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Analysis of Differences in Lymphocyte Subsets and CD4+ T Cells Immune Activation Between Re-detectable Positive and Non-re-detectable Positive COVID-19 Convalescent Patients

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1 Original submission

1 comment · 25 Nov 2023

Mahdi Shabani Reviewer

MAJOR REVISION 25 Nov 2023

In this study, the authors investigated the profile of CD4+ and CD8 T cells, B cells, and NL cells in 11 healthy controls and 66 RP (re-detectable positive) and NRP (non-re-detectable positive) COVID-19 convalescent patients. In addition, anti-SARS-CoV2 RBD IgG was tested in the included subjects. The design of the study could be interesting; however, the main goal of the study is not clear and needs to be explained further. Each patient's molecular test results were checked 2 days before discharge. Among 66 COVID-19 patients, 22 and 44 were NRP and RP, respectively. Why is the number of RP patients so high? Most COVID-19 patients tested negative on molecular testing after 14 days had elapsed from symptom onset. What control is used to avoid false positives in real-time PCR? There are a few points that also need to be addressed by the authors:

1- What is the novelty of the work? There are some publications on this topic, as listed below:
• BMC Infect Dis 21, 646 (2021). https://doi.org/10.1186/s12879-021-06278-2.
• Front. Immunol., 21 September 2023, https://doi.org/10.3389/fimmu.2023.1223730
• Ann Transl Med. 2020 Sep;8(18):1145. doi: 10.21037/atm-20-5479.
2- Please update the references. Most of them are from 2021-2022. In addition, please include the new reference in your discussion.
3- Please provide more detailed data on the real-time PCR results for SARS-CoV-2. Please present the CT of RP and NRP patients at 7 and 14 days. Please compare the fold change of the target gene in the studied groups.
4- The brief descriptions of real-time PCR and chemiluminescence tests in the MS could be informative.
5- Which programs were used for flow cytometry data analysis? Please present the gating strategy for the flow cytometry data as well as the representative data and plot of selected patients.
6- Please clarify the normality of the data in the statistical analysis.
7- What is the reference for subdividing the patients into mild, moderate, below symptoms, and above symptoms?
8- Please improve the level of the discussion.

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Revision 1

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Dear Reviewer:

Thank you for your letter and for the reviewers comments concerning our manuscript entitled “Analysis of differences in lymphocyte subsets and CD4+ T cells immune activation between re-detectable positive and non-re-detectable positive COVID-19 convalescent patients” (ID 140942).Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and have made correction which we hope meet with approval.Revised portion are marked in red in the paper. The main corrections in the paper and the responds to the reviewer’s comments are as flowing:

Q1: The primary objectives of the study on anti-SARS-CoV-2 RBD IgG are not yet clear and require further clarification.

R1: The aim of this study was to explore immunological factors related to RP. (It has been added to line 12 of the original text). Activated CD4+ T cells may play a crucial role in specific antibody responses. (It has been added to line 36/306 of the original text). Previous studies had found a significant correlation between SARS CoV-2-specific CD4+T Cell (%) and RBD-IgG, and the CD4+ T cells (%) is significantly higher in the RBD-IgG antibody response group compared to the non-response group. Therefore, we analyzed the correlation between immune cell subsets and RBD-IgG titers in COVID-19 convalescent patients, RP and NRP. (It has been added to line 289-292 of the original text).

Q2: Among 66 COVID-19 patients, 22 and 44 were NRP and PR, respectively. Why the number of PR patients is so high?

R2: From February 1 to May 5, 2020, Shenzhen's epidemic prevention and control policies required COVID-19 patients to undergo another 14-day centralized quarantine after discharge. Nucleic acid testing was conducted on days 1, 3, 7, and 14

of the quarantine period. If the nucleic acid test was positive and confirmed by laboratory review, the patient was classified as RP and required continued isolation and treatment. If all nucleic acid tests during the isolation period were negative, the patient was classified as NRP and could be released from isolation. According to management requirements, RP needed to continue isolation and monitoring, while NRP could be released from isolation. Therefore, the sample collection for recurrent cases was limited. The results of this study do not represent the recurrence rate. (It has been added to line 91 of the original text).

Q3: Most COVID-19 patients were negative in molecular testing after 14 days from symptoms onset. What is the control for avoiding false positives in the real-time PCR?

R3: On the day of sample collection, a commercial reagent kit (Zhongshan Daan Biotec) was used for Real-time RT-qPCR detection of the N and Orf1ab genes of SARS-CoV-2. The kit includes an endogenous internal standard for monitoring specimen collection, nucleic acid extraction, and PCR amplification processes. Additionally, 1 group of positive quality control and 3 groups of negative quality control were set in the test to avoid the occurrence of false positive results. After 45 cycles, the Ct value of N and Orf1ab genes ≤40 was considered positive. If a single gene is positive, the test needs to be repeated, and if the Ct value of the repeated test is still ≤40, it was judged positive. (It has been added to line 98-107 of the original text).

Q4: Novelty of the Paper.

R4: Previous studies on the characteristics of innate and adaptive immune responses in SARS-CoV-2 infection have helped us understand the potential pathogenesis of COVID-19. However, most studies have focused on the peripheral immune responses in clinical cases, especially severe COVID-19 patients , there is limited research on the immune features of re-detectable positive patients, and whether the reasons for re-detectable positive are related to differences in the immune function of COVID-19 convalescent patients needs confirmation. Currently, most studies focus on the clinical features and epidemiological data analysis of RP. Therefore, in this study, COVID-19

convalescent patients were divided into NRP and RP, with HCs as controls. The immune characteristics of the three groups were compared, and the correlation between RBD-IgG titers and CD4+ T cell activation markers in the case group was analyzed. (It has been added to line 51-55 and 249-260 of the original text).

Q5: Please update the references. Most of them are 2021-2022. In addition, please include the new reference in your discussion.

R5: The references have been updated, and new literature has been included in the discussion.

Q6: The brief descriptions of real-time PCR and Chemiluminescence tests in the MS could be informative.

R6: 3.2.1 Nucleic acid testing

On the day of sample collection, a commercial reagent kit (Zhongshan Daan Biotec) was used for Real-time RT-qPCR detection of the N and Orf1ab genes of SARS-CoV-2. The kit includes an endogenous internal standard for monitoring specimen collection, nucleic acid extraction, and PCR amplification processes. Additionally, 1 group of positive quality control and 3 groups of negative quality control were set in the test to avoid the occurrence of false positive results. After 45 cycles, the Ct value of N and Orf1ab genes ≤40 was positive. If the single-gene positive specimen is required, the test needs to be repeated, and if the Ct value of the repeat test is ≤40, it was considered positive. (It has been added to line 97-107 of the original text).

3.2.3 Antibody detection

IgG in the receptor-binding domain of the spike protein(S-RBD-IgG) on the surface of SARS-CoV-2 was detected by Chemiluminescence microparticle immunoassay (CMIA) technology (Caris200 automatic chemiluminescence instrument, Beijing Wantai Biotech), and determined by cut-off index (COI). The cut-off value for seropositivity is set according to the manufacturer's instructions, and samples with a COI of >1 are considered positive. The test has been certified by the National Medical Products Administration of China as an in vitro diagnostic reagent. (It has been added to line 118-125 of the original text).

Q7: Which programs were used for flow cytometry data analysis? Please present the gating strategy of flow cytometry data as well as the representative data and plot of selected patients.

R7: Data from flow cytometry were analyzed Diva 6 and flowjo10. 6.2. (It has been added to line 127 of the original text).

The flow cytometry gating strategy of lymphocyte subsets of HC, NRP and NR was shown in **Figure 1.** (Figure 1 was added. It has been added to line 170 of the original text).

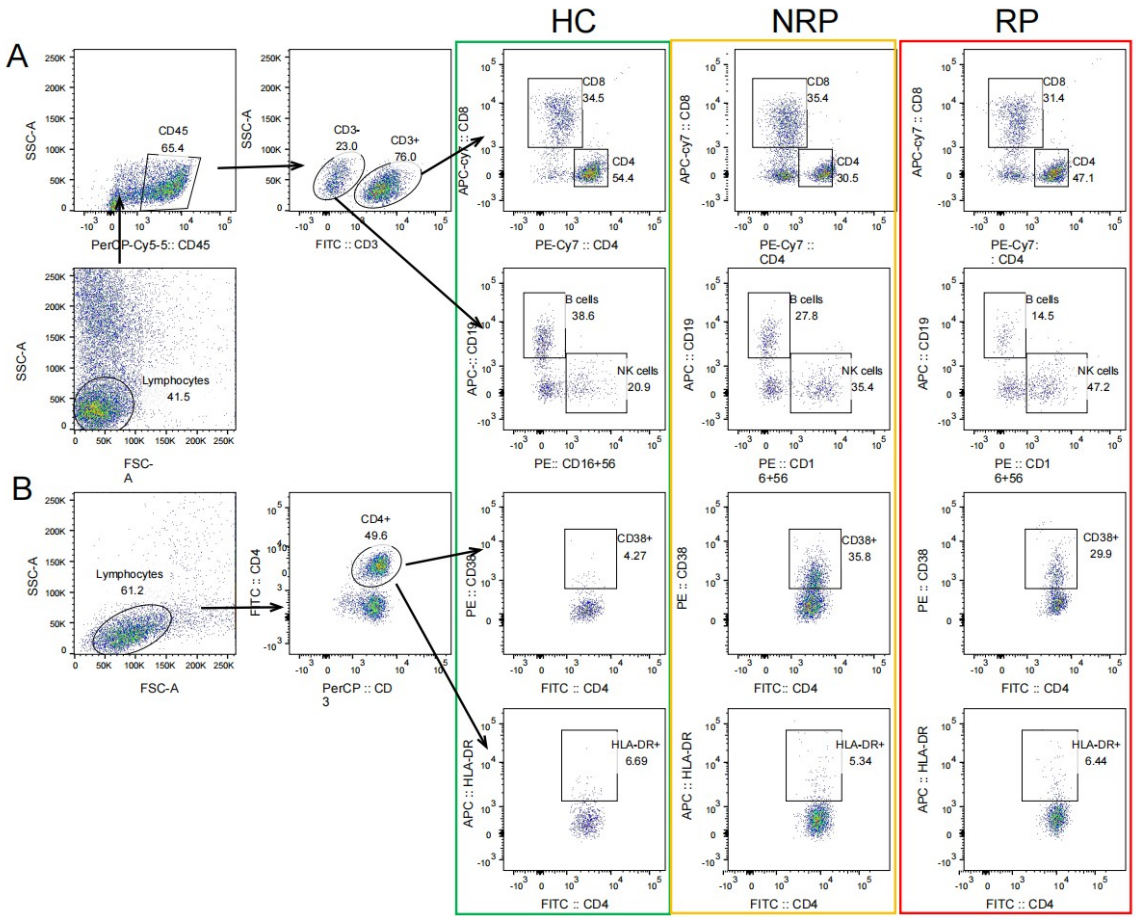


Figure 1A. Flow cytometry gating strategy of CD3+, CD4+, CD8+, B cells and NK cells in HC、NRP and RP; **Figure 1B**. Flow cytometry gating strategy of CD4+ CD38+ and HLA-DR+ in HC、NRP and RP.

Q8:Please clarify the normality of data in the statistical analysis.

R8: The S-W test was used to test the normality of continuous data, in which the percentages of CD3, CD4, CD8 and CD38 percentage met the normality distribution, and the comparison between the two groups was compared by bilateral unpaired student T-test, ANOVA test was used between the three groups, and LSD was used for post-hoc multiple comparison. The values of NK cells, B cells, CD4+HLA-DR+ percentage, and RBD-IgG did not satisfy normality, and the Mann-Whitney-U test was used for comparison between the two groups, the Crewskal-Wallis test was used for comparison between the three groups, and the significance values were adjusted by the Bonferroni correction method for multiple tests. (It has been added to line 135-143 of the original text).

Q9: What is the reference for subdividing the patients into mild, moderate, below symptoms, and above symptoms?

R9: The diagnostic criteria for symptom grading are based on the "Diagnosis and Treatment Guidelines for Novel Coronavirus Pneumonia in China(12)", categorizing the clinical severity at the first hospitalization into asymptomatic, mild, moderate, and severe. Based on this criterion, we classify patients into mild and below, and severe and above.(It has been added to line 92-96 of the original text).

Q10: Please amend the discussion to a higher level.

R10: The discussion section of the paper has been revised.

Previous studies on the characteristics of innate and adaptive immune responses in SARS-CoV-2 infection had helped us understand the potential pathogenesis of COVID-19 (13-15). However, most studies had focused on the peripheral immune responses in clinical cases, especially severe COVID-19 patients (16-17), there was limited research on the immune features of re-detectable positive patients, and whether the reasons for re-detectable positive are related to differences in the immune

function of COVID-19 convalescent patients needs confirmation. Currently, most studies had focused on the clinical features and epidemiological data analysis of RP(10-11). Therefore, in this study, COVID-19 convalescent patients were divided into NRP and RP, with HCs as controls. The immune characteristics of the three groups were compared, and the correlation between RBD-IgG titers and CD4+ T cell activation markers in the case group was analyzed.

Compared to NRP, the B cells (%) is lower in RP. Previous studies had found that T lymphocyte subsets participate in the specific immune response to SARS-CoV-2. B lymphocytes were associated with the production of specific antibodies and were a key component of the human immune system's fight against SARS-CoV-2 (15,18-19). This study found that in the three groups of HCs, NRP, and RP, the B cells (%) decreases sequentially, with the B cells (%) in RP significantly lower than in HCs (Fig 2B). Additionally, the B cells (%) in COVID-19 convalescent patients was also significantly lower than in HCs (Fig 2A). Andrei A et al. (20) found that, compared to HCs, patients at different stages of infection showed varying degrees of decrease in B cell numbers, with hospitalized patients had lower B cell numbers than convalescent patients. Combining the above results, we speculate that the percentage of B cells in RP was lower than in NRP, which may be related to the re-detectable positive of convalescent patients.

The CD4+CD38+ and CD4+HLA-DR+ T cells (%) in RP show significant differences compared to HCs and NRP. The CD4+CD38+ and HLA-DR+ T cells (%) in COVID-19 patients increased significantly after infection (21-23),this is consistent with our results (Fig 3A). However, the difference analysis of the two activation indicators among the three groups of HCs, NRP and RP has not been reported. We found that the CD4+CD38+ cells (%) in both RP and NRP were higher than those in HCs, and the CD4+CD38+ cells (%) in M-RP were also higher than those in S-RP (Fig 3C). Furthermore, the CD4+HLA-DR+ T cells (%) in RP was significantly higher than in HCs, and it was higher in M-RP than in single recurrent cases. Additionally, the CD4+HLA-DR+ (%) increased sequentially in the four groups of

HCs, NRP, S-RP, and M-RP (Fig 3). Li et al. (24) and Zhang et al. (25) had also demonstrated that peripheral blood in recurrent patients exhibits a higher inflammatory immune response. This suggests that compared to NRP, CD4+ T cells in RP remain in a highly activated state.

There was a significant positive correlation between the CD4+HLA-DR+ T cells (%) and RBD-IgG titer in COVID-19 convalescent patients. Previous studies had found a significant correlation between SARS CoV-2-specific CD4+T Cell (%) and RBD-IgG (26), and the CD4+ T cells (%) was significantly higher in the RBD-IgG antibody response group compared to the non-response group (27). Therefore, we analyzed the correlation between immune cell subsets and RBD-IgG titers in COVID-19 convalescent patients, RP and NRP. The results showed a significant positive correlation between the RBD-IgG titer and the CD4+HLA-DR+ T cells (%) in both the COVID-19 convalescent patients and NRP (Figure 4A, B). In the COVID-19 convalescent patients, the RBD-IgG titers in the HLA-DR^{+High} group were significantly higher than that in the HLA-DR^{+Low} group, suggesting that activated CD4+ T cells may play a crucial role in the specific antibody response.

Q11: Please specify more detailed data on Real-time PCR of SARS-CoV-2 results. Please present the CT of PR and NPR patients at 7 and 14 days. Please compare the fold change of the target gene in the studied groups.

R11: We are sorry that this analysis is impossible. During the monitoring process, results with CT values >40 are considered negative and are not included in the statistical analysis. Only when the CT value is ≤40, it is recorded. Therefore, each RP has only one accurate CT value record, and the CT values of NRP cannot be statistically analyzed, making it impossible to compare the fold change of the target gene in the studied groups.

Self-correction section

When the results were rechecked, the p-values of some of the results were modified, but there was no effect on the conclusions.

The P values of the three groups (ANOVA test and Crewskal-Wallis test) were adjusted the LSD and Bonferroni correction method for multiple tests, and the p values of some results were modified. P=0.017, 0.017, 0.014, 0.015, 0.013(line 174, 177, 182, 206 and 209)

ANOVA test was used between the three groups, and LSD was used for post-hoc multiple comparison. The percentages of NK cells, B cells, CD4+HLA-DR+ and RBD-IgG titer did not satisfy normality, and the Mann-Whitney-U test was used for comparison between the two groups, the Crewskal-Wallis test was used for comparison between the three groups, and the significance values were adjusted by the Bonferroni correction method for multiple tests. (It has been added to line 137-143 of the original text).

We would like to take this opportunity to thank you for all your time involved and this great opportunity for us to improve the manuscript. We hope you will find this revised version satisfactory.

Sincerely,

The Authors

2 Revision 1

0 comments

No public comments in this round.