Evaluation of the Levels of Peripheral CD3⁺, CD4⁺, and CD8⁺ T Cells and IgG and IgM Antibodies in COVID-19 Patients at Different Stages of Infection

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ABSTRACT  Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection affects the stimulatory levels of cellular-mediated immunity, which plays an essential role in controlling SARS-CoV-2 infection. In fact, several studies have shown the association of lymphopenia with severe COVID-19 in patients. The aim of this study is to investigate the response of the immune system, including cell-mediated immunity and antibody production, during different stages of SARS-CoV-2 infection. Peripheral blood and serum samples were collected from patients with moderate infection, patients under medication (hospitalized), patients who had recovered, and healthy individuals (n = 80). Flow cytometry analysis was performed on peripheral blood samples to determine the cellular immunity profile of each patient. The data showed a significant reduction in the levels of CD3⁺, CD4⁺, and CD8⁺ T cells and CD45⁺ cells in the moderate and under-medication groups, suggesting lymphopenia in those patients. Also, enzyme-linked immunosorbent assay (ELISA) was conducted on the serum samples to measure the levels of antibodies, including IgM and IgG, in each patient. The results revealed a significant increase in the levels of IgM in the moderate infection and under-medication patients, thus indicating the production of IgM during the first week of infection. Furthermore, changes in the levels of IgG were significantly detected among recovered patients, indicating therefore a remarkable increase during the recovery stage of SARS-CoV-2 infection and thus a strong humoral-mediated immunity. In summary, the results of this study may help us to understand the main role of the cellular immune responses, including CD3⁺, CD4⁺, and CD8⁺ T cells, against SARS-CoV-2 infection. This understanding might support the development of SARS-CoV-2 treatments and vaccines in the near future.

IMPORTANCE  Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in late 2019 in China. This virus is a serious threat to people not only in China but also worldwide, where it has been detected in over 222 countries. It has been reported that ~3.4% of SARS-CoV-2-infected patients have died. The significance of our study relies on the fact that an enzyme-linked immunosorbent assay and flow cytometry were used to measure the levels of antibodies and cellular immune response, respectively, from clinical samples of patients infected with SARS-CoV-2.  

KEYWORDS  SARS-CoV-2, CD4, CD8, T cells, IgG, IgM, COVID-19

Many years after the emergence of severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003 and the Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012, a new coronavirus spread in China, causing numerous cases of pneumonia in December 2019 (1). The novel coronavirus was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This virus has rapidly spread and has affected numerous countries worldwide. The World Health Organization (WHO) declared the COVID-19 outbreak a pandemic in March 2020, and the number of infected individuals continues to rise globally. The severity of COVID-19 varies from asymptomatic to severe pneumonia, and the death rate is generally higher in elderly and patients with underlying medical conditions. The pathogenesis of COVID-19 is still not fully understood, and the development of effective treatments and vaccines is a top priority. The immune response plays a crucial role in controlling SARS-CoV-2 infection, and understanding the immune response could help in the development of treatments and vaccines. In this study, we investigated the cellular and humoral immune responses in different stages of COVID-19 infection.
respiratory syndrome coronavirus 2 (SARS-CoV-2), and the disease was defined as coronavirus infectious disease-19 (COVID-19) (2). Approximately 107 million confirmed cases of COVID-19 and 2 million deaths from it have been reported since December 2019, according to World Health Organization reports (2).

The SARS-CoV-2 disease spectrum varies from asymptomatic infection, to mild disease in the upper respiratory tract or moderate pneumonia, to severe illness with dyspnea and respiratory distress requiring hospitalization and organ support (3, 4). Most reported cases of SARS-CoV-2 are mild to moderate (5, 6). The ratio of patients who require hospitalization increases among individuals above 65 years old and those with underlying medical conditions, such as hypertension, obesity, cardiovascular disease, and diabetes mellitus (5, 6).

The pathogenesis of acute respiratory viral infections and the severity of disease could be modulated either directly by the virus, dysregulated immune responses, or both (7, 8). Several studies have reported that SARS-CoV-2 infection has a greater impact on CD8+ T cells than other lymphocytes (7, 9). Other studies have suggested evidence that adaptive immunity, particularly T cells, is critical for preventing severe disease progression in mild and severe COVID-19 cases (5, 9–11). CD4+ T cells specific against SARS-CoV-2 spike protein have shown their strong participation in acute infection (12). In other settings, Ki67+ CD8+ T cells have shown increased numbers in patients with severe COVID-19 (13). Moreover, patients who have recovered from severe SARS-CoV-2 infections have shown strong humoral and cellular immune responses (14). Also, memory CD4+ and CD8+ T cells have been detected in patients who have recovered from COVID-19 (9, 15). One study has demonstrated that there is an association between the complete restoration of peripheral lymphocytes for patients who have recovered from COVID-19 and viral clearance (16). The duration of immunological memory and whether it can provide protective immunity are still uncertain (9, 15). Although a correlation between the severity of COVID-19 disease and neutralizing antibodies is absent (15), some variants of SARS-CoV-2 have shown their capability to escape the neutralizing humoral immunity that is induced by vaccination (17). In fact, it was suggested that T cell response can mitigate the severity of COVID-19 infection, whereas the presence of neutralizing antibodies is correlated with protective immunity against reinfection with SARS-CoV-2, particularly in nonhuman primates (18, 19).

The reduction of T cells causes lymphopenia, which is very common in various respiratory viral infections (9, 20). Several studies have demonstrated that lymphopenia and inflammatory cytokine storms are associated with severe COVID-19 infections. However, although lymphopenia is not a characteristic feature of COVID-19 infection, the duration of lymphopenia could be more persistent than in other similar coronavirus infections (9, 21–24). The characterization of the immune responses to and immunopathogenicity of COVID-19 infection therefore requires further investigations. Also, variations in neutralizing antibodies and peripheral cell composition, including CD3+, CD4+, and CD8+ cells, should be assessed during different stages of COVID-19 infection. This will gain insight toward understanding the immune responses against SARS-CoV-2.

In this study, peripheral blood samples were collected from COVID-19 patients to compare and identify cellular immune responses, including CD3+, CD8+, and CD4+ T cell production, between different COVID-19 patient groups classified as having moderate infection, hospitalized (under medication), and recovered from COVID-19. Healthy individuals were enrolled as negative controls. IgG and IgM antibodies were measured in the four classified groups.

RESULTS

Cell-mediated immunity. The cellular and humoral responses against SARS-CoV-2 infection were investigated in this study. The response of T cells to COVID-19 infection was analyzed with 80 blood samples from patients with PCR-confirmed COVID-19 in different categories: 20 with moderate infection, 20 under medication, 20 recovered, and 20 healthy. Since both T helper CD4+ cells and T cytotoxic CD8+ cells are involved
during viral infections, including coronavirus (25, 26), their levels were determined in each patient’s sample.

Flow cytometry analysis was performed on all of the blood cells from all categories except the healthy category, in which only 15 samples were included, to generate the profile and levels of immune cells subsets in each sample. The level of immune cells was evaluated by calculating the median percentage of cells obtained from each sample. Then, a correlation between the level of immune cells and different groups’ status (categories) was determined. The results revealed different levels of CD3⁺, CD4⁺, CD8⁺, CD16⁺ CD56⁺, CD19⁺, and CD45⁺ between the groups. Also, the results showed different ratios of CD4⁺ to CD8⁺ T cells: this ratio compares the portion of helper CD4⁺ T cells to killer CD8⁺ T cells.

As shown in Table 1 and Fig. 1, significant differences in the levels of CD3⁺, CD4⁺, CD8⁺, and CD45⁺ cells between the study groups (P < 0.05 by analysis of variance [ANOVA]) were observed. The cells were significantly higher in the healthy group compared to the moderate infection and under-medication individuals. However, there was an insignificant difference in the levels of CD3⁺ cells between the healthy and recovered patients (P < 0.05 by Tukey’s honestly significant difference [HSD] post hoc test) (Table 2 and Fig. 1).

Regarding the other components of the immune profile, including CD16⁺, CD56⁺, CD19⁺, and CD4⁺/CD8⁺ T cell ratio, the data show an insignificant difference between the study groups, as shown in Tables 1 and 2 and Fig. 1 (P < 0.05 by ANOVA) (P < 0.05 by Tukey’s HSD post hoc test).

Furthermore, a correlation matrix plot was used to assess the level of correlation between different immune cells. A strong correlation was found between CD4⁺ and CD45⁺ cells, CD3⁺ and CD4⁺ cells, CD8⁺ cells, and CD45⁺ cells. Correlations were also found with CD8⁺ and CD4⁺ cells and CD45⁺ cells. In addition, a correlation was observed between CD19⁺ and CD45⁺ cells, as shown in Fig. S1 in the supplemental material. The results revealed that CD3⁺, CD4⁺, CD8⁺, CD45⁺, and CD19⁺ cells had a strong relationship and high level of correlation with each other in a specific manner as a response to SARS-CoV-2 infection.

**Antibody production.** In addition to the T cell response, production of IgG and IgM antibodies against SARS-CoV-2 infection were investigated in this study by enzyme-linked immunosorbent assay (ELISA). ELISA is a serological test used to detect the presence of the immunoglobulins IgG and IgM against SARS-CoV-2 (27, 28), targeting the spike and nucleocapsid proteins (S and N, respectively). Four categories were involved and included the following numbers of patients: 20 who were healthy, 20 with moderate infection, 20 under medication, and 20 who had recovered.

As seen in Fig. 2, the levels of IgM in the moderate infection, under-medication, and recovered groups were significantly higher than those in the healthy group (P < 0.001 by ANOVA). Although IgM levels showed an insignificant difference between the moderate infection and under-medication groups, both had significantly higher levels than the recovered individuals (P < 0.001 by ANOVA). IgG, on the other hand, has shown similar results to IgM in terms of their presence across the categories. IgG levels were significantly lower in the healthy group compared to the other groups, including the individuals in the moderate infection, under-medication, and recovered groups (Fig. 2) (P < 0.001 by ANOVA). Although IgG levels in the moderate infection and under-medication groups were significantly higher than those in the healthy group, they were significantly lower than those in the recovered individuals (P < 0.001 by ANOVA). The levels of IgG in the moderate infection group showed a significant decrease compared to the under-medication group as well as the recovered group. The IgG levels in the under-medication group were significantly higher than in the moderate infection group but significantly lower than those in the recovered group (P < 0.001 by ANOVA). Finally, patients who have recovered from COVID-19 showed the highest level of IgG in comparison to the other groups (P < 0.001 by ANOVA). Interestingly, the levels of IgG antibody in the recovered, moderate infection, and under-medication groups were higher than IgM antibody levels in the same groups.
Moreover, the levels of IgG increased as the recovery state developed from healthy, to moderate infection, to under medications, to recovered. The recovered group exhibited the highest levels of IgG immunity after infection among different groups, thus indicating an increase in the levels of memory immunity, as illustrated in Fig. 3. The levels of IgM, on the other hand, decreased as recovery advanced. The levels of IgM were higher in the moderate infection group and decreased as the patients were recovering (Fig. 3).

In summary, we could determine the role of CD3⁺, CD4⁺, and CD8⁺ T cells, as well as CD45⁺ cells, as they showed impaired cellular immunity in infected patients. The results for IgM and IgG antibodies indicated, on the other hand, a strong humoral immune response in SARS-CoV-2 patients.

| TABLE 1 ANOVA results |
|-------------------------|
| **ANOVA parameter** | **Sum of squares** | **df** | **Mean square** | **F** | **Significance** |
| **Lymph events** | | | | | |
| Between groups | 2,421,353.063 | 3 | 807,117.688 | 1.503 | 0.222 |
| Within groups | 3.653E7 | 68 | 537,164.062 | | |
| Total | 3.895E7 | 71 | | | |
| **Bead events** | | | | | |
| Between groups | 2.371E7 | 3 | 7,904,048.484 | 1.771 | 0.161 |
| Within groups | 3.034E8 | 68 | 4,461,942.383 | | |
| Total | 3.271E8 | 71 | | | |
| **CD3⁺** | | | | | |
| Between groups | 6,666,720.701 | 3 | 2,222,240.234 | 6.575 | 0.001 |
| Within groups | 2.298E7 | 68 | 337,975.802 | | |
| Total | 2.965E7 | 71 | | | |
| **CD3⁺ CD8⁺** | | | | | |
| Between groups | 1,060,627.022 | 3 | 353,542.341 | 5.248 | 0.003 |
| Within groups | 4,580,919.774 | 68 | 67,366.467 | | |
| Total | 5,641,546.796 | 71 | | | |
| **CD3⁺ CD4⁺** | | | | | |
| Between groups | 2,025,826.300 | 3 | 675,275.433 | 5.271 | 0.003 |
| Within groups | 8,711,165.749 | 68 | 128,105.379 | | |
| Total | 1.074E7 | 71 | | | |
| **CD3⁺ CD4⁺ CD8⁺** | | | | | |
| Between groups | 127.978 | 3 | 42.659 | 1.484 | 0.227 |
| Within groups | 1,954.833 | 68 | 28.748 | | |
| Total | 2,082.812 | 71 | | | |
| **CD16⁺ CD56⁺** | | | | | |
| Between groups | 139,806.392 | 3 | 46,602.131 | 0.716 | 0.546 |
| Within groups | 4,424,094.281 | 68 | 65,060.210 | | |
| Total | 4,563,900.673 | 71 | | | |
| **CD19⁺** | | | | | |
| Between groups | 109,535.879 | 3 | 36,511.960 | 0.583 | 0.628 |
| Within groups | 4,260,738.831 | 68 | 62,657.924 | | |
| Total | 4,370,274.711 | 71 | | | |
| **CD45⁺** | | | | | |
| Between groups | 9,692,126.568 | 3 | 3,230,708.856 | 4.423 | 0.007 |
| Within groups | 4.967E7 | 68 | 730,438.296 | | |
| Total | 5.936E7 | 71 | | | |
| **CD4⁺/CD8⁺ ratio** | | | | | |
| Between groups | 0.280 | 3 | 0.093 | 0.178 | 0.911 |
| Within groups | 35.677 | 68 | 0.525 | | |
| Total | 35.957 | 71 | | | |
FIG 1 Box plot showing the level of immune cells (lymph events, bead events, CD3\(^+\), CD3\(^+\)CD8\(^+\), CD3\(^+\)CD4\(^+\), CD3\(^+\)CD4\(^+\)CD8\(^+\), CD16\(^+\)CD56\(^+\), CD19\(^+\), CD45\(^+\), and CD4\(^+\)/CD8\(^+\) T cell ratio) of the study group was evaluated using flow cytometry. The box plot shows the data distribution and shows the outliers. The line in the middle represents the median of the percentage of cells obtained from each sample. Results were considered significant at \( P < 0.05 \).
### TABLE 2 Tukey’s HSD post hoc test for multiple comparisons

| Dependent variable | Patient status | Mean difference (I – J)* | SE | Significance | 95% CI* | Lower bound | Upper bound |
|--------------------|----------------|--------------------------|----|--------------|--------|-------------|-------------|
|                    | I              | J                        |    |              |        |             |             |
| Lymph events       | Healthy        | Moderate                 | 384.161 | 259.632 | 0.455 | -299.64 | 1,067.96 |
|                    | Recovered      |                          | 244.867 | 253.146 | 0.768 | -421.85 | 911.58   |
|                    | Under meds     |                          | -67.752 | 247.770 | 0.993 | -720.31 | 584.80   |
|                    | Moderate       | Healthy                 | -384.161 | 259.632 | 0.455 | -1,067.96 | 299.64 |
|                    | Recovered      |                          | -139.294 | 244.683 | 0.941 | -783.72 | 505.13   |
|                    | Under meds     |                          | -451.913 | 239.117 | 0.242 | -1,081.68 | 177.85 |
|                    | Recovered      | Moderate                | -244.867 | 253.146 | 0.768 | -911.58 | 421.85   |
|                    | Under meds     | Moderate                | 394.294 | 244.683 | 0.941 | -505.13 | 783.72   |
|                    | Under meds     | Under meds              | -312.619 | 232.058 | 0.537 | -923.79 | 298.56   |
|                    | Under meds     | Recovered               | 67.752 | 247.770 | 0.993 | -584.80 | 720.31   |
|                    | Moderate       | Recovered               | 451.913 | 239.117 | 0.242 | -177.85 | 1,081.68 |
|                    | Under meds     | Recovered               | 312.619 | 232.058 | 0.537 | -298.56 | 923.79   |
| Bead events        | Healthy        | Moderate                | -1,058.447 | 748.285 | 0.495 | -3,029.22 | 912.32 |
|                    | Recovered      |                          | -330.958 | 729.590 | 0.969 | -2,252.49 | 1,590.58 |
|                    | Under meds     |                          | -1,451.324 | 714.098 | 0.186 | -3,332.06 | 429.41 |
|                    | Moderate       | Healthy                 | 1,058.447 | 748.285 | 0.495 | -912.32 | 3,029.22 |
|                    | Recovered      |                          | 727.489 | 705.199 | 0.732 | -1,129.81 | 2,584.79 |
|                    | Under meds     |                          | -392.877 | 689.159 | 0.941 | -2,207.93 | 1,422.17 |
|                    | Recovered      | Healthy                 | 330.958 | 729.590 | 0.969 | -1,590.58 | 2,252.49 |
|                    | Under meds     | Healthy                 | -727.489 | 705.199 | 0.732 | -2,584.79 | 1,129.81 |
|                    | Under meds     | Moderate                | -1,120.366 | 668.814 | 0.345 | -2,881.83 | 641.10 |
|                    | Under meds     | Recovered               | 1,451.324 | 714.098 | 0.186 | -429.41 | 3,332.06 |
|                    | Moderate       | Recovered               | 392.877 | 689.159 | 0.941 | -1,422.17 | 2,207.93 |
|                    | Under meds     | Recovered               | 1,120.366 | 668.814 | 0.345 | -641.10 | 2,881.83 |
| CD3⁺               | Healthy        | Moderate                | 868.04561* | 2.05943E2 | 0.000 | 325.6492 | 1,410.4402 |
|                    | Recovered      |                          | 473.35372 | 2.00798E2 | 0.095 | -55.4920 | 1,062.1994 |
|                    | Under meds     |                          | 676.41171* | 1.96534E2 | 0.005 | 158.7955 | 1,194.0280 |
|                    | Moderate       | Healthy                 | -868.04561* | 2.05943E2 | 0.000 | -1,410.4402 | -325.6492 |
|                    | Recovered      |                          | -394.69189 | 1.94085E2 | 0.186 | -905.8578 | 116.4741 |
|                    | Under meds     |                          | -191.63389 | 1.89671E2 | 0.744 | -691.1731 | 307.9054 |
|                    | Recovered      | Healthy                 | 394.69189 | 1.94085E2 | 0.186 | -116.4741 | 905.8578 |
|                    | Under meds     | Healthy                 | -191.63389 | 1.89671E2 | 0.744 | -691.1731 | 307.9054 |
|                    | Under meds     | Moderate                | 203.05799 | 1.84071E2 | 0.689 | -281.7340 | 687.8500 |
|                    | Under meds     | Recovered               | -676.41171* | 1.96534E2 | 0.005 | -1,194.0280 | -158.7955 |
|                    | Moderate       | Recovered               | 191.63389 | 1.89671E2 | 0.744 | -307.9054 | 691.1731 |
|                    | Under meds     | Recovered               | -203.05799 | 1.84071E2 | 0.689 | -687.8500 | 281.7340 |
| CD3⁺ CD8⁺          | Healthy        | Moderate                | 353.59616* | 91.94472 | 0.001 | 111.4398 | 595.7525 |
|                    | Recovered      |                          | 213.99839 | 89.64765 | 0.089 | -22.1082 | 450.1049 |
|                    | Under meds     |                          | 259.02400* | 87.74409 | 0.022 | 27.9309 | 490.1171 |
|                    | Moderate       | Healthy                 | -353.59616* | 91.94472 | 0.001 | -595.7525 | -111.4398 |
|                    | Recovered      |                          | -139.59777 | 86.65066 | 0.379 | -367.8111 | 88.6156 |
|                    | Under meds     |                          | -94.57216 | 84.67975 | 0.680 | -317.5947 | 128.4504 |
|                    | Recovered      | Healthy                 | -213.99839 | 89.64765 | 0.089 | -450.1049 | 22.1082 |
|                    | Under meds     | Healthy                 | 139.59777 | 86.65066 | 0.379 | -88.6156 | 367.8111 |
|                    | Under meds     | Moderate                | 45.02561 | 82.17987 | 0.947 | -171.4129 | 261.4641 |
|                    | Under meds     | Recovered               | -259.02400* | 87.74409 | 0.022 | -490.1171 | -27.9309 |
|                    | Moderate       | Recovered               | 94.57216 | 84.67975 | 0.680 | -128.4504 | 317.5947 |
|                    | Under meds     | Recovered               | -45.02561 | 82.17987 | 0.947 | -261.4641 | 171.4129 |

**CD3⁺ CD4⁺**  
Healthy  
Moderate  
Recovered  
Under meds  
Moderate  
Recovered  
Under meds  
Moderate  
Recovered  
Under meds  

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### TABLE 2 (Continued)

| Dependent variable | Patient status<sup>a</sup> | Mean difference (I − J)<sup>b</sup> | SE | Significance | 95% CI<sup>c</sup> | Lower bound | Upper bound |
|--------------------|-----------------------------|----------------------------------|----|-------------|-----------------|------------|------------|
| CD3<sup>+</sup> CD4<sup>+</sup> CD8<sup>+</sup> | Healthy                    | 3.05867                           | 1.89935 | 0.380 | −1.9437 | 8.6010 |
| Moderate          | Recovered                  | 1.27025                           | 1.85190 | 0.902 | −3.6071 | 6.7146 |
| Under meds        | Healthy                    | −0.37562                          | 1.81258 | 0.997 | −5.1494 | 4.3982 |
| Recovered         | Healthy                    | −1.78842                          | 1.78999 | 0.750 | −6.5027 | 2.9259 |
| Under meds        | Healthy                    | −3.43429                          | 1.74927 | 0.212 | −8.0414 | 1.1728 |
| Recovered         | Healthy                    | −1.27025                          | 1.85190 | 0.902 | −6.1476 | 3.6071 |
| Moderate          | Under meds                 | 1.78842                           | 1.78999 | 0.750 | −2.9259 | 6.5027 |
| Healthy           | Recovered                  | −1.64586                          | 1.69763 | 0.767 | −6.1170 | 2.8252 |
| Moderate          | Under meds                 | 0.37562                           | 1.81258 | 0.997 | −4.3982 | 5.1494 |
| Healthy           | Recovered                  | 3.43429                           | 1.74927 | 0.212 | −1.1728 | 8.0414 |
| CD3<sup>+</sup> CD16<sup>+</sup> CD56<sup>+</sup> | Healthy                    | 126.61435                         | 90.35717 | 0.503 | −111.3609 | 364.5896 |
| Moderate          | Recovered                  | 58.26147                         | 88.09777 | 0.911 | −173.7684 | 290.2913 |
| Under meds        | Healthy                    | 91.21676                          | 86.22907 | 0.716 | −139.8862 | 318.3197 |
| Recovered         | Healthy                    | −126.61435                        | 90.35717 | 0.503 | −364.5896 | 111.3609 |
| Under meds        | Healthy                    | −68.35288                         | 85.15452 | 0.853 | −292.6259 | 155.9200 |
| Recovered         | Healthy                    | −35.39759                         | 83.21764 | 0.974 | −254.5693 | 183.7741 |
| Moderate          | Recovered                  | 58.26147                         | 88.09777 | 0.911 | −290.2913 | 173.7684 |
| Under meds        | Healthy                    | 68.35288                         | 85.15452 | 0.853 | −155.9200 | 292.6258 |
| Recovered         | Healthy                    | 32.95529                          | 80.76092 | 0.977 | −179.7461 | 245.6567 |
| Moderate          | Recovered                  | 35.39759                         | 83.21764 | 0.974 | −183.7741 | 254.5693 |
| CD19<sup>+</sup> | Healthy                    | 9.14118                           | 88.67331 | 1.000 | −224.3992 | 242.6816 |
| Moderate          | Recovered                  | 23.15000                          | 86.45797 | 0.993 | −204.5558 | 250.5858 |
| Under meds        | Healthy                    | 95.64048                          | 84.62213 | 0.672 | −127.2303 | 318.5112 |
| Recovered         | Healthy                    | −9.14118                          | 88.67331 | 1.000 | −242.6816 | 224.3992 |
| Moderate          | Recovered                  | 14.00882                          | 83.56761 | 0.998 | −206.0846 | 234.1023 |
| Under meds        | Healthy                    | 86.49930                          | 81.66683 | 0.715 | −128.5880 | 301.5866 |
| Recovered         | Healthy                    | −23.15000                         | 86.45797 | 0.993 | −250.8558 | 204.5558 |
| Moderate          | Recovered                  | −14.00882                         | 83.56761 | 0.998 | −234.1023 | 206.0846 |
| Under meds        | Healthy                    | 72.49048                          | 79.25589 | 0.797 | −136.2471 | 281.2281 |
| Recovered         | Healthy                    | −95.64048                         | 84.62213 | 0.672 | −318.5112 | 127.2303 |
| Moderate          | Recovered                  | −86.49930                         | 81.66683 | 0.715 | −301.5866 | 128.5868 |
| CD45<sup>+</sup> | Healthy                    | 1,009.16992*                      | 3.02759E2 | 0.007 | 211.7891 | 1,806.5508 |
| Moderate          | Recovered                  | 560.00302                         | 2.95195E2 | 0.239 | −217.4567 | 1,337.4628 |
| Under meds        | Healthy                    | 869.87267*                        | 2.88927E2 | 0.019 | 108.9214 | 1,630.8240 |
| Recovered         | Healthy                    | −1,009.16992*                     | 3.02759E2 | 0.007 | −1,806.5508 | −211.7891 |
| Moderate          | Recovered                  | −449.16690                        | 2.85326E2 | 0.400 | −1,200.6356 | 302.3018 |
| Under meds        | Healthy                    | −139.29725                        | 2.78836E2 | 0.959 | −873.6734 | 595.0789 |
| Recovered         | Healthy                    | −560.00302                        | 2.95195E2 | 0.239 | −1,337.4628 | 217.4567 |
| Moderate          | Recovered                  | 449.16690                         | 2.85326E2 | 0.400 | −302.3018 | 1,200.6356 |
| Under meds        | Healthy                    | 309.86965                         | 2.70605E2 | 0.663 | −402.8265 | 1,022.5658 |
| Recovered         | Healthy                    | −869.87267*                       | 2.88927E2 | 0.019 | −1,630.8240 | −108.9214 |
| Moderate          | Recovered                  | 139.29725                         | 2.78836E2 | 0.959 | −595.0789 | 873.6734 |
| CD4<sup>+</sup>/CD8<sup>+</sup> ratio | Healthy | 0.11329                           | 0.25659 | 0.971 | −0.5625 | 0.7891 |
| Recovered         | Healthy | 0.12905                           | 0.25018 | 0.955 | −0.5299 | 0.7880 |
| Under meds        | Healthy | 0.17610                           | 0.24487 | 0.889 | −0.4688 | 0.8210 |
| Recovered         | Healthy | −0.11329                          | 0.25659 | 0.971 | −0.7891 | 0.5625 |
| CD4<sup>+</sup>/CD8<sup>+</sup> ratio | Recovered | 0.01576                           | 0.24182 | 1.000 | −0.6211 | 0.6526 |

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DISCUSSION

Cellular immune responses play an essential role in virus clearance and disease severity (29–31). One study on SARS-CoV has demonstrated the requirement of specific T cell responses for the treatment of SARS-CoV-infected mice (29). In addition, several studies have shown that cell-mediated immunity, mainly cytotoxic (CD8<sup>+</sup>), is an essential factor in eliminating numerous virus-infected cells during severe infections (reviewed in reference 26). On the other hand, a study has demonstrated that depletion of helper T cells (CD4<sup>+</sup>) in SARS-CoV-infected mice increases the duration of viral clearance, whereas there was no effect when CD8<sup>+</sup> T cells were depleted (30). This indicates the important role of CD4<sup>+</sup> T cells in controlling SARS-CoV infection, as well as other viral infections (30, 32). Specific CD4<sup>+</sup> T cells were detected in peripheral blood samples of COVID-19 patients (33). Several studies have investigated the T cell responses of COVID-19 patients; however, most of them were performed on a single patient or small number of patients (7). Besides, information regarding the correspondence between levels of T cell responses (CD4<sup>+</sup> and CD8<sup>+</sup>) during different COVID-19 stages is scarce—hence the importance of this study. This study was performed using peripheral blood samples from 80 individuals with different stages of SARS-CoV-2 infection. Flow cytometry was used to detect the populations of CD4<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup> T cells. Also, specific IgM and IgG antibodies were detected using ELISA. Differences were observed with population levels of CD4<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup> T cells at different stages of SARS-CoV-2 infection.

The results of the current study indicated a significant reduction in the levels of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells in the moderate infection and under-medication groups compared to the healthy group. This finding is in agreement with what was observed by previous studies, as the results showed a low count of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells in COVID-19 patients, particularly in moderate and severe cases (20, 34, 35). However, the recovered COVID-19 patients in this study showed an increase in CD3<sup>+</sup>, CD8<sup>+</sup>, and CD4<sup>+</sup> T cell levels in comparison to the moderate infection and under-medication groups. Therefore, it can be deduced that SARS-CoV-2 infection could affect T cell population counts in the peripheral blood, most frequently with severe cases (11, 34). When the T cell levels were compared between the group that recovered from COVID-19 and healthy controls, they showed an insignificant difference. Restoration of T cells in the recovered patients could probably be an indication of viral clearance (16). Moreover, the CD45<sup>+</sup> level was decreased significantly in the moderate infection and under-medication groups in the present study. This is similar to what was observed in previous studies that showed a decline in the level of CD45<sup>+</sup> in severe COVID-19 patients (34, 36). CD45<sup>+</sup> is an important transmembrane phosphatase involved in T cell activation and signaling (36). It has been demonstrated that the expression of CD45<sup>+</sup> on T cells was decreased in HIV infection (36). This suggests that CD45<sup>+</sup> could be used also as an indicator for assessment of the severity of COVID-19 disease (36).

The reduction in T cells, mainly CD4<sup>+</sup> and CD8<sup>+</sup>, is common between severe COVID-19 cases (9). This attribute was also observed in the present study with the moderate infection and under-medication groups, suggesting that there is a relationship between

| Table 2 (Continued) | Patient status<sup>a</sup> | Mean difference (I - J)<sup>b</sup> | SE | Significance | 95% CI<sup>c</sup> |
|---------------------|--------------------------|-----------------------------|----|-------------|------------------|
|                     | I                        | J                           |    |             | Lower bound  | Upper bound    |
| Recovered           | Under meds               | 0.06280                     | 0.23632 | 0.993     | -0.5596      | 0.6852          |
|                     | Healthy                  | -0.12905                    | 0.25018 | 0.955     | -0.7880      | 0.5299          |
|                     | Moderate                 | -0.01576                    | 0.24182 | 1.000     | -0.6526      | 0.6211          |
|                     | Under meds               | 0.04704                     | 0.22934 | 0.997     | -0.5570      | 0.6511          |
|                     | Healthy                  | -0.17610                    | 0.24487 | 0.889     | -0.8210      | 0.4688          |
|                     | Moderate                 | -0.06280                    | 0.23632 | 0.993     | -0.6852      | 0.5596          |
|                     | Recovered                | -0.04704                    | 0.22934 | 0.997     | -0.6511      | 0.5570          |

<sup>a</sup>Moderate, patients with moderate infection; Under meds, patients under medication (hospitalized).

<sup>b</sup>* indicates the mean difference is significant at the 0.05 level.

<sup>c</sup>95% CI, 95% confidence interval.
COVID-19 disease severity and the occurrence of lymphopenia (34). Although lymphopenia is a common feature of many respiratory viral infections, such as human rhinovirus, SARS-CoV, and MERS-CoV, it is more persistent with SARS-CoV-2, likely due to selection of those T cell lineages rather than the other ones. This is by affecting CD4+ T cells, CD8+ T cells, B cells, and natural killer cells (9, 20), a phenomenon found in this study as well. Detection of lymphopenia in COVID-19 patients might thus affect the cell-mediated response, which has a principal role in eliminating viral infection. In addition, lymphopenia affects other immune cells, including B cells, innate immune cells, and natural killer cells. In fact, there was an insignificant difference in the ratio of CD4+ to CD8+ T cells between the groups, although CD4+ and CD8+ T cells play a vital role in maintaining immune function and viral clearance in the body. This finding was also observed in another study that showed an insignificant difference in the CD4+/CD8+ T cell ratio between COVID-19 patients and healthy individuals (25).

**FIG 2** Illustration of IgG and IgM levels in different groups of patients. The levels of IgG and IgM were determined using ELISA in three different groups: (A) moderate group, (B) under-medication group, and (C) recovered group. Results were considered significant at P < 0.001 (**).
The immunoglobulins IgG and IgM are usually generated by the adaptive immune system in response to infections (37). IgG and IgM can be specific and serve as diagnostic markers for detecting infections such as COVID-19 (38). The results of this study showed that the levels of IgG and IgM antibodies are increased in SARS-CoV-2 patients (Fig. 3). This finding is identical to the results of another group that showed a positive IgG titer in patients infected with SARS-CoV-2 during the first 3 weeks after symptom onset, although IgM showed a slight decrease in the third week (39). The latter was also observed in our study, as IgM levels started to be generated in the moderate infection and under-mediation groups, and then the levels began to decline in the recovered individuals, suggesting that IgM could be produced first in response to the SARS-CoV-2 infection at the early stage since it can be expressed without isotype switching. Moreover, other studies have reported that IgM levels increased in patients in the first week of infection, with a high peak in the second week prior to a great reduction near background levels in most patients. IgG antibody, on the other hand, was observed after 1 week of infection and was maintained at a high level for a long period of time after the infection occurred (40, 41). The latter results are similar to what was observed in this study, as IgG levels showed an increase in the moderate stage of infection, reaching the highest level in the recovery stage. High IgG levels were maintained until day 20. This indicates that the patients have developed a humoral immune response against COVID-19 within 7 to 10 days after they have had the infection. Moreover, these results suggest that the IgM antibodies were produced as the body is still
infected; however, when the body starts to recover, the IgG antibody starts to be produced, as reported earlier (42). The production of IgM and IgG antibodies during infection and the increase in IgG in the recovery stage suggest also that IgM and IgG may have neutralized the SARS-CoV-2 and that IgG antibodies created immunity for the body to fight current and future infections.

Another study has investigated IgG and IgM responses to SARS-CoV-2 nucleocapsid (N) and spike (S) proteins in severe and nonsevere cases after infection (43). The study reported that the response of IgM against S and N proteins increases after the onset of symptoms, reaching a peak in the second week of infection in some patients. The response of IgG against N and S proteins, however, continued to increase in the third week in some patients. The response of IgG to S protein was higher in moderate cases in comparison to severe cases in the third week. The response of IgG against N protein, on the other hand, was significantly higher in severe cases, with this difference attributed to the fact that the increase in IgG in response to S protein correlated with a reduction in C-reactive proteins in severely infected patients (43). In our study, we have noticed a similar pattern where levels of IgG increased considerably. Therefore, it would be interesting to investigate the levels of IgG and IgM in response to N proteins as we have only measured the levels of IgG and IgM against the S protein in this set of patients. Also, quantitative detection of IgG and IgM could help in evaluating the severity of the disease as well as could establish a dynamic that can help in predicting prognosis.

In conclusion, our data present the responses of cell-mediated and humoral-mediated immunity in both infected and recovered SARS-CoV-2 patients. The results support the concept of lymphopenia in patients with moderate infection and under medication, as confirmed in other studies (9, 20). On the other hand, our study confirmed the presence of strong humoral-mediated immunity in the recovered cases. It could be speculated that SARS-CoV-2 infection negatively affected the activation and proliferation of T cells, while the production of antibodies occurred independently of T cells. The latter represents the role of humoral immunity in controlling SARS-CoV-2 infection and in providing the required protection, which may be investigated in future studies for their long-term presence. Furthermore, our results can aid in the future development of SARS-CoV-2 treatments and vaccines. In addition, our results can enhance the discovery of anti-virus-specific T cell clones and the development new immunotherapeutic “weapons” to fight this threatening disease.

MATERIALS AND METHODS

Ethical considerations. A total of 80 samples (n = 80) were collected with informed consent under ethical approval from the Institutional Review Board (no. 20-162) of King Fahad Medical City, Riyadh, Kingdom of Saudi Arabia (KSA).

Sample collection and processing. Eighty samples were collected from patients (49 male and 31 female, complete age range of 21 to 91 years, and average age of 51 years) at the Prince Mohammed Bin Abdulaziz Hospital (PMAH), Riyadh, KSA. None of the included patients had received a SARS-CoV-2 vaccine. The SARS-CoV-2 diagnosis was confirmed by reverse transcriptase PCR (RT-PCR) (bioMérieux Diagnostics). The cohort of the study included healthy individuals (>15 years old) with no history of respiratory illnesses, smoking, or obesity, patients with a moderate stage of infection (>15 years old, with samples collected between 4 and 9 days [average of 6.5 days] from the day of reporting COVID-19 positive), patients under medication who received medical treatments upon admission (hospitalized) (>15 years old, with samples collected between 10 and 14 days [average of 12 days] from the day of reporting COVID-19 positive), and recovered patients (>15 years old, with samples collected between 14 and 20 days [average of 17 days] from the day of reporting COVID-19 positive).

Blood samples (two tubes from each patient) from patients with confirmed SARS-CoV-2 diagnosis as well as from SARS-CoV-2-negative patients (who had no symptoms of SARS-CoV-2 infection and no identifying information) from the PMAH were utilized in this study. The first fresh blood tube was directly utilized in the flow cytometry experiment. For the ELISA, the serum from the second blood tube was isolated and stored at −80°C until use.

Flow cytometry. Each patient’s sample was placed in a BD Trucount tube that was labeled with the sample accession number. Ten microliters of BD Multitest 6-color TBKN reagent was added, followed by 50 μL of a well-mixed EDTA-anticoagulated whole-blood sample. The tube was then capped and vortexed gently to be mixed. The sample was incubated for 15 min in the dark at room temperature (20 to 25°C). Thereafter, 450 μL of 1× BD fluorescence-activated cell sorter (FACS) lysing solution was added. The sample was capped and vortexed gently and then incubated for 10 min in the dark at room temperature (20
to 25°C. The sample should have been acquired within 1 h of lysing. The sample of viable total cells was ready to be analyzed on the flow cytometer. In each sample, the levels that represent the percentages of total cell numbers of CD3⁺CD56⁻ CD4⁺CD8⁻ T cells, CD19⁺ B lymphocytes, and CD56⁺ cells were calculated for each patient. Analysis was performed using the BD FACSCont® clinical software.

**ELISA.** In order to detect the presence of the spike and nucleocapsid (S and N) proteins of the SARS-CoV-2 virus, commercially available SARS-CoV-2 IgG antibody and SARS-CoV-2 IgM antibody detection kits (from BGI, catalogue no. 0601038 and 0601039, respectively) were used as per the manufacturer’s instructions. All reagents were incubated at room temperature for half an hour prior to use. Washing buffer was diluted at a ratio of 1:20 with distilled water. Serum samples were diluted at a ratio of 1:10 in PBS. Each well was washed five times using 300 µL washing buffer with the assistance of an automated microplate washer (Wellwash, Thermo Fisher). A blank well where no liquid was added was included. The coated plate was incubated at 37°C for 60 min. The plate was washed five times using 300 µL washing buffer. Then 50 µL of both substrates A and B were added to each well and mixed thoroughly. The plate was incubated at 37°C for 10 min from light prior to quenching the reaction using 50 µL of stop buffer added to each well. The absorbance was measured immediately at a 450-nm wavelength using a Multiskan microplate reader purchased from Thermo Scientific.

**Statistical analysis.** Statistical analysis was done using R Studio version 1.4.1103 and IBM SPSS 16.0. Correlation and data exploration were performed to understand the variables. ANOVA, which is a collection of methods for comparing multiple means across different groups, was done for all four categories across variables. A *post hoc* comparison HSD Tukey’s test was performed to find the differences between the categories. A two-sided *P* value of < 0.05 in flow cytometry data and a *P* value of < 0.001 in ELISA data were considered statistically significant with a 95% confidence level.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.**

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