Measurement of SARS-CoV-2-specific Humoral and Cellular Immunity in COVID-19 Convalescent Health Care Workers in Iraq

Hasan Ali, I*, Sahib Abdulamir, A1

1. Department of Microbiology, Al-Nahrain University College Of Medicine, Baghdad, Iraq

Corresponding Author: israa2579@gmail.com

Abstract

SARS-CoV-2 belongs to Coronaviridae family and is responsible for a global pandemic. Yet, available knowledge is limited regarding an adaptive immunity in convalescent individuals. Here we studied 191 convalescent COVID 19 health care workers (HCW), and evaluated the cell mediated immunity of 122 recovered HCW and the level of anti RBD IgG antibodies of 181 recovered HCW; the cellular and humeral immune responses were profiled over time from 1 to 8 months post recovery with varying disease severity using MTT proliferation assay and Enzyme linked immunosorbent assay (ELISA). Analysis of lymphocyte proliferation with S1 protein in mild-moderate and severe HCW reveal insignificant difference with increasing in, maximum and third quartile from 1 to 8 months post COVID 19 recovery. Antibody levels in mild-moderate and severe recovered HCW were insignificantly different with post COVID 19 recovery (P>0.05), the median, maximum and Q3 values of anti RBD IgG were close to each other over the time intervals from 1 to 8 months post recovery. These data suggests many convalescent HCW enrolled in this study were re exposed to the virus without development of symptoms indicate the role of cell mediated and humoral immunity in preventing symptomatic reinfection. This study reveals that a robust immunity developed after mild, moderate and severe COVID 19 disease and could last for several months post recovery.

Keywords: Severe Acute Respiratory Syndrome (Sars-Cov-2), Coronavirus Disease 2019 (Covid 19), Cell Mediated Immunity, Humoral Immunity
1. Introduction

In December 2019, severe acute respiratory syndrome 2 (SARS-CoV-2) emerged in Wuhan china as a series of cases with pneumonia and variety of symptoms that collectively termed coronavirus disease 2019 (COVID 19). Then globally spread and the World Health Organization declared this is a worldwide pandemic (1,2).

SARS-CoV-2 belongs to Coronaviridae family and has two main structural protein, nucleo-protein which found inside the virus and spike (S) protein that is protruding from viral surface. The S glycoprotein is a large trimeric glycoprotein consisting of polypeptide chain (from 1100 to 1600 residues in length) and responsible for cell attachment and viral fusion (3,4) the spike protein used as a target for characterizing the immune response to SARS-CoV-2 (5) It is divided into two regions S1 and S2 subunits. The S1 subunit is a V shaped polypeptide with four distinct domains, Domains A, B, C, and D, and Domain B functioning as the receptor-binding domain (RBD) (6) many studies shows that the virus attached to the cells by interaction of RBD with cellular receptor angiotensin-converting enzyme 2 (ACE2) (6,7) followed by viral fusion into the cell then active viral replication and release of the virus from lung cells leading to development of symptoms (8).

COVID 19 is characterized by fever, headache, dry cough, dyspnea and pneumonia. Although most SARS-CoV-2 infections are not severe but some patients require hospitalization (9).

Host immune system make SARS-CoV-2 specific antibodies and elicit T cells that can bind to viral proteins through their antigen receptors then begin to secret molecules that help control the infection.

Single cell RNA sequence (scRNA-seq) analysis of bronchoalveolar lavage (BAL) fluid of COVID-19 patients revealed an increase in CD8 T cell infiltrate with clonal expansion (10).The recovery from disease indicate development of adequate adaptive immunity that successful in fight of infection (11) and dysregulated in host immune response to viral infection result in immunopathology (12)(13).

It is found that disease severity associated with lymphocytopenia and increased in the level of pro-inflammatory cytokines such as interleukin 6 (IL-6) interleukin-5 (IL-5), interleukin13 (IL-13) (14–16).
Acute respiratory distress syndrome (ARDS) may develop from excessive inflammation and lymphocytopenia. Cell destruction lead to the patients may be required mechanical ventilator for several weeks and even death (17).

Protective immunity mainly arise from T cell that detected in blood of convalescent COVID 19 patients with antiviral activity (18,19) and in recovery patients with asymptomatic to mild disease, SARS-CoV-2 specific antibody start to decrease after 2-3 months from recovery (20) these antibody can neutralize the virus and preventing infection (21).

Health care workers are more susceptible to infection and reinfection than other fractions of population as close contacts with the virus (22) thus they are needed for longitudinal studies with longer time frames to discover and analyze the key features of SARS-CoV-2 adaptive immunity.

In this study blood was collected from convalescent health care workers to investigate for how long SARS-CoV-2 humoral and cellular immunity could last in the circulation after recovery from COVID-19.

2. Materials and methods

2.1. Human subjects

Convalescent COVID-19 health care workers were selected from different stages of disease (mild, moderate and severe) and according to WHO classification (23).

The WHO defined the stages of disease as following: 1- severity of disease associated with oxygen saturation ≤ 89% at rest and sign of respiratory distress such as severe dyspnea and chest pain. 2- mild-moderate COVID 19 patient require non hospitalization and associated with oxygen saturation higher than 90% at rest and symptoms such as fever, headache, cough, shortness of breath, pneumonia, loss of smell and taste, nausea, vomiting and diarrhea.

Convalescent HCW (n=191) were assigned to one of four groups of period study. Samples collected from patients after one month post COVID 19 recovery (n=45), up to 25 were in mild-moderate stage and 20 were in severe stage. Samples collected from patients after three months post recovery, up to 25 were in mild-moderate stage and 20 were in severe stage. Samples collected
from patients after five months post COVID 19 recovery (n=53), up to 34 were in mild-moderate stage and 19 were in severe stage. Samples collected from patients after eight months post COVID 19 recovery (n=48), up to 26 were in mild-moderate stage and 22 were in severe stage.

Up to 5 ml of whole blood were collected from each donor, up to 2 ml in serum separator tube to be used in ELISA and up to 3 ml in citrated tube for PBMC isolation.

2.2. Peripheral blood mononuclear cell (PBMC) isolation

PBMC were isolated from citrated blood by density gradient sedimentation as previously described(24) to prepare a pure population ready for use in MTT assay. To PBMC isolation, whole blood was diluted with RPMI 1640 (US biological, USA) media then gently layered above equal volume of lymphosep (biowest, France) in Falcon tube and centrifuged for 20 min at 400G. At the end, erythrocytes and granulocytes that have higher density than mononuclear cells sediment to the bottom of tubes. Mononuclear cells form a creamy thin layer observed at interface between plasma and lymphosep. Mononuclear cells were gently removed by automatic pipette and washed twice using washing buffer (PBS and 2% fetal bovine serum) or RPMI 1640 media. Complete RPMI 1640 media was added to pelleted cells to make the final lymphocyte suspension. The cells was counted and the viability percentage estimated using hemocytometer and Trypan blue staining.

2.3. Recombinant SARS-CoV-2 S1 preparation

Recombinant SARS-CoV-2 S1 (elabscience, USA) at concentration 50 ug/ml was diluted with phosphate buffer saline (PBS) (bioworld, USA) to concentration 10 ug/ml then stored at -20 c in aliquots to minimize freezing thawing cycles.

2.4. Proliferation assay (MTT assay)

As described in a previous study(25), the final PBMC concentration was adjusted to be 1×10^6 cell/ml and approximately 13×10^4 cells in 135 ul complete RPMI 1640 media was plated for each COVID 19 convalescent individuals. Then incubated with recombinant SARS-CoV-2 S1 protein at concentration 1 ug/ml for 3 days at 37 C. Negative control wells were lacked the recombinant
protein. Then centrifugation followed by aspirate of the media. 50 ul of MTT (elabscience, USA) was added to each well according to instruction kit and incubated for 3 hour at 37 C. At the end of time, the supernatant was removed by automatic pipette then added 100 ul of dimethyl sulfoxide (DMSO) and shaking for 10 min then mixing by pipetting to completely dissolve of formazan crystals. Then reading using ELIZA reader at 490 wavelength.

Proliferation of cells = (OD of stimulated cell with protein/ OD of negative control) x 100%

2.5. Serology

Anti SARS-CoV-2 RBD IgG antibody were detected using Enzyme linked immunosorbent assay (ELISA) (Sunlong biotech, Cat num. SL321Hu_1, China). The procedure was done according to manufacturer’s protocol. Firstly, 100 ul of diluted samples and standard were added into appropriate wells and incubated for one hour at 37 C. Liquid was removed and 100 ul of biotin conjugated antibody (1X) was added into each well for 30 min at 37 C. The solution was removed and washed three times with 300ul of wash buffer (1x). The remaining liquid was removed by snapping the plate onto absorbent paper. 100ul of streptavidin conjugated HRP (1x) was added to each well and incubated for 30 min at 37 C. Then the washing process was repeated for 5 times. 90ul of TMB substrate was added to each well and incubated for 15 minutes at 37 C. Then 50ul of stop solution was added to each well and mixed by tapping the side of plate. The plate was read using microplate reader at 450 nm.

3. Results

3.1. SARS-CoV-2 cell mediated immunity started from one month and continued to eight months post-recovery

To assess virus specific cell mediated immunity, peripheral blood mononuclear cells were isolated from 122 convalescent health care workers (HCW) then treated with SARS-CoV-2 S1 protein
followed by MTT assay to measure the proliferation of SARS-CoV-2 -memory cells. SARS-CoV-2 cell mediated immunity was found to be developed in convalescent health care workers after one month post recovery and was established after eight months post recovery.

For 28 convalescent subjects at one month post recovery (17 non severe cases and 11 severe cases), the mean values of proliferation index (PI) with S1 protein were 55.53 and 17.25 for severe and mild-moderate cases, respectively and were significantly higher in severe cases ($P > 0.05$); this suggests development of specific SARS-CoV-2 immune cells as shown in figure 1.

For 39 convalescent subjects at eight months post recovery (20 severe cases and 19 non severe cases), the result showed that mean values of proliferation index with S1 protein for severe and mild-moderate cases were 114.34 and 140.13, respectively as shown in figure 2. This suggests an established SARS-CoV-2 specific cellular immunity after eight months from COVID 19 recovery.

![Figure 1. PI with S1 protein of one month recovered HCW](image-url)
3.2. Characteristics of SARS-CoV-2 cell mediated immunity in mild-moderate convalescent subjects

Cell mediated immunity in a total of 72 mild-moderate convalescent subjects were analyzed. As shown in figure 3. Mean values of lymphocytes proliferation index with S1 protein were 17.2553, 40.6836, 40.4574, and 140.13 for one month, three months, five months and eight months respectively. Intriguingly, Mean value of proliferation index for eight months were higher than mean value of PI of one month, three months and five months. Mean values of PI for three month and five month were higher than one month.

Then we compared the lymphocyte proliferation using boxplot. A boxplot is a standardized way of displaying the distribution of data based on a five number summary (“minimum”, first quartile (Q1), median, third quartile (Q3), and “maximum”). And it can tell about outliers and what their values are. Boxplot showed that the maximum and third quartile (Q3) were increasing steadily from one month to eight months post-recovery; however, the median, unlike the mean values, were not increasing steadily as shown in figure 4. However, there was insignificant difference between lymphocytes proliferation in one month and three month (p = 0.26), one month and five months (p = 0.18) and one month and eight month (p = 0.08).
Figure 3. Cell mediated immunity of mild-moderate COVID 19 recovered HCW

Figure 4. Lymphocytes proliferation of mil-moderate COVID 19 recovered HCW using a boxplot

3.3. Characteristics of SARS-CoV-2 cell mediated immunity in severe COVID 19 convalescent subjects:
Cell mediated immunity in a total of 50 convalescent severe subjects were analyzed. The mean values of PI with S1 protein were 55.5325, 222.3081, 97.4961 and 114.3402 for one month, three months, five months and eight months, respectively. Lymphocytes proliferation index for three month was higher than PI of one month, five months and eight months as shown in figure 5. There was insignificant difference between one month and three months (p = 0.4) and one month and five months (p = 0.41) and one month and eight months (p = 0.3).

Then CMI of severe cases were compared using a boxplot. The result showed that the maximum and Q3 of lymphocytes PI were highest in 5-month followed with 8-month groups as shown in figure 6.

Figure 5. PI with S1 protein of severe COVID 19 recovered HCW
Figure 6. PI with S1 of severe COVID 19 recovered HCW using a boxplot

3.4. Convalescent COVID 19 health care workers exhibited persistent anti RBD IgG antibodies till 8 months after recovery

In order to investigate antibody response against SARS-CoV-2 over time, a total of 181 samples were obtained from symptomatic COVID 19 health care workers collected after one month, three months, five months and eight months post recovery.

The serum samples were analyzed for IgG recognizing RBD of spike SARS-CoV-2 using a quantitative indirect ELISA. The mean values of anti RBD IgG for one month, three months, five months and eight months were 4.86, 3.8, 4.48 and 4.35, respectively. Antibody titer was sustained at high levels for eight months post recovery. However, mean values of serum SARS-CoV-2 IgG were not significantly different with post-recovery time (P > 0.05) as shown in figure 7.
Figure 7. Anti RBD antibody of 1, 3, 5 and 8 months of COVID 19 recovered HCW

3.5. Severe convalescent COVID 19 HCW exhibited persistent high mean anti RBD IgG antibody levels and increasing trend of maximum and third quartile levels

Antibody titer of a total of 79 severe convalescent HCW were analyzed. The mean value of antibody titer for one month, three months, five months and eight month were 5.03, 4.47, 3.73, and 4.76, respectively. Intriguingly, post-recovery groups of severe convalescent subjects showed approximately similar levels of anti RBD IgG as shown in fig. 8. There was insignificant difference in mean level of anti RBD IgG between one month and three month groups (p = 0.71), one month and five months groups (p = 0.36) and one month and eight month groups (p = 0.85) Suggesting that antibody titer in severe health care worker were not affected much by post COVID 19 recovery time. However, the maximum and Q3 values of anti RBD IgG were strangely found to have an increasing trend with post-recovery duration as these values were higher in 8-months and 5-months than earlier time intervals as shown in figure 9.
Figure 8. Anti RBD IgG titer of severe convalescent COVID 19 HCW

Figure 9. Anti RBD IgG titer of severe convalescent HCW using a boxplot
3.6. Mild-moderate convalescent HCW exhibited persistent moderate mean values of anti RBD IgG antibodies with comparable maximum and third quartile values

Antibody titer of a total of 102 convalescent HCW were analyzed. Intriguingly, mild groups showed approximately similar amount of anti RBD IgG. The mean values of antibody titer were 3.7925, 3.7265, 4.4353 and 3.7785 for one month, three months, five months and eight months, respectively as shown in figure 10.

There was insignificant difference between one month and three months (p = 0.88), one month and five months (p = 0.35) and one month and eight months (0.91). For the boxplot analysis, again the median, maximum and third quartile values were close to each other over the time intervals from 1 to 8 months post recovery as shown in figure 11. These findings indicate that humoral immunity in mild-moderate convalescent HCW were not waning quickly along with post COVID 19 recovery time.

![Figure 10. Anti RBD IgG titer of mild-moderate convalescent COVID 19 HCW](image-url)
Figure 11. Anti RBD IgG titer of mild-moderate convalescent COVID 19 HCW using a boxplot

3.7. The interplay between disease severity and cell mediated immunity

SARS-CoV-2 CMI in a total of 50 severe cases and 72 mild-moderate cases were analyzed. Using Anova test, PI was compared between mild-moderate and severe cases over the post-recovery time intervals, the findings showed that lymphocytes proliferation was insignificantly correlated with disease severity (p=0.36). As demonstrated in figure 12.

Nevertheless, by using T test, CMI of mild-moderate and severe cases in each time of study were analyzed. In one month (11 severe cases versus 17 mild-moderate cases), the result showed mean value of PI for severe cases (55.53) were significantly higher than mean value of PI for mild-moderate cases (17.25) (p = 0.05) as shown in figure 13.

In three months (15 mild-moderate and 9 severe), the result showed severe cases had insignificantly higher lymphocytes proliferation (p = 0.27) as noticed in figure 14.

In five months (21 mild-moderate cases and 10 severe cases), the result showed severe cases had insignificantly higher lymphocytes proliferation than mild cases (p = 0.17) as showed in figure 15.
In eight months (20 severe cases and 10 mild-moderate cases), the result showed severe cases were insignificantly had lower PI with S1 protein ($p = 0.68$) as shown in figure 16.

![Graph showing PI with S1 protein for different severity levels over time](image1)

Figure 12. PI with S1 protein of mild-moderate and severe convalescent COVID 19 HCW

![Graph showing PI with S1 protein for mild and severe convalescents](image2)

Figure 13. PI with S1 protein of mild-moderate and severe of one month convalescent COVID 19 HCW
Figure 14. PI with S1 protein of mild-moderate and severe of three months convalescent COVID 19 HCW

Figure 15. PI with S1 protein of mild-moderate and severe of five months convalescent COVID 19 HCW
Figure 16. PI with S1 protein of mild-moderate and severe of eight months convalescent COVID 19 HCW

3.8. The interplay between disease severity and the titer of SARS-CoV-2 anti RBD IgG

Antibody titers of 102 mild-moderate cases and 79 severe cases were compared. These cases based on the time of recovery were divided into four groups: one month post recovery (22 non-severe cases and 20 severe cases), three months post recovery (23 non severe cases and 20 severe cases), five months post recovery (31 non severe cases and 19 severe cases) and eight months post recovery (26 non severe cases and 20 severe cases).

Using T test, antibody titer of mild-moderate and severe cases were compared in each group. There was no significant higher antibody response in severe cases in one month (p = 0.36), three months (P = 0.29), five months (p = 0.3) and in eight months (0.09). as shown in figures 17, 18, 19, 20.
Figure 17. Antibody titers of mild-moderate and severe cases of one month post recovery

Figure 18. Antibody titer of mild-moderate and severe cases of three months post COVID 19 recovery
Figure 19. Antibody titer of mild-moderate and severe cases of five months post COVID 19 recovery.

Figure 20. Antibody titer of mild-moderate and severe cases of eight months post COVID 19 recovery.
4. Discussion

SARS-CoV-2 is globally spread and in the absence of effective treatment, it is critical to understand the key features of adaptive immunity to develop effective strategies to control on pandemic. Yet the knowledge about adaptive immunity remains limited. Here we report two branches of adaptive immunity, cell mediated immunity (CMI) via MTT assay and humoral immunity by indirect ELISA in convalescent health care workers (HCW) after mild, moderate and severe COVID-19 disease.

It was observed that PBMC proliferation with S1 protein was at higher level compared with PBMC proliferation without S1 protein. These finding suggest S1 protein is a strong stimulator to immune cells. A previous report stated that T cells produce abundant levels of IFN-Y once exposed to SARS-CoV-2 RBD (26). So, S1 protein is suited to detect and analyze SARS-CoV-2 immune response. The SARS-CoV-2-specific CMI was remarkably primed after one month from recovery. Data from previous research found that SARS-CoV-2-specific T cells are detected in the blood of COVID-19 patients and in recovery patients even in the absence of SARS-CoV-2-specific circulating antibodies (27,28) these data are consistent with our results suggesting that these cells most likely have a pivotal role in the protection and recovery from COVID-19.

The current study indicated that there is a significant proliferation of peripheral blood lymphocytes with S1 protein compared with lymphocytes proliferation without S1 protein in convalescent subjects after eight months from recovery. Suggesting the potential of SARS-CoV-2 infection in generating long term immunity against the virus.

The difference in lymphocytes proliferation with S1 protein among study intervals (1, 3, 5, and 8 months post-recovery) in mild-moderate convalescent HCW was insignificant, the increasing trend in the level of SARS-CoV-2-specific lymphocytes was evident. Similar to mild-moderate cases, the severe convalescent groups showed consistently high with an increasing trend of cellular immunity specific to SARS-CoV-2 cells. Consistent with other studies, T cells were found to generate in asymptomatic as well as severe COVID-19 cases with production of high level of antiviral cytokines and cytotoxic activities (29,30). And high levels of T cells demonstrated in peripheral blood of severe COVID-19 patients with acute respiratory distress syndrome along with production of remarkable levels of IFN y and TNF-α in response to viral antigen (31). Altogether,
the findings of the current study prove that convalescent HCW were in continuous re-exposure to the virus without necessarily development of symptoms. This explains why the cellular immunity in convalescent HCW was increasing instead of waning with time after infection. And this shows that SARS-CoV-2 memory cell have a role in preventing symptomatic re-infection/lowering the severity of disease for at least 8 months after primary symptomatic infection.

In one month, severe cases showed significant higher lymphocyte proliferation than in mild-moderate cases (P=0.05). In three months, five months and eight months intervals, lymphocyte proliferation of severe cases was a bit higher than in mild-moderate cases, but statistically this difference was not significant.

These findings indicate that cellular immunity has a role in COVID 19 severity and the severity of the disease affects as well the course of activation of the cellular immunity. It was found that viral antigens of SARS-CoV-2 proportionally activate alveolar macrophages that trigger production of a wave of pro-inflammatory cytokines and chemokines such as IL 6 and IFN y that in turn activate T cells to destroy viral infected cells (32). However the difference in the activation level of cellular immunity between severe and mild-moderate groups was not remarkable; this might be attributed to the fact that most severe cases that participated in this study were not critical cases and many of them had saturation of oxygen above 80% who may mount immune response similar to moderate cases.

Anti-spike SARS-CoV-2 antibody were detected in COVID 19 recovered patients (33). These antibodies may block the binding of RBD to ACE2 receptor and prevent entry of virus to the cells suggesting these antibodies have a role in recovery from disease and exert neutralizing effect. It is crucial to measure the level and duration of anti RBD antibodies in recovered COVID-19 patients.

A recent research to understand the longevity of humoral immunity observed that anti RBD IgG were detected in convalescent subjects after eight months post onset symptoms and the level of these antibodies decrease over time(34). In our study, anti RBD IgG antibodies in convalescent health care worker were at high levels after one month, three months, five months and eight months post recovery from symptomatic COVID 19. The level of anti RBD IgG antibodies among these different post-recovery time groups was not significantly different (p > 0.05).
In mild moderate convalescent subjects, it was observed that level of anti RBD IgG antibodies was approximately consistent and similar from 1 to 8 months post recovery. Also results analysis using boxplot showed that the median, third quartile and maximum values were close to each other in the studied recovery groups.

And in results analysis of the level of anti RBD IgG antibodies in convalescent severe cases, antibodies level was found to be consistently high with insignificant difference among 1, 3, 5, and 8 month groups; nevertheless, increasing trend of maximum and third quartile values in five months and eight months was found when compared to 1 and 3 months groups.

In consistent with other research, the duration of humeral immune response in mild moderate COVID-19 subjects was found to persist 4-8 months post infection. This is an important finding as anti RBD IgG antibodies were found to be correlated strongly with neutralizing antibody (35,36) and the current study results indicate that the studied recovered HCW subjects maintained high level of anti RBD IgG antibodies for at least 8 months which is a long period of time in term of Cornoaviridae-triggered humeral immune response. This might be attribute to the fact that recovered HCW are in continuous exposure to re-infection inside hospitals setting and multiple asymptomatic reinfections might be the reason behind the persistence of anti RBD IgG antibodies level high over 8 months after recovery. However, these results might not fit with recovered COVID-19 patients other than those HCW working in hospitals.

Both humeral and cellular immunity, screened in COVID-19 recovered HCW, found to persist at high levels for at least 8 months post-recovery. This might not apply to people who do not work in health care sector. The findings of the current study indicate that COVID-19 recovered or vaccinated HCW might not need additional doses of vaccination as like as people outside of health care sector. This actually needs further research to prove or refute this conclusion.

There is a notion that severe COVID 19 have higher viral titer and higher viral antigens leading to stronger inflammation and stronger humeral immune response especially in patients who need hospitalization (37,38). In the current study, this notion was not clear may be due to the fact that most of the involved severe HCW had short hospitalization and not critical condition. In addition, the number of the participants may not be as high as needed to reach statistical significance of difference of IgG antibodies between mild and severe cases. Overall, this study provided evidence
that mild, moderate and severe cases of recovered HCW exhibit good immunity for a relatively long time post recovery.

Taken together, the findings of the current study reveal that the convalescent health care workers with mild, moderate and severe disease exhibit a good with relatively long term humeral and cellular immunity to SARS-CoV-2 and this immunity could last more than eight months after recovery. This indicates a robust adaptive immunity which is developed by the natural infection.

Acknowledgments

We would like to thank all convalescent health care workers for their donation to participate in this study.

Funding

None

 References


5. Letko M, Marzi A, Munster V. Functional assessment of cell entry and receptor usage for


