	0	0
HCW-N	22	13	3	0	0	0	0

We used these external reference reagents to calculate the unitage of tested samples and/or to calibrate our own Internal (or secondary) assay calibrants. The latter were obtained from NHS healthcare workers exposed to SARS-CoV-2. Thus, HICC Serum-1 and HICC Serum-2 were pooled serum samples collected from RT-PCR-confirmed SARS-CoV-2-infected NHS personnel 2 months after presenting moderate symptoms of COVID-19.

2.3 Pre-Pandemic Plasma

A panel of 23 pre-pandemic plasma collected between 2016 and 2019, obtained from the National Institute of Biological Standards and Control (NIBSC), was used to set up the negative cut-off point of the quantitative immunoblotting assay, the pan-Ig N- and RBD-ELISA and the pMN assays.

2.4 Detection of Total Antibody (Pan-Ig) Against SARS-CoV-2 Spike (S) and Nucleocapsid (N) Antigens by ELISA

Two different ELISA tests were used for the detection of N-specific and S-specific antibodies. The assays were adapted from those originally described by Amanat and co-workers (29). Briefly, Nunc MaxiSorp™ flat-bottom plates were coated with 50 µl per well of 1 µg/ml of either RBD or N antigen in DPSB (-Ca^{2+}/-Mg^{2+}) and incubated overnight at 4°C. The next day, the plates were blocked with 3% milk in PBST (0.1% w/v Tween20 in PBS) for 1 hour. After removing the blocking buffer, 50 µl/well of serum samples, diluted in PBST-NFM (1% w/w non-fat milk in PBST) were added to the plates and incubated on a plate shaker for two hours at 20°C. The plates were washed three times with 200 ml of PBST, and 50 ml of HRP-conjugated goat anti-human Ig (H and L chains) (Jackson ImmunoResearch) diluted 1:3000 in PBST was added to each well and left to incubate for one hour on a plate shaker for 1 hour. Plates were washed three times with 200 µl of PBST and 50 µl/well of 1-Step Ultra TMB chromogenic substrate (Sigma) were added to the plates and the chemical reaction was stopped three minutes later with 50 µl 2N H_2SO_4. The optical density at a wavelength of 450 nm (OD450) was measured using a Biorad microplate reader.

All test runs included, in addition to the test sample dilutions, an internal calibrant dilution series (HICC Serum 2), a single dilution of a positive control per plate (NIBSC 20/130), a negative control sample (NIBSC 15/288) per plate and a blank control (no primary antibody or sample). All samples were tested in duplicate and the duplicate readings were used to fit the standard curve. The blank readings were subtracted from the serum sample values. The IC_{50} values of each sample dilution series were determined and expressed as relative potency respect to the Internal Calibrant, for which a unitage in ELISA binding units was calculated using the WHO International Standard 20/136 as a reference. Details of how these were calculated are described in the 'Results' section.

2.5 Roche Elecsys® Electrochemiluminescence Immunoassay (ECLIA)

Samples were tested on Roche cobas® e801 analyser at PHE Porton Down. Anti-nucleocapsid protein antibodies were detected using the qualitative Roche Elecsys® Anti-SARS-CoV-2 (ACOV2) ECLIA (Product code: 09203079190), whilst anti-RBD antibodies were detected using the quantitative Roche Elecsys® Anti-SARS-CoV-2 S (ACOV2 S) ECLIA (Product code 092892751902), as previously described (30, 31). Both assays detect total antibodies (IgG, IgA and IgM). All kits were calibrated based on a two-point calibration curve according to the manufacturer's instructions, with daily QC performed per reagent pack. Anti-NP results are expressed as a cut-off index (COI) value, with a COI ≥1 interpreted as positive. Anti-spike results are expressed as units per ml (U/ml), with results of ≥ 0.8 U/ml interpreted as positive and a quantitative range of 0.4 to 2,500 U/ml.

2.6 Detection of SARS-CoV-2-S, -RBD and -N specific Antibodies Using a Multiplex Bead Flow Cytometry Platform, Luminex™ Platform

Detection of serum IgG reactive to SARS-CoV-2 N, S and RBD (receptor binding domain) antigens was done using a Luminex based assay following the methods previously described (32, 33). The amino acid sequences used derived from the S ectodomain

TABLE 2 | Cohorts. Classification of participants according to the serological screening of the sera by the ECLIA, multiplex Micros-sphere, and lateral flow assays.

Assay Platform	**Antigen/Isotype**	**Patients**			**Seropositive Staff**			**Seronegative Staff**		
		Pos.	**Neg.**	**ND**	**Pos.**	**Neg.**	**ND**	**Pos.**	**Neg.**	**ND**
Luminex	N	36	2	0	22	2	0	0	38	1
	S	36	2	0	19	5	0	0	38	1
	RBD	36	2	0	18	6	0	0	38	1
Roche	RBD	34	2	2	18	6	0	0	36	3
	N	34	2	2	18	6	0	0	36	3
Lateral Flow	IgG	35	2	1	20	4	0	0	39	0
	IgM	19	15	1	15	9	0	0	39	0

derived from the BetaCoV/Wuhan/WIV04/2019 sequence. All samples were tested in duplicate and all test runs included a serum positive control, a serum negative control, and BSA and LPS antigen controls as blanks. Results outputs were expressed in MFI units. A machine training algorithm was used to assign a final serological classification to all the samples studied, as described previously (33). This method assigns a SARS-CoV-2 serological status considering the values the IgG values (MFI) for the three antigens. The negative cut off values for N-, RBD- and S-specific IgG assays were set up at 1604, 456 and 1896 respectively.

2.7 SARS-CoV-2 Pseudotype-Based Microneutralisation Assay (pMN)

Virus neutralising antibodies were detected and quantified by a pseudotype-based neutralisation assay based on a lentiviral system that enables the generation of replication-defective recombinant human immunodeficiency virus (HIV) displaying the Spike protein of SARS-CoV-2 on their viral envelope, as previously described (34, 35). Briefly, HEK293T cells were seeded in 10 cm^2 cell culture dishes at a density to achieve 70% confluency after 24 hours for next day transfection. HEK293T cells were maintained in DMEM (Dulbecco Minimum Essential Medium) containing 10% foetal bovine serum and 1% penicillin/streptomycin, at 37°C and 5% CO_2. Cell maintenance was done by three cell passages per week.

On the day of transfection, the culture medium was replaced with fresh complete DMEM. Cells were transfected with 1000 ng of pcDNA-SARS-CoV-2 Spike plasmid, 1000ng of HIV 8.91 gag/pol plasmid and 1500ng of pCSFLW luciferase plasmid, using FuGENE HD (Promega, UK), at a 1:3 ratio (plasmid:FuGENE HD). The culture media was harvested 48 hours post-transfection and filtered through a 0.45μm filter. The filtered pseudotype virus (PV) was aliquoted, titrated and stored at -80°C. Titration of PVs was carried out in a 96 well white plate typically using doubling serial dilutions. Pre-transfected HEK293T target cells expressing human ACE2 and TMPRSS2 were seeded at 10^4 cells per well and plates were incubated for 48 hours prior to the addition of Bright-Glo reagent (Promega, UK) and reading the result in a luminometer.

For detecting and quantifying neutralising antibodies, serial doubling dilutions of the plasma samples in complete DMEM were performed from an initial 1/40 dilution. SARS-CoV-2 PVs were added at $5x10^5$ – $5x10^6$ RLU/ml in each well and the plates incubated for 1 hour in at 37°C, 5% CO_2 incubator. Post incubation, pre-transfected HEK293T target cells expressing human ACE2 and TMPRSS2 were seeded at 10^4 cells per well and plates were incubated for 48 hours prior to the addition of Bright-Glo reagent and assaying using a luminometer.

In addition to the test sample dilutions, all test runs included dilution series of an external calibrant (NIBSC 20/162) or an internal calibrant (HICC Serum 2) and a single dilution of a positive control per plate (NIBSC 20/130). All samples were tested in duplicate and the average of the OD values determined. The IC_{50} values of each sample dilution series were determined and expressed as relative potency respect to the Internal or External Calibrant which enabled the expression of results in International Units using the WHO International Standard 20/136 as a primary calibrator. Details of how these were calculated are described in the 'Results' section.

2.8 Semi-Automated Immunoblotting

Plasma IgG antibodies reactive against the SARS-CoV-2 Spike and Nucleocapsid proteins were analysed by immuno-blotting using the 'Jess' fully automated system (ProteinSimple; Bio-Techne) and the SARS-CoV-2 Multi-Antigen Serology Module (ProteinSimple; Bio-Techne, SA-001), following the manufacturer's instructions. Here, the 12–230 kDa Jess/Wes Separation Module was used. Briefly, the kit provides five SARS-CoV-2 recombinant viral antigens: RBD, N, S1 subunit, S2 subunit and S (S1+S2). The antigens were electrophoretically separated according to their molecular weight to create a ladder for capture of reactive antibodies. Two microlitres of plasma samples diluted 1:10 in diluent buffer were loaded. For the secondary antibody, ready to use HRP-conjugated goat anti-human IgG, IgA or IgM antibody was used. Digital image of chemiluminescence of the capillary was captured with the Compass Simple Western software (version 4.1.0, Protein Simple), that automatically calculated chemiluminescence intensity of each single antigen binding signal. Results could be visualized as electropherograms representing peak of chemiluminescence intensity and as lane view from signal of chemiluminescence detected in the capillary. To control for differences in signal between experiments, a reference sample, the NIBSC Anti-SARS-CoV-2 Antibody Diagnostic Calibrant (NIBSC 20/162) was included in each experiment. A panel of pre-pandemic plasma sera was used to calculate the negative cut-off value for each of the antigen tests (mean + 2STD). Final results of the samples were calculated by subtracting the negative cut-off value from the chemiluminescent signal of the sample.

2.8 Lateral Flow IgG/IgM

A rapid detection kit (Accu-Tell COVID-19 IgG/IgM Antibody Test) for SARS-CoV-2 was used following manufacturer's instructions and compared with other antibody detection platforms. Briefly, 5μl of heat-inactivated serum were added to the antigen test cassettes followed by 2 drops of the supplied PBS. After an incubation of 30 min at 20°C, the results were recorded. A positive IgG or IgM result was indicated by the appearance of a band for either of the isotypes included in the assay. Tests were valid only if a control band appeared in the device.

2.9 Electroporation-Dependent Neutralisation Assay (EDNA)

Electroporation was performed using the Neon Transfection System (Thermo Fisher). Vero ACE2/TMPRSS2 cells (36) were washed with PBS and resuspended in Buffer R (Thermo Fisher) at a density of 1×10^6 cells per ml. For each electroporation reaction 0.5×10^6 cells (10.5 μl) were mixed with 2μl of serum to be delivered. The mixture was taken up into a 10 μl Neon Pipette Tip and electroporated using the following settings: 1400V, 20ms, 2 pulses. Electroporated cells were transferred to medium supplemented with 10% serum without antibiotics. 1.5×10^4 electroporated cells were seeded into 96-well plates in triplicates and after 24h transferred to containment level 3 laboratory.

Supernatants were removed and all wells washed with PBS to remove any remaining antibodies that could interfere with virus entry. Cells were infected at moi = 1 in DMEM supplemented with 2% FBS and antibiotics and incubated for 24h to allow for a single replication cycle. The virus used was a derivative of the Wuhan virus, SARS-CoV-2/human/Liverpool/REMRQ0001/202, isolated by Lance Turtle (University of Liverpool) and David Matthews and Andrew Davidson (University of Bristol). After incubation, plates were immediately frozen at -70°C to help with cell lysis. Next, plates were thawed at 4°C and 1 volume of lysis buffer (0.25% Triton-X100, 50mM KCl, 100mM Tris-HCl pH 7.4, glycerol 40% and RNAsecure from Invitrogen at 1/100) was added to wells and mixed gently by pipetting. After 5min of lysis, cell lysates were transferred to PCR plates and virus inactivated at 95°C for 5min. RT-qPCR was performed with Luna® Universal Probe One-Step kit (E3006, NEB) following manufacturer recommendations. Primer/probe for genomic viral RNA were CDC-N2 (IDT 2019-nCoV RUO kit). Primer probe for 18S control were described previously (37). SARS-CoV-2_N_Positive control RNA from IDT (10006625) was used as standard for the viral genomic N reactions. For 18S rRNA standard, DNA was synthesized and kindly gifted by Jordan Clarks and James Stewart (University of Liverpool). Final concentrations of 500nM for each primer and 125nM for the probe were used. RT-qPCR reactions were run on ABI StepOnePlus PCR System (Life Technologies) with following program: 55°C for 10min, 95°C for 1min and then 40 cycles of 95°C denaturation for 10sec and 60°C extension for 30sec. RNA copy numbers were obtained from standards and then genomic copies of N normalised to 10^{10} copies of 18S. Finally, all data was normalized to 100% to PBS electroporated cells.

2.10 Statistical Methods

We calculated log IC_{50} values to summarise the RBD-specific and N-specific antibodies as measured by ELISA and neutralising antibody titre as measured by neutralisation. Log IC_{50} values were estimated by fitting four parameter log-logistic regression dose response curves in the R package drc (38). The four parameters of this curve are the minimum response, the maximum response, the log of the dilution halfway between the two (IC_{50}), and the gradient at the IC_{50}. Our models actually estimated the natural log of IC_{50} values because it improved model convergence and produced normally distributed values for downstream analyses.

To ensure IC_{50} values were comparable, a single gradient, minimum, and maximum value was estimated for dose response curves of all samples. To minimise noise between experimental runs the gradient, minimum, and maximum parameters were estimated based on a random subset of 200 samples and fixed for all other samples. Graphical checks showed that these parameters produced curves that fit the observed data well. We observed that this parameter fixing decreased the variance in estimated log IC_{50} values for calibrants.

Samples and calibrants could be assigned an international unitage based on their potency relative to the international standard NIBSC 20/136 which has been assigned an arbitrary unitage of 1000 IU/ml. Unitage for a sample was expressed as

$$\text{Units of sample} = \text{Calibrant units} \times \text{Sample } IC_{50} / \text{Calibrant } IC_{50}$$

In practice, the unitage of calibrants was quantified in international units as shown above and the unitage of samples was calculated based on their potency relative to a calibrant with a known international unitage. The reason for this two-step process is that the international standard was not available until December 2020.

To assign international units to the calibrants, these were run in duplicate alongside the international standard and relative potencies and international units were calculated as described above. The assumption of parallel curves was verified by comparing the AIC of models which allowed separate gradients to those which did not.

The Spearman's rank correlation coefficient for variables pairs and Mann-Whitney U tests were calculated using R (39).

3 RESULTS

The primary objective of this work was to identify relevant biomarkers of humoral immunity that can serve to differentiate severe from mild/asymptomatic COVID-19 cases and also could be used as potential Correlates of Protection (CoP) of COVID-19. In this study we analysed antibody-based parameters present in serum or plasma of convalescent patients and compared these antibody levels to those in seropositive and seronegative health-care workers (HCW).

3.1 Clinical Details of Patients and Healthcare Workers Included in this Study

The participants of this study were classified into three cohorts: a) Patients; b) Seropositive HCW; and c) Seronegative HCW (**Table 2**) using the criteria described in the methods section. Any participant displaying a positive result by any of the screening tests was considered seropositive. A large proportion of hospitalised patients (82%) presented a clinical score of 4 (Severe Pneumonia) (**Table 1B**). Approximately half of these patients (47%) presented septic shock or sepsis (clinical scores of 7 and 6), 38% developed ARDS (Acute Respiratory Distress Syndrome) or severe pneumonia (clinical score 5 and 4) and only two patients presented moderate pneumonia (clinical score 3). Only two patients were asymptomatic. All patients (but not all HCW) had a positive PCR diagnostic result and all patients, except two, presented SARS-CoV-2-specific antibodies. Clinical scores of seropositive HCW ranged between 1 and 3. A third of these individuals (33%) presented moderate pneumonia (clinical score of 3), 50% showed mild disease (clinical score 2) and 12.5% were asymptomatic. In contrast, the majority of seronegative HCW were asymptomatic (59%) or presented with symptoms of mild disease (33%) and only 3 individuals presented moderate pneumonia.

3.2 Calibration and Standardisation of Antibody Assays

Whenever possible, we defined candidate humoral CoP in units relative to the WHO International Standard. Quantitative antibody assays (pMN, RBD ELISA, N ELISA) were calibrated using our internal reference antiserum (HICC S2) or an external

calibrant. At the start of this work, the WHO International Standard, NIBSC 20/136 had not yet been developed and we calibrated our internal standard against the NIBSC 20/162 calibrant reagent, which was available at that time. In some instances, we used the latter reagent directly as our assay calibrant in the neutralisation (nAb) assay. This reagent, NIBSC 20/162, was assigned 1000 units for the pMN, the pan-Ig N and the pan-Ig S assays. The results of the pMN, N-ELISA and RBD-ELISA, were converted to IU or Binding Antibody Units (BAU) of the WHO International Standard (NIBSC 20/136) once the latter became available.

In order to calculate the Unitage of the HICC reference sera (used as Internal Calibrants), we tested in the same assay serial dilutions of the HICC reference sera and NIBSC reagents. After preparing the corresponding calibration curves (**Figure 1**), we performed a parallel line analysis. Such analysis supported the mathematical derivation of a unitage value for our internal calibrants from the NIBSC 20/162 reagent. Thus, HICC Serum-2 was assigned a value of 504 BAU/ml for the pan Ig N-ELISA, 98 BAU/ml for the pan Ig-G RBD ELISA and 76 IU/ml for the pMN assay. The results of each sample tested by these assays were expressed in the corresponding units as follows:

$$\text{Units of sample} = (IC_{50}\text{of Test sample}/IC_{50}\text{of calibrant}) \times \text{unitage of the calibrant}$$

When the WHO International Standard for anti-SARS-CoV-2 immunoglobulin 20/136 became available, the conversion into International Units (IU) and Antibody Binding Units (BAU) specific for N-, RBD-, and S- antigens (N-BAU,RBD-BAU, S-BAU) was calculated by multiplying the values (units) of samples by a factor F, which is the ratio of the IC_{50} of NIBSC 20/162 relative to the IC_{50} of the International Standard 20/136. Thus, all results of the pMN and the Pan-Ig ELISA tests included in this study are expressed in IU and BAU relative to the WHO International Standard, respectively.

3.3 Antibody Biomarkers of COVID-19 Immunity as Potential CoP

The convalescent serum or plasma samples from these three cohorts were analysed by a range of assays that measure antibody-based biomarkers of immunity: a) a pseudotype-based microneutralisation assay (pMN); b) a Luminex IgG assay specific for N, S and RBD; c) a pan-Ig ELISA for N and RBD; d) a multiplex antigen (S, S1, S2, N and RBD) immunoblotting assay for IgG, IgM and IgA; e) a commercial lateral flow assay for rapid detection of SARS-CoV-2-specific IgG and IgM; and f) a commercial electrochemiluminescence assay (ECLIA).

We analysed the data of all these assays and determined individual correlations of all these measurements with one another and with the clinical severity scores assigned to the individuals the samples derived from. These analyses (**Figure 2A**) showed four main clusters of assay correlations. The first cluster is represented by IgA Immunoblotting assays for S, S1, S2 and RBD (**Figure 2B**). A second group is formed by IgG assays based on Spike-derived antigens and pMN assay (**Figure 2B**). The third cluster is formed by N-specific assays (**Figure 2C**). The intracellular neutralisation assay (EDNA) correlated positively with N-specific IgG and IgA binding assays. Due to the shorter duration of IgM than IgG and IgA in blood following a viral infection, it was not surprising that the IgM assay results of convalescent sera did not show positive or negative correlations with the IgG, IgA, pMN assays, intracellular neutralisation or clinical severity. Overall, clinical severity correlated positively with nAb data, S/RBD, N antibody binding measurements. As expected, nAb data correlated more strongly with S-specific and RBD-specific binding antibodies than with N-specific antibody levels, indicating N-specific antibodies maybe a good biomarker of previous infection and its severity but not necessarily the best surrogate of nAb.

Having established the general correlations of the biomarkers under study in these convalescent samples, we dissected in more detail the data generated by these assays.

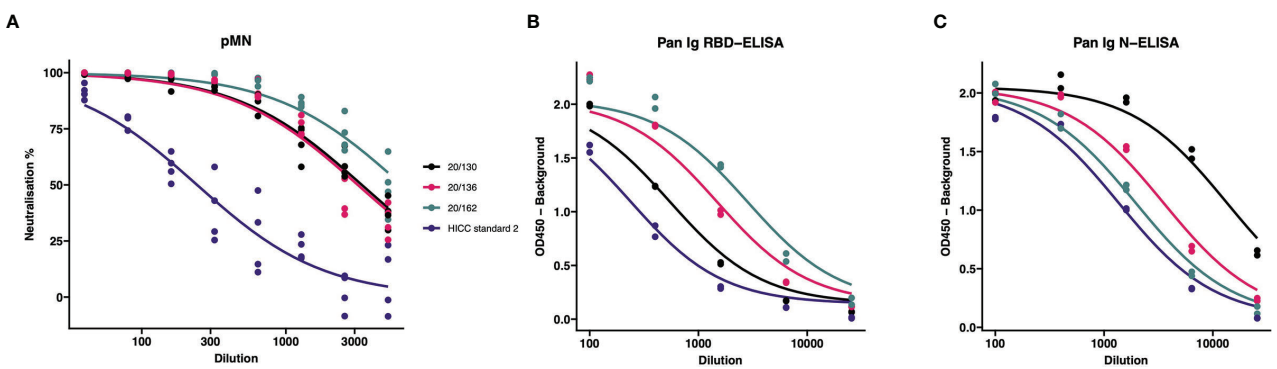

FIGURE 1 | Comparison of internal calibrants and International Standard for neutralization and binding assays. The IC_{50} from these curves were used to calculate the **(A)** International units (IU); **(B)** RBD-specific Binding Antibody Units (BAU); and **(C)** N-specific Binding Antibody Units (BAU). Calibrants were run four times at each dilution for pMN and twice for ELISA tests.

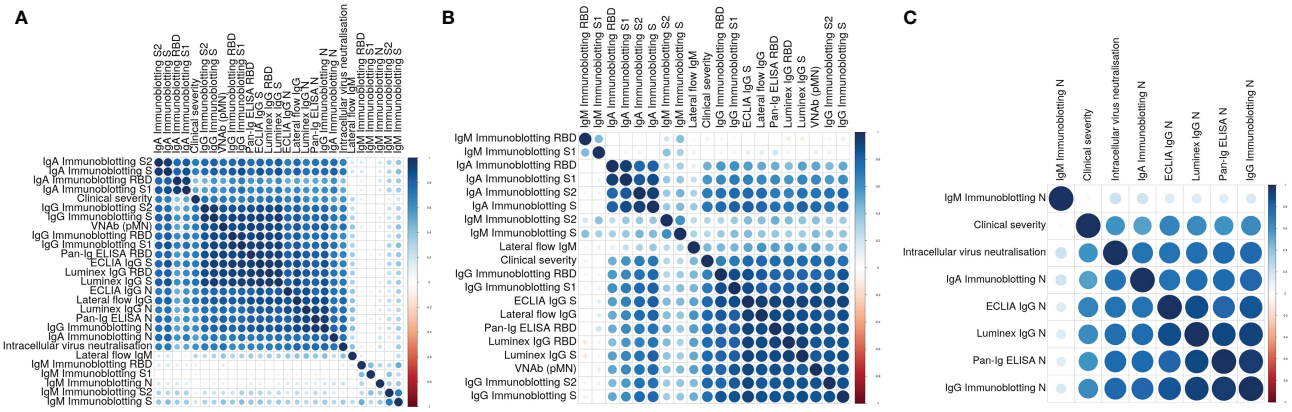

FIGURE 2 | **(A)** Correlation plot showing pairwise Spearman's rank correlation between assays. Darker and larger points indicate stronger correlations. Blue indicates positive and red indicates negative correlations. Assays are ordered by hierarchical clustering so that assays with similar relationships are together. **(B)** Correlation plot showing pairwise Spearman's rank correlation between clinical severity, pMN and S-specific antibody assays. Darker and larger points indicate stronger correlations. Blue indicates positive and red indicates negative correlations. Assays are ordered by hierarchical clustering so that assays with similar relationships are together. **(C)** Correlation plot showing pairwise Spearman's rank correlation between clinical severity, intracellular neutralization and N-specific antibody assays. Darker and larger points indicate stronger correlations. Blue indicates positive and red indicates negative correlations. Assays are ordered by hierarchical clustering so that assays with similar relationships are together.

3.4 SARS-CoV-2 Antigen Specific Antibody Responses

The antibody screening assays used to classify the serum samples from patients and HCW produced concordant results with a few exceptions. All samples from patients were positive by all three IgG assays except those from patient 37 and patient 50, which tested negative by all three assays. These patients were asymptomatic and their clinical histories revealed that they were already hospitalised for other conditions before becoming infected with SARS-CoV-2. Only a small group of seropositive HCW samples produced discordant results. Thus, HCW s198, s223, s286, s370, s398 and s423 presented positive results only by some of the assays but none of these results were strongly positive. As expected, a proportion of the convalescent samples from patients and HCW did not test positive by the IgM lateral flow assay (data not shown).

The samples were classified into the three cohorts by the screening assays were analysed by the 'HICC in-house' pan-Ig ELISA for RBD and N antigens. The results were largely consistent with those of the Luminex, lateral flow assay and ECLIA tests. Only a few discrepancies were noted. Thus, all sero-negative HCW samples tested negative by the N and RBD pan-Ig ELISA, except s195, s296 and s269 samples. HCW samples s195 and s196 were positive for RBD, presenting values of 4.4 and 2.5 RBD-BAU/ml respectively, just above the negative cut-off value (2 RBD-BAU/ml), but were negative by the N ELISA (negative cut-off value of 4 N-BAU/ml). Sample s269 which had 14.3 N-BAU/ml (negative cut-off value 7.7 N-BAU/ml) but was negative for RBD. As expected, all patients' samples tested positive against both antigens and presented high values (mean 414.5 RBD-BAU/ml; mean 316 N-BAU/ml), except patient 37, which tested negative for both antigens. Patient 50, which tested negative by all screening serological assays, presented low antibodies to RBD (20.9 N-BAU/ml) and N (26.3 N-BAU/ml) antigens. Results of the pan-Ig N and pan-Ig RBD from seropositive HCW were also in line with the Luminex, lateral flow and ECLIA tests. The average antibody levels of seropositive HCW, 118.9 RBD-BAU/ml and 234.6 N-BAU/ml, were significantly lower than those found in patients (Mann-Whitney U test, RBD: U=717, $p<0.001$; N U=630, $p=0.006$). The same samples that produced conflicting results by the serological screening assays, namely s198, s223, s286, s370, s398 and s423, produced very low N- and RBD-BAU results.

3.5 Virus Neutralising Antibody Responses Measured by Pseudotype Based Micro-Neutralisation

The pMN results revealed a significant difference in neutralising antibody titres (nAb) between the three cohorts (**Figure 3**). As expected, the seronegative HCW sera presented very low nAb (mean 5.3 IU/ml). In contrast, seropositive HCW presented moderately high nAb levels (mean value 379 IU/ml), whereas patients presented a three-fold higher level (1029 IU/ml). Of note is that three seronegative HCW (s38.2, s38.1, s228) had low nAb but these were above 24.2 IU/ml (mean of negative HCW+ 2STD). This pMN value is well above 6 IU/ml, a negative cut-off value for this assay calculated from a small panel of pre-pandemic sera (mean + 2SD) suggesting that these individuals could have been exposed to SARS-CoV-2, despite antibody binding assays showing negative values for all of them. Five seropositive HCW samples (s223, s286, s370, s398 and s423) had nAb below 6 IU/ml, which was consistent with the low values obtained in the serology screening tests and the pan-Ig ELISA. All of these individuals were asymptomatic or had mild disease without pneumonia, except HCW 370 who had moderate pneumonia. It would be interesting to investigate the frequency

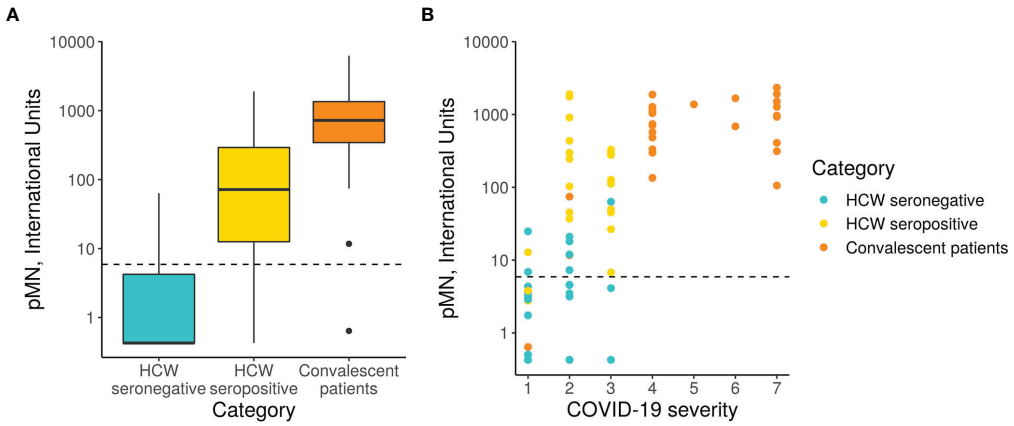

FIGURE 3 | pMN virus neutralisation in International Units by cohort and COVID-19 severity. **(A)** Boxplot showing the difference between cohorts. **(B)** Scatterplot showing neutralisation against disease severity. The dotted line shows the 95% upper CI calculated from pre-pandemic sera, 5.9 International Units.

of these cases and understand the biological meaning of these results in these particular individuals.

A more detailed analysis of the data revealed a very strong correlation between clinical severity score and nAb (**Figure 3**, Spearman's rank correlation = 0.71). As previously indicated in Section 3.1, the majority of hospitalised patients (clinical scores > 4) presented very high nAb levels, the majority above 200 IU/ml, and in some cases reached values as high as 2117 IU/ml. However, two patients' samples, those from patients, 37 and 50, had levels of nAb as low as 0.63 and 11 IU/ml respectively. These samples also had low values in the ELISA and Luminex assay as discussed in the previous section. Patient 17 presented low antibody levels by both assays but remain above the positive threshold and consistent with this, presented moderate nAb levels (74 IU/ml). Consistent with the correlation observed between severity and nAb levels, patients 17, 37 and 50 had clinical scores of 1 and 2.

The nAb data distribution in infected HCW is more widespread, ranging between 0.426 to 2092 IU/ml, including asymptomatic cases of COVID-19 (clinical score of 1) to moderate pneumonia with Sp>90% (clinical score of 3). Some of the samples had nAb levels as high as those observed in most severe cases of COVID-19. As expected, the seronegative group of HCW presented very low nAb levels ranging between 0.426 to 18 IU/ml with clinical scores of 1 or 2.

3.6 SARS-CoV-2 Antigen Display by Automated Microfluidics Western Blot Analysis

In order to dissect the specificity of the antibody response to Spike (S) and nucleoprotein (N) of SARS-CoV-2 and to identify additional candidate biomarkers of immunity, we used a semi-automated immunoblotting assay (Jess, Protein Simple, Biotechne) based on the separation of protein antigens in a polyacrylamide gel matrix contained in a capillary tube. This microfluidics assay sequentially processed diluted plasma samples, conjugated antibodies, washing buffers and chemiluminescent reagents sequentially through microfluidics. A final chemiluminescent reaction is read by the device and translated into a luminometry intensity signal which can be analysed quantitatively. The results are visualised as traditional western blotting lane format or analytically the data outputs as densitometric units for quantitative antigen specific responses. We utilised this assay qualitatively (immuno-blotting images) and quantitatively (using total luminometry units) to screen and confirm antibody specific responses to the intact Spike protein and its subunits, S1, S2, and RBD as well as the SARS-CoV-2 N antigens. Furthermore, antibody isotype (IgM, IgG, and IgA) and IgG subtype responses (IgG1 to 4) were measured.

3.6.1 Quantitative Data

The results of IgG responses (**Figure 4**) were consistent with the findings described above for the antigen binding assays (Luminex, Lateral Flow, ECLIA and ELISA assays). The IgG antibody responses of COVID-19 patients showed significantly higher median Chemiluminescent Intensity Units (CIU) values than those of seropositive HCWs. Of note is the wide range of N-specific and S-specific IgG CI measurements, of both patients and seropositive HCW.

For the most part, the results of the IgA antibody immuno-blotting mirrored those of the IgG responses although the median CIU was significantly lower than for IgG, with values below 10,000 CIU as opposed to the IgG values for the same antigen in the range of 300,000 CIU. The IgA responses to the Spike subunits, RBD, S1 and S2 of seropositive HCWs against N and Spike were markedly lower than those exhibited by the IgG responses while not surprisingly, IgM responses were heterogeneous in this cross-sectional convalescent study, presenting negative or close to '0' median CIU values for all three cohorts against all five antigens. Although the time of sampling was approximately 3-5 months following exposure or hospitalisation, IgM was clearly detected in a very few individuals.

3.6.2 Qualitative Data – Antigenic Specificity of the Antibody Response

Analysis of the immuno-blotting electropherograms revealed that the relative antigen response of individual sera was

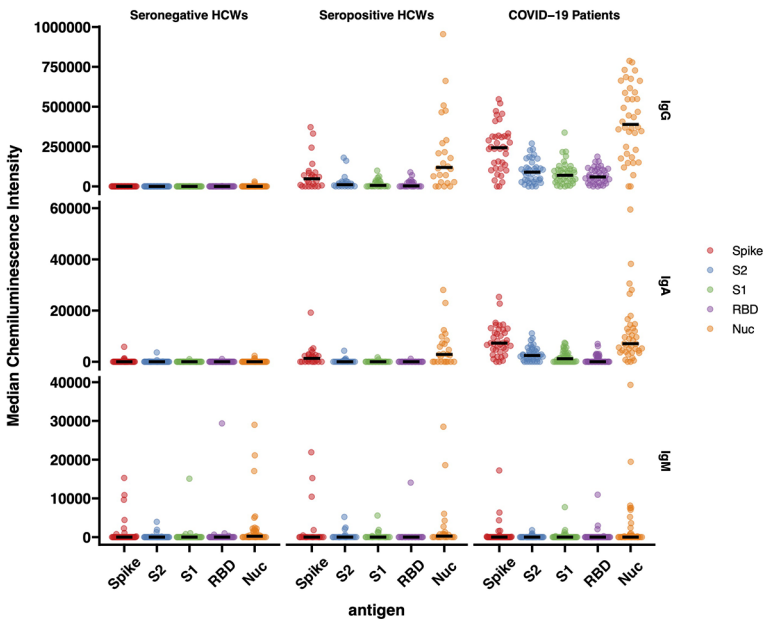

FIGURE 4 | IgG, IgA and IgM responses against Spike, RBD, S1, S2 and N antigens of SARS-CoV-2. The image displays the Median Chemiluminescence Intensities of antigen-specific IgG (top), IgA (middle) and IgM (bottom) of seronegative HCWs (left), seropositive HCWs (middle) and COVID-19 patients (right). A panel of pre-pandemic sera was used to calculate the negative cut-off value for each antigen (mean + 2STD) and then subtracted from the chemiluminescent signal of each sample.

heterogeneous across patients and seropositive HCW revealing four distinct patterns of antigen specific responses. Representative results of these patterns are presented in **Figure 5**. Most patients' samples showed equally strong IgG reactivities against both N and S antigens (High N=S). However, a number of samples displayed weaker signals against both antigens (patients p17, p37, p50) (Low N=S), whereas some presented predominantly an anti-N reactivity (p40) (N>S) and some had predominantly anti-S-specific antibodies (p8) (N<S) (**Figure 3B**). This heterogeneity of the antigen specificity of the IgG response was also evident in the results of seropositive HCW samples. Again, these four categories could be distinguished according to N/S ratios: a) N = S (high) (HCW 361.1); b) N > S (HCWs s24.1, s25.1, s38.1, s38.2, s117.1, s224.1, s414); c) N < S (HCWs s249.1, s408) and d) N = S (low) (HCWs s4.1, s198.1, s223.1, 254.1, s286.1, s418.1, s423.1, s439.1). Similarly, these patterns were also identified in the IgA responses, although uniformly lower than the IgG responses in all individuals, especially in the seropositive HCW.

There were very few samples giving a positive result in the IgM immuno-blotting assay and this signal was very low in magnitude except for one sample in each of the cohorts. For this reason the antigenic specificity patterns of the IgM response differed significantly from the IgG and IgA responses. Most positive IgM samples in the patient cohort were N-specific (n=7), albeit the detection signal of the electropherogram was weak in 6 of them, and only one of the samples also had an S-specific IgM signal. Some of the seropositive HCW samples detected the Spike (s25.1, s308.1 and s398.1) and its subunits RBD, S1, S2 (s398) as well as N (s398 and s418). One sample presented a strong N response (HCW s418) suggestive of a recent exposure to SARS-CoV-2 or to a common cold coronavirus which was not detected by the primary screening serological tests used in this study.

3.7 Intracellular Neutralisation Assay (EDNA)

To explore the potential use of biomarkers indicative of TRIM-21 based mechanisms of immunity we applied the EDNA assay to our cohorts' samples. We electroporated Vero ACE2/TMPRSS2 cells with sera from each patient and seropositive HCW and ten seronegative HCW samples for reference. In the presence of electroporated N-binding antibodies, virus replication was inhibited. The results of these analyses (**Figure 6**) indicate that patients' sera are more effective at inhibiting virus replication than sera from the seropositive HCW. All but one patients' samples were positive by the EDNA (**Figure 6B**). In contrast, some seropositive HCW tested negative or produced a low positive result (**Figure 6C**), whereas all tested seronegative HCW did not affect virus replication, as expected (**Figure 6D**). Interestingly, patients and seropositive HCW samples with the strongest inhibition of virus replication had the highest levels of anti-N antibodies such as s414 or p32, confirming that the observed intracellular neutralisation is mediated by anti-N antibodies. Importantly, these results highlight that traditional neutralization assays, fail to measure the potential contribution of anti-N antibodies present in SARS-CoV-2 positive sera.

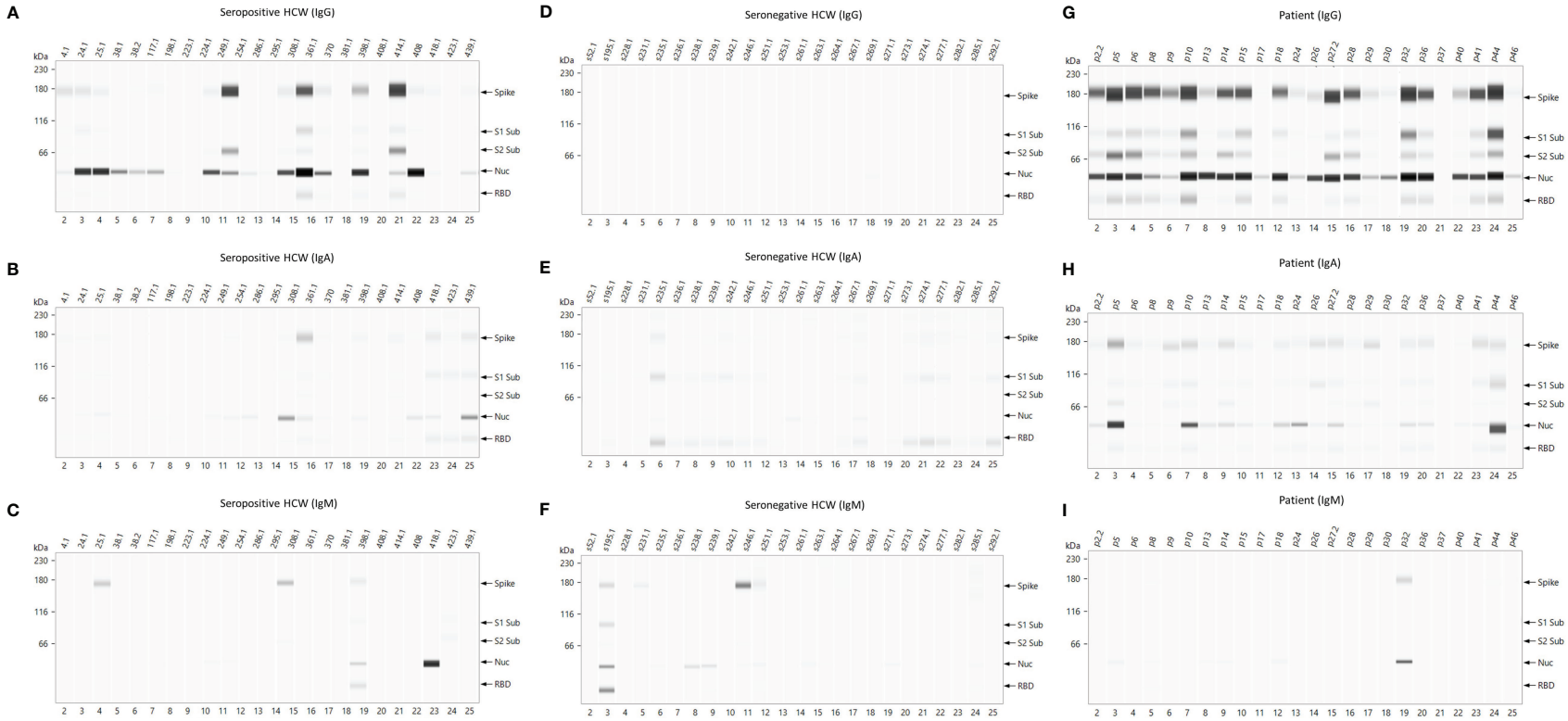

FIGURE 5 | Representative electropherograms of IgG, IgA and IgM antibody responses to Spike, RBD, S1, S2 and N antigens of SARS-CoV-2 in Health Care Workers and Patients. Thefigure shows antigen-specific IgG **(A, D, G)**, IgA **(B, E, H)** and IgM **(C, F, I)** antibody reactivities of seropositive HCWs (left panel), serongative HCWs (middle panel), and patients (right panel).

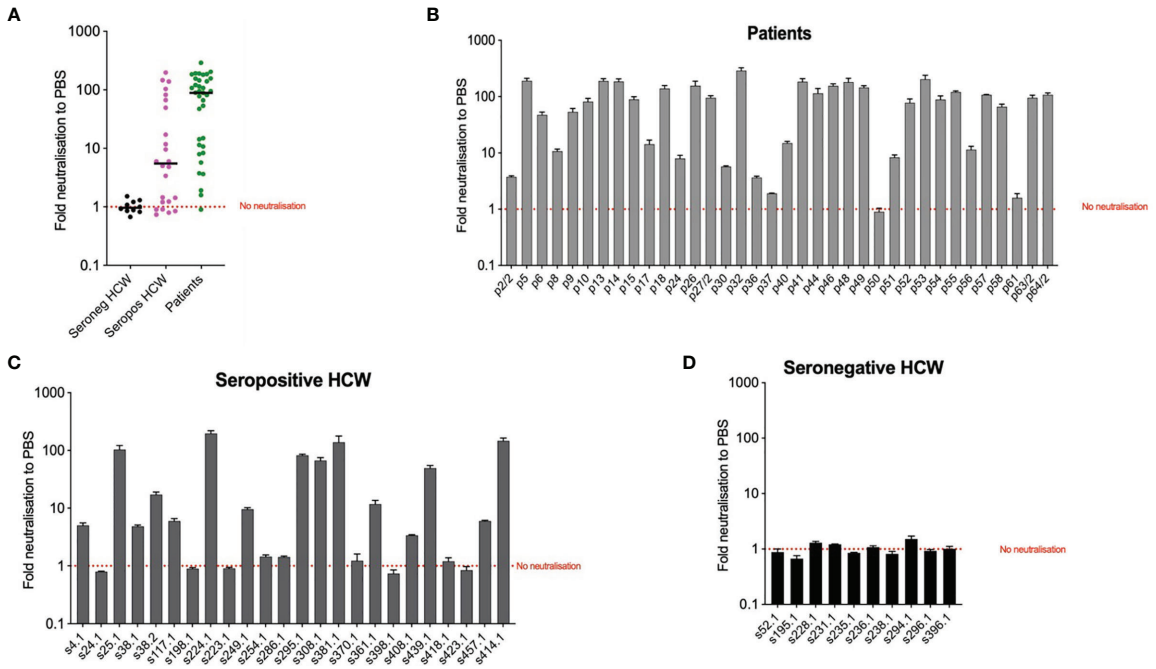

FIGURE 6 | Intracellular neutralisation data from EDNA assay. The results are expressed in genome copies relative to 18S and percentage normalised to PBS. Panel **(A)** depicts median values of EDNA results of the three cohorts, expressed as Fold neutralisation relative to PBS; Panels **(B–D)** correspond to individual EDNA results of patients, seropositive HCW and seronegative HCW respectively.

4 DISCUSSION

The 'Humoral Immune Correlates of COVID-19 Project (HICC)' (https://www.hicc-consortium.com/) was established to identify humoral biomarkers of immunity and develop standardised assays to determine the thresholds of antibody responses that correlated with protection against SARS-CoV-2 infection or with severe COVID-19 disease requiring hospitalisation. Before conducting serological analysis of vaccine breakthrough or re-infection cases with which to define correlates of protection we wanted to define first those candidate antibody biomarkers by cross-platform comparison of a range of antibody-based assays, such as nAb and SARS-CoV-2 antigen-specific (N-, S-, S1-S2- N- and RBD) binding antibodies (Pan-Ig, IgG, IgM, IgA) in convalescent serum or plasma samples from COVID-19 hospitalised patients and seropositive and seronegative HCW (**Table 3**). The main findings of the present study confirmed: a) that there is a strong positive correlation between clinical severity and SARS-CoV-2-specific antibodies; b) that there is a strong correlation between nAb and S- and RBD-specific antibody levels; c) that intracellular neutralisation correlated very well with N-specific antibody levels; and d) that there are different antigen-specific reactivity patterns of IgG, IgA and IgM in seropositive samples. We used the WHO International Standard (NIBSC 20/136) to quantify some of these antibody-based parameters in International Units (IU) for neutralisation assays and Binding Antibody Units (BAU) for ELISA. The adoption of common results reporting unitage in IU and BAU as those described in this study would eventually facilitate comparative analyses of data generated by immunogenicity studies performed by different teams in different parts of the world.

The criteria for the serological classification of the convalescent samples used in this study were based on a UKAS-accredited Luminex assay benchmarked for COVID serological screening (33) and another two well-stablished serological assays (AccuTell lateral flow IgG/IgM; Roche ECLIA) (30, 40, 41). The results of these tests were consistent with other additional tests (pan-Ig N and RBD ELISA; immuno-blotting) described in this paper. As expected, only samples with low antibody levels produced some discrepancies due to the positive-negative cut-off of each particular assay. Such discrepancies could also be due to the previously described cross-reactivity between the N antigens of SARS-CoV-2 and the seasonal human common cold coronaviruses (42) which was also consistent with our analysis of pre-pandemic serum samples.

Consistent with published data (8, 11, 43, 44), we found a very strong correlation between nAb, as measured by the pMN assay, and severity of disease. Evidence from epidemiological studies and vaccine clinical trials indicated that nAb correlate with immunity against COVID-19 (45, 46). However, knowledge of the early immunopathologic events that trigger severe COVID-19 disease is still incomplete, in particular the role of complement system its interaction with early antibody responses.

Various studies indicate that the high nAb levels found in severe COVID-19 patients are a consequence of the high and

TABLE 3 | Summary of the main features of the antibody-based assays used in this study.

Antibody assays	Isotype/ Sub-isotype	Functional	Turnaround	Throughput	Suitable for Standardisation (WHO Standard)	Potential to derive a Correlate of Protection
Pan Ig ELISA - N	IgG+IgA+IgM	No	24 hours	Medium	Yes	Unknown
Pan Ig ELISA - S	IgG+IgA+IgM	No	24 hours	Medium	Yes	Possible
ECLIA (total Antibody) - N	IgG+IgA+IgM	No	24 hours	Medium-High	Likely	Unknown
ECLIA (total antibody) - S	IgG+IgA+IgM	No	24 hours	Medium-High	Likely	Possible
Multiplexed Bead Flow Cytometry – Luminex (IgG/IgA/IgM)	IgG/IgA/IgM	No	6 hours	Medium-High	Potentially	Possible
Semi-automated Immunoblotting	IgG/IgA/IgM	No	4 hours	Medium-High	Potentially	Possible
Lateral Flow IgG/IgM	IgG/IgM	No	30 mins	Low	No	No
EDNA	N/A	Yes; TRIM-21 mediated CTL	48 hours	Low	Not possible at the moment	Unknown
pMN	N/A	Yes; Virus neutralisation	48 hours	Low	Yes	Reasonably likely

IgG+IgA+IgM, indicates the three isotypes are detected simultaneously in the assay. IgG/IgA/IgM, indicates that each isotype generates a separate reading.

persistent viral replication, high virus load and that the marked expression of viral antigens in the host, or alternatively, a consequence of a dysregulated immune response leading to antibody-mediated immunopathology. Studies by Garcia-Beltran and co-workers suggested the latter and that the antibody response profile in severe patients, characterised by high nAb to IgG-RBD ratios, is a consequence of such dysregulation. These authors suggested that the use of specific antibody response metrics could be useful to discriminate between immuno-pathological antibody responses from those that would lead to protective immunity. Our study does not address this question but proposes different antibody-based assays, parameters and standardised methods that could facilitate comparative data analysis of humoral immunity. Use of these antibody response metrics could be applied to serum or plasma samples in vaccination efficacy/efficiency or re-infection studies in order to elucidate thresholds of protective immunity.

COVID-19 serological studies published to date show a positive correlation between Spike-specific antibodies and nAb (47). Accordingly, our study showed that nAb of COVID-19 convalescent sera correlated very strongly with Spike-specific IgG and IgA binding antibodies. This can be potentially very advantageous for assessing protective immunity in clinical trials or in immuno-surveillance programmes, as evidence supporting the use of nAb as a biomarker of COVID-19 immunity continues to grow (48). Indeed, some of the S-specific antibody binding assays used in our study are quantitative, reproducible, suitable for calibration to the international standard and high-throughput. The latter is a distinct advantage over the more laborious and time-consuming neutralisation assays. However, validity of these correlations need further evaluation as other reports indicate the importance of IgM and IgA contribution to virus neutralisation, and that the nAb/IgG ratio correlate with 30-day survival (11).

Our data also showed a strong correlation between nAb, disease severity and N-specific IgG and IgA antibody levels in convalescent samples. The intracellular neutralisation data generated by the EDNA assay represents an indirect evidence that the TRIM-21 mediated mechanism of immunity could play a relevant role in protection against COVID-19. The output of the EDNA assay from SARS-CoV-2 infected cells previously electroporated with serum from patients or HCW showed a significant reduction of virus replication. This was proportional to the N-specific antibody levels of the sera. Previously, it has been shown that antibody-antigen complexes are rapidly degraded in the cytosol by TRIM21 and the proteasome (17, 49). If N protein is degraded inside an antigen presenting cell, this provides peptides for MHC-I presentation. Indeed, studies have shown that cytotoxic T-cell immunity to virally-infected cells requires internalization and cross-presentation of virus-antibody complexes by dendritic cells (50). It has been previously shown that TRIM21 uses anti-N antibodies to degrade the nucleoprotein of LCMV, promote cytotoxic T-cells and clear mice of infection (18). As indicated earlier, longitudinal analyses of antibody levels from patients and HCW will help to determine the relevance of this anti-SARS-CoV-2 immunity mechanism and how N-specific antibodies might contribute to either protective immunity or immuno-pathology. Here we provided experimental evidence that sera from COVID-19 convalescent patients and seropositive HCW, but not those from seronegative HCW, neutralised effectively SARS-CoV-2's infectivity intracellularly and that these measurements correlated very strongly with anti-N antibody levels (**Figure 2**).

The analysis of the antigen specificity of serum IgG and IgA of patients and HCW showed an overall immuno-dominance of N- and S-specific antibodies over S1, S2 and RBD antigens. However, we observed, consistent with other studies (51), that the N/S ratios were not always homogeneous. Further analyses of the evolution of antigen-specific antibody responses of our cohorts over time will help to interpret the relationships between these metrics and the clinical outcome of SARS-CoV-2 infection.

Our data indicate that serum IgA responses paralleled those of IgG in terms of antigen specificity, albeit the magnitude is

significantly lower. Mucosal IgA might represent a critical component of the immune response against COVID-19 (52, 53) as it contributes to virus neutralisation (54). Interestingly, studies have stablished a correlation between serum IgA levels and severity, with mild COVID-19 cases, such as those occurring in the young, showing secretory IgA responses with little detection of IgA in serum (55). In our study we have found IgA in convalescent samples collected 3-5 months post-infection but consistent with Sterlin's findings (54) the levels were significantly reduced relative to IgG titres. However, Varadhachary and co-workers (53) have detected peak IgA levels in saliva at 3 months post-infection suggesting the kinetics of IgA in serum and mucosal surfaces are different. In our study we did not measure mucosal IgA and thus we were unable to establish their correlation with serum IgA but further efforts should be aimed at elucidating how these two isotypes evolve in time in different body compartments in order to define an IgA-based biomarker of protection.

After a viral infection, IgM responses are usually the first to appear in serum and this is the case too for COVID-19 (56, 57). Our data indicates that IgM are easily detected only in a few individuals from the patients and seropositive HCW cohorts. Some studies report IgM lasting up to at least 3 months post-infection (56, 58) and it is therefore not surprising that IgM was detected in some of our convalescent samples.

A cornerstone of our study was the use of the WHO International Standard for anti-SARS-CoV-2 immunoglobulin (20/136) to benchmark neutralising antibody responses and to relate other binding assays to our findings with this standard. We expressed in International Units (IU) and Binding Antibody Units (BAU) the results of the most commonly used serological assays (25). The objective of this approach was to adopt the WHO International Standard unitage to quantify the levels of cardinal serological (antibody) biomarkers of COVID-19 immunity in order to facilitate cross-comparison of immunogenicity data, which ultimately will facilitate the derivation of Correlates of Protection against COVID-19. This may become increasingly important for bio-regulatory approval of SARS-CoV-2 vaccines in future months. Indeed, the emergence and rapid spread across the globe of COVID-19, prompted the rapid development of vaccines against this disease. However, the vast amount of scientific data arising from clinical trials and epidemiological studies addressing COVID-19 immunity have not yet translated into an unequivocal definition of a reliable CoP. The vaccines that are now being used across the globe were licensed on the basis of vaccine efficacy data obtained in placebo controlled clinical trials. These are very costly and they depend on the rates of natural infections occurring in the populations to which the vaccinated participants belong. However, more vaccines are needed to meet the global public health demands, even more so with the emergence of SARS-CoV-2 variants of concern with proven ability to escape the antibody responses developed against vaccines or previous infections (https://www.sciencedirect.com/science/article/pii/S0092867421002981). But the exposure of participants to natural infections in placebo-controlled clinical trials, are increasingly difficult to justify. Furthermore, recruitment of seronegative volunteers will become more and more complicated with the continuing rise of SARS-CoV-2 seroprevalence in the global human population. In these circumstances, non-inferiority clinical trial designs and immuno-bridging using an existing vaccine as a comparator would seem to be favoured. The definition of a CoP in International Units would help assess clinical efficacy of COVID-19 vaccines on the basis of analyses of immunogenicity data, rather than relying on evaluating clinical efficacy. Recent studies point to nAb as a reliable indicator of vaccine induced immunity (48). However, the majority of these studies used disparate assays and units to define antibody protection thresholds. The use of an International Unit for this purpose would enable comparative analyses of immunogenicity data to be made facilitating the derivation of CoP. It is important to note, that the WHO International Standard is not intended to be used as a day-to-day reagent, but rather, as a primary calibration reagent against which secondary standards should be calibrated. Thus, in our study, we calibrated our HICC sera against the WHO standard and used these HICC sera as our secondary calibration reagents to derive the unitage of the samples we tested in our assays.

In conclusion, we have identified a range of assays and biomarkers of COVID-19 immunity that will be used to define CoP in future studies using serum and plasma samples sequentially collected from these or similar cohorts, or notably, from vaccination breakthrough or re-infection cases. Such studies would need to extend their focus to SARS-COV-2 variants of concern that have been emerging since the beginning of the pandemic. The emergence of these strains with enhanced transmissibility, pathogenicity and antigenicity represents another challenge for vaccine manufacturers and regulators, and developing methods for standardising assays for comparison of Nab against VOC should be a priority.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Study approved by Research Ethics Committee Wales, IRAS: 96194 12/WA/0148. Amendment 5. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JC-O: Coordinating the laboratory work; analysis of results; conception of the manuscript; writing the manuscript. DW: Statistical analysis. MF: Semiautomated immunology-blotting; analysis of results. AC: ELISA, Lateral flow testing; sample

processing; analysis off results; PS: Blood processing, samples archiving, ELISA testing. AN: ELISA set up; pseudo neutralisation assay. MiP: Laboratory set up; ELISA set up. LO: Semi-automated immunoblotting. DC: pseudoneutralization assays. MMN: pseudoneutralization assay. PP: Statistical analysis. NJT: pseudoneutralization. DP: Design constructs, expression and purification of recombinant RBD. PN: Design constructs, expression and purification of recombinant RBD. RW: Design constructs, expression and purification of recombinant RBD. RD: Luminex assay. SK: Sample selection and provision pre-pandemic sera. AO: Electrochemiluminescence assays. AS: Electrochemiluminescence assays. AS: Electrochemiluminescence assays. TB: Electrochemiluminescence assays. AA: EDNA assay. LCJ: EDNA assay. MaP: Consultation on Standardization of serological assays. WS: Grant holder; assessing manuscript; scientific direction. HB: Clinical Lead; clinical assessment; coordination clinical work. JLH: Scientific direction. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: DAW, MF, RW and JH are affiliated to the company DIOSynVax. JC-O holds a position as vaccinology consultant at Oxford Expression Technologies, Ltd.

The remaining authors 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.

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Response and Duration of Serum Anti-SARS-CoV-2 Antibodies After Inactivated Vaccination Within 160 Days

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Background: A vaccine against coronavirus disease 2019 (COVID-19) with highly effective protection is urgently needed. The anti-severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antibody response and duration after vaccination are crucial predictive indicators.

Objectives: To evaluate the response and duration for 5 subsets of anti-SARS-CoV-2 antibodies after vaccination and their predictive value for protection.

Methods: We determined the response and duration for 5 subsets of anti-SARS-CoV-2 antibodies (neutralizing antibody, anti-RBD total antibody, anti-Spike IgG, anti-Spike IgM, and anti-Spike IgA) in 61 volunteers within 160 days after the CoronaVac vaccine. A logistic regression model was used to determine the predictors of the persistence of neutralizing antibody persistence.

Results: The seropositivity rates of neutralizing antibody, anti-RBD total antibody, anti-Spike IgG, anti-Spike IgM, and anti-Spike IgA were only 4.92%, 27.87%, 21.31%, 3.28% and 0.00%, respectively, at the end of the first dose (28 days). After the second dose, the seropositivity rates reached peaks of 95.08%, 100.00%, 100.00%, 59.02% and 31.15% in two weeks (42 days). Their decay was obvious and the seropositivity rate remained at 19.67%, 54.10%, 50.82%, 3.28% and 0.00% on day 160, respectively. The level of neutralizing antibody reached a peak of 149.40 (101.00–244.60) IU/mL two weeks after the second dose (42 days) and dropped to 14.23 (7.62–30.73) IU/mL at 160 days, with a half-life of 35.61(95% CI, 32.68 to 39.12) days. Younger participants (≤31 years) had 6.179 times more persistent neutralizing antibodies than older participants (>31 years) (P<0.05). Participants with anti-Spike IgA seropositivity had 4.314 times greater persistence of neutralizing antibodies than participants without anti-Spike IgA seroconversion (P<0.05).

Conclusions: Antibody response for the CoronaVac vaccine was intense and comprehensive with 95.08% neutralizing seropositivity rate, while decay was also obvious after 160 days. Therefore, booster doses should be considered in the vaccine strategies.

Keywords: COVID-19, SARS-CoV-2, neutralizing antibody, anti-SARS-CoV-2 antibody, CoronaVac

INTRODUCTION

Vaccines are expected to be the most effective and economical means to prevent and control coronavirus disease 2019 (COVID-19) (1). Immunity to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) induced through either natural infection or vaccination has been shown to provide some degree of protection against reinfection/infection and reduce risk of clinical case fatality (2). Nevertheless, basic questions remain about the mechanism of protection against the disease, the degree of protection that results in asymptomatic infection, and the duration of vaccine-induced humoral and cellular immunity (3–5). Potential differences between different COVID-19 vaccines also remain obscure. There is ongoing transmission of increasingly concerning viral variants that may escape control by both vaccine-induced and convalescent immune responses. Therefore, an understanding of the correlation between vaccine-induced immunization and protection against COVID-19 is urgently needed to assist in the future deployment of vaccines. A critical current challenge is to identify the immune correlate(s) of protection from SARS-CoV-2 infection to evaluate whether an individual is protected based on an immunological marker. Although antibodies are produced, an effective immune response requires the generation of long-lived memory B and T cells. Strong evidence of a protective role for serum neutralizing antibodies exists in really world (6–9). Khoury DS et al. suggested that the neutralization level is highly predictive of immune protection and estimated that the neutralizing antibody level for 50% protection from infection equates to approximately 54 international units (IU)/mL, which is equivalent to 20% of the mean titer in convalescent subjects (2). This study provides an evidence-based model of SARS-CoV-2 immune protection that will assist in developing vaccine strategies. In the real world, the response and duration for anti-SARS-CoV-2 antibody and immune protection after vaccination are crucial predictive indicators that need to be assessed. Here, we evaluated the dynamic response and duration of 5 subsets of anti-SARS-CoV-2 antibodies (neutralizing antibody, anti-RBD total antibody, anti-Spike IgG, anti-Spike IgM, and anti-Spike IgA) after a complete vaccine schedule in 61 volunteers within 160 days and speculated that the protection was based on the dynamic neutralizing antibody levels.

METHODS

Study Design and Participants

We enrolled participants from the Xiamen Boson Biotech Co., Ltd., Fujian, China, who were vaccinated with the first standard dose (0.5 mL per dose) of the inactivated CoronaVac vaccine (Sinovac Life Sciences, Beijing, China) in January 2021 and the second vaccine dose 28 days later. The neutralizing antibody, anti-RBD total antibody (total antibody against the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein), anti-Spike IgG (Immunoglobulin G antibody against the spike protein), anti-Spike IgM (Immunoglobulin M antibody against the spike protein), and anti-Spike IgA (Immunoglobulin A antibody against the spike protein) were determined to evaluate immune response and duration at 7-day intervals over 9 visits (0 to 56 days post-vaccine) and additional 2 visits (130 and 160 days post-vaccine). The exclusion criteria included those participants with previous or later SARS-CoV-2 infection, with allergy to any ingredient included in the vaccine, who had received any blood products in the past 4 months, who had received any research medicines or vaccines in the past month, who had uncontrolled epilepsy or other serious neurological diseases, with acute febrile disease, with the acute onset of chronic diseases, with uncontrolled severe chronic diseases, and who were unable to comply with the study schedule. Finally, 61 participants were enrolled in our study. The ages of the participants ranged from 25 to 57, with a median age of 37, and 44 (72%) volunteers were women.

This study was approved by the Institutional Ethics Committee of Zhongshan Hospital, Medical College of Xiamen University, and was in compliance with national legislation and the Declaration of Helsinki guidelines. All participants provided written informed consent.

Laboratory Assays

Approximately 3 mL of blood was collected in coagulation tubes from all participants who had fasted for at least 8 h. The blood samples were centrifuged at 3,000 ×g, and the upper serum layer was analyzed for the 5 subsets of anti-SARS-CoV-2 antibodies within 6 h of sampling using the reagent matching Autolumo A2000 plus system, which functions based on a chemiluminescence microparticle immunoassay (Anto Biological Pharmacy Enterprise Co., Ltd., Zhengzhou, China). The resulting chemiluminescent reaction was measured as relative light units (RLU). Detection experiments were performed according to the manufacturer's instructions. The neutralizing antibody assay was based on the one-step competitive method. SARS-CoV-2-specific neutralizing antibodies in the sample bind to an HRP-labeled RBD antigen, which neutralizes the binding of ACE2 (coated on the microparticles) and the RBD antigen. The HRP-labeled RBD antigen not neutralized by SARS-CoV-2-specific neutralizing antibodies forms a complex with ACE2 on the microparticles. The RLU were inversely proportional to the amount of SARS-

CoV-2 neutralizing antibody in the sample. The neutralizing antibody titer was calibrated and traceable to the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin and was recorded as IU/mL (10). Based on a 50% protection from SARS-CoV-2 infection, <54.00 IU/mL was considered negative, and ≥54.00 IU/mL was considered positive (2). The anti-RBD total antibody titer was recorded as arbitrary units (AU)/mL based on a 4-parameter fitting method in which the calibration curve was established with the calibrator concentration as the horizontal axis and the calibrator RLU value as the vertical axis, <8.00 AU/mL was considered negative, and ≥8.00 AU/mL was considered positive. The anti-Spike IgG, anti-Spike IgM, and anti-Spike IgA titers were recorded S/CO (RLU of samples to be tested/cutoff), S/CO <1.00 was considered negative, and S/CO ≥1.00 was considered positive.

Statistical Analysis

The statistical analysis was carried out using IBM SPSS statistics version 26 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism version 8.00 (GraphPad Software, San Diego, CA, USA). Continuous variables that did not follow a normal distribution are reported as medians with interquartile ranges (IQRs). Repeated-measures ANOVA was conducted to assess the differences in antibody titers over time. The Mann-Whitney U test was used for the group comparisons. The trajectory of antibody titers was fitted by a multilevel model with random intercepts and random slopes. The half-life of antibody titers in subjects was assessed over time using the same multilevel modeling approach in R version 3.6.3 (2). A logistic regression model was used to determine the predictors of neutralizing antibody persistence. A p value <0.05 was considered statistically significant.

RESULTS

Anti-SARS-CoV-2 Antibody Response and Duration After Vaccination

We determined the levels of neutralizing antibody, anti-RBD total antibody, anti-Spike IgG, anti-Spike IgM, and anti-Spike IgA in 61 participants within 160 days after vaccination. The neutralizing antibody response had minimal response at two weeks after the first dose. The seropositive rate for neutralizing antibody was only 4.92% (3/61) (95% CI, 0.00% to 10.50%) at 28 days after the first dose based on the cutoff value of 54.00 IU/mL. Encouragingly, the seropositivity rate rapidly increased after the second dose, rising to 52.46% (32/61) (95% CI, 39.60% to 65.40%) in one week (35 days) and reaching a peak of 95.08% (58/61) (95% CI, 89.50% to 100.00%) at two weeks (42 days) (only 3 participants without response). The peak was maintained for 1 week and began to decrease three weeks after the second dose (49 days). After 160 days, the seropositive rate dropped to only 19.67% (12/61) (95% CI, 9.40% to 29.90%) (**Figure 1A**). The level of neutralizing antibody increased from a base value of 5.65 (2.15–8.22) IU/mL to 15.18 (10.46–21.89) IU/mL at the end of the first dose (28 days). After the second dose, the level of neutralizing antibody rapidly increased and reached a peak of 149.40 (101.00–244.60) IU/mL at two weeks (42 days). The level of neutralizing antibody also began to decline three weeks after the second dose (49 days) and dropped to only 14.23 (7.62–30.73) IU/mL at 160 days (**Table 1**). To measure decay in neutralizing antibody levels, we fitted a model of exponential decay and analyzed the half-life. The neutralizing antibody half-life was 35.61 (95% CI, 32.68 to 39.12) days after vaccination within 160 days (**Figure 1A**).

For the anti-RBD total antibody, the seropositive rate was 27.87% (17/61) (95% CI, 16.30% to 39.40%) after the first dose (28 days). Notably, the seropositivity rate rapidly increased after the second dose, rising to 83.61% (51/61) (95% CI, 74.00% to 93.20%) within one week (35 days) and reaching a peak of 100.00% (61/61) within two weeks (42 days), which was maintained for another 2 weeks (56 days). However, the seropositive rate remained at only 54.10% (33/61) (95% CI, 41.20% to 67.00%) at 160 days (**Figure 1B**). The dynamic titer of the anti-RBD total antibody was similar to the seropositivity rate. After the first dose, the anti-RBD total antibody level slightly increased, from a base value of 0.00 (0.00–0.00) AU/mL to 1.68 (0.00–11.79) AU/mL at 28 days (P<0.001). After the second dose, it rapidly increased and reached a peak of 131.30 (70.16–229.20) AU/mL within the two weeks (42 days), then began to decline three weeks later after the second dose (49 days), and dropped to 11.57 (4.44–20.68) AU/mL at 160 days (**Table 1**). The anti-RBD total antibody half-life was 36.46 (95% CI, 33.48 to 40.02) days after vaccination within 160 days (**Figure 1B**). The response and duration for anti-Spike IgG after vaccination were similar to those of the anti-RBD total antibody. The seropositive rate for anti-Spike IgG was 21.31% (13/61) (95% CI, 10.70% to 31.90%) after the first dose. After the second dose, the seropositive rate rose to 77.05% (47/61) (95% CI, 66.20% to 87.90%) in one week (35 days), reached a peak of 100.00% (61/61) in two weeks (42 days) and was maintained for another 2 weeks (56 days). After 160 days, the seropositive rate was still 50.82% (31/61) (95% CI, 37.90% to 63.10%) (**Figure 1C**). Within 160 days after vaccination, the anti-Spike IgG half-life was 30.33 (95% CI, 28.20 to 32.80) days (**Figure 1C**).

The response and duration for anti-Spike IgM and anti-Spike IgA were much different from the above, exhibiting an obviously lower seroconversion and shorter duration. The anti-Spike IgM seropositive rate was only 3.28% (2/61) (95% CI, 0.00% to 7.90%) at 28 days after the first dose. After the second dose, the peak seropositive rate was 59.02% (36/61) (95% CI, 46.30% to 77.10%) at 42 days and immediately decayed without maintenance. At 130 days after the first dose, the positive rate precipitously dropped to a minimum of 3.28% (2/61) (95% CI, 0.00% to 7.90%) (**Figure 1D**). The anti-Spike IgM half-life was 13.54 (95% CI, 11.84 to 15.82) days within 56 days after vaccination (**Figure 1D**). Similarly, anti-Spike IgA seroconversion was not observed 28 days after the first dose. After the second dose, the highest IgA seropositive rate was only 31.15% (19/61) (95% CI, 19.20% to 43.10%) at two weeks (42 days) and immediately decayed. At 130 days, the anti-Spike IgA seropositivity disappeared (**Figure 1E**). The dynamic levels of anti-Spike IgM

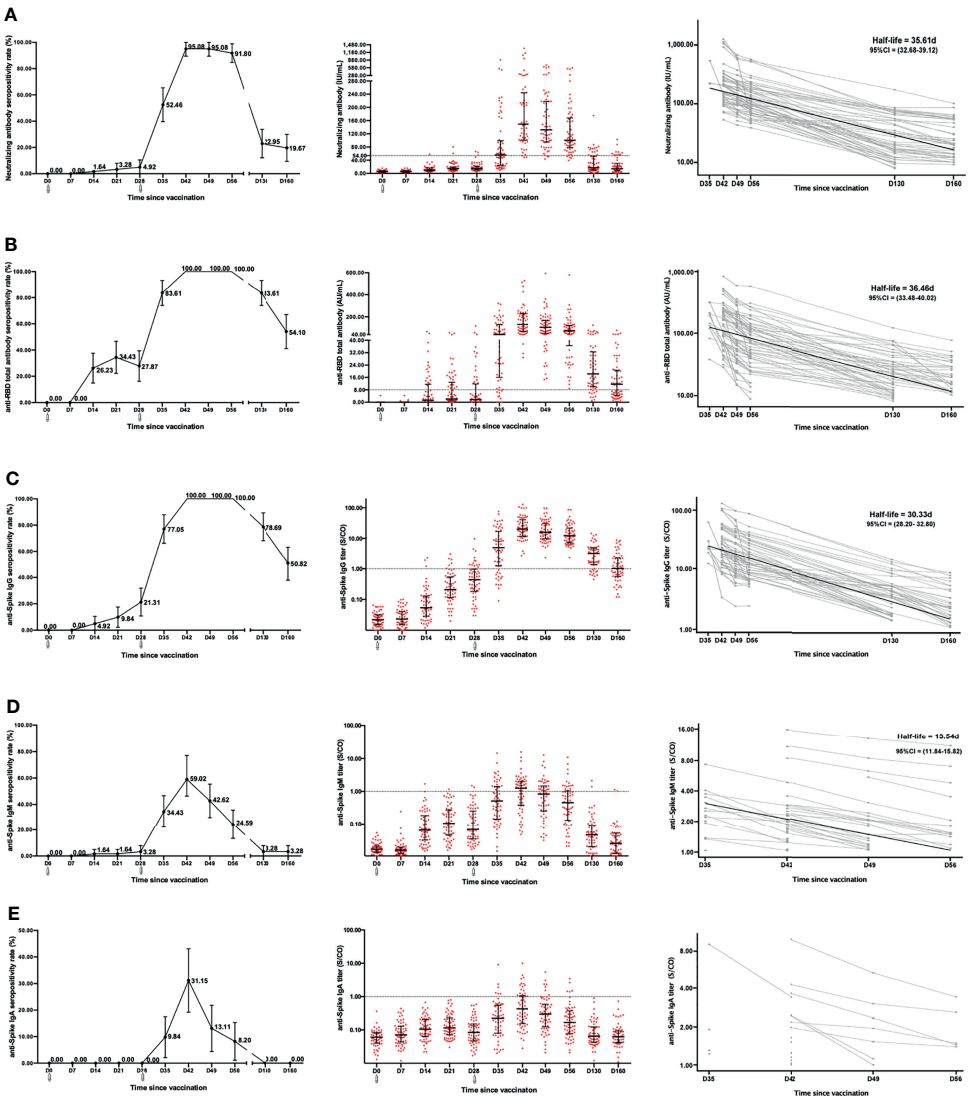

FIGURE 1 | Anti-SARS-CoV-2 antibody response and duration after vaccination over time. The levels and half-lives of neutralizing antibody **(A)**, anti-RBD total antibody **(B)**, anti-Spike IgG **(C)**, anti-Spike IgM **(D)**, and anti-Spike IgA **(E)** were determined after vaccination over time. There were significant differences with repeated-measures ANOVA in all of antibodies ($P < 0.05$). The decay half-lives for individuals were estimated using a linear mixed effects model with censoring of titers below the positive threshold. Receive vaccine .

and anti-Spike IgA were very low within 160 days after vaccination (**Table 1**).

Factors Associated With the Duration of the Neutralizing Antibody Response

Logistic regression was used to analyze the significance of sex, age, anti-Spike IgA response and anti-Spike IgM response in the persistence of neutralizing antibodies at 160 days. Age and anti-Spike IgA response were indeed independent factors ($P<0.05$). Younger participants (≤31 years) had a higher likelihood of neutralizing antibody persistence than older participants (>31 years), with an odds ratio of 6.179. Participants with anti-Spike IgA seropositivity had a higher likelihood of a persistence of neutralizing antibody than participants without anti-Spike IgA seroconversion, with an odds ratio of 4.314 (**Table 2**).

DISCUSSION

The dynamics of immunity and protection after vaccination are the basis for formulating vaccine strategies. The immune response after vaccination includes humoral and cellular immunity. Attenuated vaccines use a two-dose strategy to achieve a high antibody response. In our study, 61 participants who received the first dose of the CoronaVac inactivated vaccine indued a very low level of neutralizing antibody, anti-RBD total

TABLE 1 | Level of anti-SARS-CoV-2 antibody over time after vaccination.

Antibody	D0	D7	D14	D21	D28	D35	D42	D49	D56	D130	D160	*P*
Neutralizing antibody (IU/mL)	5.65 (2.15–8.22)	5.74 (2.15–8.41)	10.63 (6.92–16.94)	14.34 (10.80–19.40)	15.18 (10.46–21.89)	55.78 (24.51–99.23)	149.40 (101.00–244.60)	131.90 (95.04–218.10)	100.50 (77.89–168.20)	17.12 (10.18–52.36)	14.23 (7.62–30.73)	<0.001
Anti-RBD total antibody (AU/mL)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	1.28 (0.00–11.53)	2.14 (0.92–12.93)	1.68 (0.00–11.79)	40.83 (16.05–130.40)	131.30 (70.16–229.20)	106.50 (48.77–168.60)	72.84 (36.58–122.90)	18.32 (10.08–32.54)	11.57 (4.44–20.68)	<0.001
Anti-Spike IgG (S/CO)	0.02 (0.02–0.03)	0.02 (0.02–0.04)	0.05 (0.03–0.13)	0.21 (0.12–0.55)	0.44 (0.19–0.98)	5.00 (1.23–16.98)	20.25 (11.72–41.02)	15.92 (9.73–30.87)	12.17 (7.25–21.94)	3.21 (1.35–4.83)	1.02 (0.57–2.25)	<0.001
Anti-Spike IgM (S/CO)	0.02 (0.01–0.02)	0.02 (0.01–0.02)	0.06 (0.03–0.18)	0.11 (0.04–0.27)	0.07 (0.03–0.25)	0.51 (0.14–1.42)	1.25 (0.38–2.03)	0.83 (0.25–1.46)	0.46 (0.13–1.03)	0.04 (0.02–0.09)	0.02 (0.01–0.05)	<0.001
Anti-Spike IgA (S/CO)	0.06 (0.04–0.08)	0.07 (0.04–0.13)	0.11 (0.06–0.21)	0.12 (0.07–0.23)	0.08 (0.05–0.15)	0.22 (0.08–0.55)	0.43 (0.15–1.06)	0.30 (0.12–0.59)	0.17 (0.08–0.38)	0.07 (0.04–0.12)	0.06 (0.04–0.09)	<0.001

The level of antibody was recorded as medians with interquartile ranges (IQRs). Repeated measures ANOVA was constructed to assess the differences.

TABLE 2 | Factors associated with duration of neutralizing antibody.

	Persistence time M (IQR) (day)	P_1	Persistence rate at 160 days % (n/N)	Odds ratio (95%CI)	P_2
Sex		0.971			
Female	95 (88–118)		23.8% (10/42)	1.0	
Male	95 (88–102)		12.5% (2/16)	0.416(0.066–2.609)	0.349
Age group		0.015			
>31	95 (88–95)		11.9% (5/42)	1.0	
≤31	95 (95–125)		43.7% (7/16)	6.179 (1.454–26.266)	0.014
Anti-Spike IgA		0.158			
Negative	95 (88–102)		12.8% (5/39)	1.0	
Positive	95 (88–125)		36.8% (7/19)	4.314(1.020–18.246)	0.047
Anti-Spike IgM		0.662			
Negative	95 (88–118)		13.0%(3/23)	1.0	0.494
Positive	95 (88–95)		25.7%(9/35)	1.782(0.340–9.354)	

M, medians. IQR, interquartile range.

1. The Mann-Whitney U test was constructed to assess the differences in the persistence over time.

2. A logistic regression model was used for the predictors of the persistence of neutralizing antibodies.

antibody, anti-Spike IgG, anti-Spike IgM, and anti-Spike IgA levels. However, all of the antibody levels increased rapidly after the second dose and reached a peak within two weeks (42 days); the neutralizing antibody seropositivity rate was 95.08%, and the seropositivity rate for anti-RBD total antibody or anti-Spike IgG was 100%. On the other hand, the decay of the antibody was obvious. The neutralizing antibody, anti-RBD total antibody, anti-Spike IgG and anti-Spike IgM half-lives were 35.61 days, 36.46 days, 30.33 days and 13.54 days, respectively. The seropositivity rates of the neutralizing antibody and the anti-RBD total antibody were only 19.67% and 54.10% on 160 days after vaccination. Our results showed that the immune response to the vaccine was intense and comprehensive, but the decay was obvious.

The neutralization level is an important predictor of vaccine efficacy (11). Immunity to SARS-CoV-2 induced through either natural infection or vaccination has been shown to afford a degree of protection against reinfection/infection or to reduce the risk of clinically significant outcomes (12, 13). Seropositive recovered COVID-19 patients had an 89% protection from reinfection, and vaccine efficacies against infection were reported to be 50 to 95% (4, 14, 15). In addition, the passive transfer of neutralizing antibodies can prevent severe SARS-CoV-2 infection in multiple animal models (16, 17), and Regeneron has recently reported similar data in humans (18). Neutralizing antibody levels are highly predictive of immune protection, which may wane with time as neutralizing antibody levels decline (2). In our study, the dynamic response and duration of neutralizing antibodies at various time points after vaccination were measured to evaluate the efficacy of the vaccine. The neutralizing antibody was traceable to the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin. The threshold of the neutralizing antibody level for 50% protection was considered 54.00 IU/mL (2). The seropositive rate of the neutralizing antibody was only 4.92% at the end of the first dose (28 days), and the level of neutralizing antibody was also only 15.18 IU/mL. It is quite clear that effective protection from infection was hard to obtain after one dose. However, the seropositive rate of neutralizing antibody rose to 52.46% in one week (35 days) and reached a peak of 95.08% in two weeks (42 days), which is a high level of antibody after the second dose. After vaccination, the majority of adult individuals could produce neutralizing antibodies to prevent infection.

On the other hand, the duration of immunity after vaccination is vital and is used to estimate the protective effects of vaccination. The plasma-derived hepatitis B vaccine maintains a satisfactory protection for 20–31 years after the initial immunization (19), protective immunity after pertussis vaccination wanes after 4–12 years (20), and the protection conferred by influenza vaccination is generally thought to last less than one year, which necessitates annual revaccination (21). Recent studies have identified a gradual decline in the neutralization titer for up to 8 months after SARS-CoV-2 infection (22–24). In our study, the neutralizing antibody started to decline three weeks post vaccination (49 days) and dropped to 14.23 IU/mL on the 160th day, at which point the seropositive rate was only 19.67%. Based on the threshold of the neutralizing antibody level for 50% protection from infection (54.00 IU/mL), the ability to protect against infection became poor at 160 days after vaccination, indicating that booster doses should be considered in future vaccine strategies.

In addition, protection from severe SARS-CoV-2 infection is another effect of vaccines. It was reported that the neutralizing antibody level for 50% protection from severe infection was equivalent to 3.0% of the mean titer in convalescent subjects (equating to approximately 8.10 IU/mL) (2). Our results showed that the neutralizing antibody level was 14.23 IU/mL at 160 days. It is possible that vaccine recipients could still obtain sustained protection from severe infection after vaccination for 160 days. Moreover, our results showed that the seropositive rates for anti-RBD total antibody and anti-Spike IgG were still 54.10% and 50.82%, respectively, at 160 days. In addition, protective effects also involve B cell memory and T cell responses, which may be more durable and may play a larger role later after infection or vaccination (22, 24).

The decay of anti-SARS-CoV-2 antibody levels is closely associated with vaccination efficacy. It has been reported that the decay of vaccine-induced neutralization ability was similar to that observed after natural SARS-CoV-2 infection (2, 21). In our study, the half-life of neutralizing antibody, anti-RBD total antibody, anti-Spike IgG and anti-Spike IgM was 35.61days, 36.46days, 30.33days and 13.54days, respectively. The decay of vaccine-induced anti-Spike IgG and anti-Spike IgA were shorter than that reported after natural SARS-CoV-2 infection (24). The anti-RBD total antibody and anti-Spike IgG half-lives were similar to those with Pfizer BNT162b2 or Moderna mRNA-1273 vaccination (25). CoronaVac has been approved for emergency use in several countries and was crucial for curbing the pandemic (26); the efficacies have been reported to be 50%, 65%, 78% and 91% in clinical trials in several countries (4, 27). Many factors affect the efficacy, including optimization of dose, schedule and boosters, as well as the sex, age and even race of the recipient. In our study, we further conducted a sustained multifactor analysis of neutralizing antibody levels to understand the factors influencing of neutralizing antibody persistence. Our results showed that age and the anti-Spike IgA response were indeed independent factors influencing neutralizing antibody persistence. Younger participants had a higher likelihood of persistent neutralizing antibody than older participants (6.179 times). Participants with anti-Spike IgA seropositivity had a higher likelihood of neutralizing antibody persistence than participants without anti-Spike IgA seroconversion, with an odds ratio of 4.314. In our study, anti-Spike IgA seroconversion did not occur at the end of the first dose (28 days). After the second dose, the anti-Spike IgA seropositive rate was 31.15% at two weeks (42 days) and started to decay at 49 days. After 130 days, anti-Spike IgA seropositivity disappeared, which is different from the case with SARS-CoV-2 natural infection, which resulted in less decay at 1.3 and 6.2 months after natural infection (22). Specific IgA serum concentrations have been found to decrease notably 1 month after symptom onset, but neutralizing IgA remained detectable in the saliva for a longer time (days 49 to 73 post symptoms) (28). The early SARS-CoV-2-specific humoral response is dominated by anti-Spike IgA antibody responses (29), and IgA antibodies have been shown to bind to the RBD of SARS-CoV-2 and to neutralize the virus (30). Higher concentrations of serum IgA are associated with better persistence of neutralizing antibodies.

This study had some limitations. First, we enrolled only 61 uninfected volunteers, which is a relatively limited number of participants. Second, effective vaccines must elicit a diverse repertoire of antibodies (humoral immunity) and CD8+ T-cell responses (cellular immunity). Unfortunately, the immune cell response and evolution were not evaluated in this study due to the lack of effective cell preservation. Third, comparing with the gold standard for neutralization assay that is a cell-based assay based on either real virus or pseudovirus, the neutralization assay we used is limited in determining the true neutralizing capacity of antibodies. Fourth, one flaw in the study sampling was the large interval between 58 days and 130 days. Finally, due to the effective prevention and control of the epidemic in China, the protective efficacy of the vaccine could not be verified.

In conclusion, our results indicated that the immune response was activated in all participants after COVID-19 vaccination. The majority of adult individuals could produce neutralizing antibodies after vaccination, which could have a certain protective effect against SARS-CoV-2 infection. However, the antibody titer was severely attenuated, and booster doses should be considered in vaccine strategies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study was approved by the Institutional Ethics Committee of Zhongshan Hospital of Xiamen University, School of

Medicine, Xiamen University, and complied with national legislation and the Declaration of Helsinki guidelines. Informed consent was obtained according to the institutional guidelines. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Q-YX is the first author. T-CY and X-ML are joint corresponding authors. T-CY designed the trial and the study protocol and critically reviewed and revised the manuscript. Q-YX and X-ML worked as coprincipal investigators of this trial and drafted the manuscript. Q-YX was responsible for data collation. J-HX and YX were responsible for statistical analysis and validation. Z-JJ, M-JW, and Y-YL contributed to sample collection, sorting and verification. W-LL, J-HX, X-ML, and Q-YX were responsible for laboratory analyses and monitored the trial. All authors read and approved the final manuscript.

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The remaining authors 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.

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Two Opposing Roles of SARS-CoV-2 RBD-Reactive Antibodies in Pre-Pandemic Plasma Samples From Elderly People in ACE2-Mediated Pseudovirus Infection

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A novel coronavirus designated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged and caused an outbreak of unusual viral pneumonia. Several reports have shown that cross-reactive antibodies against SARS-CoV-2 also exist in people unexposed to this virus. However, the neutralizing activity of cross-reactive antibodies is controversial. Here, we subjected plasma samples from SARS-CoV-2-unexposed elderly Korean people (n = 119) to bead-based IgG antibody analysis. SARS-CoV-2 S1 subunit-reactive IgG antibody analysis detected positive signals in some samples (59 of 119, 49.6%). SARS-CoV-2 receptor-binding domain (RBD)-reactive antibody levels were most significantly correlated with human coronavirus-HKU1 S1 subunit-reactive antibody levels. To check the neutralizing activity of plasma samples, the SARS-CoV-2 spike pseudotype neutralizing assay was used. However, the levels of cross-reactive antibodies did not correlate with neutralizing activity. Instead, SARS-CoV-2 pseudovirus infection was neutralized by some RBD-reactive plasma samples (n = 9, neutralization ≥ 25%, $P \leq 0.05$), but enhanced by other RBD-reactive plasma samples (n = 4, neutralization ≤ -25%, $P \leq 0.05$). Interestingly, the blood plasma groups with enhancing and neutralizing effects had high levels of SARS-CoV-2 RBD-reactive antibodies than the plasma group that had no effect. These results suggest that some SARS-CoV-2 RBD-reactive antibodies from pre-pandemic elderly people exert two opposing functions during SARS-CoV-2 pseudovirus infection. In conclusion, preformed RBD-reactive antibodies may have two opposing functions, namely, protecting against and enhancing viral infection. Analysis of the epitopes of preformed antibodies will be useful to elucidate the underlying mechanism.

Keywords: SARS-CoV-2, pre-pandemic samples, cross-reactive antibodies, receptor binding domain (RBD), neutralizing activity

INTRODUCTION

Four seasonal human coronaviruses (HCoVs), the alphacoronaviruses HCoV-229E and HCoV-NL63 and the betacoronaviruses HCoV-HKU1 and HCoV-OC43, are globally distributed and usually cause mild upper respiratory tract illness and common cold (1, 2). At the end of 2019, however, a novel coronavirus belonging to the *Betacoronavirus* genus designated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged and caused an outbreak of unusual viral pneumonia (2, 3). In addition, the emergence of additional new variants with mutated receptor-binding domains (RBDs) with increased ACE2 binding affinity has produced a health emergency (1, 4, 5). Interestingly, SARS-CoV-2 shares homologous sequences with common coronaviruses. Therefore, there have been various studies to determine whether the immune responses to HCoV infection affect the severity of coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2. A study suggested that $HCoV^+$ SARS-CoV-2–infected hospitalized patients had less severe COVID-19 illness with lower odds for intensive care unit admission and higher survival probability than $HCoV^-$ SARS-CoV-2–infected hospitalized patients (6). In a study of T cell immunity to SARS-CoV-2, 44% of blood samples of unexposed subjects produced interferon-γ after stimulation by SARS-CoV-2 spike protein, the RBD protein or nucleocapsid protein (7). These studies show that preexisting memory $CD4^+$ T cells reactive to HCoVs can cross-react with corresponding homologous sequences of SARS-CoV-2 and can affect COVID-19 patient disease severity (8). In a study of B cell immunity to SARS-CoV-2, it was also identified that there were immunoglobulin G (IgG) antibodies reactive to SARS-CoV-2 spike protein in some unexposed samples (9–11). Furthermore, studies have revealed an increased level of OC43 spike protein-reactive IgG antibodies after SARS-CoV-2 infection, suggesting that preexisting memory B cells targeting the epitope of SARS-CoV-2 homologous with the common cold virus can be boosted by SARS-CoV-2 infection (11, 12). However, there is controversy regarding whether cross-reactive antibodies have neutralizing activity against SARS-CoV-2 (9–12). In previous studies, the cross-reactive IgG antibodies against total spike proteins of SARS-CoV-2 were quantified, and the neutralizing activities of the spike protein cross-reactive IgG antibodies were analyzed (9, 12). Furthermore, homology between HCoV and SARS-CoV-2 is higher in the spike S2 subunit than in the spike S1 subunit. Thus, most of the cross-reactive IgG antibodies target the S2 subunit (9, 10). However, the S1 subunit, especially the RBD, is responsible for the direct binding of the spike protein with ACE2, and antibodies targeting the S1 subunit are the main source of neutralizing activity (13, 14). Thus, the relationship between analyzed cross-reactive antibody levels and neutralizing activities can vary because total spike protein-reactive antibodies have been analyzed (9, 12).

MATERIALS AND METHODS

Plasma Samples

Total plasma samples (n = 119) were collected from Korean elderly people attending Chosun University Hospital and Chonnam National University Medical School in Korea before the COVID-19 pandemic (from June 2014 to June 2019). The donors of the plasma samples do not have any comorbidities. All experiments were performed in accordance with the relevant guidelines and regulations. Information related to their age and sex is reported in **Supplementary Table 1**.

Cell Lines

HEK293T cells were from the American Type Culture Collection (ATCC, Cat. CRL-3216) and cultured in 10% fetal bovine serum (FBS, HyClone, Cat. SH3008403)-supplemented Dulbecco's modified Eagle's medium (DMEM, HyClone, Cat. SH30243.01) at 37°C and with 5% CO_2. HEK293T-ACE2 cells were established *via* infection of VSV G-pseudotyped lentivirus packaging human ACE2 encoded by pWPI-IRES-Puro-Ak-ACE2 [Addgene, Cat. 154985, kindly provided by Inchan Kwon (Gwangju Institute Science and Technology)]. Pseudotype production was performed as described in the Method Details below. The ACE2 expression level was maintained under 10 μg/ml puromycin. ACE2 expression was confirmed through surface staining (R&D Systems, Cat. FAB933A-100) and inhibition assay by soluble ACE2 protein (*In vivo*gen, Cat. fc-hace2).

Bead-Based IgG Antibody Analysis

Before bead-based IgG antibody analysis, protein biotinylation was performed with EZ-Link™ NHS-LC-LC-Biotin (*Thermo Fisher* Scientific, Waltham, MA, USA, Cat. 21343) according to the manufacturer's protocols. Dialysis was conducted using a Slide-A-Lyzer™ MINI Dialysis Device Kit (Thermo Fisher Scientific, Cat. 69558) for removing unreacted biotinylation reagent. Dialysis using PBS was repeated twice at RT for 2 h, followed by dialysis at 4°C for 12 h and dialysis at RT for 1 h with gentle stirring. Biotinylated proteins were stored in 50% glycerol and 0.02% sodium azide buffer at -20°C. HCoV-OC43 spike S1 subunit protein (AcroBIOSYSTEMS, Cat. SIN-V52H5), HCoV-HKU1 spike S1 subunit protein (SinoBiological, Cat. 40021-V08H), HCoV-229E spike S1 subunit protein (SinoBiological, Cat. 40601-V08H), HCoV-NL63 spike S1 subunit protein (SinoBiological, Cat. 40600-V08H), SARS-CoV-2 spike S1 subunit protein (SinoBiological, Cat. 40591-V08H) and SARS-CoV-2 RBD protein (SinoBiological, Cat. 40592-V08B) were used for biotinylation. For bead-based IgG antibody analysis, Dynabeads M-280 Streptavidin (< 200 μg, Invitrogen, Carlsbad, CA, USA, Cat. 11205D) were coated with biotinylated proteins (10 μg/ml) for 2 h at 4°C and washed twice with a solution of 0.1% bovine serum albumin (BSA) (BOVOGEN, *Melbourne, Australia, Cat.* BSAS 0.1) in PBS (pH 7.4) for 5 min at 4°C. After bead isolation, the beads were incubated with plasma samples (diluted 1:400 in PBS/0.1% BSA). After incubation overnight at 4°C, washing was performed twice with PBS (pH 7.4) containing 0.05% Tween-20. Isolated beads were incubated with secondary antibodies (diluted at 0.4 μg/ml in PBS/0.1% BSA), and R-phycoerythrin Affinipure F(ab')2 fragment goat anti-human IgG antibodies (Jackson ImmunoResearch, *West Grove, PA, USA*, Cat. 109-116-170) were incubated overnight at 4°C. Washing was performed twice with PBS containing 0.05% Tween-20. The beads were analyzed on a BD *FACSCanto II* (*Becton, Dickinson and*

Company, *Franklin Lakes*, NJ, United States) and analyzed with FlowJo™ v10.7.1 (*Becton, Dickinson and Company*) data analysis software. A serially diluted anti-SARS-CoV-2 RBD neutralizing antibody (AcroBIOSYSTEMS, Cat. SAD-S35) was used to quantify plasma IgG antibodies. Therefore, 1 AU was defined as an amount equivalent to 1 ng of anti-RBD antibody.

Enzyme-Linked Immunosorbent Assay (ELISA)

Human anti-SARS-CoV-2 S1 RBD IgG antibody ELISAs (BioVendor, Cat. RAI009R) were used to compare the limit of detection (LOD) and limit of quantitation (LOQ) with the bead assay, according to the manufacturer's protocols. For calculation of the LOD and LOQ, serially diluted anti-SARS-CoV-2 RBD neutralizing antibody (AcroBIOSYSTEMS) was used.

SARS-CoV-2 Spike Pseudotype Neutralizing Assay and Quantification

Lentiviral SARS-CoV-2 Spike pseudotypes were generated with a spike-pseudotyped lentiviral kit from BEI Resources (Cat. NR-53816) as described previously (15). Briefly, 4.0×10^6 HEK293T cells were cultured overnight and transfected with 10 μg of pHDM SARS-CoV-2-Spike glycoprotein with a C-terminal 21 amino acid deletion (BEI Resources, Cat. NR-53742), a lentiviral backbone with the Luc2 gene (BEI Resources, Cat. NR-52516) and lentiviral packaging plasmids (BEI Resources, Cat. NR-52517, NR-52518, NR-52519) using FuGENE® HD (Promega, Cat. E2312). Thirty-six hours after transfection, the culture medium was refreshed, and the supernatant was harvested 72 h after transfection. The supernatant was clarified by centrifugation at 500×g for 10 min and filtration through a 0.45 μm filter (Advantec, Cat. 25CS045AS). The filtered media were concentrated through a lenti-X™ concentrator (Takara, Cat. 631232) as described in the manufacturer's guidelines. Lentiviral VSV G pseudotypes packaging human ACE2 were produced under the same platform with a lentiviral backbone plasmid encoding human ACE2 (Addgene, Cat. 154985). These pseudotypes were stored at -80°C until infection.

The titer of lentiviral SARS-CoV-2 pseudotypes that selectively infect HEK293T-ACE2 cells was determined by measuring relative luciferase units (RLUs) as described previously (15). For infection, HEK293T and HEK293T-ACE2 cells were seeded in 50 μl at 1.0×10^4 cells/well in a 96-well cell culture plate. The next day, HEK293T or HEK293T-ACE2 cells were treated with polybrene (5 μg/ml). After the treatment, 1.0×10^6 RLUs/well of pseudoviruses were preincubated with serially diluted plasma, neutralizing antibody or anti-HA antibody, which was a negative control (Sigma-Aldrich, Cat. H9658), for 1 h at 37°C before infection. Forty-eight to sixty hours post infection of the plasma-virus or antibody-virus mixture, a luciferase assay was performed as described in the manufacturer's protocol (Promega, Cat. E1501).

Quantification and Statistical Analysis

The significance of differences between two groups was analyzed using unpaired Student's t-tests. Pearson's correlation was performed using Origin2021 software (Origin Lab Corporation) to determine associations between two continuous variables. To compare the sensitivity of the bead-based IgG antibody assay with that of the ELISA, the LOD and LOQ were calculated using a serially diluted anti-SARS-CoV-2 RBD neutralizing antibody (AcroBIOSYSTEMS). For the determination of the LOD and LOQ values, the limit of blank (LOB) was considered. The LOB was calculated by the mean of the blank + 1.645 (standard deviation of the blank), and the LOD was calculated by $LOD = 3.3 \times \sigma / S$, where S is the slope of the calibration curve, and σ is the standard deviation of the Y-intercept. The LOQ was calculated by $LOQ = 10 \times \sigma / S$ (16). Standard dilution series obtained for the ELISA and bead-based IgG antibody analysis were used to determine the LOD and LOQ. For linear regression to obtain a calibration curve, data with a range higher than the LOB were analyzed using Origin2021 software (Origin Lab Corporation). All data are expressed as the mean ± SD. For all statistical tests, *P*-values ≤ 0.05 were considered significant.

RESULTS

In this study, we first validated the sensitivity of the bead-based IgG antibody assay. RBD-specific bead-based IgG antibody analysis showed lower LODs (5.68 ng/ml) and LOQs (17.2 ng/ml) than RBD-specific ELISA (LOD = 151.1 ng/ml, LOQ = 456.9 ng/ml) (**Figure 1A**).

With the developed bead-based IgG antibody analysis system, we analyzed plasma samples of Korean elderly people (average age 73.1 ± 5.3, n = 119) collected before the SARS-CoV-2 pandemic outbreak (**Supplementary Table 1**). In order to antibody quantification, RBD-specific IgG antibodies were used as a standard (**Supplementary Figure 1**). In the initial analysis, we used S1 subunit proteins of SARS-CoV-2, HCoV-229E, NL63, HKU1 and OC43 for analysis of S1 subunit-reactive IgG antibodies. In the analysis, we used nonconjugated beads to remove signals from the nonspecific binding of plasma antibodies. The analyzed data showed that the mean signal of HCoV-229E (25,038 AU/ml) was higher than those of HCoV-HKU1 (20,351 AU/ml), HCoV-OC43 (9,574 AU/ml), HCoV-NL63 (1,902 AU/ml), and SARS-CoV-2 (519 AU/ml) (**Figure 1B**). In addition, most elderly people in Korea (more than 95%) have IgG antibodies against the S1 subunit of common coronaviruses, including HCoV-229E (119 of 119; 100%), HCoV-HKU1 (118 of 119; 99.2%), HCoV-OC43 (117 of 119; 98.3%), and HCoV-NL63 (114 of 119; 95.8%). Interestingly, even though we analyzed IgG antibodies with the S1 subunit, there were many positive signals from SARS-CoV-2 S1 subunit-reactive IgG antibody analysis (59 of 119; 49.6%) (**Figure 1B**).

In fact, SARS-CoV-2 shares homologous sequences with common coronaviruses. Thus, to determine which coronaviruses most significantly affect cross-reactive antibodies, we analyzed the correlation between SARS-CoV-2 S1 cross-reactive IgG antibody levels and other coronavirus S1-reactive IgG antibody levels. The results showed that HCoV-OC43 S1 subunit-reactive IgG antibody levels were most highly correlated

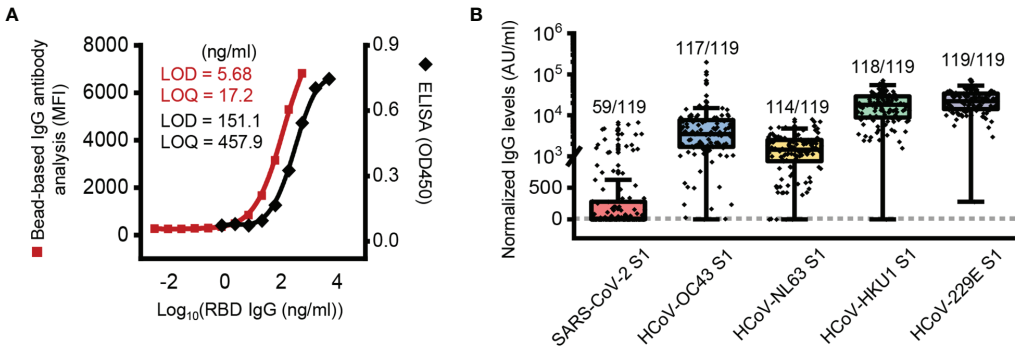

FIGURE 1 | Presence of SARS-CoV-2 S1 subunit reactive antibody in pre-pandemic plasma samples. **(A)** Limit of detection (LOD) and limit of quantification (LOQ) of the ELISA and bead-based IgG antibody analysis. **(B)** Box plot of normalized IgG antibody levels of pre-pandemic samples against each protein. The dotted line represents a threshold set 2-fold above the LOD (11.36 AU/ml). AU, arbitrary unit; 1 AU/ml is equivalent to 1 ng/ml of the anti-RBD antibody.

with SARS-CoV-2 S1 cross-reactive IgG antibody levels ($P < 0.001$, $r = 0.458$) (**Figure 2A**). Thus, our results also showed that HCoV-OC43 infection may mainly contribute to the generation of SARS-CoV-2 cross-reactive antibodies, as shown in a previous study (12). In addition, previous reports have suggested that HCoV-OC43 spike protein-reactive IgG antibodies can affect the disease severity of COVID-19 (11). In addition to S1 subunit-reactive antibody analysis, we also analyzed SARS-CoV-2 RBD cross-reactive IgG antibodies with the plasma samples showing the top 40 SARS-CoV-2 S1 subunit-reactive highest signals for reliable correlation analysis. The levels of SARS-CoV-2 S1 subunit-reactive IgG antibodies had a positive correlation with SARS-CoV-2 RBD-reactive IgG antibody levels ($P < 0.001$, $r = 0.423$) (**Figure 2A**). However, a previous study showed no correlation between SARS-CoV-2 spike protein-reactive IgG antibodies and RBD-reactive IgG antibodies in pre-pandemic plasma samples (12), unlike our data. This discrepancy may be caused by the different analysis systems in which we analyzed SARS-CoV-2 S1 subunit-reactive antibodies. We performed correlation matrix analysis to identify the correlation

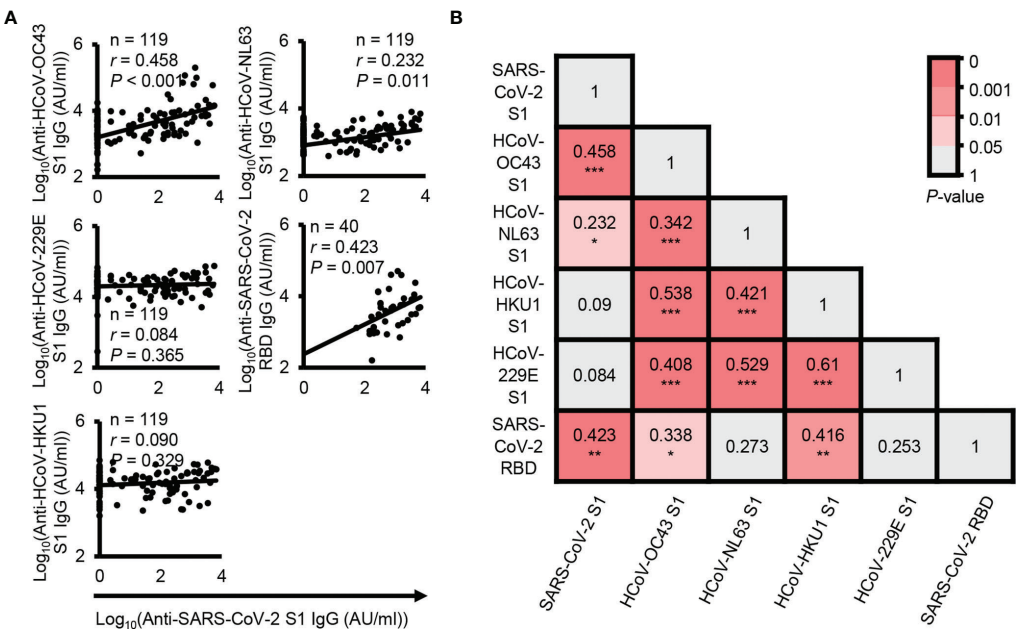

FIGURE 2 | Correlation analysis between SARS-CoV-2 cross-reactive antibody levels and other coronavirus reactive antibody levels. **(A)** Correlation analysis between the levels of plasma IgG antibodies against the SARS-CoV-2 S1 subunit protein and other HCoV S1 subunit proteins. **(B)** Correlation analysis between the levels of plasma IgG antibodies against the SARS-CoV-2 RBD protein and other HCoV S1 subunit proteins. *r* and *P* represent Pearson's correlation coefficient and its associated *P*-value, respectively. *$P \leq 0.05$; **$P \leq 0.01$; and ***$P \leq 0.001$.

patterns between HCoV or between HCoV and SARS-CoV-2 S1 subunit-reactive IgG antibodies. Interestingly, the results showed a high correlation between anti-HCoV antibodies ($P < 0.001$, $r \geq 0.342$), but SARS-CoV-2 S1 subunit-reactive antibodies showed a high correlation only with HCoV-OC43 S1 subunit-reactive IgG antibodies ($P < 0.001$, $r = 0.423$) (**Figure 2B**). In addition, SARS-CoV-2 RBD reactive IgG antibody levels correlated significantly with HCoV-HKU1 S1 subunit reactive IgG antibody levels ($P < 0.01$, $r = 0.416$) (**Figure 2B**). This result possibly indicates that high cross-reactivity between HCoVs can affect protective B cell immunity against different common coronavirus infections. However, antibodies against most HCoV S1 subunits have nonsignificant cross-reactivity with SARS-CoV-2 S1 subunits. For the above reasons, protective B cell immunity against newly emerged SARS-CoV-2 infection may be low in unexposed elderly people.

To check the neutralizing activity of SARS-CoV-2 S1 subunit and RBD cross-reactive IgG antibodies, we generated an ACE2-overexpressing HEK293T cell line because ACE2 has been identified as a receptor for SARS-CoV-2 (**Supplementary Figure 2A**). With the generated cell lines, we successfully infected the cells with recombinant pseudotype virus containing SARS-CoV-2 spike proteins, while HEK293T cells were not infected with the recombinant pseudotype virus (**Figure 3A**). In addition, SARS-CoV-2 spike protein-ACE2 specific binding-mediated infection was confirmed by RBD-neutralizing antibody and ACE2 protein-mediated inhibition of the infection (**Figure 3B** and **Supplementary Figure 2B**). In neutralizing activity analysis, we divided the analyzed samples into four groups using median split (3.63 = $\log_{10}$(AU/ml) for RBD and 2.87 = $\log_{10}$(AU/ml) for S1 subunit) (**Figure 3C**). Interestingly, there were low levels of cross-reactive antibodies against the S1 subunit and high levels of cross-reactive antibodies against the RBD, even though the RBD is a part of the S1 subunit. A previous report also showed a similar result (17, 18), which is possibly caused by hidden epitopes in the S1 subunit structure that are exposed on the RBD without other structural domains of the S1 subunit. Unexpectedly, there was no correlation between neutralizing activity and SARS-CoV-2 S1 subunit and RBD cross-reactive IgG antibody levels (**Figure 3D**) and no difference in neutralizing activity among the four divided groups (**Figure 3E**).

Interestingly, some plasma samples showed significant neutralizing activity (n = 9, neutralization ≥ 25%, $P \leq 0.05$), while others significantly enhanced pseudotype virus infection (n = 4, neutralization ≤ -25%, $P \leq 0.05$) (**Figure 4A** and **Supplementary Figure 3**). Besides, the groups which affected the viral infection, showed significantly higher reactivity to RBD (**Figure 4B**). These results reveal there was no correlation between the levels of cross-reactive S1 subunit- and RBD-specific IgG antibodies and neutralizing activity. This may occur because RBD-reactive antibodies in pre-pandemic plasma samples have two opposing functions, namely, inhibiting and enhancing viral infection.

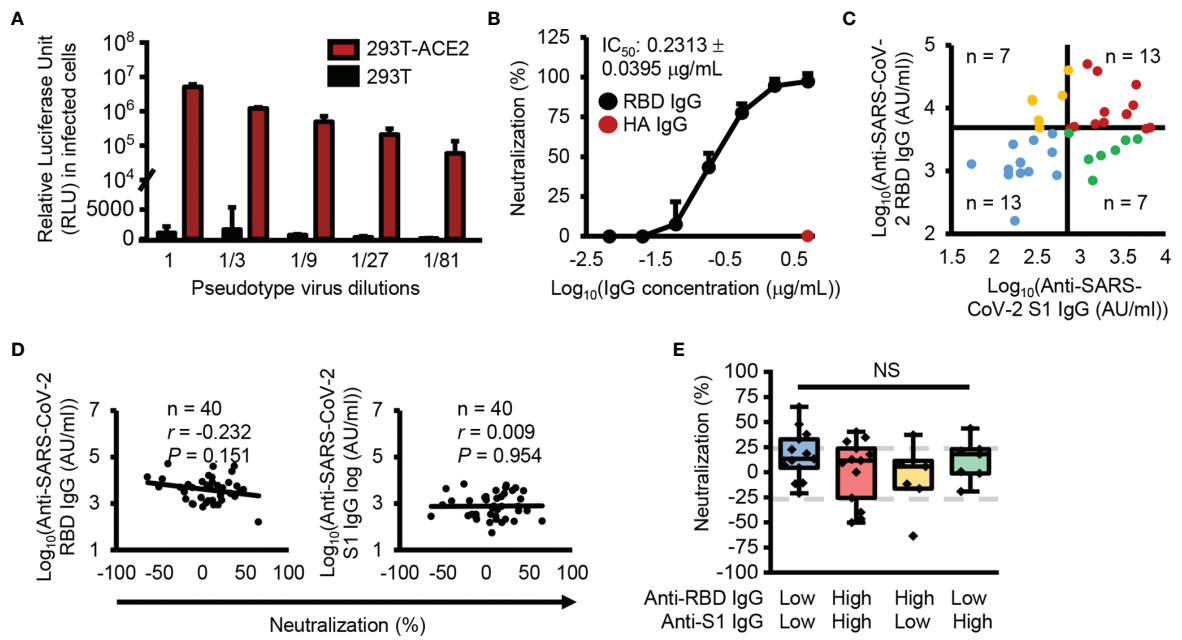

FIGURE 3 | No correlation between antibody levels cross-reactive SARS-CoV-2 S1 subunit or RBD and neutralization activity. **(A)** Infection with SARS-CoV-2 pseudotype viruses. **(B)** Confirmation of RBD specific infection with anti-SARS-CoV-2 RBD (black dots) or anti-HA (red dots) antibodies. **(C)** Dot plot showing the four groups divided using median split (3.63 = $\log_{10}$(AU/ml) for RBD and 2.87 = $\log_{10}$(AU/ml) for S1 subunit). **(D)** Correlation analysis between anti-SARS-CoV-2 S1 subunit and anti-RBD IgG antibody levels and neutralization. **(E)** Box plot representing the neutralization activity level. Gray dotted lines denote 25% neutralization (upper) and -25% neutralization (lower). Pearson's correlation coefficient (*r*) and its associated *P*-value (*P*). NS, not significant (unpaired Student's t-test).

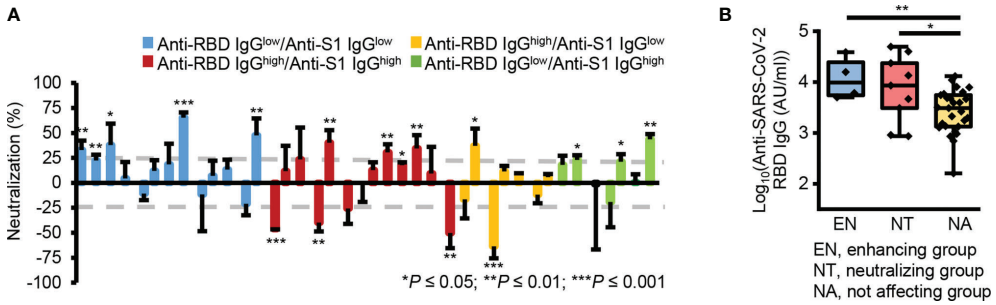

FIGURE 4 | Two opposing roles of RBD-reactive antibodies in SAR-CoV-2 infection. **(A)** Bar graph of the neutralizing activity of individual samples. Gray dotted lines denote 25% neutralization (upper) and -25% neutralization (lower). **(B)** Box plot representing the RBD-reactive antibody levels in three groups according to the nature of ACE2-mediated SARS-CoV-2 viral infection. EN, enhancing group; NT, neutralizing group; NA, not affecting group. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$ (unpaired Student's t-test); NS, not significant (unpaired Student's t-test).

DISCUSSION

It is controversial whether the preformed B cell immunity to common coronavirus infection affects COVID-19 disease severity. Although the presence of SARS-CoV-2 spike protein cross-reactive IgG antibodies in some pre-pandemic samples is consistently observed in many reports (9, 10, 12), the neutralizing activity of these antibodies is controversial (9, 11, 12, 19). A study measured the neutralizing activity of spike protein cross-reactive IgG antibodies by the SARS-CoV-2 pseudotype virus infection system. However, the cells used for infection were HEK293T cells, so the results of the study may not be mediated by neutralizing ACE2-mediated viral entry (9). In the viral entry of SARS-CoV-2, binding between open-state RBD to ACE2 is required at the first step (20–22), so the ACE2-mediated *in vitro* infection system is required to confirm receptor-specific infection by SARS-CoV-2. Thus, we used the ACE2-mediated pseudotype viral infection system.

In this study, approximately half (49.6%) of pre-pandemic elderly people who live in Korea had SARS-CoV-2 S1 subunit cross-reactive plasma IgG antibodies, which were significantly positively correlated with HCoV-OC43 S1 subunit reactive IgG antibodies. Correlation analysis between HCoV S1 subunit-reactive IgG antibodies showed a high correlation. This correlation suggests the sharing of preformed B cell immunity between common coronaviruses, which may explain why a mild state appears during common coronavirus infection. In our data, the correlation analysis between HCoVs and SARS-CoV-2 S1 subunit-reactive IgG antibodies showed that HCoV-OC43 S1 subunit-reactive IgG antibodies are only significantly related to SARS-CoV-2 S1 subunit-reactive IgG antibodies. Interestingly, when the epitope was narrowed to RBD, SARS-CoV-2 RBD reactive IgG antibody levels also correlated significantly with HCoV-HKU1 S1 subunit reactive IgG antibody levels.

In addition to the detection of cross-reactive antibodies, we also assessed antibody-mediated protective immunity by using a pseudotype virus-mediated *in vitro* infection system. Even though we narrowed down the epitopes, such as using the S1 subunit instead of the total spike protein, the SARS-CoV-2 S1 subunit cross-reactive IgG antibody levels were not correlated with neutralizing activity. Furthermore, the SARS-CoV-2 RBD cross-reactive IgG antibody levels were also not correlated with neutralizing activity. Another important point in this report is that some SARS-CoV-2 S1 subunit cross-reactive IgG antibodies seemed to enhance pseudotype virus infection. This phenomenon was shown in a previous report even though this enhancement was not mentioned in the text (12). In our data, many of the reactive samples (n = 9) significantly inhibited pseudotype virus infection, but some samples (n = 4) significantly enhanced the *in vitro* infection. This result possibly means that specific epitopes are important for neutralizing activity. Thus, based on these data, some HCoV-exposed people may be susceptible to SARS-CoV-2 infections without consideration of preformed T cell immunity. This possibly led to variations of neutralizing activity of RBD-reactive pre-pandemic plasma samples.

A limitation of this study is that only samples from elderly people were analyzed. Thus, the characteristics of the cross-reactive antibodies described in this study cannot be generalized to all pre-pandemic cross-reactive antibodies against SARS-CoV-2 pseudovirus infection, such as those in young people. Another is that this study was unable to analyze for possible age-dependent changes in the function of SARS-CoV-2 cross-reactive antibodies. However, it is important to note that elderly people have been probably infected multiple times by common coronaviruses (23). Thus, plasma samples from the elderly may be suitable for analyzing differences in cross-reactivity of antibodies to the common coronavirus and SARS-CoV-2. In addition, the cross-reactive antibodies are possibly involved in antibody-dependent cell-mediated cytotoxicity and complement-dependent toxicity. However, in this paper, we focused on the neutralizing activity of the cross-reactive antibodies. A future study will be required to characterize the other functions of these cross-reactive antibodies to understand better the roles of cross-reactive antibodies in pre-pandemic samples.

In this report, we found that cross-reactive antibodies against the SARS-CoV-2 S1 subunit were present in the plasma of some elderly Korean people. Interestingly, the levels of cross-reactive

antibodies against the SARS-CoV-2 S1 subunit were not correlated with neutralizing activity. Furthermore, even the levels of RBD cross-reactive antibodies were not correlated with neutralizing activity. However, RBD-reactive antibody levels were significantly higher in the groups displaying inhibition and enhancement of viral infection than in the non-affecting group. Thus, our data indicate that the preformed RBD-reactive antibodies have two opposing roles in SARS-CoV-2 infection. Analysis of the epitopes of preformed antibodies will be useful to understand the mechanism by which RBD-reactive antibodies enhance or inhibit SARS-CoV-2 infection in future studies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by institutional review board of Seoul National University (IRB No. E2104/002-012). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

S-GP conceived this study. S-GP and K-YS designed study and the experiments. M-RS interpreted the data. K-YS, G-HK, and S-EB performed experiments and analyzed the data. K-YS and G-HK wrote the manuscripts. KC, JL, BK, and KL collected patient samples and data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Identification of Distinct Immune Cell Subsets Associated With Asymptomatic Infection, Disease Severity, and Viral Persistence in COVID-19 Patients

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The cell-mediated protective and pathogenic immune responses to SARS-CoV-2 infection remain largely elusive. Here we identified 76 distinct cell subsets in the PBMC samples that were associated with various clinical presentations of COVID-19 using scRNA-seq technology coupled with a deep and comprehensive analysis of unique cell surface markers and differentially expressed genes. We revealed that (TRAV1-2^{+}CD8^{+}) MAIT cells and (NCAM1^{hi}CD160^{+})NK cells significantly enriched in the asymptomatic subjects whereas (LAG3^{+}CD160^{+}CD8^{+})NKT cells increased in the symptomatic patients. We also observed that (CD68^{-}CSF1R^{-}IL1B^{hi}CD14^{+})classical monocytes were positively correlated with the disease severity. Moreover, (CD33^{-}HLA-DMA^{-}CD14^{+})classical monocytes and (CLEC10A^{-}S100A9^{lo})pDC were associated with the viral persistence. The GO and KEGG analyses identified enriched pathways related to immune responses, inflammation, and apoptosis. These findings may enhance our understanding of the immunopathogenesis of COVID-19 and help develop novel strategies against SARS-CoV-2 infection.

Keywords: SARS-CoV-2, COVID-19, asymptomatic infection, disease severity, viral persistence, singlecell RNA sequencing (scRNA-seq)

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INTRODUCTION

The pandemic of COVID-19 has posed unprecedented challenges to the international communities. As of January 28, 2022, there have been 364,191,494 confirmed cases of COVID-19, including 5,631,457 deaths worldwide reported to the World Health Organization (1). Despite the ongoing vaccination programs, the emerging variants of SARS-CoV-2 have resulted in the devastating surge of COVID-19 cases in several regions and countries, which reminds us that we still have a tremendous task to fight the SARS-CoV-2 infections and control this devastating pandemic.

The clinical presentations of SARS-CoV-2 infection are highly variable, ranging from asymptomatic infections to critical conditions (2–13). One of these reports by a living systematic review of 86 studies in different populations and settings suggested that approximately 20%-31% of SARS-COV-2 infected individuals remained asymptomatic state (AS) during the follow-up period (5). Among the symptomatic patients (SM), approximately 80% of them showed mild or moderate diseases (MD) whereas 20% displayed severe conditions (SD). While the previous studies have suggested that both the host factors and viral mutations may contribute to the diverse manifestations of the COVID-19 (14–17), the underlying molecular mechanisms remain to be further dissected and elucidated. An increasing number of studies have demonstrated the involvement of T cells, B cells, NK cells, monocytes, neutrophils, and inflammatory macrophages in the pathogenesis of COVID-19 patients with moderate or severe diseases (18–25), suggesting that host immune responses play important roles in the pathogenesis of COVID-19. On the other hand, only a few studies have reported the immune responses in the asymptomatic individuals (AS) (26–29). It is conceivable that examining the differences in the immune responses between the AS subjects and the SM patients may help understand the protective and pathogenic immune responses to SARS-CoV-2 infection. In addition, some of the hospitalized patients were tested positive for the nucleic acid of SARS-CoV-2 by RT-PCR but did not become negative for a longer period of time (>45 days, herein designated as long-term nucleic acid test positive, LTNP) whereas others turned into negative for the viral nucleic acid in a shorter period of time (≤45 days, designated as short-term nucleic acid test positive, STNP). However, the immune cells alternations in the LTNP and STNP patients remain largely unknown.

In this study, we employed the scRNA-seq technology coupled with a deep and comprehensive analysis of unique cell surface markers and differentially expressed genes to profile 51 PBMC samples from eleven HC individuals, five AS subjects and 33 SM patients. We identified 76 distinct immune cell subsets in the PBMC samples and revealed a large number of distinct immune cell subsets that were associated with various clinical presentations and viral persistence in the COVID-19 patients. These findings have shed new light on understanding the immunopathogenesis of COVID-19 and may help develop novel strategies against SARS-CoV-2 infection.

MATERIALS AND METHODS

Patient Cohort and Case Definition

This study was reviewed and approved by the Ethics Committee of The First affiliated Hospital of Xi'an Jiaotong University (XJTU1AF2020LSK-015) and The Renmin Hospital of Wuhan University (WDRY2020-K130). All participants enrolled in this study offered the written informed consent by themselves or their surrogates. The definition and classification of all COVID-19 patients in this study follow the Guidelines of the World Health Organization and the "Guidelines on the Diagnosis and Treatment of the Novel Coronavirus Infected Pneumonia" developed by the National Health Commission of People's Republic of China (30–32). We collected 53 samples of peripheral blood mononuclear cells (PBMCs), including 42 COVID-19 patient samples and 11 healthy controls (HC) samples (**Figure 1A** and **Table S1**). Two samples were excluded in this study, of which COV077 was a fatal case whereas COV166 was an immunocompromised case. The number of patients included five asymptomatic subjects (AS, n=5) and 33 symptomatic patients (SM, n=33) consisting of 13 moderate disease (MD, n=13), 10 severe disease (SD, n=10), and 10 SD recovery (SDR, n=10), and two samples collected at two different time points during hospitalization from patient C-19 and C-26, respectively. The SM group was further divided into the long-term nucleic acid test positive (LTNP, n=12) and the short-term nucleic acid test positive (STNP, n=21) sub-groups. In this study, based on the clinical observation that most of the COVID-19 patients hospitalized in the Renmin Hospital in Wuhan became negative for the nucleic acid test within 45 days, we therefore defined the STNP was ≤45 days whereas the LTNP was >45 days (**Table S1**). The demographic features, clinical laboratory testing results and other relevant information were provided in **Table S1**.

Preparation of Single-Cell Suspensions

The frozen PBMCs were retrieved from the liquid nitrogen storage tank and thawed in a 37°C water bath, followed by washing with 10mL of 90% DMEM+10% FBS in a 15-mL polypropylene tube and then centrifuged at 500g for 20 min. The supernatant was removed (repeated this step twice). The cell pellet was resuspended with 500μl of 1x PBS with 0.04% BSA (A1933-25G, SIGMA), followed by adding 5ml of 1x Red blood cell (RBC) lysis buffer (130-094-183, 10x stock solution, Miltenyi Biotech) and incubated at room temperature for 10 min to lyse the remaining red blood cells. After incubation, the cell suspension was centrifuged at 500g for 20 min at room temperature and then resuspended in 100μl of Dead Cell Removal MicroBeads to remove dead cells using Miltenyi Dead Cell Removal Kit (130-090-101-1, Miltenyi Biotech). The live cells were collected and resuspended in 1x PBS with 0.04% BSA and centrifuged at 300g for 3 min at 4°C (repeated twice). The cell pellet was resuspended in 50μl of 1x PBS with 0.04% BSA. The cell viability was measured by the trypan blue exclusion method and confirmed to be 85% or higher. The cell number of the single cell suspension was counted using a Countess II

Automated Cell Counter and the final concentration was adjusted to 700-1200 cells/μl.

Chromium 10x Genomics Library Construction and Sequencing

Approximately 5000 single cells each sample were captured using the Chromium Single-Cell 5' kit (V1) according to the manufacturer's instructions (PN-1000020, 10x Genomics), followed by cDNA amplification and library construction performed according to the standard protocols. The libraries were sequenced on an Illumina NovaSeq 6000 sequencing platform (paired-end multiplexing run, 150bp) by LC-Bio Technology Co. Ltd., (HangZhou, China) at a minimum depth of 20,000 reads per cell. To avoid batch effects, the scRNA-seq data sets were generated by the same operators at the same laboratories using the standard operation protocols (SOPs) for cell dissociation, library preparation and sequencing.

Single-Cell RNA-Seq Data Processing

The sequencing results were demultiplexed and converted to FASTQ format using Illumina Bcl2fastq2 software (v2.20, Illumina). Sample demultiplexing, barcode processing and single-cell 5' gene counting were completed by using the Cell Ranger pipeline (https://support.10xgenomics.com/single-cellgeneexpression/software/pipelines/latest/what-is-cell-ranger, version 3.1.0) and the scRNA-seq data were aligned to Ensembl genome GRCh38 reference genome. A total of 222,457 single cells captured from eleven healthy controls and 42 COVID-19 patient samples were processed using 10x Genomics Chromium Single Cell 5' Solution (PN-1000006, 10x Genomics). The Seurat (version 3.1.1) was used for dimensional reduction, clustering, and analysis of scRNA-seq data (33). Overall, 207,718 cells passed the quality control threshold: all genes expressed in less than one cell were removed, number of genes expressed per cell >500 as low cut-off, UMI counts less than 500, and the percentage of mitochondrial-DNA derived gene-expression < 25%. To visualize the data, we further reduced the dimensionality of all 207,718 cells by Seurat and used t-Distributed Stochastic Neighbor Embedding (t-SNE) to project the cells into 2D space. The steps included: (1) Using the LogNormalize method of the "Normalization" function of the Seurat software to calculate the expression level of genes; (2) The principal component analysis (PCA) was performed using the normalized expression level, within all the PCs, the top 10 PCs were used to do clustering and t-SNE analysis; (3) Using weighted Shared Nearest Neighbor (SNN) graph-based clustering method to find clusters. The marker genes for each cluster were identified with the "bimod"(Likelihood-ratio test) with default parameters *via* the FindAllMarkers function in Seurat. This selects marker genes that were expressed in more than 10% of the cells in a cluster and the average log (Fold Change) of greater than 0.26. To further avoid interference of putative multiplets (where more than one cell was loaded into a given well on an array), cells in a defined cluster that had high expression of more than one cell type canonical marker gene were filtered to ensure the data quality. In detail, we identified nine major cell types using the canonical markers (T cell: $CD3E^+$; NK: $KLRD1(CD94)^+$ or $CD16A^+$; Monocyte: $CD14^+$ or $CD16A^+$ or LYZ^+; B cell: $CD20(MS4A1)^+$; Dendritic cells: $CD16A^+$ or $CD83^+$ or LILRA4 (CDF85g) or LYZ^+ or $CD1C^+$; Platelet: $GP9^+$ or $ITGA2B(CD41)^+$; Neutrophil: $CD177^+$ or LYZ^+; Plasma cell:$CD38^+$; Stem cell:$CD34^+$) and then excluded any cells that expressed more than one canonical marker genes, which could not be classified into one type. Parameters used for graph-based clustering follow: FindNeighbors with parameter reduction = pca, dims = 1:10 and FindClusters with parameter resolution = 0.8. "Cellranger aggr" in Seurat was used to integrate the samples. As a result, a total of 119,799 cells were used for the final analysis in this study. The nine cell types were integrated for further sub-clustering. After integration, genes were scaled to unit variance. Scaling, principal component analysis and clustering were performed as described above.

Analysis of Differentially Expressed Genes and Functional Enrichment

The analysis of differentially expressed genes (DEGs) between each pair of cells from different groups (e.g., the asymptomatic, symptomatic, and healthy control groups) was performed using "bimod" with default parameters in Seurat. DEGs were filtered using a minimum log2 (fold change) of 0.26, a P value <0.05 and >10% of cells expressed in at least one group. To further understand the associations and function of the DEGs, GO and KEGG pathway analysis was performed using the OmicStudio tools at https://www.omicstudio.cn/tool. DEGs with a log2 mean expression difference ≥0.26 enriched in GO or KEGG pathways were considered as significant candidate biomarkers or pathways.

Clinical and Laboratory Tests

All clinical and laboratory tests were conducted in the Renmin Hospital of Wuhan University, including the tests of SARS-COV-2-specific IgM (Cat#20203400769, YHLO Biotech) and SARS-COV-2-specific IgG (Cat#20203400770, YHLO Biotech) antibodies.

Statistical Analysis

All data and statistical analyses were performed using SPSS (Statistical Package for the Social Sciences, Version 23.0 software, SPSS Inc.). R (https://www.cran.r-project.org, Vienna, Austria) and GraphPad Prism 8.0.2 (GraphPad Software, San Diego, USA) were also used for analysis in this study. Categorical variables were described as frequency rates and percentages, whereas continuous variables were described using mean, median, and inter quartile range (IQR) values. Difference analysis of HC vs AS and STNP vs LTNP were conducted using two-groups comparison strategy, whereas multiple groups comparison strategy was employed for analysis of HC vs AS vs SM and HC vs AS vs MD vs SD vs

SDR. The cell cluster analysis was performed using cell abundance data to identify the distinct cell subsets associated with various clinical presentations. The gene expression analysis was performed to identify the differential expression genes (DEGs). We also analyzed the data distribution in each group. Independent group t-tests were performed for two-groups comparisons when the data were normally distributed; otherwise, the Mann-Whitney test was used. For three-groups comparison, One-way Anova test was conducted when the data were normally distributed and homoscedasticity, otherwise, Kruskal-Wallis was employed. In addition, Bonferrion correction was used for the multiple- groups comparison. Of note, Bonferrion correction (Bonferroni adjustment) include the following steps: First, divide the desired alpha-level by the number of comparisons; Second, calculate the p-value and evaluate the significance. SPSS employed a mathematically equivalent adjustment in this study for pairwise comparisons. The Bonferrion correction was performed by taking the observed (uncorrected) p-value and multiply it by the number of comparisons. Proportions for categorical variables were compared using the $\chi 2$ test, whereas the Fisher exact test was employed when the data were limited. For unadjusted comparisons, a two-sided α of less than 0.05 was considered statistically significant. Correlation analyses

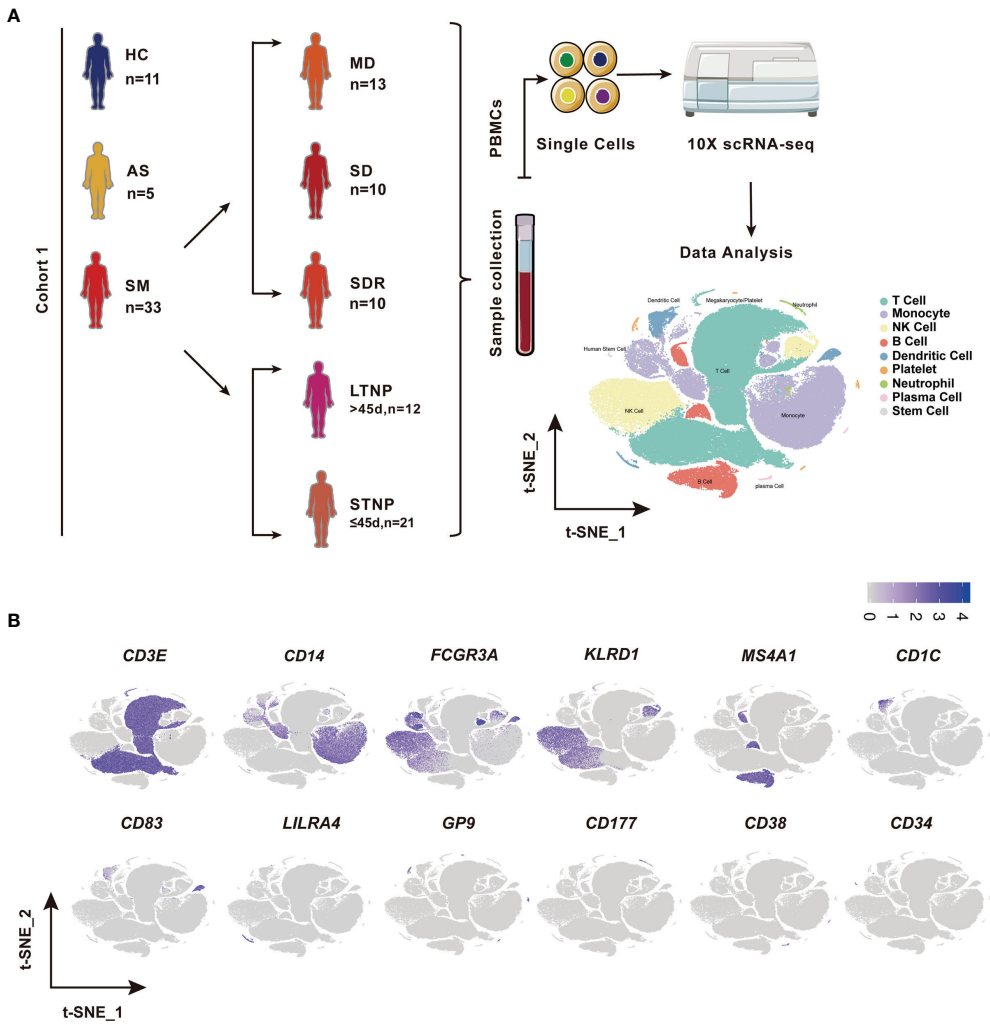

FIGURE 1 | Identification of nine cell types in the peripheral blood mononuclear cells (PBMCs). **(A)** A schematic diagram of the study design for single-cell RNA sequencing (scRNA-seq). Peripheral blood mononuclear cells (PBMCs) were collected from eleven healthy controls (HC) and 39 SARS-CoV-2 infected individuals followed by scRNA-seq using the 10x Genomics platform. The T-distributed Stochastic Neighbor Embedding (t-SNE) plot of 119,799 cells showing nine cell types (i.e., T cells, Monocytes, NK cells, B cells, Dendritic cells, platelets neutrophils, plasma cells, and stem cells) identified from this study. **(B)** The t-SNE plots showing the expression of canonical cell marker genes used for identification of the nine cell types described above. Each dot represents a single cell. The plots are color-labelled based on the expression level of the respective marker gene in log scale, which was calculated *via* LogNormalize method of the "NormalizeData" function of the Seurat software. HC, Healthy controls; AS, Asymptomatic subjects; SM, Symptomatic patients; MD, Moderate disease; SD, Severe disease; SDR, Recovery patients from SD; LTNP, Long term nucleic acid test positive; STNP, Short term nucleic acid test positive.

were performed using Spearman, whereas Mann-Whitney and Wilcoxon tests were employed for unpaired and paired comparisons, respectively. The details of the statistical analysis were provided in the respective figure legends.

RESULTS

Identification of 76 Cell Subsets in the PBMCs of COVID-19 Patients by scRNA-Seq

To identify the immune cell alternations in the peripheral blood of COVID-19 patients with various clinical presentations, we performed the droplet-based scRNA-seq to profile the immune cell landscape in 51 PBMC samples collected from eleven healthy controls (HC), five asymptomatic individuals (AS), and 33 symptomatic patients (SM) with moderate diseases (MD, n=13) or severe diseases (SD, n=10), and the patients recovered from SD (SDR, n=10), as well as three samples collected at two different time points during hospitalization from patient C-19 and C-26, respectively (**Figure 1A**). In addition, this study cohort included 12 long-term nucleic acid test positive (LTNP) patients, 21 short-term nucleic acid test positive (STNP) patients. (**Figure 1A** and **Table S1**). The demographic features, clinical characteristics and the laboratory testing results of these study subjects were presented in the **Supplementary Materials** (**Table S1**). After a series of stringent high-quality filtering and removal of multiplets, a total of 119,799 single cells captured from all the participants were used for the final data analysis. As shown by the t-Distributed Stochastic Neighbor Embedding (t-SNE) plot, we first identified nine major cell types, including T cells (*CD3E*), B cells (*MS4A1*), monocytes (*CD14, FCGR3A*), natural killer (*KLRD1*), dendritic cells (*CD1C, CD83, LILRA4*), platelets (*GP9*), neutrophils (*CD177*), plasma cells (*CD38*), and stem cells (*CD34*) using the unique marker genes (**Figures 1A, B**). We also examined the distribution patterns of these cell types in each of the study sub-groups (**Figure S1A**) and calculated the relative abundance of these cell types in each sample, respectively (**Figure S1B**). We then identified 24 cell clusters from the 119,799 single cells, including ($CD8^+GZMK^-$)naïve T cells (cluster 0), ($CD7^{hi}$)NK cells (cluster 1), ($CD4^+GATA3^+GPR183^+$) naïve CD4 T cells (cluster 2), ($CD3^+KLRD1^+$)NKT cells (cluster 3), ($CD4^{hi}CD68^+CD14^+$) classical monocytes (cluster 4), ($GZMK^+CD8^+$)effector/memory CD8 T cells (cluster 5), ($CD4^{lo}CSF1R^-CD33^-CD14^+$)classical monocytes (cluster 6), ($CD14^+CD16^+$)intermediate monocytes (cluster 7), ($CD4^-CD8^-$) double negative T cells (cluster 8), ($CCR7^+$)naïve B cells (cluster 9), ($Siglec10^+CD16^+$)non-classical monocytes (cluster 10), ($CD7^{lo}$) NK cells (cluster 11), ($CD4^{lo}CSF1R^-CD33^+CD14^+$)classical monocytes (cluster 12), ($CD27^+$)memory B cells (cluster 13), ($CD4^{hi}CD68^-CD14^+$)classical monocytes (cluster 14), ($CD14^-CD16^-$)immature monocytes (cluster 15), ($CD1C^+$)myeloid DC (mDC) (cluster 16), ($ITGA2B^+$)platelets (cluster 17), ($MKI67^+$) proliferation T cells (cluster 18), ($CD83^{hi}$)mDC (cluster 19), ($LILRA4^+$)plasmacytoid DC (pDC) (cluster 20), ($CD177^+$) neutrophils (cluster 21), ($CD38^{hi}IGHG4^+$)plasma cells (cluster 22), and ($CD34^+$)stem cells (cluster 23) (**Figures 2A–C** and **Table S2**), and calculated the relative abundance of the 24 cell clusters in each of the samples, respectively (**Figure S2A**). Furthermore, we identified 76 distinct cell subsets from the nine cell types with a combination of unique cell surface markers and the differentially expressed genes, including sixteen T cell subsets, twelve monocyte subsets, twelve dendritic cell subsets, eight NK cell subsets, ten B cell subsets, six platelet subsets, five neutrophil subsets, four plasma cell subsets, and three stem cell subsets (**Figures 2B, C**, **S2B**, and **Table S2**). We also calculated the relative abundance of the cell types, clusters and subclusters described above (**Tables S3** and **S4**), which allowed us to compare the cell alternations between various groups of individuals and understand their associations with the pathogenesis of COVID-19.

Distinct Immune Cell Subsets Associated With Asymptomatic Infection of SARS-COV-2

To understand the protective and pathogenic immune responses to SARS-CoV-2 infection, we first compared the percentage of each cell type between the healthy controls (HC) and the asymptomatic subjects (AS). We found that ($TRAV1\text{-}2^+CD8^+$) MAIT cells, ($NCAM1^{hi}CD160^+$) NK cells, ($CD4^{lo}CSF1R^-CD33^-CD14^+$)classical monocytes and ($CD33^-HLA\text{-}DMA^-CD14^+$) classical monocytes increased significantly in the AS group and were positively correlated with the AS (**Figures 3A, B** and **Table 1**). We further analyzed the differentially expressed genes (DEGs) in these cell subsets (**Table S5**). The heatmap in **Figure 3C** showed the average level of 51 DEGs in the (TRAV1-2+CD8+)MAIT cells from the HC and AS individuals, of which twelve genes (such as *CD6*, *CD69* and *KLRB1*) were related to immune responses, and five genes (such as *KLRB1*, *KLRG1*, and *GNLY*) were linked to cytotoxicity. Of note, the Killer Cell Lectin Like Receptor B1(KLRB1) and the Killer Cell Lectin Like Receptor G1 (*KLRG1*), which have been suggested to be involved in innate immune responses, NK cell-mediated cytotoxicity and T cell activations (34, 35), were overexpressed in the AS subjects (**Figure 3E**). The heatmap in **Figure 3D** showed the average level of 134 DEGs in the ($NCAM1^{hi}CD160^+$)NK cells from the HC and AS individuals, of which 39 genes (such as *GSK3B*, *CANX* and *KLRD1*) were related to immune responses, and 13 genes (such as *DAD1*, *ATG3*, and *TRAF2*) were linked to apoptosis pathways (**Figure 3C**). Of note, the Defender Against Cell Death 1 (*DAD1*) was overexpressed in the AS subjects (**Figure 3F**). Additional analysis of Gene Ontology (GO) and KEGG pathways revealed significant enrichments in viral transcription (**Figure S3C**). These findings suggest that the innate immune responses may play important roles in controlling the SARS-CoV-2 infections in the AS individuals.

Immune Cell Subsets Associated With the Symptomatic COVID-19 Patients

To identify the potential cell subsets that may contribute to the development of clinical symptoms in COVID-19 patients, we compared the percentage of each cell type between the HC, AS and symptomatic (SM) groups, respectively, and detected 21 cell

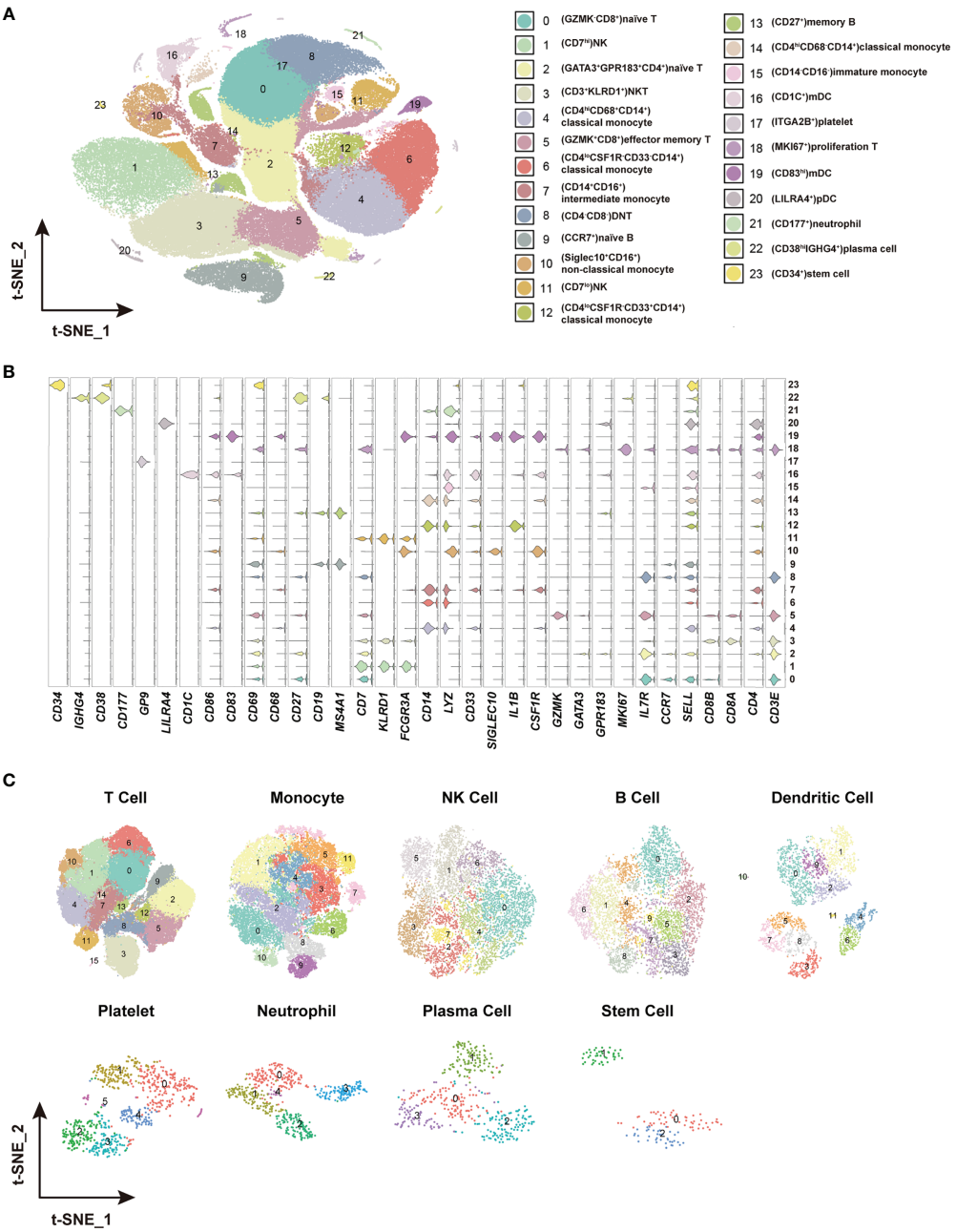

FIGURE 2 | Identification of distinct cell clusters and sub-clusters in the PBMCs. **(A)** The t-SNE plot showing 24 cell clusters with separate color labels and marked by signature gene. **(B)** The violin plots showing the expression pattern of the selected cell markers in each of the 24 clusters. The marker gene expression level was calculated *via* LogNormalize method of the "NormalizeData" function of the Seurat software. **(C)** The t-SNE plots showing the sub-clusters of T cells, monocytes, NK cells, B cells dendritic cells, platelets, neutrophils, plasma cells and stem cells.

subsets that either increased or decreased significantly in these groups (**Figures 4A**, **S3A** and **Table 1**). In particular, we observed that (LAG3+CD160+CD8+)NKT cells, ($FOXP3^+IL2RA^+IL7R^+CD4^+$)Treg cells, ($GATA3^+GPR183^+CD4^+$)naïve T cells, ($GATA3^+CCR6^-S1PR1^+CD4^+$)naïve T cells, ($MKI67^+$) proliferation T cells, ($CCR6^+CD4^+$)Th17 cells, and ($LAMP1^+CD4^+CD8^+$)pro-NKT cells increased significantly whereas ($CD4^-CD8^-$)DNT (the double negative T cells), ($CSF1R^+CD86^-CD14^+$) classical monocytes, ($CXCL8^+CSF1R^-IL1B^-CD14^+$)classical monocytes, ($AIF1^+BCL2^+CD8^+$)naïve T cells, ($TRGC1^+CD4^-CD8^-$)γδT cells, ($CD4^{lo}CSF1R^-CD33^-CD14^+$)classical monocytes, ($CD68^+IL1B^{lo}CD14^+$)classical monocytes, ($CD33^-HLADMA^-CD14^+$)classical monocytes, ($CD7^{lo}$)NK cells, ($IGHD^+CD27^+CD80^-$)memory B cells, ($CD1C^+$)mDC, ($LILRA4^+$)pDC, and

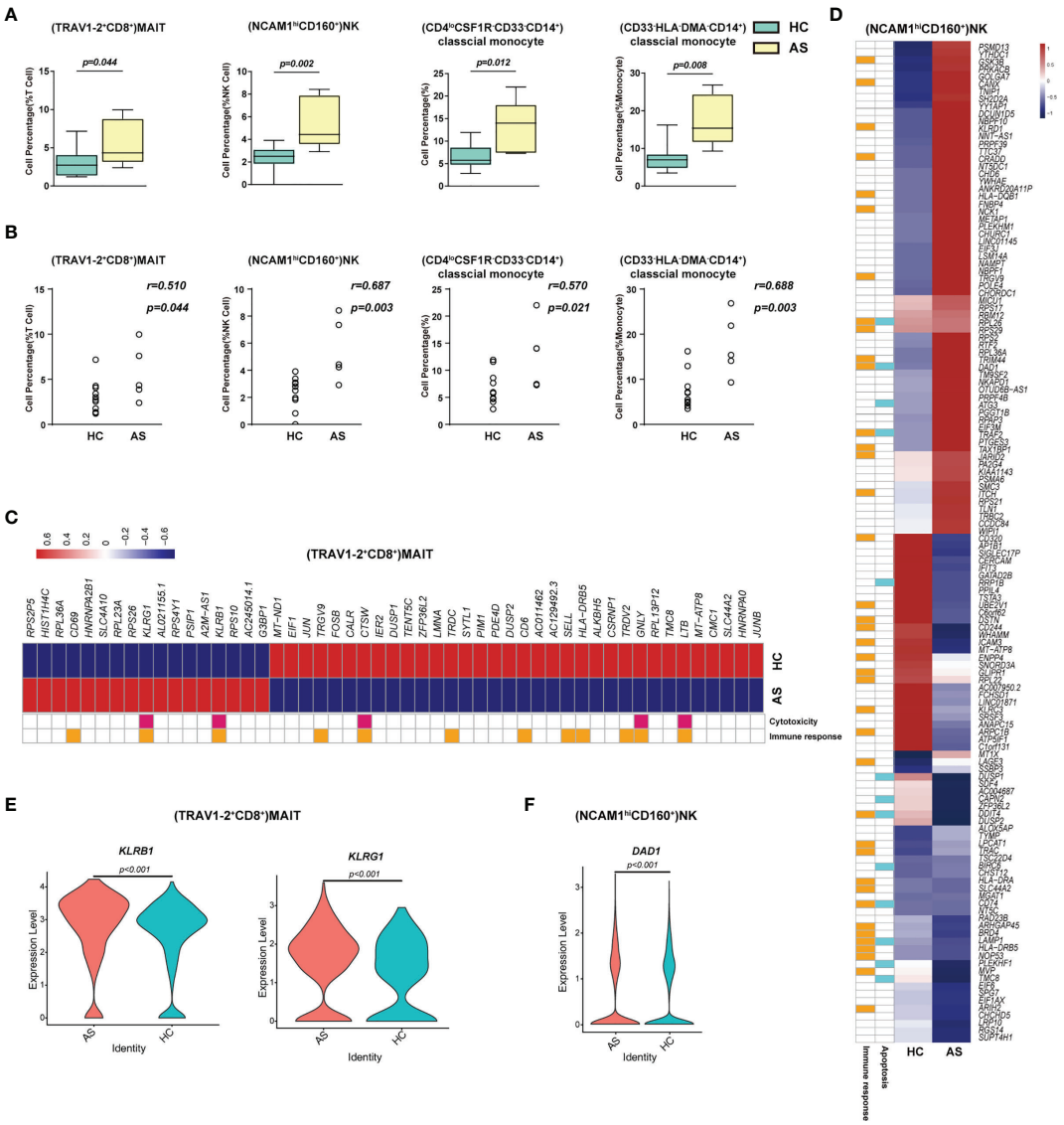

FIGURE 3 | Immune cell subsets associated with asymptomatic COVID-19 patients. **(A)** The Box and whisker plots showing the percentage of four cell clusters that had significant differences ($p < 0.05$) HC and AS groups. The horizontal lines, box and whiskers correspond to median values, interquartile range (IQR) and minimum/maximum value, respectively. **(B)** Spearman rank order correlation analysis showing the percentage of the four cell types described above are positive associated with the asymptomatic state **(C)** A Heatmap showing the average level of the differentially expressed genes (DEGs) in the (TRAV1-2$^+$CD8$^+$)MAIT cells with $p < 0.05$ and log2 fold change (FC) ≥0.26 in HC and AS. The gene function (immune responses and cytotoxicity) is indicated in the heatmap. The gene expression was calculated *via* LogNormalize method of the "NormalizeData" function of the Seurat software. **(D)** A Heatmap showing the average level of the DEGs in the (NCAM1^{hi}CD160$^+$)NK cells with $p < 0.05$ and log2 fold change (FC) ≥0.26 in HC and AS. The gene function (immune responses and apoptosis) is indicated in the heatmap. The gene expression was calculated *via* LogNormalize method of the "NormalizeData" function of the Seurat software. **(E)** The violin plot showing the expression levels of two representative DEGs (*KLRB1* and *KLRG1*) in (TRAV1-2$^+$CD8$^+$)MAIT cells, which was involved in cytotoxicity and/or immune responses. **(F)** The violin plot showing the expression levels of one representative DEG (*DAD1*) in (NCAM1^{hi}CD160$^+$)NK cells, which was involved in apoptosis and/or immune responses (also see **Figure S3**). $p < 0.05$ was considered significant. The samples included HC (n=11) and AS (n=5).

(CLEC10A$^-$S100A9$^-$)pDC decreased significantly in the SM patients compared with the AS subjects (**Figures 4A** and **S3A**). We further analyzed the differentially expressed genes (DEGs) in these cell subsets to understand the immune responses and pathogenesis of SARS-CoV-2 infection. Here, we showed a representative heatmap with the average level of 89 DEGs in the (LAG3+CD160+CD8+)NKT cells from the HC, AS and SM individuals, of which 35 genes (e.g. *CXCR4*, *IFNG* and *XCL2*) were related to immune responses, and eight genes (*RHOB, PMAIP1*, *CXCR4, MCL1, IFNG, LGALS1, DDIT4* and *TNFAIP3*) were linked to apoptosis pathways (**Figures 4B, C**). Additional analysis of Gene Ontology (GO) and KEGG pathways revealed a number of DEGs that were associated with viral transcription, response to cytokines, interferon-gamma-mediated signaling

TABLE 1 | Distinct cell subsets associated with various clinical presentations of SARS-CoV-2 infection.

Cell types and subsets	AS vs HC	SM vs AS	SD vs MD	LTNP vs STNP
($CD4^-CD8^-$)DNT		↓	↓	
($TRGC1^-CD4^-CD8^-$)DNT			↓	
($AIF1^+BCL2^+CD8^+$)naïve T				↓
($GATA3^+CCR6^-S1PR1^+CD4^+$)naïve T		↑	↑	
($GATA3^+GPR183^+CD4^+$)naïve T		↑	↑	
($MKI67^+$)Proliferation T			↑	↓
($TRGC1^+CD4^-CD8^-$)γδT			↓	
($TRAV1\text{-}2^+CD8^+$)MAIT	↑		↓	
($FOXP3^+IL2RA^+IL7R^+CD4^+$)Treg		↑	↑	
($LAG3^+CD160^+CD8^+$)NKT		↑	↑	
($LAMP1^+CD4^+CD8^+$)pro-NKT			↑	
($CD7^{lo}$)NK		↓	↓	
($NCAM1^{hi}CD160^+$)NK	↑		↑	
B cells			↓	
($CD24^-ITGAE^+CD180^+$) marginal zone B cells				↓
($IGHD^+CD27^+CD180^-$) memory B		↓		
Plasma cells				↓
($CD68^-CSF1R^-IL1B^{hi}CD14^+$) classical monocytes			↑	↓
($CD4^{lo}CSF1R^-CD33^-CD14^+$) classical monocytes	↑	↓		
($CD4^{lo}CSF1R^-CD33^+CD14^+$) classical monocytes			↑	↓
($CD33^-HLA\text{-}DMA^-CD14^+$) classical monocytes	↑	↓	↓	↑
($CSF1R^+CD86^-CD14^+$) classical monocytes		↓	↓	
($CXCL8^+CSF1R^-IL1B^-CD14^+$) classical monocytes		↓	↓	↑
($CD14^{lo}CD16^{lo}$)immature monocytes			↓	↑
($CD40^+CLEC9A^-$)mDC			↑	
($CD83^{hi}$)mDC			↑	
($CX3CR1^{hi}CD14^{hi}$)mDC			↑	
($S100A12^-CX3CR1^{lo}$)mDC			↓	
($TNFRSF8^+$)mDC			↑	
($CLEC10A^-S100A9^{lo}$)pDC			↓	↑
Neutrophils				↑
(MMRN1$^-$PDZK1IP1-) activated platelets				↓

HC, Healthy controls; AS, Asymptomatic subjects; SM, Symptomatic patients; LTNP, long-term nucleic acid test positive patients; STNP, short-term nucleic acid test positive patients; hi, high; lo, low; ↑= cell percentage increased and/or showing positive correlation; ↓=cell percentage decreased and/or showing negative correlation.

pathway, TNF signaling pathway, Th1 and Th2 cell differentiation, Th17 cell differentiation, antigen processing and presentation, and other pathways (**Figures 4E, F** and **S3B–D**). Of note, all the genes associated with the apoptosis pathways were up-regulated in the SM group, indicating that (LAG3+CD160+CD8+)NKT cells may play a critical role in the pathogenesis of COVID-19.

Immune Cell Subsets Associated With the Disease Severity of COVID-19

To delineate the immune cell subsets associated with the disease severity of COVID-19, we compared the percentage of each cell type, cluster and sub-cluster between HC, AS, MD, SD, and SDR, respectively. We identified 27 distinct cell subsets that increased or decreased significantly in these groups, 23 of which were positively or negatively correlated with the disease severity by the Spearman rank order correlation analysis (**Figures 5A, B**, **S4A, B** and **Table 1**). When we compared the SD with MD, we observed that ($GATA3^+CCR6^-S1PR1^+CD4^+$)naïve T, ($GATA3^+GPR183^+CD4^+$)naïve T, ($MKI67^+$)Proliferation T, ($FOXP3^+IL2RA^+IL7R^+CD4^+$)Treg, ($LAG3^+CD160^+CD8^+$) NKT, ($LAMP1^+CD4^+CD8^+$)pro-NKT, ($NCAM1^{hi}CD160^+$)NK, ($CD68^-CSF1R^-IL1B^{hi}CD14^+$)classical monocytes, ($CD4^{lo}CSF1R^-CD33^+CD14^+$)classical monocytes, ($CD40^+CLEC9A^-$)mDC, ($CD83^{hi}$)mDC, ($CX3CR1^{hi}CD14^{hi}$)mDC and ($TNFRSF8^+$) mDC increased significantly, whereas ($CD4^-CD8^-$)DNT, ($TRGC1^-CD4^-CD8^-$)DNT, ($TRGC1^+CD4^-CD8^-$)γδT, ($TRAV1\text{-}2^+CD8^+$)MAIT, ($CD7^{lo}$)NK, ($CD33^-HLA\text{-}DMA^-CD14^+$)classical monocytes, ($CSF1R^+CD86^-CD14^+$)classical monocytes, ($CXCL8^+CSF1R^-IL1B^-CD14^+$)classical monocytes, ($CD14^{lo}CD16^{lo}$)immature monocytes, ($S100A12^-CX3CR1^{lo}$) mDC, and ($CLEC10A^-S100A9^{lo}$)pDC decreased significantly (**Figures 5A, B**, **S4A, B**, and **Table 1**). Here, we showed ($CD68^-CSF1R^-IL1B^{hi}CD14^+$)classical monocytes as a representative cell subset that was positively correlated with the disease severity (**Figures 5A, B**).

To investigate whether ($CD68^-CSF1R^-IL1B^{hi}CD14^+$)classical monocytes were associated with the disease progression, we examined two SM patients who had PBMC samples collected at two different time-points, including patient #1 (a fatal patient): COV012 (time-point 1) and COV077 (time-point 2); and patient #2 (an SD patient at the recovery stage): COV029 (time-point 1) and COV126 (time-point 2). We observed that ($CD68^-CSF1R^-IL1B^{hi}CD14^+$)classical monocytes dramatically increased in both time points in the fatal patient (COV012→ COV077), and the second time point was higher than the first one (**Figure 5C**). In contrast, for the SD patient at the recovery stage (SDR) (COV029→COV126), the percentage of ($CD68^-CSF1R^-IL1B^{hi}CD14^+$)classical monocytes at the second time point

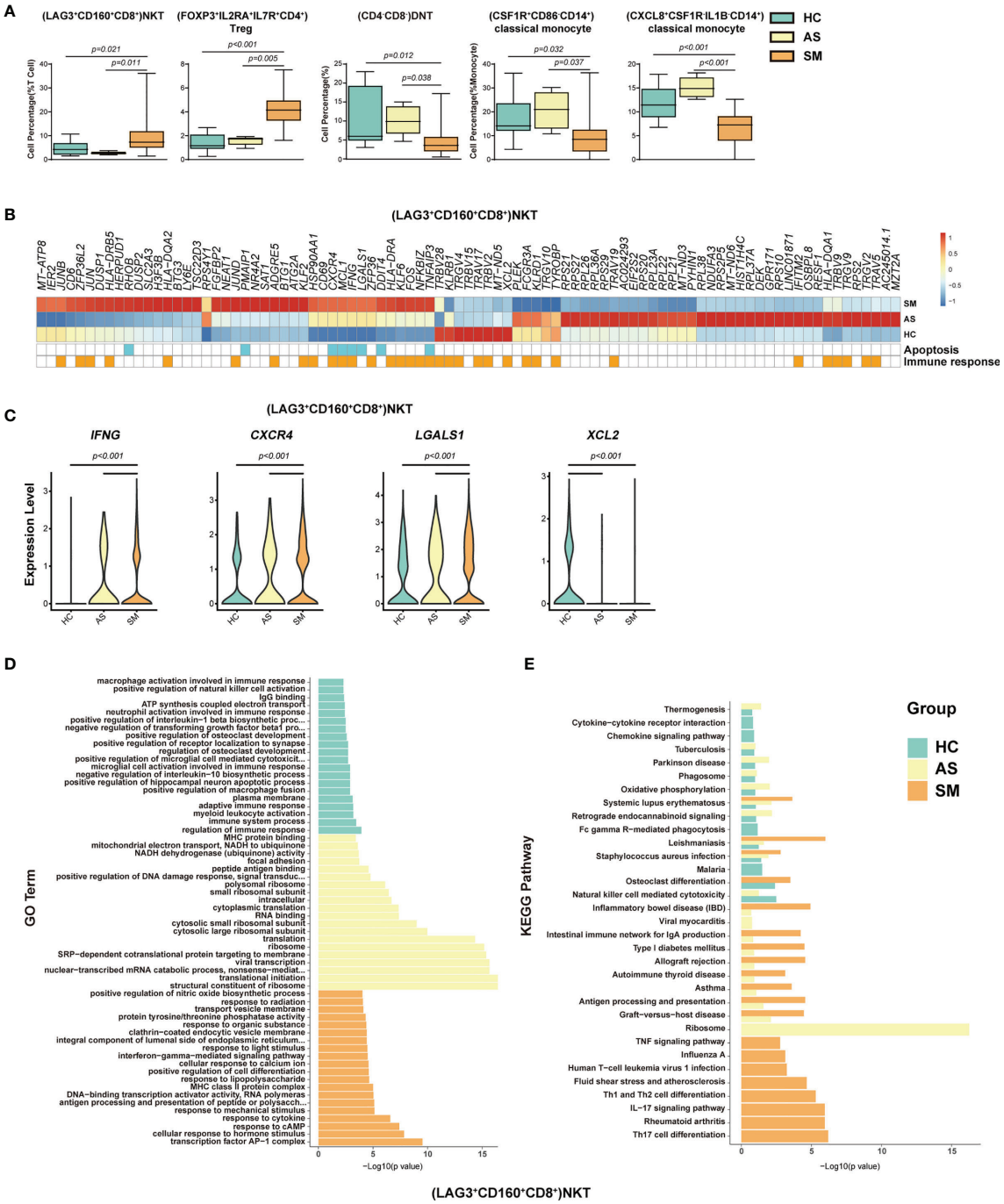

FIGURE 4 | Immune cell subsets associated with symptomatic COVID-19 patients. **(A)** The Box and whisker plots showing the percentage of five distinct cell clusters that exhibited significant differences ($p < 0.05$) between HC, AS, and SM groups. The horizontal lines, box and whiskers correspond to median values, interquartile range (IQR) and minimum/maximum value, respectively. **(B)** A Heatmap showing the average level of the DEGs in the (LAG3$^+$CD160$^+$CD8$^+$)NKT cells with $p < 0.05$ and log2 fold change (FC) ≥0.26 in HC, AS and SM. The gene function (immune responses and apoptosis) is indicated in the heatmap. The gene expression was calculated *via* LogNormalize method of the "NormalizeData" function of the Seurat software. **(C)** The violin plots showing the expression levels of four representative DEGs (*IFNG, CXCR4, LGALS1*, and *XCL2*) in (LAG3$^+$CD160$^+$CD8$^+$)NKT cells, which were involved in apoptosis and/or immune responses. **(D)** Gene Ontology (GO) analysis. Top 20 significant GO terms sorted by – Gene Ontology (GO) analysis. Top 20 significant GO ter$^+$CD160$^+$CD8$^+$)NKT cells. **(E)** KEGG pathway analysis. Top 20 significant KEGG pathways sorted by –log10 (p value) from HC, AS, and SM were shown in (LAG3$^+$CD160$^+$CD8$^+$)NKT cells (also see **Figure S3**). $p < 0.05$ was considered significant. The samples included HC (n=11), AS (n=5) and SM (n=35, total 33 patients and two patients were sampled twice at different disease stages).

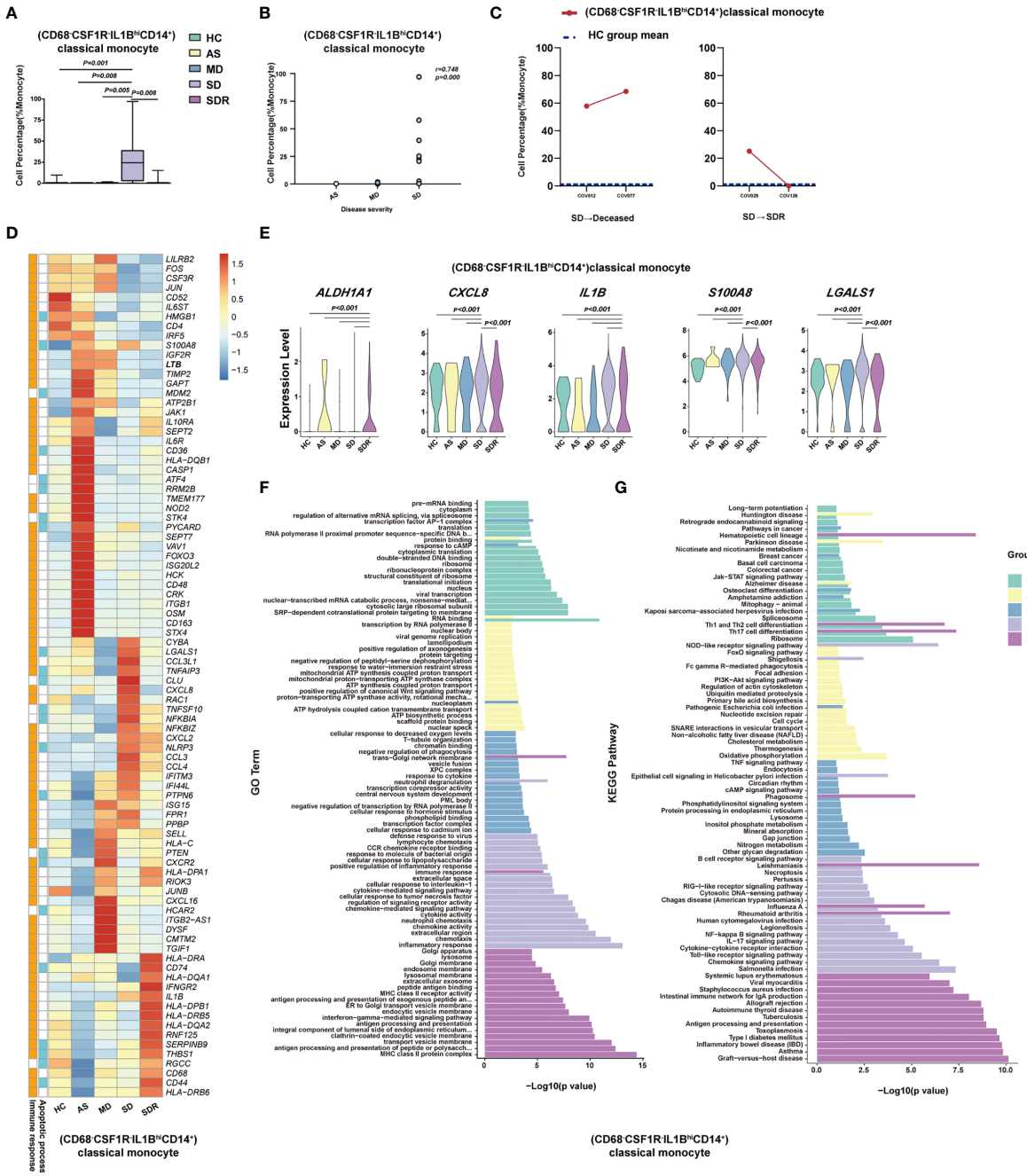

FIGURE 5 | (CD68-CSF1R-IL1BhiCD14+)classical monocyte correlated with disease severity of COVID-19 patients. **(A)** The Box and whisker plots showing the percentage of (CD68$^-$CSF1R$^-$IL1B^{hi}CD14$^+$)classical monocytes that showed significant differences ($p < 0.05$) between the HC, AS, MD, SD and/or SDR groups. The horizontal lines, box and whiskers correspond to median value, interquartile range (IQR) and minimum/maximum value, respectively. **(B)** Spearman rank order correlation analysis showing the significant associations between (CD68$^-$CSF1R$^-$IL1B^{hi}CD14$^+$)classical monocytes with the disease severity. **(C)** The alternation trend of (CD68$^-$CSF1R$^-$IL1B^{hi}CD14$^+$)classical monocytes in the fatal patient (COV012/COV077) and one recovery SD patient (COV029/COV126). **(D)** The Heatmap showing the average level of the DEGs in the (CD68$^-$CSF1R$^-$IL1B^{hi}CD14$^+$)classical monocytes with $p < 0.05$ and log2 fold change (FC) ≥0.26 in HC, AS, MD, SD, and SDR. The gene function (immune responses and apoptosis) is indicated in the heatmap. The gene expression was calculated *via* LogNormalize method of the "NormalizeData" function of the Seurat software. **(E)** The violin plots showing the expression levels of five representative DEGs (*ALADH1A1, CXCL8, IL1B, S100A8*, and *LGALS1*) in (CD68$^-$CSF1R$^-$IL1B^{hi}CD14$^+$)classical monocytes, which were involved in apoptosis and/or immune responses. **(F)** Gene Ontology (GO) analysis. Top 20 significant GO terms sorted by –log10 (p) value from HC, AS, MD, SD, and SDR were shown, respectively. **(G)** KEGG pathway analysis. Top 20 significant KEGG pathways sorted by –log10 (p value) from HC, AS, MD, SD, and SDR were shown, respectively (also see **Figure S4**). $p < 0.05$ was considered significant. The samples included HC (n=11), AS (n=5), MD (n=13), SD (n=11, one patient was sampled twice at different disease stages) and SDR (n=11, one sample was sampled at different disease stages).

resumed to the level similar to the healthy control (HC) group (**Figure 5C**), suggesting that ($CD68^-CSF1R^-IL1B^{hi}CD14^+$) classical monocytes may play an important role in the pathogenesis of COVID-19.

We also analyzed the DEGs in these cell subsets and presented a representative heatmap showing the average level of 88 DEGs in the ($CD68^-CSF1R^-IL1B^{hi}CD14^+$)classical monocytes in the HC, AS, MD, SD and SDR individuals. Most of the DEGs were related to immune responses and apoptosis pathways (**Figure 5D**), such as *ALADH1A1, CXCL8, IL1B, S100A8*, and *LGALS1* (**Figure 5E**). The DEGs were also detected in other cell subsets, such as ($CD4^-CD8^-$)DNT cells, ($TRGC1^+CD4^-CD8^-$)γδT cells, ($CD4^{lo}CSF1R^-CD33^+CD14^+$)classical monocytes and ($CLEC10A^-S100A9^{lo}$) pDC (**Figure S4C, D**). Additional analysis of the GO and KEGG pathways revealed a number of DEGs that were involved in chemotaxis, inflammatory and immune responses, and the signaling pathways of chemokine, NF-kappa B, IL-17, Toll-like receptor and apoptosis (**Figures 5F, G** and **S4E, F**).

Immune Cell Subsets Associated With the Viral Persistence of SARS-CoV-2 Infection

To understand the immune cell alternations associated with the LTNP and STNP, we compared the percentage of each of the major cell types and 76 cell subsets, and found that the LTNP subjects had significant increases in ($CD33^-HLA\text{-}DMA^-CD14^+$) classical monocytes, ($CXCL8^+CSF1R^-IL1B^-CD14^+$)classical monocytes, ($CD14^{lo}CD16^{lo}$)immature monocytes, and ($CLEC10A^-S100A9^-$)pDC and neutrophils, as well as significant decreases in ($AIF1^+BCL2^-CD4^+$)naive T cells, ($MKI67^+$) proliferation T cells, ($CD24^-ITGAE^+CD180^+$)marginal zone B cells, plasma cells, ($CD68^-CSF1R^-IL1B^{hi}CD14^+$)classical monocytes, and ($CD4^{lo}CSF1R^-CD33^+CD14^+$)classical monocytes (**Figure 6A**, **S5A**, and **Table 1**). The Spearman rank order correlation analysis suggested that ($CD33^-HLA\text{-}DMA^-CD14^+$) classical monocytes, ($CLEC10A^-S100A9^-$)pDC, ($CXCL8^+CSF1R^-IL1B^-CD14^+$)classical monocytes, ($CD14^{lo}CD16^{lo}$)immature monocytes, and neutrophil were positively correlated with LTNP, whereas ($CD24^-ITGAE^+CD180^+$)marginal zone B cells and ($MMRN1^-PDZK1IP1^-$)activated platelets were negatively correlated with LTNP (**Figure 6B**, **S5B**, and **Table 1**). Additional analysis of DEGs on two representative cell subsets found that the expression level of *S100A8, S100A9, S100A12, CXCL8, KIF6, IFITM2, IFITM3*, and *IL1B* significantly increased in STNP whereas *CD74, CD52, HLA-DRB5, IL17RA, TNFSF10, IFI30, ITGA4*, and *LILRP1* significantly increased in LTNP in the ($CD33^-HLA\text{-}DMA^-CD14^+$)classical monocytes (**Figure 5C, D**). On the other hand, *HLA-DRB5, S100A4, HLA-DQA2, BTG1, PECAM1, IFITM3, S100A8, S100A9, CD48, CD68*, and *SIGLEC6* significantly increased in LTNP in the ($CLEC10A^-S100A9^-$)pDC (**Figures 6C, D**). The GO and KEGG analysis showed significant enrichments in antigen processing and presentation, neutrophil aggregation, chemokine production, Th1 and Th2 cell differentiation and other pathways (**Figures 6E, F**). To our knowledge, this is the first scRNA-seq study showing the immune cell alternations associated with the viral persistence of SARS-CoV-2 infection.

DISCUSSION

In this study, we identified a huge number of distinct cell subsets that were associated with asymptomatic infection, disease severity, and viral persistence in COVID-19 patients. In particular, we revealed that ($TRAV1\text{-}2^+CD8^+$)MAIT cells and ($NCAM1^{hi}CD160^+$)NK cells were significantly enriched in the AS subjects compared with the HC individuals (**Figure 4A**, **Table 1** and **Table S2**). On the other hand, ($TRAV1\text{-}2^+CD8^+$) MAIT cells were negatively correlated with the disease severity in COVID-19 patients (**Table 1**). The mucosa-associated invariant T (MAIT) cells were suggested to be associated with the mucosa immunity to a variety of microbial infections (36–39). In addition, recent studies reported that MAIT cells significantly declined in COVID-19 patients with severe diseases and resumed to normal level when the disease was resolved (40–42). These findings suggest that ($TRAV1\text{-}2^+CD8^+$)MAIT cells might be involved in the protective immune responses against SARS-CoV-2 infection.

Natural killer (NK) cells are important components of the innate and adaptive immune responses and have been suggested to play protective or pathogenic roles in the pathogenesis of human diseases, including SARS-CoV-2 infections (43–49). In this study, ($NCAM1^+CD160^+$)NK cells increased significantly in all of the SARS-CoV-2 infected individuals compared to the HC (**Figures 3A, B**, and **Table 1**) and were positively correlated with the disease severity (**Figure S4B**), suggesting that this NK cell subset may play both protective and pathogenic roles in the pathogenesis of COVID-19. In contrast, ($CD7^{lo}$)NK cells decreased significantly in all of the SARS-CoV-2 infected individuals compared to the HC and were negatively correlated with the disease severity (**Figure S4B**), indicating that this NK cell subset may function differently from ($NCAM1^+CD160^+$)NK cells. Moreover, our results also suggest that ($CD4^{lo}CSF1R^-CD33^-CD14^+$) and ($CD33^-HLA\text{-}DMA^-CD14^+$) classical monocytes were involved in the innate immune responses against SARS-CoV-2 infections. Therefore, it is possible that robust innate immune responses may control the virus replication and allow sufficient time to mount acquired immune responses, resulting in an asymptomatic infection and disease-free state in the AS subjects.

Natural killer T cells (NKT) and regulatory T cells (Treg) have been suggested to play important roles in immune responses to viral infections and tumors (50–58). In this study, ($LAG3^+CD160^+CD8^+$)NKT and ($FOXP3^+IL2RA^+IL7R^+$)Treg cells increased significantly in the SM patients (**Figure 4B**). In addition, we observed a trend towards increasing levels of these cell subsets in the SD patients. The differentially expressed genes (DEGs) in the ($LAG3^+CD160^+CD8^+$)NKT cells (e.g. *IFNG, CXCR4, LGALS1* and *XCL2*) and ($FOXP3^+IL2RA^+IL7R^+$) Treg cells (e.g. *CCR7, IFI16, TNFRSF4* and *TNFRSF18*), which were linked to immune responses, inflammation and apoptosis, suggest that these cells may contribute to the development of clinical symptoms and the subsequent disease progression (**Figures 4C–F** and **S3B–D**). Since ($LAG3^+CD160^+$ $CD8^+$)NKT cells were also positively correlated with the disease severity, suggesting that it may function as a double edge sword, playing

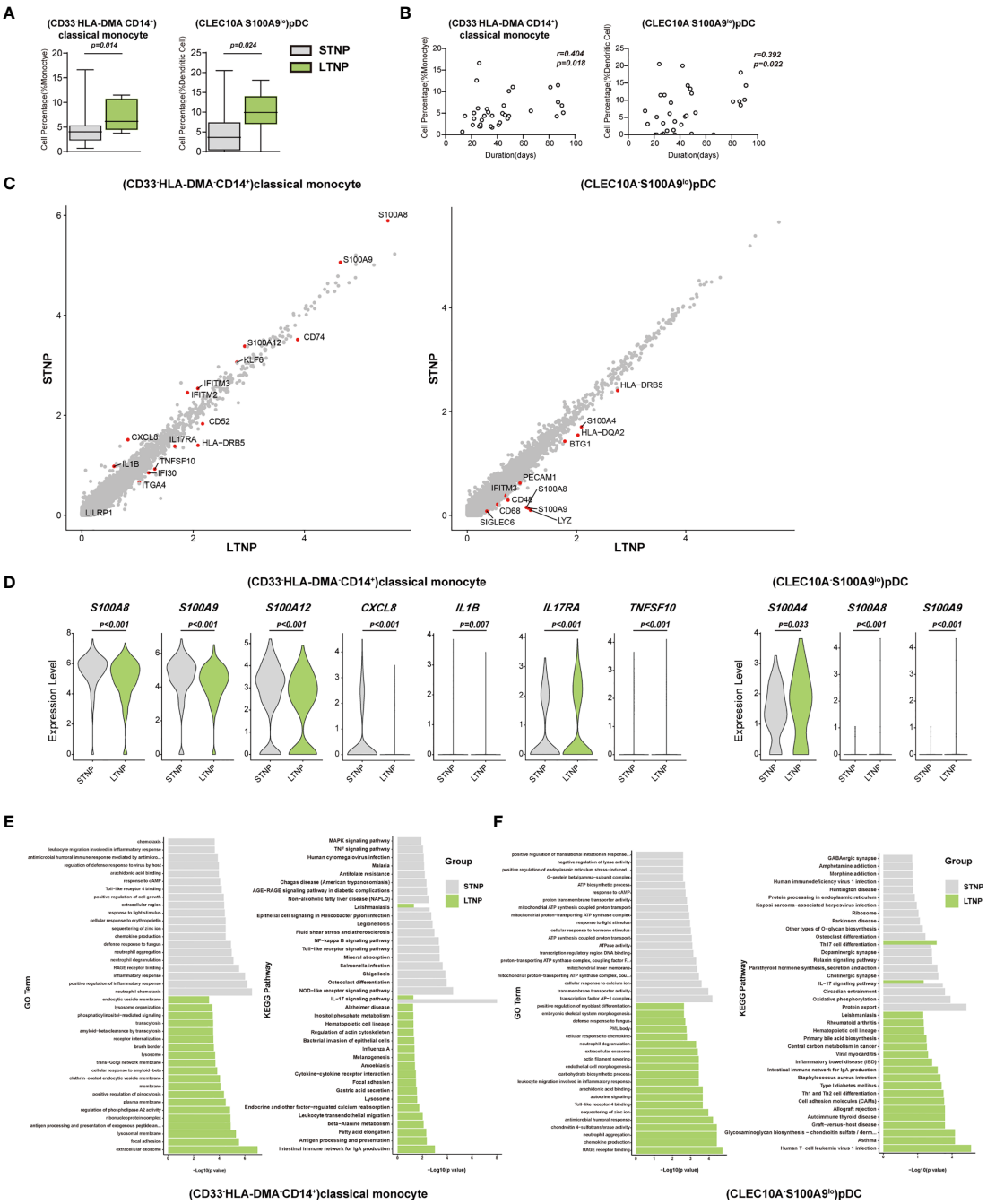

FIGURE 6 | (CD33-HLA-DMA-CD14+)classical monocytes and (CLEC10A-S100A9lo)pDC were positively correlated with the viral persistence. **(A)** The Box and whisker plots showing the percentage of (CD33$^-$HLA-DMA$^-$CD14$^+$)classical monocytes and (CLEC10A$^-$S100A9^{lo})pDC that had significant differences ($p < 0.05$) between the STNP and LTNP groups, respectively. The horizontal lines, box and whiskers correspond to median values, interquartile range (IQR) and minimum/maximum value, respectively. **(B)** Spearman rank order correlation analysis showing the (CD33$^-$HLA-DMA$^-$CD14$^+$)classical monocytes and (CLEC10A$^-$S100A9^{lo})pDC were positively correlated with the viral persistence. **(C)** The M-versus-A (MA) plots showing the differentially expressed genes (DEGs) in the (CD33$^-$HLA-DMA$^-$CD14$^+$) classical monocytes and (CLEC10A$^-$S100A9^{lo})pDC with $p < 0.05$ and log2 fold change (FC) ≥0.26 in STNP and LTNP. The gene expression was calculated *via* LogNormalize method of the "NormalizeData" function of the Seurat software. The X and Y axis represent log (1+average expression value), respectively. **(D)** The violin plots showing the expression levels of seven representative DEGs (*S100A8, S100A9, S100A12, IL17RA, TNFSF10, CXCL8*, and *IL1B*) in (CD33$^-$HLA-DMA$^-$CD14$^+$)classical monocytes and three representative DEGs (*S100A4, S100A8, S100A9*) in (CLEC10A$^-$S100A9^{lo})pDC. **(E)** Gene Ontology (GO) and KEGG analysis for (CD33$^-$HLA-DMA$^-$CD14$^+$)classical monocytes. Top 20 significant GO terms and KEGG pathways sorted by –log10 (p value) from STNP and LTNP were shown. **(F)** GO and KEGG analysis for (CLEC10A$^-$S100A9^{lo}) pDC. Top 20 significant GO terms and KEGG pathways sorted by –log10 (p value) from STNP and LTNP were shown (also see **Figure S5**). $p < 0.05$ was considered significant. The samples included LTNP (n=12) and STNP (n=21).

both protective and pathogenic roles in the pathogenesis of COVID-19.

Myeloid cell subsets, such as monocytes, macrophages, dendric cells and neutrophils have been suggested to be involved in a variety of inflammatory responses, including the pathogenesis of COVID-19 (19, 25, 59–67). In this study, we identified a large number of myeloid cell subsets that were associated with the disease severity and viral persistence in the COVID-19 patients (**Table 1**). Notably, ($CD4^{lo}CSF1R^{-}CD33^{-}CD14^{+}$), ($CD33^{-}HLA\text{-}DMA^{-}CD14^{+}$), ($CSF1R^{+}CD86^{-}CD14^{+}$) and ($CXCL8^{+}CSF1R^{-}IL1B^{-}CD14^{+}$) classical monocytes decreased significantly in the SM patients (**Table 1**), suggesting that these cell subsets may negatively associated with the disease symptoms. On the other hand, ($CD68^{-}CSF1R^{-}IL1B^{hi}CD14^{+}$) and ($CD33^{-}HLADMA^{-}CD14^{+}$) classical monocytes were positively correlated with the disease severity and were associated with aggregation of neutrophils. Of note, ($CD68^{-}CSF1R^{-}IL1B^{hi}CD14^{+}$) and ($CD33^{-}HLADMA^{-}CD14^{+}$) classical monocytes as well as neutrophils dramatically increased in the fatal patient (COV077). Moreover, these cells overexpressed a number of cytokines, chemokines, acute-phase proteins and other proinflammatory factors (e.g. *CCL3, CCL4, CXCL2, CXCL8, IL1B, S100A8, TNFSF10, LGALS1*), suggesting that these cell subsets might be associated with the cytokine storm and other pathological processes. On the other hand, ($CLEC10A^{-}S100A9^{lo}$) pDC were negatively correlated with the disease severity of COVID-19 patients.

Another novel and important aspect of this study was the profiling of PBMCs from the COVID-19 patients with short and long duration of viral persistence (i.e. STNP and LTNP). In particular, ($CD33^{-}HLA\text{-}DMA^{-}CD14^{+}$)classical monocytes and ($CLEC10A^{-}S100A9^{-}$)pDC were found to be positively correlated with LTNP (**Figure 6A**). Moreover, we detected a panel of DEGs (e.g. *S100A8, S100A9, S100A12, CXCL8, KIF6, IFITM2, IFITM3*, and *IL1B*) that may discriminate the LTNP from the STNP patients (**Figures 6C, D**). HLA-DMA is a member of the HLA class II alpha chain paralogues and plays a critical role in the antigen presentations (68, 69). The enrichment of ($CD33\text{-}HLA\text{-}DMA\text{-}CD14^{+}$)classical monocytes may weaken the antigen presentation ability and antiviral immune responses, and subsequently result in the prolonged viral persistence in the LTNP patients.

In conclusion, this study identified a large number of distinct immune cell subsets that were associated with various clinical presentations and viral persistence. Our findings may enhance our understanding of the immunopathogenesis of COVID-19. In addition, our huge datasets will become a valuable resource for future studies in the scientific communities.

DATA AVAILABILITY STATEMENT

The raw sequence data reported in this paper have been deposited in GEO repository, under accession code GSE165080 and are publicly accessible at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE165080. Other supporting raw data are available from the corresponding author upon reasonable request. Source data are provided with this paper.

ETHICS STATEMENT

This study was reviewed and approved by the Ethics Committee of The First affiliated Hospital of Xi'an Jiaotong University (XJTU1AF2020LSK-015) and The Renmin Hospital of Wuhan University (WDRY2020-K130). All participants enrolled in this study offered the written informed consent by themselves or their surrogates. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: CZ and BS. Methodology and Investigation: XW, HB, JM, HQ, QZ, FH, TJ, WM, YZ, XC, XQ, ML, JX, JH, YW, XD, YL, TH, CF, CG, and CZ. Resources: CZ, BS, BHZ, and KH. Writing: CZ, BS, BJZ, XR, XW, HB, JM, TJ, and WM. Review and editing: CZ, HB, JM, BJZ, XR, and BS. Supervision: CZ, BS, BHZ, KH, CF, and DL. Funding: CZ and BS. Project administration: CZ and BS. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.812514/full#supplementary-material

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Age-Associated Seroprevalence of Coronavirus Antibodies: Population-Based Serosurveys in 2013 and 2020, British Columbia, Canada

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Background: Older adults have been disproportionately affected during the SARS-CoV-2 pandemic, including higher risk of severe disease and long-COVID. Prior exposure to endemic human coronaviruses (HCoV) may modulate the response to SARS-CoV-2 infection and contribute to age-related observations. We hypothesized that cross-reactive antibodies to SARS-CoV-2 are associated with antibodies to HCoV and that both increase with age.

Methods: To assess SARS-CoV-2 unexposed individuals, we drew upon archived anonymized residual sero-surveys conducted in British Columbia (BC), Canada, including before SARS-CoV-2 emergence (May, 2013) and before widespread community circulation in BC (May, 2020). Fifty sera, sex-balanced per ten-year age band, were sought among individuals ≤10 to ≥80 years old, supplemented as indicated by sera from March and September 2020. Sera were tested on the Meso Scale Diagnostics (MSD) electrochemiluminescent multiplex immunoassay to quantify IgG antibody against the Spike proteins of HCoV, including alpha (HCoV-229E, HCoV-NL63) and beta (HCoV-HKU1, HCoV-OC43) viruses, and the 2003 epidemic beta coronavirus, SARS-CoV-1. Cross-reactive antibodies to Spike, Nucleocapsid, and the Receptor Binding Domain (RBD) of SARS-CoV-2 were similarly measured, with SARS-CoV-2 sero-positivity overall defined by positivity on ≥2 targets.

Results: Samples included 407 sera from 2013, of which 17 were children ≤10 years. The 2020 samples included 488 sera, of which 88 were children ≤10 years. Anti-Spike antibodies to all four endemic HCoV were acquired by 10 years of age. There were 20/407

(5%) sera in 2013 and 8/488 (2%) in 2020 that were considered sero-positive for SARS-CoV-2 based on MSD testing. Of note, antibody to the single SARS-CoV-2 RBD target was detected in 329/407 (81%) of 2013 sera and 91/488 (19%) of 2020 sera. Among the SARS-CoV-2 overall sero-negative population, age was correlated with anti-HCoV antibody levels and these, notably 229E and HKU1, were correlated with cross-reactive anti-SARS-CoV-2 RBD titres. SARS-CoV-2 overall sero-positive individuals showed higher titres to HCoV more generally.

Conclusion: Most people have an HCoV priming exposure by 10 years of age and IgG levels are stable thereafter. Anti-HCoV antibodies can cross-react with SARS-CoV-2 epitopes. These immunological interactions warrant further investigation with respect to their implications for COVID-19 clinical outcomes.

Keywords: SARS-CoV-2, COVID-19, antibodies, humoral immune response, endemic coronavirus, cross-reactive antibodies, COVID-19 severity

INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the causative agent of the Coronavirus Disease 2019 (COVID-19) pandemic, responsible for hundreds of millions of known infections worldwide since its emergence in December 2019 (1). While younger children more often experience mild or asymptomatic infections, older adults are disproportionately affected by COVID-19 (2, 3), with a strong association between morbidity and mortality (4). Older adults also have an increased risk of prolonged symptoms following COVID-19, or "long COVID syndrome". In contrast, COVID-19 rarely causes serious disease in children, emphasizing the urgent need to better understand the pathogenesis of age-dependent disease. Several hypotheses have been proposed to describe the age-related differences in COVID-19 severity (3), including comorbidities, immune senescence, differential expression of the SARS-CoV-2 host receptor angiotensin-converting enzyme 2 (5), as well as variation in the inflammatory immune response; however, none appear to account for the strong correlation between COVID-19 severity and age. Additionally, immunological interactions due to prior exposure to closely-related pathogens may also modulate the immune response to SARS-CoV-2, which may potentially enhance or dampen protective immune responses and contribute to COVID-19 severity.

In addition to the betacoronavirus SARS-CoV-2, humans are also susceptible to at least six other viruses within the alphacoronavirus and betacoronavirus genus of the *Coronaviridae* family (6). Among these, SARS-CoV-1 and Middle East Respiratory Syndrome (MERS-CoV) are two highly pathogenic betacoronaviruses, which are associated with elevated morbidity and mortality rates among those infected (7). In contrast, there are four endemic human coronaviruses (HCoVs), two alphacoronaviruses (HCoV-NL63 and HCoV-229E) and two betacoronaviruses (HCoV-HKU1 and HCoV-OC43), which circulate seasonally and typically only cause mild upper respiratory tract infections (8–10). Initial infection generally occurs during childhood, with virtually everyone believed to have some immunity against HCoVs by adolescence (9, 11). However, HCoV immunity wanes (12), hence reinfections are common, typically occurring every 2-3 years throughout a person's lifetime (6, 13).

Observational studies have identified the presence of SARS-CoV-2 cross-reactive immune responses in pre-pandemic individuals unexposed to SARS-CoV-2, including cross-reactive T cells (14), B cells (15, 16), and antibody responses (17–20). To date, the source of these cross-reactive immune responses, as well as their potential negative or positive implications during a SARS-CoV-2 infection are still poorly understood. As HCoVs are highly similar to SARS-CoV-2 (6), it has been speculated that the cross-reactive SARS-CoV-2 antibody responses seen in unexposed individuals might be associated with pre-existing HCoV antibodies.

We hypothesized that cross-reactive antibodies against SARS-CoV-2 are associated with the presence of HCoV antibodies in SARS-CoV-2-unexposed individuals and that both increase with age. To assess the age-associated seroprevalence of HCoV and SARS-CoV-2 cross-reactive antibodies among SARS-CoV-2 unexposed individuals, we drew upon archived samples of anonymized residual sero-surveys previously conducted according to a cross-sectional, age-stratified protocol in British Columbia, Canada before SARS-CoV-2 emergence (May, 2013) or its broad pandemic circulation (May, 2020).

MATERIALS AND METHODS

Study Population

As part of risk assessment for emerging respiratory pathogens, the British Columbia Centre for Disease Control (BCCDC) previously established a sero-survey protocol to assess changes in sero-positivity with time (21). As part of this protocol, anonymized residual sera are periodically procured in an age-based fashion from the only outpatient laboratory network

(formerly BC Biomedical Laboratories, now LifeLabs) servicing the Lower Mainland, BC (i.e. Greater Vancouver Area, including the Fraser Valley), where ~60% of the provincial population resides and community attack rates are expected to be high (21). For the current study, remaining archived samples primarily collected during prior sero-surveys for influenza (May, 2013) and SARS-CoV-2 (May, 2020) were used (21).

For this study, remaining archived specimens were included in the study if at least 200µL were available. Accompanying characteristics included age and sex. The original May 2013 sero-survey protocol sought up to 50 samples per ten-year age band <10, 10-19, 20-29, 30-39, 40-49, 50-59, 60-69, 70-79, ≥ 80 years of age; whereas, the May 2020 protocol sought 100 serum samples among those <5 and 5-9 years and per ten-year age band thereafter. When available, up to 50 sera per age-band and season were randomly selected (sex-balanced) where more than 50 were an option. Where <50 sera per age band were available, notably among children, additional samples were supplemented from a similarly conducted sero-survey in March 2020. As the samples from 2013 were collected prior to the COVID-19 pandemic, they were assumed to be from persons unexposed to SARS-CoV-2 and all deemed "true SARS-CoV-2 sero-negatives". Of note, SARS-CoV-2 circulation was well-suppressed in BC through spring 2020 with sero-surveys in both March and May 2020 showing overall <1% sero-positivity at most (21).

We further assessed 40 anonymized residual samples identified from the serial SARS-CoV-2 sero-surveys, similarly-conducted March (N=11), May (N=9) and September 2020 (N=20) sero-surveys, respectively. All 40 samples were known to be positive for antibodies to at least one of three SARS-CoV-2 antigens (Spike, S1 Receptor Binding Domain (RBD), Nucleocapsid) using commercial Health Canada-approved chemiluminescent immuno-assays (CLIA) (21). Among these, 20/40 had been identified as positive for at least two CLIA targets (2, 4 and 14 from March, May and September, respectively), while the remaining 20/40 were positive only for one CLIA target. Among these, the 19 that were found to be SARS-CoV-2 sero-positive according to the Meso Scale Discovery (MSD) testing algorithm (described below) are included as a subset of positive controls for comparison, when appropriate.

Original sero-surveys and use of specimens for these investigations were approved by the Clinical Research Ethics Board of the University of British Columbia (H20-00653).

Meso Scale Discovery (MSD) Multiplexed Pan-Coronavirus Immunoassay

To simultaneously detect and quantify antibody levels against six coronaviruses (all except MERS), we utilized a highly sensitive multiplexed chemiluminescent immunoassay from MSD (V-PLEX Coronavirus Panel 2). Multi-spot plates spotted with purified antigens were used for the detection of IgG antibodies against Spike, S1 RBD, and Nucleocapsid of SARS-CoV-2, as well as Spike of SARS-CoV-1 and alpha-HCoVs (229E and NL63) and beta-HCoVs (HKU1 and OC43). Assays were performed according to the manufacturer's protocol.

Briefly, multi-spot plates were initially incubated with MSD Blocker A for 30 minutes, then washed off. Reference standard, controls, and serum samples (diluted 1:5000 in Diluent 100) were added and incubated on the plates for 2 hours. Plates were washed and MSD SULFO-TAG Anti-Human IgG detection antibody was added, incubated for an hour, and then washed again. Finally, MSD Gold Read Buffer B was added to the plate and signals were immediately measured on the MSD QuickPlex SQ120 instrument. All incubation steps were carried out at room temperature while shaking at 700rpm, and all wash steps were performed three times with MSD Wash Buffer, prior to addition of the subsequent reagents.

Raw data generated was processed using MSD Discovery Workbench software (Version 4.0), then imported into RStudio (Version 1.2.5033) to interpret signal cut-off values. SARS-CoV-2 cut-off thresholds for reactivity provided by the manufacturer are as follows: anti-SARS-CoV-2 Spike values above 1960 AU/mL, anti-SARS-CoV-2 Nucleocapsid values above 5000 AU/mL, and anti-SARS-CoV-2 S1 RBD values above 538 AU/mL. Samples above cut-off values for at least two of three SARS-CoV-2 targets (S1 RBD and/or nucleocapsid, and/or spike) were considered serologically reactive using this MSD immunoassay (i.e. overall status sero-positive) based on our previous validation (22).

Because everyone is expected to have been exposed to HCoV by adolescence, we opted to use the lower 95% confidence interval of the geometric mean antibody titres among the SARS-CoV-2 negative population (which includes all of the 2013 population and the SARS-CoV-2 sero-negatives from 2020) to define positive signal cut-offs for HCoV. Consequently, the positivity cutoffs assigned for HCoVs were as follows: 1700 AU/mL for HCoV-229E Spike, 900 AU/mL for HCoV-HKU1, 270 AU/mL for HCoV-NL63, and 2000 AU/mL for HCoV-OC43. As only 1 epitope (Spike) was included for HCoVs, sero-positivity for HCoVs was defined based on positivity for the Spike epitope alone.

Statistical Analysis

All statistical analyses were conducted on R (Version 3.6.2) and RStudio (Version 1.2.5033). Processed data from the MSD immunoassay was visualized using the *ggplot2* (Version 3.3.3) and *ggpubr* (Version 0.6.0) packages on RStudio. Kruskal-Wallis, Wilcoxon rank sum, Spearman's correlation calculations were conducted using both *ggpubr* and *stats* (Version 3.6.2) packages. Correlation matrices were made using *corrplot* (Version 0.92). Geometric mean antibody levels were calculated on *rcompanion* (Version 2.4.1). Antibody trends over chronological age were fitted using a locally weighted regression to fit a curve between points on *ggpubr*. Using the *stats* package, multivariable linear regression models were built, model diagnostics and model fit were assessed, and distribution of residuals were evaluated to determine whether assumptions for linear models were met. Box-Cox transformations on *MASS* (Version 7.3-54) were used to determine the optimal transformation methods. All regression analyses were carried out using natural log-transformed antibody levels. P-values less than 0.05 were considered statistically significant.

RESULTS

Study Population

A total of 935 sera were assessed from individuals ranging 0-99 years of age. A summary of the age and sex distribution of individuals is listed in **Table 1**, according to the 10-year age bands from our sampling. Of the 935 sera, 895 were used for sero-prevalence estimation and 40 were previously identified as sero-positive for SARS-CoV-2 on at least one commercial CLIA. There were 407 samples procured from 2013 and 528 from 2020. Excluding the 40 sera from 2020 that were sero-positive for SARS-CoV-2 on commercial platforms, 488 samples from the 2020 season were used including 463 from May 2020, and 25 from March 2020. Overall, the 2013 population tested was 62.4% (254/407) female, while the 2020 population (March and May taken together) was 50.8% (248/488) female.

Of the 40 sera previously identified as sero-positive on at least one CLIA platform, we identified 19/40 as sero-positive by a validated MSD algorithm (22), which requires a positive result on 2 of the 3 SARS-CoV-2 targets tested. 1/19 was previously positive for only one CLIA target and was a sample collected in March 2020, and the remaining 18/19 were previously positive for at least two CLIA targets (4 were collected in May and 14 were collected in September 2020). We included these 19 MSD-positive specimens as our SARS-CoV-2 sero-positive controls.

HCoV Antibodies Across Age, and by Sex and Year

Geometric mean IgG antibody titres for HCoV-229E, HKU1, NL63, and OC43 among SARS-CoV-2 negative individuals (N=887) from both 2013 (407/407; pre-pandemic) and 2020 (480/488; sero-negative for SARS-CoV-2) are shown in **Figure 1**. Initial seroconversion to all four HCoV was seen in all pediatric individuals ≤10 years of age, and sero-reactivity against HCoV was stable across age groups thereafter.

We stratified the population by three age categories to compare HCoV antibody levels between age groups, biological sex (male and female) and years as a proxy for different respiratory seasons (2013 and 2020): children (≤10 years old), pre-teens (adolescents) to adults (11-69 years old), and elderly adults (≥70 years old). Discrete categories for age groups were selected based on biological reasons and because we found that by 10 years of age, almost 100% of individuals had IgG to all 4 HCoV above the lower 95% confidence interval. HCoV-specific antibody levels were similar between males and females, although NL63 antibodies were significantly higher in female than male pre-teens/adults ($P<0.05$), and 229E antibodies were significantly higher in male than female pre-teens/adults ($P<0.05$) (**Figure 2A**). Males and females demonstrated very similar HCoV antibody trends across ages (**Figure 2B**), with no overall sex differences. Within the SARS-CoV-2 sero-negative population (N=887), antibody levels to HCoV showed minimal differences between the 2013 and 2020 seasons (**Figures 2C, D**). Pre-teens to adults in 2020 exhibited significantly higher levels of 229E antibodies when compared against the 2013 population ($P<0.05$), while the elderly in 2020 exhibited significantly lower levels of NL63 antibodies. Overall, no consistent differences in HCoV antibodies were observed between sampling year and sexes.

Among the SARS-CoV-2 sero-negative population (N=887), correlations were observed between age and HCoV antibody levels, specifically for HCoVs 229E and NL63 (**Figure 3A**). Overall, strong correlations between the four HCoV were seen in children (**Figure 3B**), pre-teens to adults (**Figure 3C**) and the elderly (**Figure 3D**). Additionally, age appears to be more strongly correlated with HCoV-229E and NL63 in children than in the other age categories.

Antibodies Against SARS-CoV-1 and SARS-CoV-2, Stratified by Age, Sex, and Year

We also evaluated the levels of cross-reactive IgG antibodies against SARS-CoV-1 and SARS-CoV-2 antigens in SARS-CoV-2 sero-negative persons. **Figure 4** shows the geometric mean antibody levels of target-specific SARS-CoV-2 cross-reactive antibodies among all sero-negative persons in our population (N=887), which are stable across chronological age after the age of 10 years. Anti-SARS-CoV-2 Nucleocapsid and Spike antibody

TABLE 1 | Age and sex distribution of study participants from 2013 and 2020.

	2013 (N = 407)		2020 (N = 488)		Overall (N = 895)	
	F (N = 254)	M (N = 153)	F (N= 248)	M (N = 240)	2013	2020
Median Age (IQR)	**42 (28, 66)**	**58 (30, 73)**	**40 (17, 64)**	**40 (19, 65)**	**49 (28, 69)**	**40 (18, 65)**
Age Band						
0-1	0 (0%)	0 (0%)	1 (0.4%)	3 (1.2%)	0 (0%)	4 (0.8%)
2-4	5 (2%)	2 (1.3%)	22 (8.9%)	12 (5.0%)	7 (1.7%)	34 (7%)
5-9	4 (1.6%)	6 (3.9%)	25 (10%)	25 (10%)	10 (2.5%)	50 (10%)
10-19	32 (13%)	16 (10%)	25 (10%)	25 (10%)	48 (12%)	50 (10%)
20-29	34 (13%)	14 (9%)	25 (10%)	25 (10%)	48 (12%)	50 (10%)
30-39	45 (18%)	4 (2.6%)	25 (10%)	25 (10%)	49 (12%)	50 (10%)
40-49	30 (12%)	19 (12%)	25 (10%)	25 (10%)	49 (12%)	50 (10%)
50-59	25 (9.8%)	24 (16%)	25 (10%)	25 (10%)	49 (12%)	50 (10%)
60-69	28 (11%)	20 (13%)	25 (10%)	25 (10%)	48 (12%)	50 (10%)
70-79	21 (8.3%)	29 (19%)	25 (10%)	25 (10%)	50 (12%)	50 (10%)
80+	30 (12%)	19 (12.4%)	25 (10%)	25 (10%)	49 (12%)	50 (10%)

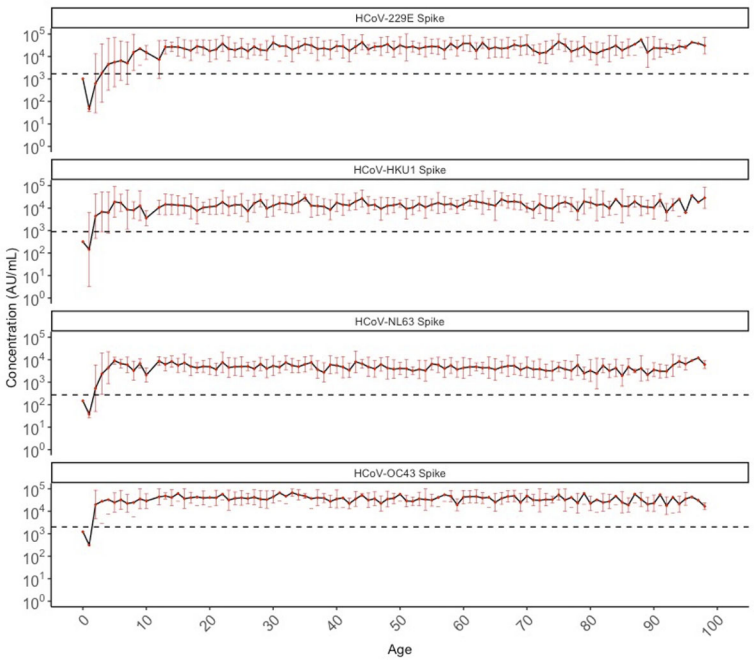

FIGURE 1 | Geometric mean IgG antibody titres against HCoV-229E, HCoV-HKU1, HCoV-NL63, and HCoV-OC43 spike in SARS-CoV-2 sero-negative populations (N = 887) by chronological age. SARS-CoV-2 sero-negative populations include all 2013 individuals (N = 407) and SARS-CoV-2 sero-negative individuals from 2020 (N = 480). Red dots indicate the geometric mean antibody titres for each HCoV among all individuals of that age (in years), with the black solid line connecting geometric means. Red bars represent upper and lower standard deviations. Black dashed lines describe the positivity cutoff for each antigen target.

levels were generally well below the manufacturer positivity cutoff; whereas, geometric mean SARS-CoV-2 RBD antibody levels meet or exceed the positivity cutoff across ages. While there are no validated cutoffs for SARS-CoV-1 positivity, geometric mean concentrations of cross-reactive antibodies appeared lower than those against SARS-CoV-2 and this was observed consistently across all ages.

We also compared cross-reactive SARS-CoV-1 and SARS-CoV-2 antibodies between sexes across age categories among the SARS-CoV-2 sero-negative individuals (N=887). Male children had significantly higher cross-reactive antibodies against SARS-CoV-1 Spike and SARS-CoV-2 Spike, Nucleocapsid, and RBD compared to female children (**Figure 5A**). Antibody differences between sexes was not observed in the pre-teen to adult group and the elderly group for any of the SARS-CoV-1 and SARS-CoV-2 targets. Notably, cross-reactive SARS-CoV-2 RBD antibodies do appear to increase slightly with age for both males (Spearman's rho = 0.24) and females (Spearman's rho = 0.29), with older ages exceeding the positivity cutoff (**Figure 5B**).

Cross reactive antibodies against SARS-CoV-1 and SARS-CoV-2 targets were similar among children in both 2013 and 2020, except for SARS-CoV-2 RBD, for which pediatric individuals in 2013 showed significantly higher levels of cross-reactivity that mostly exceeded the positivity cutoff, compared to 2020 (**Figures 5C, D**). Among adults and the elderly, cross-reactive antibody levels against SARS-CoV-1 spike and SARS-CoV-2 Spike, Nucleocapsid, and RBD in 2013 were each significantly higher than in 2020. However, with the exception of SARS-CoV-2 RBD, most remained below the positivity cutoff.

Sera from 2013 and 2020 were dichotomized as negative or positive for antibodies against SARS-CoV-2 RBD, Nucleocapsid, and Spike in **Table 2**. Notably, 4.9% (20/407) of the pre-pandemic individuals from 2013 were deemed SARS-CoV-2 positive based on exceeding cut-off values for at least two of three MSD targets. When evaluating antibodies specific to each SARS-CoV-2 target separately, 2013 pre-pandemic individuals exhibited antibodies above the positivity cutoff for Nucleocapsid (4.7%, 19/407), RBD (81%, 329/407), and Spike (0.5%, 2/407).

Among the 2020 population, 1.6% (8/488) were deemed SARS-CoV-2 sero-positive based on exceeding cut-off values for at least two of three targets as per the validated MSD algorithm. The sera from these individuals were collected in May 2020 survey, and 1/8 were children, 4/8 were pre-teens to adults, and 3/8 were from the elderly group. All 8 individuals were seropositive for Nucleocapsid and RBD, while 7/8 individuals were also sero-positive for Spike. Among the remaining 98.4% (480/488) individuals from 2020 who were sero-negative for SARS-CoV-2 by MSD testing, some individuals also exhibited cross-reactive antibodies above the cutoff for Nucleocapsid (1%, 5/480), RBD (17%, 83/480), and Spike (0.4%, 2/480). While the percent with cross-reactive antibodies against SARS-CoV-2 Nucleocapsid and Spike was similar between the 2013 and 2020 population, this differed for RBD (**Table 2** and **Figure 5D**).

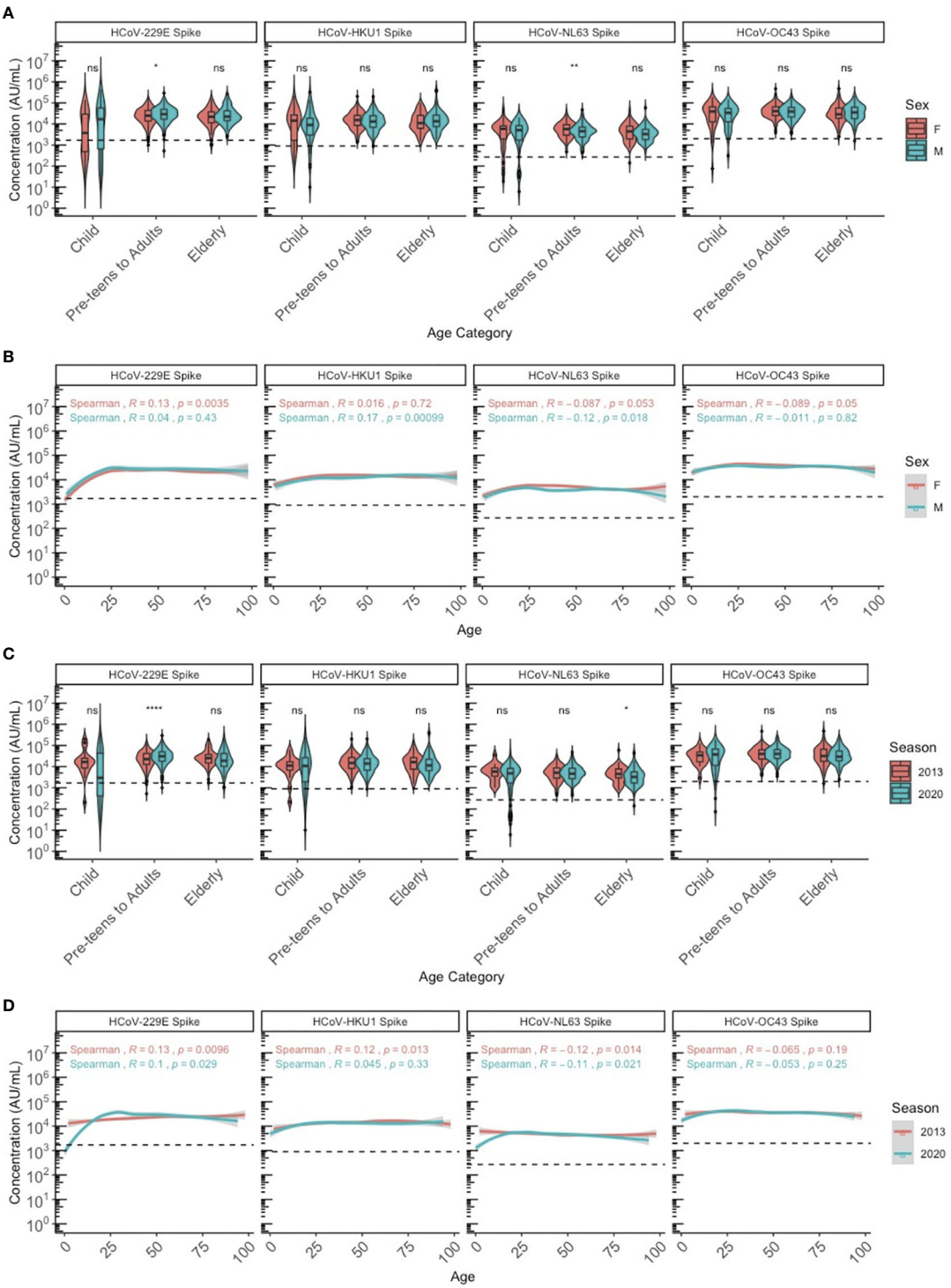

FIGURE 2 | HCoV-229E, HCoV-HKU1, HCoV-NL63, and HCoV-OC43 spike IgG antibody levels between sexes and seasons (2013 and 2020) by chronological age among SARS-CoV-2 sero-negative persons (N = 887). **(A, B)** describe the HCoV antibody levels between male and female sexes by **(A)** age category and **(B)** chronological age. **(C, D)** describe the HCoV antibody levels between 2013 and 2020 seasons by **(C)** age category and **(D)** chronological age. In **(A, C)**, age categories are stratified according to children (≤10 years old), pre-teens to adults (11-69 years old), and the elderly (≥70 years old). Black dashed lines describe the positivity cutoff for each antigen target. Wilcoxon rank sum test was used to compare antibody levels between **(A)** sexes and **(C)** seasons. Spearman's rank correlation was used to describe the relationship between antibody level and age in **(B, D)**, and Spearman's correlation coefficient rho (R) and corresponding P-values (P) are reported. ns, not significant, * = $p < 0.05$, ** = $p < 0.01$ **** = $p < 0.0001$.

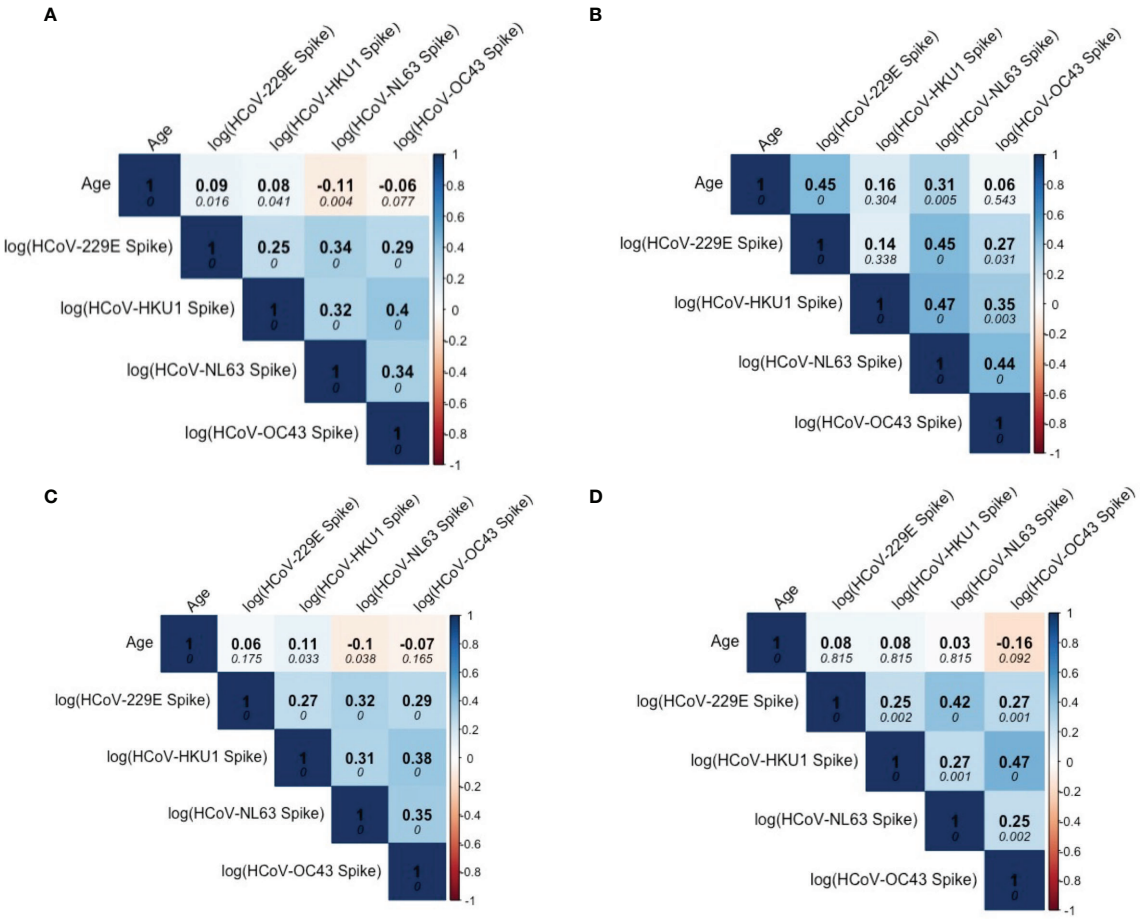

FIGURE 3 | Correlation matrix describing relationship among Age and HCoV-specific IgG antibodies in SARS-CoV-2 sero-negative persons (N = 887). **(A)** describes correlations within the entire SARS-CoV-2 sero-negative population, comprised of all 2013 individuals (N = 407) and SARS-CoV-2 sero-negative individuals from 2020 (N = 480), which are further stratified by age categories: **(B)** children (≤10 years old) (N = 587), **(C)** pre-teens to adults (11-69 years old) (N = 104), and **(D)** the elderly (≥70 years old) (N = 196). Darker colours represent stronger correlations. Spearman's correlation coefficient is reported in each matrix (top numerical value). P-values are reported in italics.

Among the series of 40 samples across serial sero-surveys in March, May and September 2020 that were positive for at least one SARS-CoV-2 target on commercial CLIA, 19 were SARS-CoV-2 sero-positive according to the MSD algorithm requiring dual positivity. These 19 individuals were sourced from the March (1/19), May (4/19), and September (14/19) 2020 surveys and comprised of 1/19 children, 16/19 preteens to adults, and 2/19 elderly. Together with the 8/488 considered SARS-CoV-2 sero-positive described above, there were a total of 27/935 (3%) individuals considered SARS-CoV-sero-2 positive in this study (two children, 20 pre-teens to adults, 5 elderly).

Association of HCoV Antibodies and SARS-CoV-2 Cross Reactive Antibodies

We assessed the association between age and HCoV antibodies with detection of SARS-CoV-2 cross-reactive antibodies exceeding cutoff values in presumably unexposed persons. Specifically, among those with SARS-CoV-2 RBD antibodies above the positivity cutoff, we evaluated the correlation between age and HCoV antibody levels vs. SARS-CoV-2 cross-reactive RBD antibody levels among the 2013 population collected pre-COVID-19 pandemic (N=329), 2020 SARS-CoV-2 sero-negatives (N=83), and SARS-CoV-2 sero-positive individuals (N=27) (**Figure 6**). Among the SARS-CoV-2 sero-negative individuals, age and HCoV antibody titres show a weakly positive relationship with SARS-CoV-2 crossreactive RBD antibodies, such that older age and higher HCoV titres are correlated with the presence of more cross-reactive SARS-CoV-2 RBD antibodies (**Figures 6A, B**). Among the four HCoVs, 229E and HKU1 exhibit the strongest correlations (**Figures 6A, B**).

In contrast, the positive association is lost in SARS-CoV-2 sero-positive individuals (**Figure 6C**), who demonstrate a weakly negative trend between SARS-CoV-2 RBD antibodies with HCoVs 229E, NL63, and HKU1, although associations were not significant and large 95% confidence intervals were observed. Lower levels of anti-SARS-CoV-2 RBD antibodies

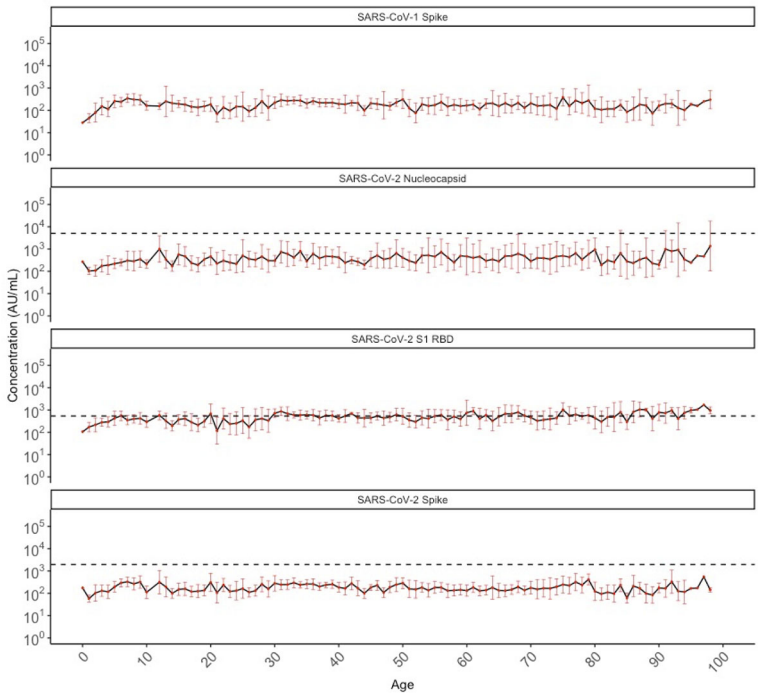

FIGURE 4 | Geometric mean IgG antibody titres against SARS-CoV-1 spike and SARS-CoV-2 nucleocapsid, S1 RBD, and spike in SARS-CoV-2 sero-negative populations (N = 887) by chronological age. SARS-CoV-2 sero-negative populations comprise of all 2013 individuals (N = 407) and SARS-CoV-2 sero-negative individuals from 2020 (N = 480). Red dots indicate the geometric mean antibody titres for each HCoV among all individuals of that age (in years), with the black solid line connecting geometric means. Red bars represent upper and lower standard deviations. Black dashed lines describe the manufacturer provided positivity cutoff for each antigen target. No cutoff is available for SARS-CoV-1 spike.

were associated with older age (R=-0.4, P=0.037). Higher OC43 titres were positively correlated with higher anti-SARS-CoV-2 RBD antibodies (R = 0.59, P=0.0019).

Multivariable linear regression was also conducted to identify whether age, sex, and HCoV antibody levels independently predict the magnitude of SARS-CoV-2 cross-reactive RBD antibodies (**Table 3**). Age, HCoV-229E titres, and HCoV-HKU1 titres were significantly associated (P<0.05) with cross-reactive RBD antibodies for the 2013 SARS-CoV-2 negative population (N=329), although only HCoV-229E remained a significant predictor of SARS-CoV-2 RBD antibody levels in the SARS-CoV-2 sero-negative population in 2020 (N=83). Among SARS-CoV-2 sero-positive individuals (N=27), only OC43 was independently associated with SARS-CoV-2 RBD antibody titres (P<0.05).

In contrast, age and HCoV antibody level did not independently predict cross-reactive Nucleocapsid titers. With only two individuals from 2013 exhibiting SARS-CoV-2 spike antibody levels above the positivity cutoff (**Table 2**), we could not conduct these analyses for that target.

HCoV Antibodies in SARS-CoV-2 Positive Individuals

Finally, we wanted to evaluate how HCoV antibody levels differ between SARS-CoV-2 sero-negative (N=887) and sero-positive (N=27) individuals. The 27 sero-positive individuals used for this analysis included the 8/488 from the 2020 cohort together with the 19 supplemental SARS-CoV-2 known positives. In addition to their higher mean SARS-CoV-2 antibody levels, these SARS-CoV-2 sero-positive individuals also had higher SARS-CoV-1, HCoV-229E, HKU1, and OC43 antibody levels (**Figure 7**).

We attempted to stratify this by age categories to evaluate differences between the SARS-CoV-2 sero-positive and sero-negative populations across ages (**Figure 7**). The small sample size of sero-positive specimens overall precluded statistical comparisons by age group but the observation that SARS-CoV-2 sero-positive individuals had higher SARS-CoV-1 and HCoV antibodies did not appear to be limited to or driven by one age category but was generally seen for all age groups.

DISCUSSION

In this study we explored age-based seroprevalence to the endemic HCoVs, HCoV-229E, HCoV-NL63, HCoV-HKU1, and HCoV-OC43 in BC, Canada. Retrospective sampling of anonymized residual sera obtained from an outpatient laboratory from 2013 (pre-COVID-19 pandemic) and early 2020 (first wave of the pandemic) was used to interrogate the association between HCoV and SARS-CoV-2 cross-reactive

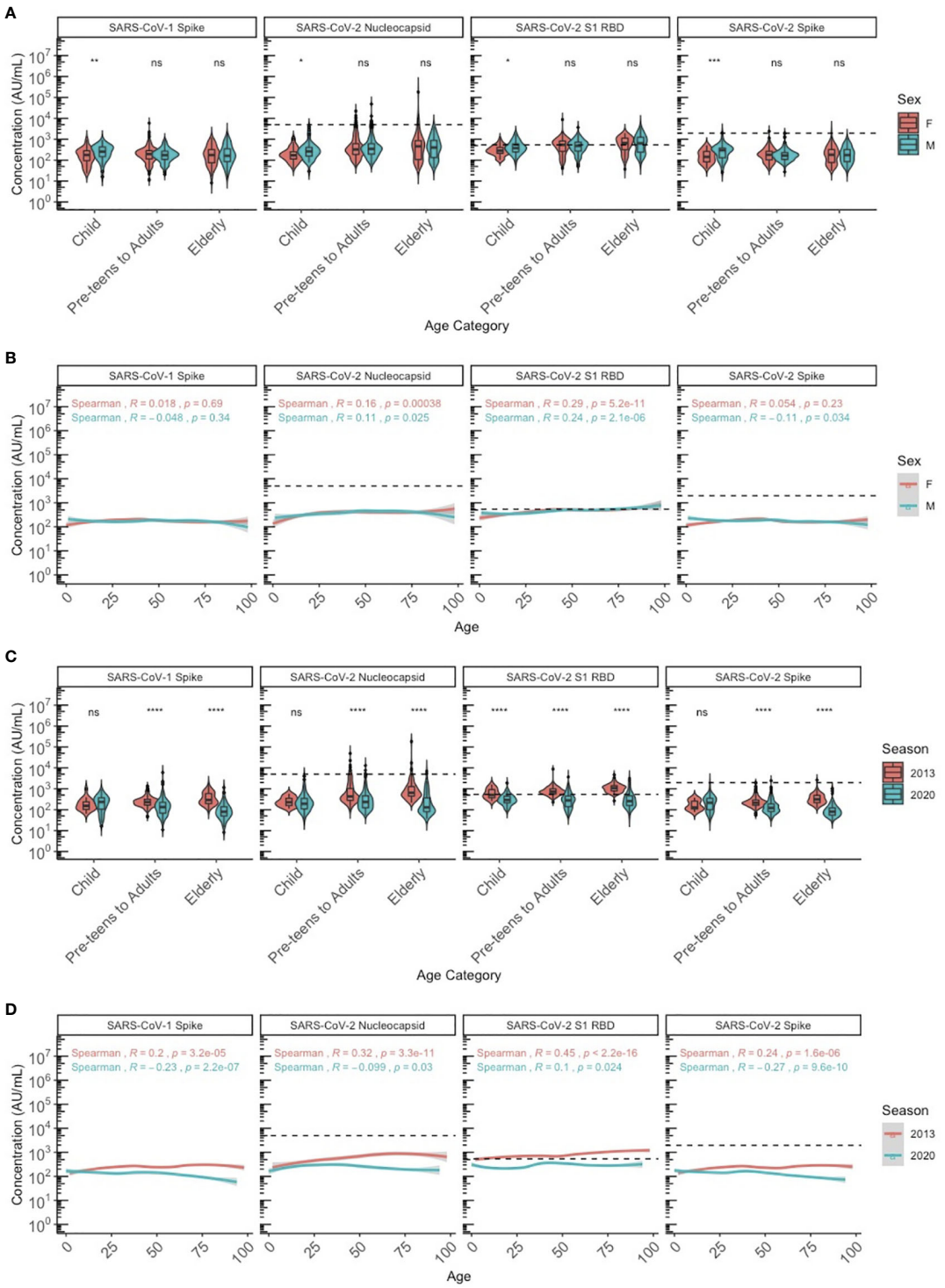

FIGURE 5 | SARS-CoV-1 Spike and SARS-CoV-2 S1 RBD, nucleocapsid and spike IgG antibody levels between sexes and seasons by age among SARS-CoV-2 sero-negative persons (N=887). **(A, B)** describe the SARS-CoV-1 and SARS-CoV-2 cross-reactive antibody levels between male and female sexes by **(A)** age category and **(B)** chronological age. **(C, D)** describe the SARS-CoV-1 and SARS-CoV-2 cross-reactive antibody levels between 2013 and 2020 seasons by **(C)** age category and **(D)** chronological age. In **(A-C)**, age categories are stratified according to children (≤10 years old), pre-teens to adults (11-69 years old), and the elderly (≥70 years old). Black dashed lines describe the positivity cutoff for SARS-CoV-2 targets. Wilcoxon rank sum test was used to compare antibody levels between **(A)** sexes and **(C)** seasons. Spearman's rank correlation was used to describe the relationship between antibody level and age in **(B, D)**, and Spearman's correlation coefficient rho (R) and corresponding P-values (P) are reported. ns, not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001 **** = p < 0.0001.

TABLE 2 | Population above positivity cut-off for SARS-CoV-2 targets in 2013 and 2020.

		2013 (N=407)	2020 (N=488)		CLIA+ (N=40)
	MSD Status	**Overall**	**Negatives**	**Positives**	**Overall**
Overall SARS-CoV-2 Sero-status	**Negative**	387 (95%)	480 (98.4%)	0	21 (52%)
	Positive	20 (4.9%)	0	8 (1.6%)	19 (48%)
Nucleocapsid	**Negative**	388 (95%)	475 (99%)	0 (0%)	21 (52%)
	Positive	19 (4.7%)	5 (1.0%)	8 (100%)	19 (48%)
RBD	**Negative**	78 (19%)	397 (83%)	0 (0%)	14 (35%)
	Positive	329 (81%)	83 (17%)	8 (100%)	26 (65%)
Spike	**Negative**	405 (99.5%)	478 (99.6%)	7 (88%)	24 (60%)
	Positive	2 (0.5%)	2 (0.4%)	1 (12%)	16 (40%)

CLIA+ = 40 samples positive for at least one SARS CoV-2 target on chemiluminescent assays supplemented.

antibodies in presumably unexposed individuals. By using a high-throughput pan-coronavirus multiplex immunoassay with age-stratified sampling, we reinforce earlier findings that priming exposures to HCoVs occur during early childhood and remain stable into older age (9, 20, 23). Furthermore, we demonstrate HCoV antibodies are associated with cross-reactive antibodies against SARS-CoV-2, notably the anti-RBD IgG.

No overall differences in population HCoV antibody levels were observed between respiratory seasons (2013 and 2020 collection years), except for 229E among adults and NL63 among the elderly. No overall sex differences were observed in

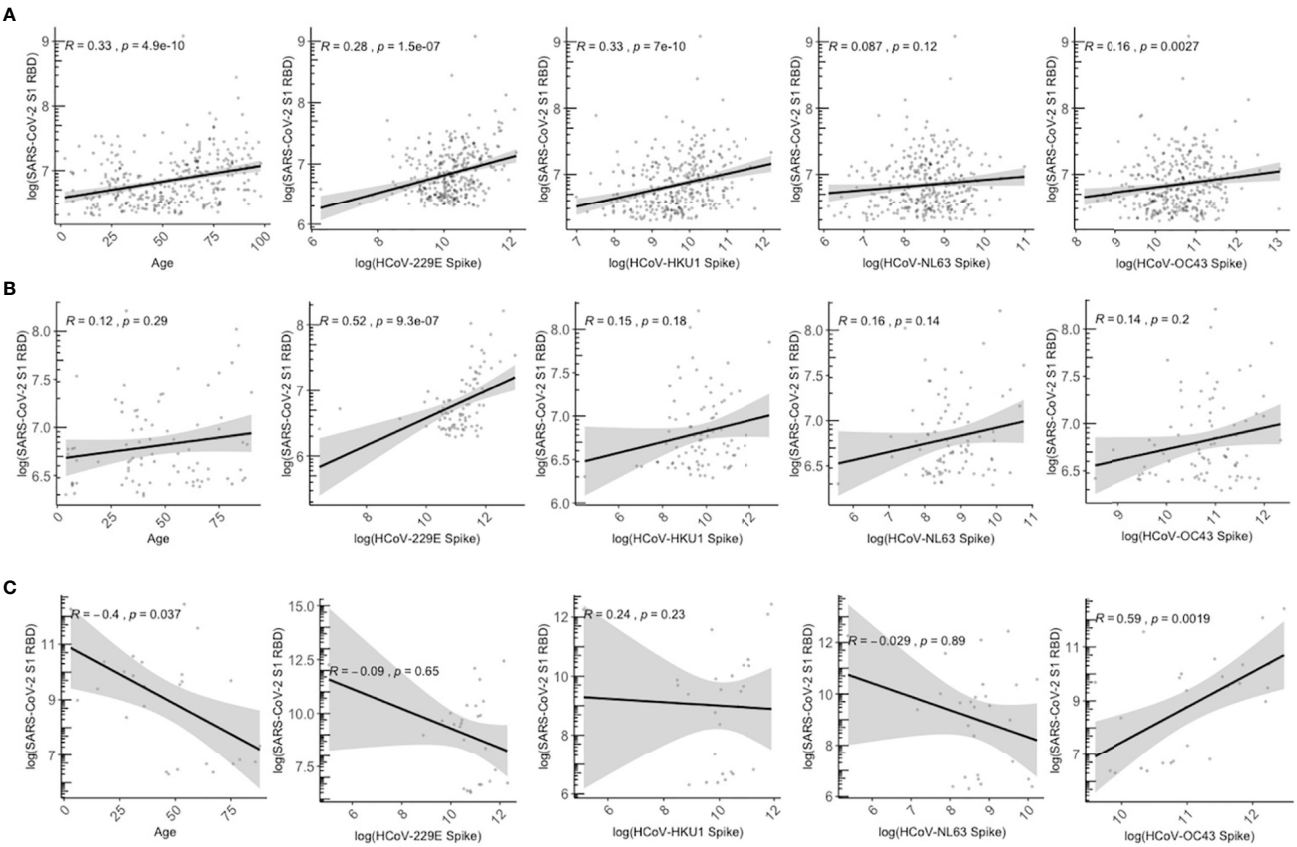

FIGURE 6 | Relationship between age and HCoV-specific IgG antibodies with SARS-CoV-2 S1 RBD antibodies among SARS-CoV-2 overall sero-negative persons who are positive for SARS-CoV-2 S1 RBD antibodies. **(A)** describes all individuals from 2013 season who have SARS-CoV-2 S1 RBD antibodies above positivity cutoff (N = 329). **(B)** describes SARS-CoV-2 sero-negative individuals from 2020 who have SARS-CoV-2 S1 RBD above positivity cutoff (N = 87). **(C)** describes SARS-CoV-2 sero-positive individuals from 2020 who also have SARS-CoV-2 S1 RBD above positivity cutoff (N = 27). SARS-CoV-2 sero-positive individuals are from the serosurvey cohort (N = 8) and the known positive controls added (N = 27). Gray shaded area indicates 95% confidence intervals. Spearman correlations were used to describe the relationship between samples, and corresponding Spearman correlation coefficient (R) and p-values are reported.

TABLE 3 | Multiple linear regression analysis of anti-SARS-CoV-2 RBD IgG antibody levels above positivity cutoff in SARS-CoV-2 sero-negative individuals (2013 and 2020) and SARS-CoV-2 sero-positive individuals, with adjustment for age, sex, and anti-HCoV IgG signals.

Variable	2013 (N = 329)		2020 (N = 83)		SARS-CoV-2 Sero-Positives (N = 27)	
	Coefficient	P-value	Coefficient	P-value	Coefficient	P-value
(Intercept)	4.009	–	3.685	–	-7.787	–
Age	0.005	<0.001	0.001	0.461	-0.029	0.127
Sex (Male)	0.035	0.372	0.021	0.818	-0.606	0.380
log(HCoV-229E Spike)	0.116	<0.001	0.0215	<0.001	0.734	0.175
log(HCoV-HKU1 Spike)	0.087	<0.001	0.065	0.183	-0.251	0.648
log(HCoV-NL63 Spike)	0.022	0.379	0.047	0.449	-0.549	0.252
log(HCoV-OC43 Spike)	0.031	0.243	-0.036	0.629	1.604	0.023
Adjusted R-squared	0.233		0.195		0.335	

HCoV antibody levels, apart from small differences among adults for 229E and NL63. Given that circulating HCoV strains may differ each season (23), and different age groups may be subjected to varying exposures, slight differences in antibody levels between seasons and sexes are expected. Nonetheless, no overall sex and seasonal differences in HCoV antibody levels across ages were consistently observed in our study population.

In addition to SARS-CoV-2 RBD, we identified cross-reactive antibodies, albeit to a lesser extent, against SARS-CoV-2 Spike and Nucleocapsid and SARS-CoV-1 Spike in 2013 and 2020, consistent with other published findings (19, 24). The greater cross-reactivity observed in anti-RBD in comparison to anti-spike is not unexpected due to the larger % CVs and 95% confidence intervals for specificity of RBD, as we previously described in our assay validations (22). Additionally, the MSD assay utilizes recombinant proteins printed onto the surface of the plate, and does not account for any post-translational modifications occurring *in vivo*. Recent *in vitro* studies have demonstrated the importance of post-translational modifications, such as glycosylation, on the immunogenicity of RBD (25). As such, cross-reactivity observed on the MSD assay may not be fully representative of biological cross-reactivity. However, we believe our findings still capture the biological interactions, due to the large differentials observed between positive and negative antibodies in our assay validations.

Interestingly, we found male children demonstrated higher levels of cross-reactive antibodies. While sex has been identified as an independent prognostic factor for COVID-19 (26), very few studies, if any, have interrogated sex differences in SARS-CoV-2 cross-reactive antibodies. However, the absence of any consistent sex differences among adults in our cohorts suggests that the increased severity observed among male COVID-19 patients is unlikely to be due to pre-existing cross-reactive SARS-CoV-2.

Notably, SARS-CoV-2 cross-reactive antibodies were consistently higher among the population in 2013 than in 2020 across all ages (**Figures 4C, D**), with a substantial proportion of the 2013 cohort having anti-RBD antibodies above the positivity cutoff. While it may be suspected that this is due to inter-run variability, our assay is well-validated and any observed cross-reactivity for RBD were unlikely due to assay inter-run factors, but rather a true phenomenon for the currently defined cutoffs. It is also important to note that the 2020 cohort was sampled during the first wave of the COVID-19 pandemic in BC, when public health measures successfully suppressed SARS-CoV-2 circulation as well as the transmission of other respiratory viruses. In this case, the 2020 population in this study may have had less exposure to other respiratory viral pathogens, including HCoVs. Further studies investigating the comprehensive contributions of respiratory viral pathogens may help explain the little difference observed in HCoV antibodies, but substantial difference in cross-reactive anti-RBD antibodies, observed between the 2013 and 2020 seasons.

Here, we show that in the presumed absence of SARS-CoV-2 exposure, age, HCoV-229E, and HKU1 antibodies, are positively correlated and significant predictors of SARS-CoV-2 cross-reactive RBD antibodies. However, the low R-squared values indicate that other unexplained factors contribute to the observed variation. Our findings add to the very few other studies that have assessed the association between alpha-CoVs and cross-reactive SARS-CoV-2 antibodies, similarly suggesting an association between HCoV-229E and cross-reactive anti-RBD IgG antibodies in unexposed individuals (9, 19).

The source of cross-reactive antibodies to SARS-CoV-2 has not been extensively studied, and the extent to which they may influence the severity of COVID-19 or long COVID remains unknown. Evidence indicates that a recent HCoV infection is associated with less severe disease, including lower intensive care unit (ICU) admission rates and a higher probability of survival (27, 28), suggesting that HCoV responses may protect against COVID-19 progression. In contrast, other studies have demonstrated that the cross-reactive antibodies do not protect against COVID-19 or neutralize SARS-CoV-2 (29, 30), suggesting a potential role for "original antigenic sin" (OAS) cannot be ruled out. In OAS, immune response to a new or evolved virus is biased by past exposures to a closely related pathogen. OAS may preferentially boost memory responses from the initial exposure, at the expense of generating new immune responses against epitopes specific to the current infection. Our findings neither confirm nor refute a role for OAS but provide further understanding of possible immunological interactions or cross-reactivity that could underpin protective or untoward effects and on that critical basis warrant further investigation.

There are several limitations to this study. As a cross-sectional sero-survey, we were only able to describe associations between

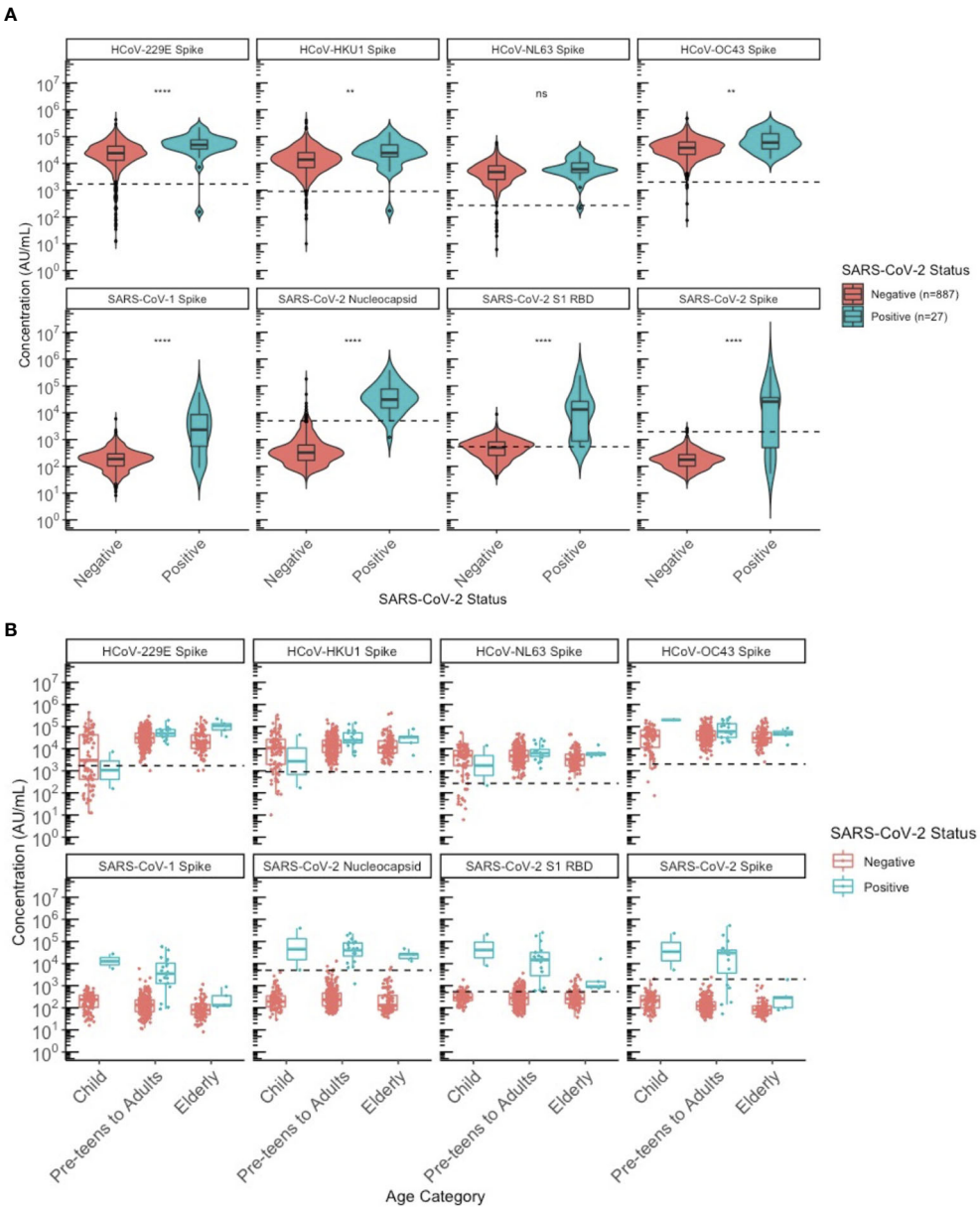

FIGURE 7 | Comparison of HCoV-specific and SARS-CoV-2 specific IgG antibody levels between SARS-CoV-2 sero-negative and sero-positive individuals. **(A)** describes the entire population from 2013 and 2020. All 2013 individuals were classified as sero-negative for SARS-CoV-2 status, regardless of whether they appeared SARS-CoV-2 sero-positive by MSD algorithm or not. **(B)** describes only the 2020 population stratified by age category: children (≤10 years old), pre-teens to adults (11-69 years old), and the elderly (≥70 years old). SARS-CoV-2 sero-positive individuals include children (N = 2), pre-teens to adults (N = 20), elderly (N = 5); similar to SARS-CoV-2 sero-negative individuals where the distribution by age category is children (N = 88), pre-teens to adults (N = 295), elderly (N = 97). Black dashed lines describe the positivity cutoff for each antigen target. ns, not significant, ** = $p < 0.01$, **** = $p < 0.0001$.

HCoV and SARS-CoV-2 cross-reactive antibodies. To further understand causation, future studies utilizing longitudinal cohorts would be highly beneficial. Delays in generating memory antibody responses and timing of the blood draws could also affect the detection of CoV specific antibodies, which may lead to an overall underestimation of seroprevalence (6, 21, 31). In addition, our study did not assess the potential protective effects of these antibody responses. For example, although RBD-binding antibodies are strongly correlated with neutralizing activity (32), gold standard neutralization assays might provide additional insights into the functional importance of SARS-CoV-2 cross-reactive antibodies between age groups. Our study also includes a small SARS-CoV-2 sero-positive population, especially when stratified by age categories, precluding our ability to conduct age-stratified comparisons between SARS-CoV-2 sero-positive versus sero-negative individuals.

In summary, our data reinforces that initial exposure and seroconversion to endemic HCoVs occurs during early childhood and that SARS-CoV-2 cross-reactive antibodies are detected among unexposed populations. Anti-HCoV antibodies appear to be associated with SARS-CoV-2 cross-reactive antibodies. Specifically, we found that HCoV-229E (and HCoV-HKU1 for 2013) antibody titres appeared to be positively associated with cross-reactive RBD antibodies for both the 2013 and 2020 SARS-CoV-2 negative populations. However, we are only able to draw associations, but not infer any causation, as our study was a cross-sectional study. Additionally, our results also demonstrate that there are likely other unexplained factors that can influence SARS-CoV-2 cross-reactive antibodies. The finding of SARS-CoV-2 cross-reactive antibodies, particularly to RBD in a large proportion of unexposed individuals, also highlights the importance of utilizing multiple targets when diagnosing SARS-CoV-2 seropositivity to improve the positive predictive value of serological diagnosis. Further investigation into the durability and functionality of the antibody response will clarify the role of cross-reactive antibodies for natural and vaccine-induced protection against SARS-CoV-2. These investigations are particularly critical now in the context of more relaxed public health measures related to SARS-CoV-2 pandemic control, particularly with the subsequent re-emergence of various respiratory viruses including HCoVs, as they may shed light on the relationship between HCoV exposures and COVID-19 disease severity and duration.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Clinical Research Ethics Board of the University of British Columbia (H20-00653). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AJ, DS, SG, and MK conceptualized the study. AJ, DS, SG, and IS were awarded funding for the project (PI AJ). DS, DP, and RR selected and contributed samples for testing. AJ, DS, SG, IS, AL, GT, and SK were involved in the overall study design. GT, AL, SK, IS, MM, AJ, and MI were involved in performing laboratory methods and analysis of results. GT and AL drafted the manuscript. All authors reviewed and edited the manuscript. AJ, IS, MK, SG, and DS critically advised on the reported conclusions. All authors contributed to the article and approved the submitted version.

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Asymptomatic SARS-CoV-2 Infection Is Associated With Higher Levels of Serum IL-17C, Matrix Metalloproteinase 10 and Fibroblast Growth Factors Than Mild Symptomatic COVID-19

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Young adults infected with SARS-CoV-2 are frequently asymptomatic or develop only mild disease. Because capturing representative mild and asymptomatic cases require active surveillance, they are less characterized than moderate or severe cases of COVID-19. However, a better understanding of SARS-CoV-2 asymptomatic infections might shed light into the immune mechanisms associated with the control of symptoms and protection. To this aim, we have determined the temporal dynamics of the humoral immune response, as well as the serum inflammatory profile, of mild and asymptomatic SARS-CoV-2 infections in a cohort of 172 initially seronegative prospectively studied United States Marine recruits, 149 of whom were subsequently found to be SARS-CoV-2 infected. The participants had blood samples taken, symptoms surveyed and PCR tests for SARS-CoV-2 performed periodically for up to 105 days. We found similar dynamics in the profiles of viral load and in the generation of specific antibody responses in asymptomatic and mild symptomatic participants. A proteomic analysis using an inflammatory panel including 92 analytes revealed a pattern of three temporal waves of inflammatory and immunoregulatory mediators, and a return to baseline for most of the inflammatory markers by 35 days post-infection. We found that 23 analytes were significantly higher in those participants that reported symptoms at the time of the first positive SARS-CoV-2 PCR compared with

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asymptomatic participants, including mostly chemokines and cytokines associated with inflammatory response or immune activation (i.e., TNF-α, TNF-β, CXCL10, IL-8). Notably, we detected 7 analytes (IL-17C, MMP-10, FGF-19, FGF-21, FGF-23, CXCL5 and CCL23) that were higher in asymptomatic participants than in participants with symptoms; these are known to be involved in tissue repair and may be related to the control of symptoms. Overall, we found a serum proteomic signature that differentiates asymptomatic and mild symptomatic infections in young adults, including potential targets for developing new therapies and prognostic tests.

Keywords: SARS-CoV-2, COVID-19, asymptomatic, serum, proteomics, inflammation, innate immunity, antibodies

INTRODUCTION

In March 2020 the World Health Organization declared the Coronavirus Disease 2019 (COVID-19) a global pandemic (1). As of March 2022 there have been roughly 445 million cases and more than 5.9 million deaths reported worldwide (2). SARS-CoV-2 is highly transmissible (3, 4) and the mortality rate is reported to be between 0.9 to 7.7%, depending on the country (5). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) frequently causes mild or asymptomatic disease, especially in young individuals (6–8) who contribute to viral transmission.

Most studies on the pathogenesis of COVID-19 have focused on severe cases [e.g (9–12)]. Although the immune response of individuals with asymptomatic and mild disease has been studied (13–16), it is much less well characterized. Indeed, many important studies characterized the immune response to severe COVID-19 by comparing to mild cases and uninfected participants (12, 17, 18). Studies of mild COVID-19 and asymptomatic SARS-CoV-2 infections have the potential to identify correlates of protection from severe disease, which could indicate new targets for therapy and prognostic tests.

Individuals with asymptomatic infections develop SARS-CoV-2 antibodies, but the magnitude of their response is lower than those with moderate to severe disease (19, 20), and similar to those with mild disease (6). On the other hand, we also learned from elegant studies that asymptomatic individuals are able to mount an efficient memory T cell response against SARS-CoV-2 during the convalescent phase (14, 15). However, the extent and characteristics of the immune response during the acute phase of the disease in asymptomatic individuals remains unclear.

Individuals with mild COVID-19 produce several of the pro-inflammatory mediators seen in individuals with severe disease, including IL-6, CXCL10, TNF-α, MCP-1 and IFN-γ(12, 21), but a prolonged duration of the inflammatory response is likely characteristic of severe cases (9). In this sense, a longitudinal immune response profile of asymptomatic and symptomatic individuals is needed to better understand their differences and the mechanisms that protect some individuals from developing symptoms.

Here, we characterized the dynamics of the early humoral and innate immune response in otherwise healthy young adults with asymptomatic or mildly symptomatic SARS-CoV-2 infection, in a subset of participants of the previously reported COVID-19 Health Action Response for Marines (CHARM) cohort study (22). The study design of the CHARM cohort, with regular antibody and PCR testing, allowed for collection of pre-infection samples, approximate identification of the beginning of the infection, and follow-up sampling of the infected participants for up to 63 days after infection in this subset.

We found similar levels of induction of SARS-CoV-2 spike (S)-specific IgG and IgM antibodies in asymptomatic and symptomatic participants, as well as similar neutralizing antibody levels. Slightly higher viral load, as approximated by Ct value, was found in symptomatic participants as compared to asymptomatic at first positive PCR (PCR+) detected. Longitudinal proteomic analysis of 92 analytes in serum revealed a subset of pro- and anti-inflammatory markers that are positively correlated with symptoms and viral load, as well as others that are exclusively associated only with either number of symptoms or with viral load. Interestingly, we found a proteomic signature associated with asymptomatic infections, that includes the analytes IL-17C, MMP-10 and FGF-23, with previously described functions in tissue repair. Overall, our findings suggest that control of symptoms during SARS-CoV-2 asymptomatic infections, as compared to mildly symptomatic infections, could be achieved by the appropriate balance of inflammatory and tissue repair associated mediators in young adults

METHODS

Cohort and Data Collection

The CHARM cohort study, which has been previously described (22), was designed to identify SARS-CoV-2 infection regardless of symptoms and to assess the host immune response during acute infection and early convalescence stages. The cohort is composed of Marine recruits that arrived at Marine Corps Recruit Depot—Parris Island (MCRDPI) for basic training between May and November 2020, after undergoing two quarantine periods. The first one was a home-quarantine, and the second a supervised quarantine starting at enrollment in the CHARM study, as previously described (22, 23).

At enrollment, participants completed a questionnaire consisting of demographic information, risk factors, reporting of 13 specific COVID-19-related symptoms or any other unspecified symptom since the previous visit, or in the previous 2 weeks in the case of the

first visit or if more that 2-weeks since the last visit had passed, temperature recording and brief medical history. At approximately weeks 0, 1, 2, 4, 6 and 8 after enrollment, additional PCR testing was performed, and the follow-up symptom questionnaire was administered. Serum samples were collected in all the visits. For the analysis presented in this study, we included a subset of participants who were PCR negative and SARS-CoV-2 seronegative at enrollment (negative for IgG RBD and S titers, at a threshold titer of 1:150) (22), had zero (negative controls) or at least one PCR detected (infected participants), and had sera available after PCR detection in the case of infected participants.

Collection of Biological Specimens and Quantitative PCR Testing

At each time point, blood was collected using serum separator tubes (SST) which were centrifuged to isolate serum (1500 x g for 10 min). Aliquots of serum were frozen at -80°C. Nares swabs were collected and kept at 4°C for SARS-CoV-2 PCR testing. All PCR assays were performed within 48 h of sample collection at the high complexity Clinical Laboratory Improvement Amendments-certified laboratories, Lab24Inc (Boca Raton, FL, USA, assays performed May 11-Aug 24, 2020) and the Naval Medical Research Center (Silver Spring, MD, USA, assays performed Aug 24 -Nov 2, 2020), using the US Food and Drug Administration-authorized Thermo Fisher TaqPath COVID-19 Combo Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Enzyme-Linked Immunosorbent Assay for Evaluation of SARS-CoV-2 RBD Specific IgG and IgM Titers

IgG and IgM SARS-CoV-2 specific antibodies in serum were evaluated using an enzyme-linked immunosorbent assay (ELISA) as previously described (22, 23). 384-well Immulon 4 HBX plates (Thermo Fisher Scientific, Waltham, MA, UA), were coated overnight at 4°C with 2 μg/mL of recombinant His-tagged spike receptor-binding domain (RBD) (Sino Biological, Beijing, China) or spike (S) protein (LakePharma, Irving, TX, USA). Plates were washed with 0.1% Tween-20 using an automated ELISA plate washer (AquaMax 4000, Molecular Devices, San Jose, CA, USA), and blocked for 1 h at room temperature with 3% milk in PBS-T. Blocking solution was removed, and serum samples diluted in 1% milk PBS-T were dispensed in the wells. At least two positive controls (sera with known IgG presence), eight negative controls (sera collected before July 14, 2019), and four blanks (no serum) were included in every assay. Plates were incubated for 2 h at room temperature, and then washed. Next, peroxidase conjugated goat F (ab')2 Anti-Human IgG (Abcam, Cambridge, UK) was added at 1:5000–1:10 000 dilutions (determined after optimization for each antibody lot) in 1% milk PBS-T, and plates were incubated for 1 h at room temperature. Plates were washed, developed using o-phenylenediamine, and the reaction was stopped after 10 min with 3M HCl. Optical density (OD) at 492 nm was measured using a microplate reader (SpectraMax M2, Molecular Devices). All serum samples were screened at a 1:50 dilution with RBD. Those samples with an OD 492 nm value higher than the average of a set of 8 negative controls plus three times their SD in the screening assay underwent titration assay (six serial 1:3 serum dilutions starting at 1:50) using S protein. Serum samples were considered positive for each assay when at least two consecutive dilutions showed higher OD 492 nm than the average of the negative controls plus three times their SD at the correspondent dilution or 0·15 OD 492 nm. Specificity was 100% on both RBD and S protein ELISA using 70 control sera obtained before July 14, 2019. At baseline, participants were only considered seropositive to SARS-CoV-2 if IgG titrations for both S and RBD ELISA gave a positive result at a minimum of 1:150 dilution.

Neutralization Assays

Studies involving infectious SARS-CoV-2 were performed at the Galveston National Laboratory as previously described (6). Two-fold serial dilutions of heat-inactivated serum at an initial dilution of 1:20, were prepared in serum free media (Minimum Essential Medium; Thermo fisher Scientific, containing 25 mM HEPES and 0.05 g/L Gentamicin sulfate) and incubated with an equal volume of mNeonGreen SARS-CoV-2 (24) for 1 hour at 37°C (200 plaque forming units/well, which results in a final multiplicity of infection of 0.005) in humidified 5% CO_2. Virus-serum mixtures were then added to Vero-E6 monolayers in 96 well optical black plates and incubated at 37°C. Plates were read using the BioTek Cytation 5 plate reader (EX 485 nm, EM 528 nm) at 48 h post-infection. Following background signal correction, virus neutralization half-maximal inhibitory serum dilution (ID50) values were determined using a 4-parameter logistic regression.

Proteomics Analysis Using OLINK Proximity Extension Assay

For proteomics, we used the commercially available Inflammatory panel from OLINK®, composed of 92 analytes. PEA was performed at the Human Immune Monitoring Center at Mount Sinai, New York, as previously described (25). Briefly, sera samples were inactivated by UV exposition for 1 h and mixed with PEA probes that are oligonucleotide-labeled antibodies used to bind to target proteins. Then, a combined extension and pre-amplification mix of reagents were added to the samples incubated, with PEA probes allowing subsequent extension by a DNA polymerase. Upon binding to the protein epitope, the paired oligonucleotide sequences are amplified through a quantitative real-time PCR (qRT-PCR) reaction. The results are shown as NPX (Normalized Protein eXpression), that is an arbitrary unit which is Log2 scale and is calculated from Ct values generated by the qRT-PCR reaction, and after data pre-processing is performed to minimize inter and intra-assay variation. The data were pre-processed by Olink using NPX Manager software. For longitudinal analysis, samples from the different time points were grouped in the following categories: "Before" infection, "First PCR+", "3-10 days", "11-21 days", "22-35 days", and "> 35 days" after infection.

RNA-Seq Processing and Analysis

Total RNA from PAXgene preserved blood was extracted using the Agencourt RNAdvance Blood Kit (Beckman Coulter) on a

BioMek FX^P Laboratory Automation Workstation (Beckman Coulter). Concentration and integrity (RIN) of isolated RNA were determined using Quant-iT^{TM} RiboGreen^{TM} RNA Assay Kit (Thermo Fisher) and an RNA Standard Sensitivity Kit (DNF-471, Agilent Technologies, Santa Clara, CA, USA) on a Fragment Analyzer Automated CE system (Agilent Technologies), respectively. Subsequently, cDNA libraries were constructed from total RNA using the Universal Plus mRNA-Seq kit (Tecan Genomics, San Carlos, CA, United States) in a Biomek i7 Automated Workstation (Beckman Coulter). Briefly, mRNA was isolated from purified 300 ng total RNA using oligo-dT beads and used to synthesize cDNA following the manufacturer's instructions. The transcripts for ribosomal RNA (rRNA) and globin were further depleted using the AnyDeplete kit (Tecan Genomics) prior to the amplification of libraries. Library concentration was assessed fluorometrically using the Qubit dsDNA HS Kit (Thermo Fisher), and quality was assessed with the Genomic DNA 50Kb Analysis Kit (DNF-467, Agilent Technologies). Following library preparation, samples were pooled, and preliminary sequencing of cDNA libraries (average read depth of 90,000 reads) was performed using a MiSeq system (Illumina) to confirm library quality and concentration. Deep sequencing was subsequently performed using an S4 flow cell in a NovaSeq sequencing system (Illumina) (average read depth ~30 million pairs of 2×100 bp reads) at New York Genome Center.

All RNA-seq data was processed in a uniform pipeline. Gene expression levels were quantified with kallisto (v0.46.0) (26), using Gencode v34 transcript annotations (27). Transcript-level quantifications were aggregated to gene level using the tximport (v1.14.2) package, and expression levels were normalized across samples using DESeq2 (28). Differential gene expression analysis was performed with DESeq2, comparing samples during the various time points during infection to baseline gene expression levels, controlling for sex and plate number to minimize batch effects. Immune cell type proportions were estimated from bulk RNA-seq using CIBERSORTx (29). In order to obtain total proportions of each major cell type, multiple cell subsets were combined by adding the component proportions (e.g. resting and activated natural killer (NK) cell categories were summed up to a single NK cell type category).

Statistics

Statistical analysis was performed with Rstudio (version 1.3.1093), R (version 4.0.2) and the Prism 9 software. Correlations between symptoms and Ct were evaluated using the Pearson's method. Distribution of ethnicity, race and sex among study groups was assessed with a Pearson's Chi-squared test followed *post-hoc* analysis based on residuals, adjusted using the Bonferroni method. Serological and Ct pairwise comparisons between Asymptomatic and Symptomatic groups were performed using the non-parametric Mann-Whitney U test.

For the PEA analysis, delta NPX (ΔNPX) values were obtained for every participant by subtracting the NPX value at baseline (before infection) from the NPX value at every time point after detection by PCR (first PCR+ and later). This implicitly controls for differences between individual baselines, allowing us to compare only the differences observed in each participant during infection, rather than using a different population as a healthy control which introduces many confounding factors. Distributions of ΔNPX values were compared using the non-parametric Mann-Whitney U test and corrected for multiple hypothesis testing by the Benjamini-Hochberg procedure at an FDR of 0.05. Of the 92 analytes measured, 66 showed any significant changes from baseline at any point during or after infection, so only these 66 analytes with differential activity were studied further. Correlations between NPX values and both number of symptoms and Ct values were computed using linear mixed models (LMMs), with the predicted slope (LMM coefficient) representing the direction and degree of correlation.

Study Approval

The CHARM study was approved by the Naval Medical Research Center (NMRC) institutional review board (IRB), protocol number NMRC.2020.0006, in compliance with all applicable U.S. federal regulations governing the protection of human participants. 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 Participants and the Naval Information Warfare Center Pacific (NIWC Pacific) Human Research Protection Program (HRPP) and received non-human participants (NHS) determination. All participants provided written informed consent.

RESULTS

Cohort Description, Symptoms, Viral Load, and Antibody Responses

The CHARM cohort study has been previously described (22, 23). With the purpose of investigating the dynamics of the early immune response in asymptomatic and symptomatic participants, we selected a subset of participants that i) were seronegative and SARS-CoV-2 PCR negative at enrollment in CHARM and ii) were PCR negative during the entire study (negative controls, n=23), or were PCR positive at least at one time point during the study (infected participants, n=149). Within the infected participants, we defined two groups: the Asymptomatic group (n=85) included participants that, between the time of diagnosis and the end of the study, reported 0 or 1 symptoms total and temperature below 100.4°F; the Symptomatic group (n=64) included participants that, between the time of diagnosis and the end of the study, reported more than 1 symptom total and/or temperature above 100.4°F. We identified 4 participants that had one symptom at one time point within the first 2 weeks after the first PCR+ (1 with headache, 1 with chill and 2 with loss of taste), and they were included in the Asymptomatic group. Therefore, the initial sample population consists of 172 participants, selected following the above criteria and included in this study in their order of enrollment and based on availability of samples, of which 88.3% reported as being males, and the age mean was 19.57 ± 2.22 years. Race and Ethnicity distribution was balanced across the participants in the

Negative Control, Asymptomatic and Symptomatic participants (**Table 1**, p-values = 0.47 and 0.21 for race and ethnicity, respectively). However, in agreement to our findings in a separate sub-study within CHARM (6), male participants were more represented than female participants in the Asymptomatic group as compared with the rest of the groups (*post-hoc* adjusted p-value = 0.03).

The distribution of symptoms over time among all participants in which infection was detected is represented in **Figure 1A**. All the 13 symptoms and temperature measurements were more frequently reported at the time of first PCR+ (**Figure 1A**), while less frequency was found at 3-10 and 11-21 days after first PCR+. Most of the symptomatic participants resolved all symptoms by 21 days after infection (**Figures 1A, B**).

Symptomatic and asymptomatic participants showed similar dynamics of viral load as measured by PCR for the S gene (**Figure 1C**), or the N or ORF1ab genes (**Figure S1A**). However, comparison of the PCR measurements at the time of the first PCR+, indicated that symptomatic participants had lower S Ct values (22.91 ± 5.05) and therefore higher viral load, than asymptomatic participants (24.96 ± 5.67, p=0.021) on average (**Figure 1D**). Results for N gene (23.38 ± 5.88 symptomatic and 24.81 ± 5.76 asymptomatic) and ORF1ab gene (23.13 ± 6.67 symptomatic and 24.77 ± 5.49 asymptomatic) Ct analysis at the time of first PCR+ followed a similar trend but did not yield statistically significant differences (**Figures S1A, B**; p = 0.095 and p = 0.0528, respectively).

Since it is known that there are sex differences in the immune response and disease manifestation due to SARS-CoV-2 infection (30), we analyzed the Ct values at the time of diagnosis in asymptomatic and symptomatic males and females. Interestingly, while symptomatic males showed significantly higher viral load than asymptomatic males for the three genes (S, N and ORF1ab), no differences were found in females regarding symptoms for any of the genes (**Figure S1C**). It is important to note that the number of females included in this analysis (4 symptomatic and 12 symptomatic) was notably lower than males, therefore the lack of significance in this comparison in females could be due to limited statistical power from small sample size data.

Then, we assessed the dynamics of the antibody response to SARS-CoV-2 infection. S-specific IgG and IgM were measured in longitudinal serum samples from symptomatic (n=55) and asymptomatic (n=84) participants. As shown in **Figure 1E**, there was high variability in the antibody titers among the participants. However, very similar longitudinal profiles were observed overall when we compared asymptomatic and symptomatic participants. Similar observations were found when we analyzed the virus neutralizing activity of the serum from a subset of participants (n=34 symptomatic and n=44 symptomatic participants, **Figure 1E**). We did not find statistically significant differences between asymptomatic and symptomatic participants when the last time point with serum available for each participant (collected 10-63 days after first positive PCR) was compared for either IgG titers (3,133; 95% CI 2415-4044 asymptomatic, and 4,295; 95% CI 3342-5534 symptomatic group) or ID50 values (92.0; 95% CI 66.6-127.4 asymptomatic and 100.0; 95% CI 71.1-140.6 symptomatic) (**Figure S1D**). No differences were found regarding sex in the levels of S-IgG specific titers or neutralizing activity (**Figure S1E**).

Proteomic Profiling Shows Three Temporal Waves of Immune Mediators That Are Resolved Early After Infection in Asymptomatic and Mild COVID-19

We performed a longitudinal Proteomic Extension Assay (PEA) on the sera of 89 infected participants (42 symptomatic and 47 asymptomatic). We also included 23 participants with no positive SARS-CoV-2 PCR test at any time point and no evidence of antibodies from previous infections as controls. First, we evaluated changes overtime of the 92 markers in the PEA panel, considering

TABLE 1 | Contingency table showing the distribution of sex, race, and ethnicity in the study groups.

	Negative Control	Asymptomatic	Symptomatic
Sex			
F (11.6%)	4 (17.4)	4 (4.7)*	12 (18.7)
M (88.4%)	19 (82.6)	81 (95.3)*	52 (81.3)
		Chi-squared p-value = 0.02; * post-hoc p-value = 0.03	
Race			
White (73.3%)	16 (69.6)	62 (72.9)	48 (75)
Black (12.2%)	2 (8.7)	11 (12.9)	8 (12.5)
Asian (2.9%)	0 (0.0)	1 (1.2)	4 (6.25)
American Indian/Alaska Native (1.7%)	0 (0.0)	1 (1.2)	2 (3.1)
Multi-racial (2.9%)	1 (4.3)	4 (4.7)	0 (0.0)
Native Hawaiian/Other Pacific Islander	0 (0.0)	0 (0.0)	0 (0.0)
Other (0.6%)	0 (0.0)	0 (0.0)	1 (1.56)
Non-specified (6.4%)	4 (17.4)	6 (7.1)	1 (1.56)
		Chi-squared p-value = 0.47	
Ethnicity			
Hispanic (33.72%)	6 (26.1)	33 (38.8)	19 (29.7)
Non-hispanic (40.7%)	11 (47.8)	29 (34.1)	30 (46.9)
Non-specified (25.58%)	6 (26.1)	23 (27.1)	15 (23.4)
		Chi-squared p-value = 0.21	

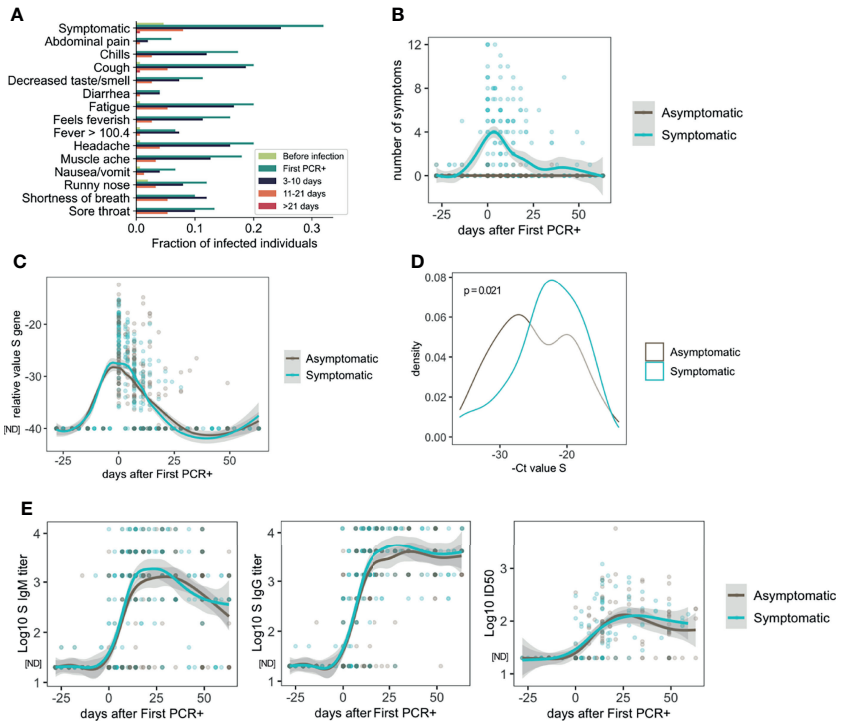

FIGURE 1 | Symptoms, viral load and antibody response in asymptomatic and mild symptomatic participants (n=85 asymptomatic and n=64 symptomatic). **(A)** Distribution of symptoms and fever reported over time. **(B)** Number of symptoms over time. **(C)** Longitudinal distribution of viral load as measured by PCR (S Ct values). **(D)** S gene PCR results at first SARS-CoV-2 positive test in asymptomatic and symptomatic participants. **(E)** Longitudinal analysis of serum IgM and IgG S-specific titers (n=85 asymptomatic and n=64 symptomatic participants), and half inhibitory infectious dose (ID50), (n=45 asymptomatic and n=46 symptomatic participants). ND, Not Detected.

all infected participants. Normalized Protein eXpression (NPX) values for each participant were analyzed as ΔNPX (NPX sample each time point – NPX baseline) (**Table S1**). The values compared are therefore always the differences in a specific participant from their own pre-infection healthy baseline. A longitudinal proteomic analysis until 60 days post first SARS-CoV-2 PCR+ showed significant changes over time for 66 of the markers from this panel (**Figure 2A**). By sorting these markers according to their time of maximum value, we identified three temporal waves of inflammatory and immunoregulatory mediators upregulated after infection (**Figures 2A, B**).

The first wave started at the time of first PCR+, coinciding with when most symptoms were reported (**Figure 1A**). As expected, this group of immune mediators is composed of inflammatory cytokines, such as IFN-γ, IL-12B, TNF-α, IL-18, IL-6, and the chemokines CXCL10, MCP-2, CXCL11, and CX3CL1, consistent with previous reports (17, 21, 31, 32) (**Figures 2A, B**). We also detected immunoregulatory markers, such as IL-10, which was previously reported as a marker of COVID-19 severity (33), and soluble PD-L1 (sPD-L1), which binds to PD-1 on the surface of effector CD8 T cells promoting their suppression or exhaustion (34, 35). IL-18 upregulation may indicate inflammasome activation, however, we did not detect an increase of IL-1β, a cytokine that is also part of this pathway (36).

The second wave (**Figures 2A, B**) is composed by a slight upregulation of relatively few mediators including IL-8, CCL3, TNF-β, Flt3L, IL-22RA1 and IL15RA. Levels of these markers showed already higher expression at first PCR+ time point with respect to their baseline, but peaked at 3-10 days after infection. IL-8 and CCL3 which have been reported as COVID-19 severity markers (21, 37–39) and TNF-β was found in less severe disease (9), which is in agreement with our findings.

Though none of the participants in this study developed severe COVID-19 and many of them resolved symptoms within days after first PCR+ (**Figure 1A**), we observed upregulation of markers of severity early during infection. However, most of those markers, represented in the first and second waves, returned to baseline levels within the first 10-35 days of diagnosis (**Figure 2A**), indicating a rapid control of the systemic inflammatory responses in this cohort.

Interestingly, the third wave (11-35 days post first PCR+) is composed of some mediators that were induced already at the time of first PCR+, but peaked at later time points (22-35 days), when most of the participants have cleared the virus (**Figures 2A** and **1C**). TGF-α and FGF-19 may be indicators of tissue repair related to the infection (**Figures 2A, B**) (40–42). One of the markers of this group is ST1A1 which plays a role in acetaminophen metabolism (43) but the cause of its increase in

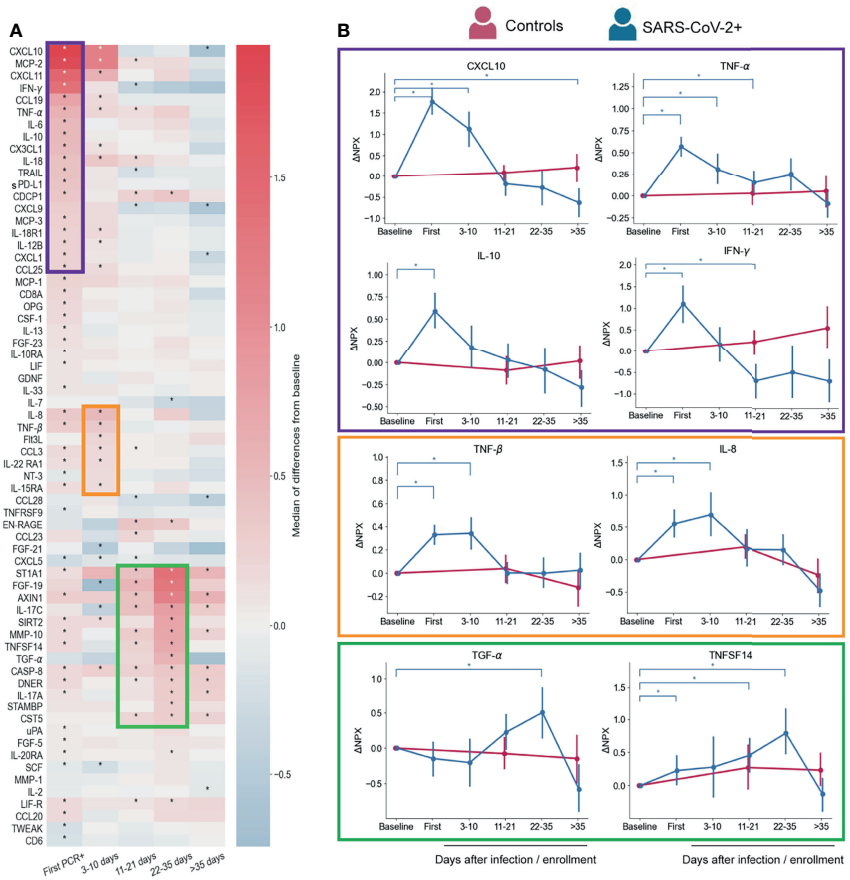

FIGURE 2 | Serum proteins measured by PEA with overall changes over time with respect to pre-infection (n=88 participants) regardless symptoms status. **(A)** Heatmap showing the proteomic signature with relative expression of the markers with significant changes overtime in infected participants. **(B)** Representative temporal profile of markers belonging to the first, second and third wave (represented by the boxes in purple, orange and green, respectively). Controls in panel B are uninfected participants (n=23) that were included in the analysis with samples collected at study enrollment (baseline), 14 days, and 56 days after enrollment. Mean and 95% CI are indicated. *p < 0.05.

circulation is unknown. Levels of IL-17A (**Figure 2A**) and TNFSF14 (**Figures 2A, B**) are enhanced early after infection and gradually increase until late time points. Both proteins have been described as markers of severe cases (11, 17, 44), although our cohort is composed of only mild and asymptomatic cases. Proteins from the third wave were likely induced by early mediators in the acute phase of the infection, and might contribute to the recovery from infection or disease since their peak of expression coincides with clearance of virus and symptoms (**Figures 1A, B**).

To further understand the inflammation serum dynamics, we utilized a blood RNA-seq dataset that was generated as part of the CHARM study to estimate proportions of circulating innate immune cells. Interestingly, proportions of monocytes, dendritic cells (DC) and NK cells were increased at the time of first PCR+ with respect to the baseline levels (**Figure S2**), which coincides with the first wave of inflammatory markers in our PEA analysis (**Figure 2**). DC and NK cells proportions were also significantly elevated at the 3-10 days time points. Chemo-attractants of NK cells, DC and monocytes (e.g. CXCL10, MCP-1, 2 and 3) (45, 46) were detected in serum as part of the first and second waves (**Figure 2**) and could explain the increased cell proportions detected by RNA-seq analysis (**Figure S2**).

Serum Immune Mediators Correlate With Number of Symptoms and Viral Load

We next used a linear mixed model (LMM) to investigate the relationship between the inflammatory profile and the number of symptoms or viral load. **Figure 3A** shows 19 analytes that are positively and 2 that are negatively correlated with the number of symptoms at the same time point. In the case of viral load, we found 11 analytes that were positively correlated and 2 that were negatively correlated (**Figure 3B**). Interestingly, there were 8 markers, most of them chemokines, which were positively correlated with both number of symptoms and viral load (**Figure 3C**). Related to this, we found a weak but significant positive correlation (Pearson's), between the number of symptoms and viral load (-Ct values for genes S, N or ORF1ab) (**Figure 3D**).

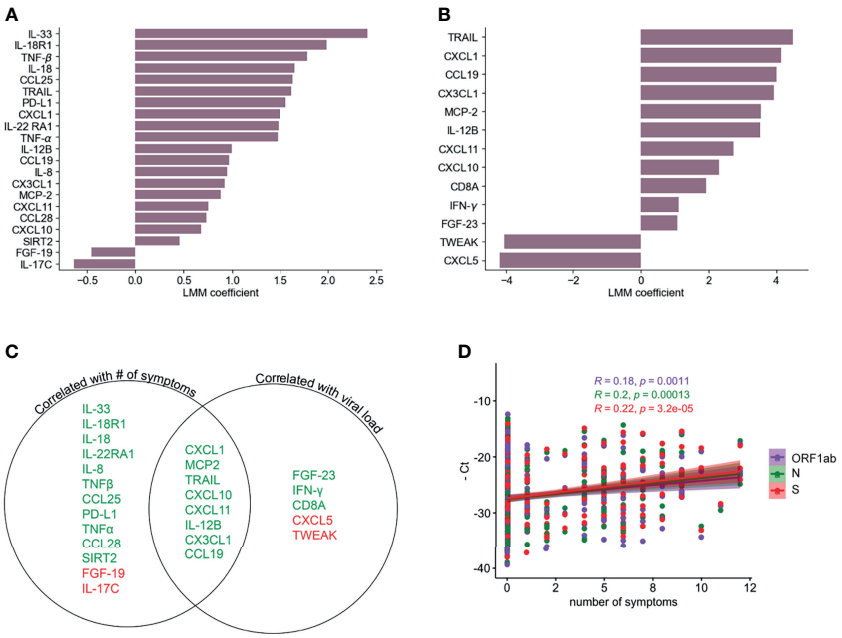

FIGURE 3 | Correlation of PEA serum markers with symptoms and viral load. LLM correlation analysis of PEA detected markers and number of symptoms **(A)** and relative viral load determined as the average of the negative Ct values of S, N and ORF1ab genes **(B)** FDR cutoff = 0.05. **(C)** Venn diagram showing the serum markers that are correlated with number of symptoms and/or viral load. Green denotes positive correlation and red denotes negative correlation. **(D)** Correlation (Pearson's) between PCR negative Ct values for ORF1ab, N and S genes and numbers of symptoms.

CXCL10, CXCL1, CXCL11, CX3CL1 were correlated with severity in previous reports (17, 21) but showed a rapid decline over time in the participants of this cohort (**Figure 2A**), similarly to the monocyte chemoattractant MCP-2, IL-12B and the death-receptor ligand TRAIL, all of which are known to be important for viral clearance (47, 48). None of the analytes that correlated only with the number of symptoms showed any trend towards significance with viral load (**Figure 3C**). Among those that were significantly correlated with viral load, IFN-γ was the only one that showed some level of correlation with the number of symptoms as well, which was significant only before multiple hypothesis correction (p= 0.000637). The positive correlation between viral load and IFN-γ is in agreement with other reports showing the importance of this cytokine in promoting direct and indirect anti-viral immunity (49).

Dynamics of Serum Immune Signatures Are Associated With Onset of Symptoms

In order to understand the longitudinal inflammatory response induced by SARS-CoV-2 infection during symptomatic and asymptomatic infections, we analyzed the differences between these groups of participants over time. Our initial analysis did not identify significant differences for any of the cytokines between those two groups after correcting for multiple hypothesis testing. However, we found a strong association between the number of symptoms at a given time point and the inflammatory landscape in serum (**Figure 3A**), which suggests that the longitudinal analysis could be obscured by the fact that symptoms do not occur at the same time (with respect to first PCR+ detection) in all participants. Therefore, we further stratified the group of Symptomatic participants according to symptom onset: the Early Symptomatic group includes those participants that reported more than 1 symptom at their time of first PCR+ (n=31, **Figure 4A**), and the Late Symptomatic group includes those that reported more than 1 symptom for the first time 3 or more days post first PCR+ (n=15) (**Figure S3A**).

Using this approach, we found that participants in the Early Symptomatic group presented 23 analytes with higher levels of upregulation than the Asymptomatic group (**Table S2**), and these differences were only detected at the first PCR+ time point, when they first reported symptoms. Among those analytes, we found the pro-inflammatory mediators CXCL10, CCL25, MCP-2, IL-8, TNF-β and the alarmin IL-33 (**Figure 4B**). We did not find significantly higher levels of induction of these analytes between the Late Symptomatic and the Asymptomatic group (**Figure S3B**). It is important to note that the Late Symptomatic group was composed of a smaller group of participants (n=15) and was very heterogeneous with regards to the time of occurrence of symptoms (**Figure S3A**), with some participants peaking at 3-10 days after first PCR+ while others peaked at 11-21 days. In addition, the Late symptomatic group tended to report on average fewer symptoms (4.89) than the Early Symptomatic group (7.15) at their peak of their symptoms (3-10 days post first PCR+ and first PCR+, respectively). This is reflected in the high variance of the presence of these analytes

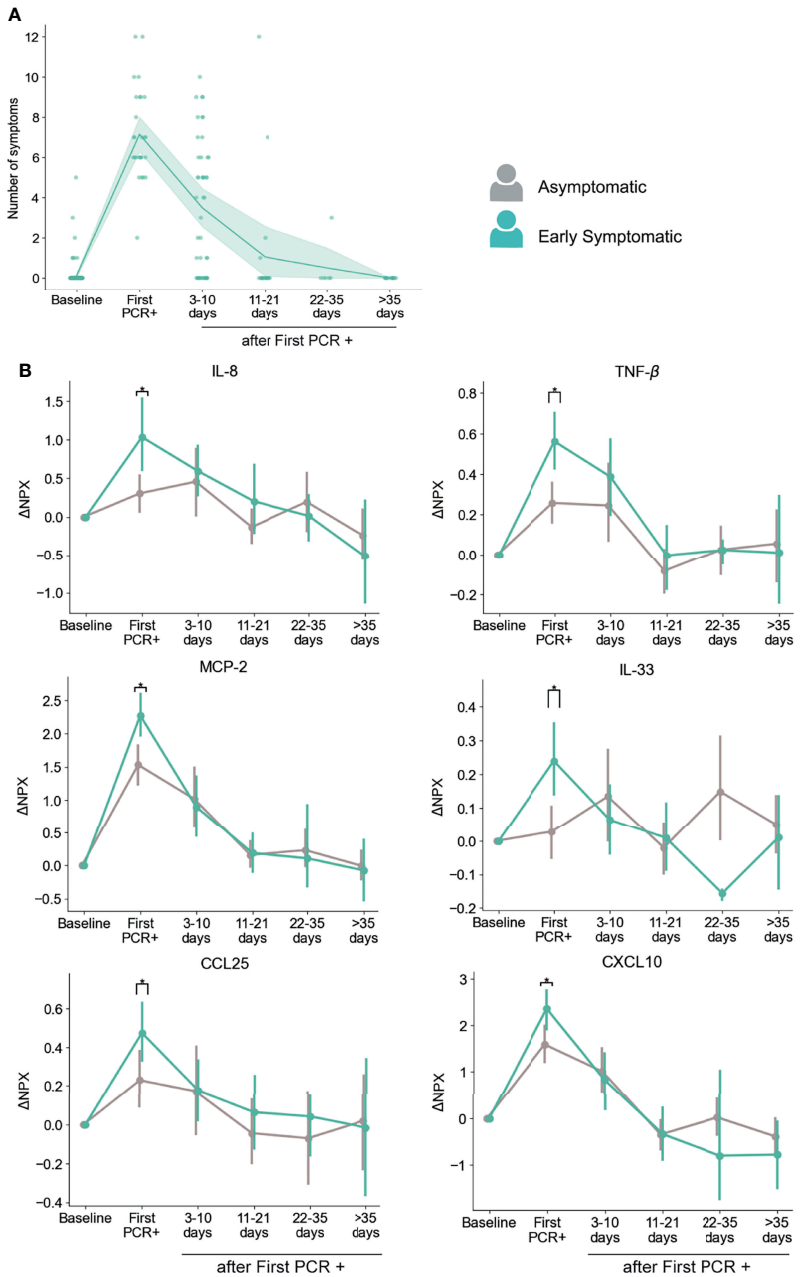

FIGURE 4 | Dynamics of serum markers with higher upregulation in Early Symptomatic participants (symptoms reported at First PCR+) than in Asymptomatic participants. **(A)** Temporal distribution of the number symptoms in Early Symptomatic participants. **(B)** Representative markers that are significantly upregulated in Early Symptomatic in comparison to Asymptomatic group of participants. Mean and 95% CI are indicated. *p < 0.05.

over time in serum (**Figure S3B**). Therefore, the Early Symptomatic group represents a clearer temporal distribution and larger sample size to compare with the Asymptomatic group. Even with the high variability found in the Late Symptomatic group, we observed several mediators, including IL-8, MCP-2, and CXCL10, that peaked 3-10 days post-first PCR+, coinciding with the time of the maximum average number of symptoms in these participants (**Figure S3B**).

To further assess the co-occurrence with symptoms for these analytes, we did an additional longitudinal analysis, in which we evaluated their levels with respect to the time of maximum number of symptoms reported by each participant. As shown in **Figure S4A**, they all peaked at the time of reporting of the maximum number of symptoms and started to decrease early after this timepoint, except for TNF-β, that was maintained longer (at 3-10 days after maximum of symptoms), and then decreased (**Figure S4A**).

In addition, we performed an independent analysis in which we included PEA data from all the PCR+ timepoints from all symptomatic participants (both Early and Late Symptomatic) at the time they experienced symptoms, and from all PCR+ timepoints in the case of the asymptomatic participants, regardless of the time after infection. CXCL10, CCL25, MCP-2, IL-8, TNF-β, TNFSF14, and IL-33 showed significantly increased levels in participants reporting symptoms at the time of sampling than in asymptomatic participants at any PCR+ time point (**Figure S4B**). Therefore, we identified multiple inflammatory mediators associated with the presence of symptoms, where their peaks coincide with the time of maximum number of symptoms reported and decrease over time as symptoms clear.

A total of 6 proteins were found to be significantly higher in females than males (TNFSF14, AXIN1, SIRT2, CASP-8, ST1A1, TRANCE) in both Asymptomatic and Symptomatic groups, in an analysis that included all samples with SARS-CoV-2 PCR+ results (**Figure S5**). Of those, AXIN1, SIRT2, ST1A1 and TRANCE showed significant upregulation in symptomatic participants only in males. However, assessment of differences with regards to presence of symptoms in females is challenging due to their low numbers in this analysis (n=16 total, n=12 with detected SARS-CoV-2 infection).

We next assessed the estimated cell proportions in the blood RNA-seq dataset in Asymptomatic and Early Symptomatic participants. Early Symptomatic participants showed significantly higher frequencies of DC at the first PCR + time point and of NK cells at 3-10 days after first PCR + (**Figure S6**) as compared to Asymptomatic participants. Interestingly, we also found significantly higher proportions of monocytes, DC, and NK cells in Early Symptomatic than in Asymptomatic participants at 22-35 days after infection. However, we did not find any significant differences at 22-35 days between these groups for any serum markers at these time points, so the connection between this increase in the proportion of these immune cells and the cytokine profile between asymptomatic and symptomatic participants is unclear. A lower proportion of neutrophils was found in the Early Symptomatic group than in the Asymptomatic group at 3-10 days after infection. A previous report found similar results when they analyzed mature neutrophils by flow cytometry, but an opposite trend in the case of immature neutrophils (16). Therefore, it is possible that the estimated neutrophil proportions in this bulk-RNA analysis correspond to mature neutrophils.

Candidate Markers That Could Give Insights on Suppression of COVID-19 Related Symptoms

As anticipated, we found a strong association between the presence of symptoms and levels of multiple inflammatory markers. Interestingly, we also identified three immune mediators that showed significantly higher levels in the Asymptomatic group than in the Early Symptomatic group at

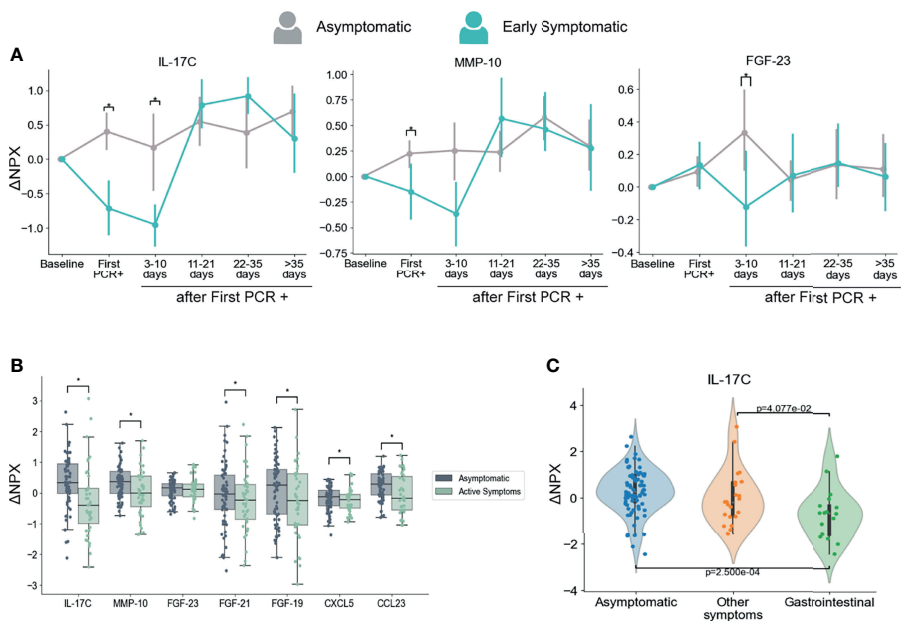

FIGURE 5 | Dynamics of serum markers that are higher in Asymptomatic participants as compared with Symptomatic participants. **(A)** Mediators that are significantly higher in Asymptomatic participants in comparison with Early Symptomatic participants. Mean and 95% CI are indicated. **(B)** Mediators with decreased levels in Symptomatic participants at the time points when they had symptoms (Active Symptoms) than in Asymptomatic participants at any time point (Asymptomatic). This analysis includes only samples collected at PCR+ timepoints and compares levels of PEA markers regardless time after first PCR+. **(C)** IL-17C is differentially regulated in participants presenting with GI related symptoms (vomiting/nausea, diarrhea and/or abdominal pain) in comparison to participants that reported other symptoms, but none of them GI related, or to Asymptomatic participants. This analysis includes only samples collected at the time of first PCR +. *p < 0.05.

the first PCR+ and/or at 3-10 days after infection. Those immune mediators are IL-17C, MMP-10, and FGF-23 (**Figure 5A**).

Next, we compared the PEA markers between all samples from symptomatic participants collected at the time they reported symptoms and were PCR+ (including Early and Late Symptomatic participants) and all samples from asymptomatic participants at the time they were PCR+. This analysis considers the presence or absence of symptoms regardless of the time after first PCR+. In agreement with our longitudinal analysis in Fig 5A, IL-17C and MMP-10 showed significantly higher levels in asymptomatic participants than in symptomatic participants at the time they reported active symptoms (**Figure 5B**). In addition, we also found significantly higher levels of CCL23, FGF-19, FGF-21 and CXCL5 in asymptomatic participants than symptomatic participants at the time point they reported symptoms (**Figure 5B**).

Moreover, SARS-CoV-2 infection induced early IL-17C, MMP-10, FGF-23 and CCL23 upregulation in Asymptomatic participants when compared to their baseline levels, while no significant changes, or a significant decrease in the case of IL-17C, was detected in Early Symptomatic participants (**Table S3** and **Figure S7**). These analytes therefore increased in serum early after infection in asymptomatic participants, yet unchanged or downregulated among Early Symptomatic participants.

IL-17C is a cytokine described as pro-inflammatory (50) and in combination with other mediators, works as an epithelial barrier against different bacterial (51, 52) and viral infections (53, 54). Interestingly IL-17C can be involved in tissue repair and protection of nerve fibers (54). CCL23, while also known as a pro-inflammatory chemoattractant (55) has been involved in angiogenesis by promoting migration of endothelial cells (56, 57). Other proteins in this group that could be related to tissue repair are the FGF-23, FGF-19 (also negatively correlated with number of symptoms, **Figure 3A**), and FGF-21 (58). CXCL5 is a neutrophil chemoattractant that has been shown to have important roles in homeostasis and wound healing (59, 60).

Given the importance of IL-17C in innate immunity and tissue repair, the clear contrast between the upregulation observed in Asymptomatic participants (**Figure S5**), the downregulation found in Early Symptomatic ones (**Figure 5**), and the negative correlation with the number of symptoms (**Figure 2A**), we sought to explore further other aspects of this cytokine. Specifically, we wondered what the mechanism of downregulation of this cytokine during symptomatic infections could be. In agreement with previous reports that indicate that IL-17C is not produced by hematopoietic cells (61), we did not find significant changes of the expression of IL-17C gene in the blood RNA-seq data from participants in the CHARM cohort in either the Asymptomatic or Early Symptomatic groups (**Table S4**).

The upregulation of IL-17C during respiratory viral infection might be produced by epithelial cells upon virus infection (62) which would explain the profile we observed in asymptomatic participants. Downregulation of IL-17C has been previously reported as a consequence of changes in the gut microbiota after treatment with antibiotics in a mouse model (63). Interestingly, COVID-19 patients have been previously reported to suffer microbiota composition modifications that are associated with the degree of severity (64, 65). Related to this, serum levels of IL-17C were found to be lower in participants with gastrointestinal (GI) symptoms than in those with no GI symptoms in hospitalized COVID-19 patients (66). To assess if there is an association between IL-17C and GI tract involvement in this cohort, we analyzed the levels of IL-17C among participants that reported GI symptoms (nausea/vomiting, diarrhea, and/or abdominal pain) at the time of first PCR+. Our results show that GI symptomatic group of participants presented significantly lower levels of IL-17C protein in the circulation in comparison to participants that presented only non-GI symptoms or those that were asymptomatic (**Figure 5C**), indicating a further association between GI symptoms and decreased levels of IL-17C. Therefore, it is possible that changes in microbiota during symptomatic infection modulates systemic circulating levels of IL-17C.

In conclusion, we identified a group of cytokines, including IL-17C, MMP-10, FGF-23, CCL23, FGF-19 and CXCL5 that are differentially regulated during asymptomatic and mild symptomatic infections and are known to be associated with tissue repair functions. We did not find significant changes in the expression of these genes in blood cells, suggesting that the expression of these proteins is regulated in tissues from respiratory or GI epithelia, and proteins are released to circulation. Given their differential patterns of expression regarding presence of symptoms and their previously described functions, we propose that they might have an important role in protecting the lung from tissue damage and subsequent clinical manifestation in asymptomatic individuals.

DISCUSSION

The immune response to severe COVID-19 has been well characterized, often by remarkable studies that included asymptomatic and/or mild symptomatic participants as control groups (11, 16, 19, 21, 67). To fully understand the pathogenesis of SARS-CoV-2 infection, it is also critically important to unravel the mechanisms that protect asymptomatic individuals from developing symptoms. Here, with a unique prospective study of healthy young adults with either mild or asymptomatic SARS-CoV-2 infection, we show the dynamics of the temporal immunity through the longitudinal analysis of the antibody response and a serum proteomic analysis.

The development of antibody responses and the levels of viral load as estimated by Ct value by PCR showed very similar profiles between asymptomatic and symptomatic participants. However, while the Ct dynamics were similar in participants with or without symptoms, slightly significantly higher viral load was found at the time of first PCR+ in symptomatic participants. Other studies have found an association between severity and viral load as well (68–71). The modest differences in our study as compared to other reports might be due to the presence of minor symptoms in many of the symptomatic participants in our cohort. We also found a positive correlation between the number of symptoms and viral

load, which might explain the fact that several markers in the PEA analysis are correlated with both number of symptoms and viral load. It is well known that the innate immune response early during infection induces the B cell response and therefore is expected to contribute to the development of antibodies (72, 73). However, in this study we did not find any significant correlations between the levels of S IgG or neutralizing antibodies elicited and the relative serum levels of any of the PEA markers during the acute phase of the infection (data not shown) as reported before (12).

In patients with severe COVID-19, there is an abundance of cytokine production, that can induce a cytokine storm in addition to a series of adverse reactions (21, 38, 67). Here, we profiled the dynamics of the inflammatory response to SARS-CoV-2 in young adults with mild and asymptomatic infection, and identified three temporal waves of inflammatory markers. Overall, the participants of our cohort presented with upregulation of markers of severe disease reported before such as IL-6, IL-8 and CXCL10 (9, 10, 21), with most of them increasing early after infection and decreased over the time (see the first and second waves in **Figure 2**). In agreement with our results, the control of inflammatory response early in SARS-CoV-2 infection is crucial to avoid severe disease (9). However, it is important to mention that a subset of previously described severe disease markers, including IL-17A, CASP-8 and TNF-α, which belong to the third wave (**Figure 2A**), remained upregulated in our study until later time points post infection (≥ 11-35 days) (17, 67). These cytokines may act synergistically to promote an anti-viral response (74, 75) and may not be enough to define severe disease. Therefore, they should be used in combination with clinical and demographic information of the cohort.

We found multiple mediators positively correlated with number of symptoms that followed a temporal profile associated with symptom onset (**Figures 4**, **S4**), with highest levels at the peak of symptoms and a subsequent decline. As expected, several of these mediators were inflammatory markers such as IL-8, TNF-β, IL-18R1, and IL-22RA1. However, other mediators in this group can exert both inflammatory and immunoregulatory functions, such as sPD-L1 and IL-33 (**Figure 3A**). sPD-L1 can be induced by pro- and anti-inflammatory cytokines, but also directly by viral infections (76, 77). IL-33 on the other hand is an alarmin that was correlated positively with SARS-COV-2 and HIV specific antibody production (78, 79), and with anti-viral cytotoxic T cell response (80).

On the other hand, the cytokine IL-17C and FGF-19 were negatively correlated with symptoms, while CXCL5 and TWEAK were negatively correlated with viral load. In contrast to our findings, CXCL5, which was also downregulated after infection in our study (**Figures 2A** and **S3C**), was reported to be induced after *in vitro* SARS-CoV-2 infection of primary lung cells (62). TWEAK was also downregulated after infection in our study (**Figures 2A**, **3B**). However, it has been previously found elevated in SARS-CoV-2 patients (81), but decreased levels were found in patients infected with HIV (82).

The fact that our correlation analysis revealed many analytes that are exclusively correlated with the number of symptoms, and not with viral load, may be attributed to an indirect induction of these markers, through an alternate pathway than the virus replication *per se*. sPD-L1 and IL-33 are probably induced by other mediators and not by viral replication, since they were not correlated with viral load. IL-33 was shown to be induced by IL-17A in γδ T cells in a mouse model of influenza infection (83). Both IL-17A and IL-33 were induced after infection here, but only IL-33 is positively correlated with number of symptoms. IL-33 is a growth factor that plays a major role in lung tissue repair by inducing the production of amphiregulin. IL-33 production is induced in influenza infected epithelial lung cells that in combination with IL-18, bind to their respective receptors (IL-18R and ST2) on regulatory T cells (Treg) (84) and/or innate lymphoid cells (ILC) (85) to promote lung tissue repair and inflammation control

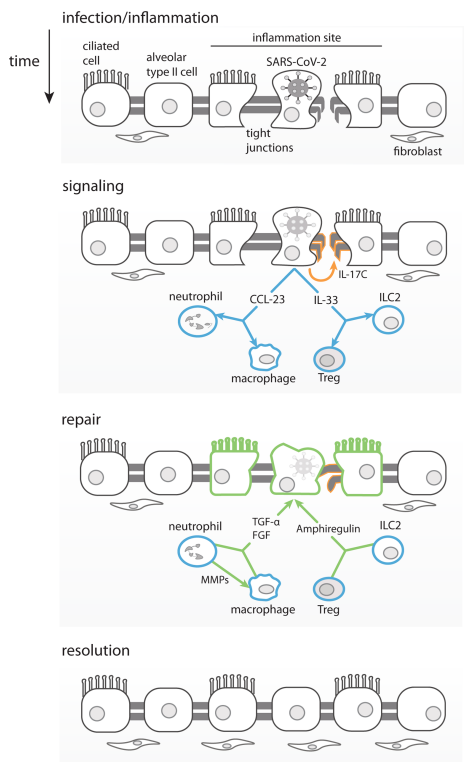

FIGURE 6 | Proposed Model for Asymptomatic SARS-CoV-2 infection. Infected lung epithelial cells induce an inflammatory response and mild damage in the pulmonal tissue barrier through tight junction disruption. The immune mediators produced in this context, such as IL-33, can stimulate ILC2 and Tregs to produce amphiregulin and promote tissue repair. IL-17C produced by epithelial cells would also contribute to the lung repair, by stimulating tight-junction proteins production. Chemokines such as CCL23 once produced by infected epithelial cells would recruit neutrophils and promote local differentiation of macrophages. MMP-10 produced by neutrophils would alternatively activate macrophages. Both, macrophages and neutrophils produce FGFs and TGF-α leading to fibroblasts recruitment, growth and promoting lung epithelial barrier repair, respectively.

(**Figure 6**). Interestingly our results show that IL-33 is the analyte which is the most positively correlated with number of symptoms.

Our PEA analysis revealed, to the best of our knowledge for the first time, a group of analytes that shows lower serum levels in participants that reported symptoms at the time of diagnosis, as compared to those who remained asymptomatic during the study-namely IL-17C, MMP-10 and FGF-23. It was proposed that IL-17C would be able to boost IL-17A production to reinforce innate host barrier (61) during influenza infection for example (84, 85). In addition to being downregulated in early symptomatic participants (in both sexes when grouped separately), IL-17C was also negatively correlated with symptoms. Importantly, IL-17C, MMP-10, FGF-23 and CCL23 are upregulated only in asymptomatic participants early after infection, which strongly suggests their role in the control of COVID-19 clinical signs. The FGFs are involved in pulmonary tissue repair if signals of fibrosis occur (86) and in the negative modulation of inflammation (87, 88). MMP-10 was reported as a negative regulator of macrophage activation (89), indicating its role in the regulation of inflammatory response (90).

These results indicate that despite the mild disease in the CHARM cohort, individuals might be experiencing some degree of temporary tissue injury caused by the infection and/or by the inflammation induced after infection. It is possible that participants from our cohort presented lung injury related to COVID-19, but we cannot discard the possibility of gastrointestinal damage as well, since individuals also reported here and elsewhere (66) experiencing diarrhea, nausea and abdominal pain. An indicator that the symptomatic individuals might have lung and/or gut tissue damage related to COVID-19 is the high levels of TNFSF14 detected by PEA, and as reported before this protein is implicated in lung fibrosis when produced by local fibroblasts acting in tissue remodeling (91) but also play a role in limiting inflammation in an animal model of chronic colitis (92). It was shown by others that even asymptomatic SARS-CoV-2 infection can cause lung injury (19, 93), but the tissue protective factors may limit the extent of lung pathology and consequently the clinical symptoms in asymptomatic and mild symptomatic participants in this study. Moreover, many of the participants of this study reported shortness of breath, fatigue and/or cough, which could be symptoms of lung damage (94). It is possible that IL-17C, MMP-10, FGF-19, FGF-21, and FGF-23 act together to guarantee viral clearance and to promote lung tissue repair in asymptomatic individuals, and the early symptomatic individuals had a delay in this response. Moreover IL-33, TGF-α and IL-17A are all upregulated in our cohort, and may also play a role in tissue repair and control of inflammation (42, 74, 87, 95).

It has been reported that TLR activation can induce IL-17C (96), and it is mostly produced by epithelial cells in lung, skin or colon (96, 97), promoting tissue repair through an unknown mechanism that involves tight junction proteins (98, 99). Tight junction proteins, such as claudins and occludins are crucial for epithelial barrier function and are composed by several classes of cytosolic, transmembrane and cytoskeletal proteins, that regulate paracellular permeability, an important physiological condition to keep normal respiration (100, 101). Therefore, the pattern of expression of IL-17C that we found with regards to symptoms, and its previously described role in tissue repair, suggests that the modulation of this cytokine might have important implications in the control of symptoms in asymptomatic participants, and in the contribution to symptom resolution in early symptomatic participants. While the upregulation of IL-17C during respiratory viral infection might be produced by epithelial cells upon virus infection (62) the mechanisms for the downregulation of serum levels in Early Symptomatic participants are unclear. One possibility could be related to changes in the composition of their microbiota as a consequence of the SARS-CoV-2 infection. Decreased expression of IL-17C by GI epithelial cells has been found upon antibiotic-induced microbiota perturbations in animals (63). Importantly, the influence of SARS-CoV-2 infection in the gut microbiome composition has been reported by multiple groups (64, 65, 102). Moreover, associations of the microbiome composition with COVID-19 severity and inflammatory markers have been identified (64, 102), highlighting the importance of gut dysbiosis in the regulation of the immune response to respiratory viral infections thorough the lung-gut axis (103, 104). In our study, we found lower levels of IL-17C in participants with GI symptoms than in those with other symptoms, supporting an association between the decreased levels of IL-17C and GI involvement, possibly as a result of microbiota changes in the gut. In agreement with this hypothesis others have reported not only IL-17C decrease as a result of changes in the microbiota, but also CCL23, MMPs and FGFs (39, 63, 105, 106). Further research to clarify the mechanisms underlying the downregulation of IL-17C during symptomatic COVID-19 will have important implications in our understanding of COVID-19 pathogenesis, which is critical for clinical management and identification of new possible targets for treatment.

We propose that SARS-CoV-2 infected respiratory epithelial cells, in asymptomatic and otherwise healthy young adults, could produce IL-17C through TLRs activation. Importantly, expression of IL-17C has been shown to be induced in epithelial cells by SARS-CoV-2 (62, 97, 107), rhinovirus (108), and bacterial infection (97). The release of IL-17C could also be induced by IL-33 and IL-17A expressed by infected cells as part of the initial inflammatory response (see model in **Figure 6**). Infected epithelial cells would also release chemokines, such as CCL23 (109), that would recruit and promote the local differentiation of monocytes/macrophages and neutrophils, which would be the main producers of FGFs, MMP-10 and TGF-α. IL-17A, IL-17C and IL-33, among others, and would help promote viral clearance and control the inflammation. In parallel, the viral infection also causes tissue damage (of lung, guts, etc.) that is repaired by IL-17C through induction of tight junction proteins, in combination with FGFs and TGF-α that would exert fibroblasts recruitment and proliferation to ultimately resulting in tissue repair (**Figure 6**).

A cross-sectional study which also focused on asymptomatic SARS-CoV-2 infections, found preferential expression of growth factors and an immune tolerance profile in blood from

asymptomatic as compared to symptomatic individuals (16). Importantly, this study also found a stronger virus-specific Th17 response in asymptomatic as compared to symptomatic participants, while Th1 and Th2 responses were similar in the two groups. It is possible that the higher levels of IL-17C in asymptomatic infections could promote the establishment of robust virus-specific Th17 responses.

Our study has an important advantage due to the use of baseline samples obtained from participants prior to infection, allowing us to study differences in immune response that occur during infection while minimizing other confounders that could induce immune mediators in the participants before infection. In fact it is known that intense physical training, which is an important component of the basic training of Marine recruits, can contribute to changes in inflammatory markers (110). To account for these possible changes due to training effects, we incorporated an uninfected control group with 3 longitudinal samples in a period of 56 days. Indeed, the control group presented significant enhancement in IL-6 at 56 days after enrollment in this study, as well as variations in other cytokines such as MCP-3, NT-3, and CASP-8 (**Table S1**) all in different time points. Another advantage of our study is that the frequent sampling and follow up allowed for identification of a high number of asymptomatic cases, which are usually difficult to detect. We are confident that the asymptomatic participants did not develop symptoms during the course of their infection since any symptoms that might have started between study visits were specifically asked about on the questionnaire. In addition, sample and data collection was conducted by the medical research team in the same location where the participants reside for their basic training at MCRDPI. If symptoms developed, the study team was made aware by medical providers. Therefore, it is highly likely that if a participant experienced any symptoms during the course of the illness, they were identified and the complaints recorded by the study team.

This study has some limitations. For example, we did not have access to participants' PBMCs or lung epithelial cells to perform a deeper analysis to understand better the mechanisms underlying the mild and asymptomatic COVID-19. Given the characteristics of the cohort (mainly white, young adults), these results might not be representative of children or older adults, of other races/ethnicities, or cases with severe COVID-19. Additionally, the female representation in the cohort was low (11.7%) and the sample size is still modest to being able to analyze the data stratifying it per symptom. Despite these challenges, we were able to find an important set of markers associated strongly with asymptomatic infection or protection from symptomatic disease. Another potential limitation is the possible presence of other respiratory infections during course of the study. However, long-term passive surveillance data, collected from January 1, 2017 to December 31, 2020, indicated a low level of the circulation of non-SARS-CoV-2 respiratory infections at MCRDPI during the time the CHARM study took place, which was dramatically reduced in 2020 as compared to previous years (unpublished data). The decrease in overall respiratory infections was probably due to the safety measures and protocols established to reduce SARS-CoV-2 spread. In addition, the timeline of the CHARM study did not overlap with the influenza season, and therefore the probability of influenza virus infections interfering with our results is minimal (111, 112). Our data depended on serial samples obtained before and during documented SARS-CoV2 infections that occurred in nearly half the participants during an 8-week study period (22). Given the high rate of SARS-CoV-2, the serial sampling and low rate of other respiratory illnesses during the study period we believe that our findings were due to SARS-CoV-2 and not the effects of other circulating viruses.

Collectively, our results show a group of immune mediators that may be pursued as potential targets for developing therapies as well as prognostic testing. To our knowledge this is the first study that shows the immune longitudinal profile of asymptomatic individuals, spanning their baseline state prior to infection through viral clearance, in combination with potential markers that are inhibited in mild symptomatic COVID-19.

DATA AVAILABILITY STATEMENT

The RNA-seq data presented in the study are deposited in the Gene Expression Omnibus repository, accession number GSE198449. The PEA data are available in **Table S5**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by NMRC.2020.0006. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS-S: performed serological assays, coordinated PEA data generation, analyzed data, prepared figures, wrote the manuscript; NS: performed statistical and data analysis, prepared figures, and wrote the manuscript; CWG, DLW, SL, RL: contributed to sample and data collection; SP and NK: performed neutralization assays; SV, SM, NM and CMM: contributed to sample preparation for PEA OLINK and serological assays; YG: contributed to data management and data analysis; VDN: supervised RNA-seq assays; XBY: contributed to sample management; M-CG: contributed to projects management; AC: prepared figures; WC, EZ: Contributed to data analysis; AB: supervised data generated from neutralization assays; OGT: supervised data analysis and contributed to scientific discussions; AGL: supervised sample and data collection and contributed to scientific discussions; SCS: supervised data generation, and data analysis and manuscript preparation; IR: analyzed data, prepared figures, wrote the manuscript, and supervised data generation, data analysis and manuscript preparation. All the authors reviewed and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.821730/full#supplementary-material

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