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Assessment of changes in immune status linked to COVID-19 convalescent and its clinical severity in patients and uninfected exposed relatives

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ABSTRACT

Introduction: The immune response during and after SARS-CoV-2 infection can be complex and heterogeneous, and it can be affected by the severity of the disease. It can also contribute to an unfavorable evolution and bring about short and long term effects. The aim of this study was to characterize the lymphocyte composition according to the severity of COVID-19, as well as its degree of relationship to the specific humoral response to SARS-CoV-2 in convalescents up to 106 days after the infection and in their exposed relatives.

Methods: An applied research was carried out with a cross-section analytical design, from March 11 to June 11, 2020 in Cuba. The sample consisted of 251 convalescents from COVID-19 over 18 years of age and 88 exposed controls who did not become ill. The B and T cell subpopulations, including memory T cells, as well as the relationship with the humoral immune response against SARS-CoV-2, were identified by flow cytometry and enzyme immunoassay.

Results: Convalescent patients, who evolved with severe forms, showed a decrease in frequency and a greater proportion of individuals with values lower than the minimum normal range of B cells, CD3 + CD4 + cells and the CD4 + / CD8 + ratio, as well as a higher frequency and a greater proportion of individuals with values above the normal maximum range of CD3 + CD8 + and NK cells. Convalescent patients with severe forms of COVID-19 that exhibited IgG / RBD titers $\geq$ 1/200 had a lower frequency of TEMRA CD8 + cells ($p = 0.0128$) and TEMRA

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CD4 + (p = 0.0068). IgG / RBD titers were positively correlated with the relative frequency of CD4 + CM T memory cells (r = 0.4352, p = 0.0018).
Conclusions: The identified alterations of B and T lymphocytes suggest that convalescent patients with the severe disease could be vulnerable to infectious, autoimmune or autotinflammatory processes; therefore, these individuals need medical follow-up after recovering from the acute disease. Furthermore, the role of T cells CD4 + CM in the production of antibodies against SARS-CoV-2 is confirmed, and it is noted that the defect of memory T cells CD8 + TEMRA could contribute to the development of severe forms of COVID-19.

1. Introduction

The infection by the novel coronavirus SARS-CoV-2, (severe acute respiratory syndrome coronavirus 2) first reported by Huang et al. (2020), is a severe health problem worldwide that has produced the death of>5 million people until October 29, 2021 (Lu et al., 2020; MINSAP, 2021).

Within the immuno-pathology of the disease, it has not only been stated that the damage may be mediated by the virus itself, but that other factors are involved. These include a hyper-inflammatory immune response, (Chen and John Wherry, 2020; Kuri-Cervantes et al., 2020; Mathew et al., 2020), the exhaustion or dysfunction of T cells (Chen and John Wherry, 2020; Zheng et al., 2020), and the development of a cytokine storm (Kuri-Cervantes et al., 2020; Mathew et al., 2020). These elements are responsible for the large clinical spectrum of COVID-19 (Kuri-Cervantes et al., 2020; Mathew et al., 2020).

A post COVID-19 syndrome having a significant clinical repercussion has been described (Greenhalgh et al.,2020). It is expressed in that the dysfunction of the immune system may subsist for up to two years after viral infections (Wiedemann et al., 2020), the magnitude of the antibody and T cell response may be diverse, discordant, and it may be influenced by the severity of COVID-19 (Wiedemann et al., 2020). Several authors have shown that the response of the memory cells to SARS-CoV-2 will play an important role in the infection, pathogenesis and protection against the disease (Mathew et al., 2020; Sekine et al., 2020).

The study focus on the identification of the degree of alterations of the immune system that may favor the presence of sequels at the short- or long-term, in convalescents from SARS-CoV-2 (Greenhalgh et al.,2020; Shuwa et al., 2021).

The aim of this study was to characterize the lymphocyte composition according to the severity of COVID-19, as well as its degree of relationship to the specific humoral response to SARS-CoV-2 in convalescents up to 106 days after the infection and in their exposed relatives.

2. Materials and methods

2.1. Subjects

We carried out an applied research at the National Medical Genetics Center, Havana, Cuba, through a cross-section analytical design, with the patients diagnosed in Cuba with COVID-19 by means of real time polymerase chain reaction (RT-PCR) from March 11 to June 11, 2020.

The sample was selected by stratified sampling with proportional allocation method (Olayiwola Olaniyi et al., 2013), and it was finally formed by 251 **convalescent individuals** (asymptomatic convalescents (A) = 67, moderate convalescents (M) = 122, severe convalescent (S) = 62), that had been sick with COVID-19 (confirmed by RT-PCR for SARS-CoV-2 from nasopharyngeal swabs) and had got epidemiological discharge, which was defined as the presence of a negative RT-PCR to SARS-CoV-2, 14 days after the first negative RT-PCR (SARS-CoV −2) that granted the clinical discharge (Ministry of Public Health, 2021a; World Health Organization, 2020a) (see Supplementary material). The inclusion and exclusion criteria of the subjects to study were exposed in Table 1. The convalescent study time was from epidemiological discharge to blood collection for the evaluation with an average of 68 days (interquartile range of 55–77 days, minimum 20 days, and maximum 106 days) (Table 2).

The COVID-19 convalescents individuals were grouped according to the clinical severity of the infection by SARS-CoV-2: Asymptomatic convalescent (A): they experienced asymptomatic COVID-19; Moderate convalescent (M): they experienced mild or moderate COVID-19; Severe convalescent (S): they experienced a severe or critical form of COVID-19.

The COVID-19 disease severity was classified, according to the guidelines for clinical management of COVID-19 of the World Health Organization (World Health Organization, 2020a) and the guidelines of the national action protocol for COVID-19 (Ministry of Public Health, 2021a) as: **Asymptomatic disease**: when patients infected with SARS-CoV-2 (positive to RT-PCR), present no signs or symptoms of the disease (see symptoms associated to COVID-19 and Table S1 in Supplementary material); **Mild disease**: when patients (positive to RT-PCR) present symptoms of COVID-19, without evidence of viral pneumonia, hypoxia or others complications; **Moderate disease**: when patients infected with SARS-CoV-2 (positive to RT-PCR) present clinical signs and imagines of pneumonia, but without signs of severity and with oxygen saturation as measured by pulse oximetry (SpO2) ≥ 90% on room air**; Severe disease**: when patients (positive to RT-PCR) present clinical signs of pneumonia (fever, cough, dyspnea, fast breathing) plus one of the following: respiratory rate > 30 breaths/min; severe respiratory distress; or SpO2 < 90% on room air; **Critical disease**: when patients (positive to RT-PCR) present the Acute Respiratory Distress Symptom (ARDS), sepsis, septic shock, multiple organ dysfunction or other severe complications (Table S1 in Supplementary material).

Table 1
Inclusion and exclusion criteria of the subjects of study.

	Convalescents Patients	Uninfected Exposed individuals
Inclusion criteria	- Age > 18 years - Of both sexes -Who accepted their participation in the study	
	- That had been sick with COVID-19 confirmed by RT-PCR for SARS-CoV-2.	- That were living in close contact** to a first-degree relative (mother, father, children) having COVID-19.
	-That had Epidemiological discharge.*	-They did not test positive to two RT-PCR for SARS-CoV-2 done in the quarantine period (14 days) since their convalescent relative was diagnosed with COVID-19. - These exposed individuals were also negative to specific antibodies against SARS-CoV-2.
Exclusion criteria	-The individuals who were not Cuban residents -The individuals who were residing outside the health area at the time of inclusion in the study. -Deceased	

*__Epidemiological discharge__: Defined as the presence of a negative RT-PCR to (SARS-CoV −2), 14 days after the first negative RT-PCR to (SARS-CoV-2) that granted the clinical discharge (Ministry of Public Health, 2021a; World Health Organization, 2020b). **__Close contact:__ Defined as a person who lived and had been in contact or within a close distance (<1.0 m) to an individual(s) with COVID-19 in a confined space for > 24 h, from 2 days before and up to 14 days after the onset of symptoms. (Ministry of Public Health, 2021a; World Health Organization, 2020b)

A group of 88 **uninfected exposed individuals** (Exposed), who were living in close contact to a first-degree relative (mother, father, children) having COVID-19, was included (Table 1 and Supplementary material). Blood samples for the study of the uninfected exposed individuals were collected the same day it was collected from their first-degree relative convalescent (minimum 42 days, and maximum 107 days from the diagnoses the convalescent relatives).

A face-to-face interview was carried out for the collection of clinical, epidemiological and social data of the patients, given directly by the patients or by their legal tutors, in the case of intellectual disability.

The variable "Duration of the disease" was defined as the time lapse between the diagnosis by RT- PCR for SARS-CoV-2 infection and the first negative RT-PCR as part of the criteria for clinical discharge (Tabla 2).

Demographics and clinical information for convalescent patients can be found in Tables 2.

2.2. Immunotyping using flow cytometry

Cellular immunotyping through flow cytometry (8 color Gallios flow cytometry, Beckman Coulter, France) was made using the peripheral blood obtained by venipuncture, with the use of the K2-EDTA anticoagulant and the lysing solution VersaLyse (BeckmanCoulter, France). According to the recommendations of the manufacturer (Beckman Coulter, France), we used the red blood cell lysis without washing. To each volume of the conjugate, we added 100 μL of blood, mixed for 3 s, and incubated it in a dark chamber for 15 min. at room temperature. Then we added 1 mL of the lysis buffer VersaLyse TM (Beckman Coulter, France) and incubated it for 10 min under the same conditions as the previous step. Finally, we immediately proceeded to the acquisition of the sample by the cytometer. The acquisition of the data was carried out through the Kaluza Acquisition v1.0 software by which we obtained a minimum of 50,000 total events. For the analysis and the report of the results we used Kaluza Analysis v1.5a. The absolute cell counts of the lymphocyte populations were performed through a dual-platform. We designed a manual and sequential window selection strategy with bi-parametric graphs (Fig. S1). For the reference values of the analyses of cellular sub-populations we used previous studies in the Cuban population (Kokuina et al, 2019).

2.2.1. Identification of B, T, NK cells

We quantified the CD 19 + lymphocytes (B cells), T CD3 + lymphocytes, T CD3 + CD4 + lymphocytes, T CD3 + CD8 + lymphocytes and CD56 + CD3- cells (NK cells). A polychromatic flow cytometry tube was used for peripheral lymphocyte immunotyping developed at the immunology laboratory of the National Medical Genetics Center (Zúñiga Rosales et al., 2020). The monoclonal antibodies conjugated with fluorochromes from MACS MiltenyiBiotec (Germany) included anti-CD45 APC-Vio770 (Clone 5B1), anti-CD19 PE-Vio700 (Clone LT19), anti-

Table 2
Demographic characteristics of Cuban individuals who suffered from COVID-19 up to three months after the SARS-CoV-2 infection, according to clinical severity and exposed relatives.

	CONVALESCENTS / COVID-19				EXPOSED RELATIVES
	Total n (%)	Asymptomatic n (%)	Moderate n (%)	Severe n (%)	Total n (%)
Distribution according to age					
Total	251 (100)	67 (26.7)	122 (48.6)	62 (25.10)	88 (100)
19–29 years old	26 (10.4)	9 (13.4)	12 (19.7)	5 (8.1)	25 (28.4)
30–39 years old	40 (16)	14 (20.9)	24 (23.8)	2 (3.2)	14 (16.0)
40–49 years old	51 (20.3)	15 (22.4)	29 (23.8)	7 (11.3)	21 (23.9)
50–59 years old	61 (24.3)	17 (25.4)	29 (23.8)	25 (40.3)	19 (21.6)
≥60 years old	73 (29)	12 (17.9)	28 (22.9)	33 (53.2)	9 (10.2)
60–69 years old	34 (13.5)	9 (13.4)	13 (10.6)	12 (19.3)	3 (3.4)
70–79 years old	19 (7.6)	2 (3)	6 (4.9)	11 (17.7)	3 (3.4)
≥80 years old	20 (7.9)	1 (1.5)	9 (7.4)	10 (16.1)	3 (3.4)
Age in years, median (IQR)	51 (39–63)	48 (34–57)[b1]	49 (30–59)[b2]	64 (51–73)	28.3 (28–54)
Females, n (%)	142 (56.6)[a]	38 (56.7)	67 (54.9)	37 (59.6)	66 (75.0)
Males, n (%)	109 (43.4)	29 (43.2)	55 (45)	25 (40.3)	22 (25.0)
Comorbidities					
High blood pressure	106 (42.2)	22 (20.7)	47 (18.7)	37 (14.7)	24 (27.3)
Obesity	21 (8.4)	3(14.3)	12 (57.1)	6 (28.6)	10 (11.4)
Diabetes mellitus	34 (13.5)	6 (17.6)	13 (38.2)	15 (44.1)	10 (11.4)
Cardiovascular diseases	23 (9.17)	1 (0.03)	9 (3.6)	13 (5.2)	3 (3.4)
Chronic pulmonary disease	43 (17.1)	12 (27.9)	19 (44.2)	12 (27.9)	0
Immunodeficiencies	10 (4)	3 (30)	3 (30)	4 (40)	1 (1.1)
Autoimmune diseases	14 (5.6)	5 (35.7)	3 (21.4)	6 (42.8)	4 (4.4)
Cancer n (%)	4 (1.6)	1(25)	1(25)	2(50)	1 (1.1)
Symptoms of the disease					
Fever	101 (40.2)	0	62 (61.4)	39 (38.6)	–
Coughing	69 (27.5)	0	45 (65.2)	24 (34.8)	–
Myalgia	2	0	2	0	–
Fatigue	55 (22)	0	35 (63.6)	20 (36.4)	–
Anosmia	2	0	2 (100)	0	–
Loss of taste	48 (19.1)	0	40 (83.3)	8 (16.7)	–
Diarrhea	4 (2)	0	2 (50)	2 (50)	–
Breathlessness (dyspnea)	61 (24.3)	0	30 (49.2)	31 (50.8)	–
Duration of the disease* (in days), median (IQR)	16 (14–19.5)	16 (14–18)	16 (14–18.5)	17 (14–24)[c]	–
Convalescent study time ** (in days), median (IQR)	68 (55–77)	64.5 (47.2–74.0)	68.5 (54–78.3)	68 (59.5–78.5)[d]	–

Legend: [a]: statistical significance using proportion comparison between both sexes in the total number of COVID-19 convalescent individuals. [b1]: statistical significance between asymptomatic patients and those severely ill, [b2]: statistical significance between moderately ill and severely ill convalescent patients, in both cases $p < 0.0001$ identified through the Mann-Whitney test. [c]: statistical significance between the duration of the disease in persons with moderate and severe forms of COVID-19 through the Mann-Whitney test. [d]: statistical significance between the duration of convalescence in individuals with asymptomatic and severe forms of COVID-19 identified through the Mann-Whitney test. * Duration of the disease: was defined as the time lapse between the diagnosis made by PCR-RT of SARS-CoV-2 infection and the first negative PCR as part of the criteria for clinical discharge. ** Convalescent study time: was defined from epidemiological discharge until day of blood sampling. IQR: interquartile range. For all tests, statistical significance was considered as $p < 0.05$.

CD3 FITC (Clone BW264/56), anti-CD4 PerCP-Vio700 (Clone M−T466), anti-CD8 APC (Clone BW135/80), anti-CD56 PE (Clone REA196), (Fig. S1).

2.2.2. Identification of the memory and naive cells

We identified the memory cells as: central memory: CM, CD45RA-CD27+, effector memory: EM, CD45RA-CD27−, terminally differentiated T effector cells (TEMRA, CD45RA + CD27 −) and naïve cells (CD45RA + CD27 +). The monoclonal antibodies used were CD8-PE-Cy7 (invitrogen, eBioscience, clone SK1), CD45 RA APC- eF780 (invitrogen, eBioscience, clone HI100), CD3/FITC (Clone BW264/56), Anti-CD27 APC, eBioscience, clone 0323, San Diego, CA, anti-CD127-PE, BD Pharmingen, Clone HIL-7R-M21, BD Biosciences (Fig. S1).

2.3. Qualitative determination of total antibodies anti-SARS-Cov-2 in the serum

We carried out the determination in the serum of total antibodies against SARS-CoV-2 through a double antigen sandwich-type ultra-immune-enzymatic assay (UMELISA ANTI SARS-CoV-2) that was standardized and validated at the Immuno-assay Center of Cuba (CIE, according to its Spanish acronym). The SARS-CoV-2 antigens were fragments from the spike protein (S) and the nucleocapsid (N) of SARS-CoV-2. (Supplementary material).

2.4. Detection of antibodies anti-RBD in the serum of patients

At the Cuban Center of Molecular Immunology (CIM, according to its Spanish acronym), we quantified the total IgG specific RBD antibodies in the serum of patients using an enzyme-linked immunosorbent assay (ELISA). The plates were coated with RBD-mFc and incubated with serial dilutions of serum samples, starting at 1:100. The experimental titers of IgG were determined (Supplementary material).

See the supplementary material for particulars of the methods.

2.5. Statistical analysis

The normal distribution of the quantitative variables was verified using the Shapiro-Wilk test. To describe the quantitative variables, the estimates were made through the median and interquartile ranges (IQR) or the mean and standard deviation, as appropriate. The 95% confidence intervals were also calculated.

To assess the statistical significance of the association between qualitative variables and the comparison of proportions between each convalescent group and between those and the exposed group the Fisher's exact test was used.

The Mann-Whitney *U* test was used for comparisons between two groups for the analysis of cell subpopulations by flow cytometry and anti-SARS-CoV-2 antibody levels. The correlation between the flow cytometric variables (cell subpopulations CD3, CD4, CD8, CD19, NK and memory cells CM, EM and TEMRA) and the IgG / RBD titers and total antibodies against SARS-CoV-2, was performed using the Spearman's rank correlation.

Using the IBM SPSS Statistics software (version 22), we carried out multivariate logistic regression analyses to evaluate the influence of age, severity and duration of the disease (we adjusted age) on the variables: CD19+, CD3+, CD3 + CD4+, CD3 + CD8+, NK. We also used the GraphPad Prism 7 (GraphPad Software, California, USA). We consider that there is statistical significance when $p < 0.05$.

2.6. Ethical issues

The research was carried out under the compliance of the regulations of the Helsinki Declaration of 2013 (World Medical Association, 2013). All cases participants in the research signed the informed consent before accepting their participation. This study is part of a research project approved by the Ethics and Research Committee of the National Medical Genetics Center, and by the advisory committee of the Ministry of Public Health of Cuba.

3. Results

3.1. Demographic and clinical characteristics according to the clinical severity of COVID-19 patients who were epidemiologically discharged

The sample was of 251 individuals who had been ill with COVID-19, and in the group of exposed persons we included 88 first-degree relatives who were exposed to the virus and did not become ill (Table 2).

The clinical forms of COVID-19, from mild to moderate, were more frequent in the convalescents (48.6%), followed by individuals with asymptomatic forms of the disease (67%) ($p < 0.0001$; 95% CI: 13.5–29.9) (Table 2). Females were predominant ($n = 142$, 56.6%, $p = 0.0385$, 95% CI: 0.73–25.1) within all patients having COVID-19 (Table 2).

The median of age was higher ($p < 0.0001$) in patients with severe forms of the disease compared to asymptomatic individuals ($p < 0.0001$) and moderate ($p < 0.0001$) (Table 2).

In convalescents that had severe forms of COVID-19, the time lapse between the diagnosis by RT- PCR of SARS-CoV-2 infection and the first negative PCR of the disease was slightly longer compared to patients with moderate COVID-19 symptoms ($p = 0.0313$) (Table 2). The Convalescent study time (adopted for the study was from epidemiological discharge until day of blood sampling collection) was of 68 days (IQR: 55.0–77.0 days, minimum 20 days, and maximum 106 days) (Table 2).

3.2. Immunotype of B, T and NK cells in convalescents according to the clinical forms of COVID-19

The absolute lymphocyte count was lower in convalescent people that had asymptomatic forms (A) and moderate forms (M) of the disease compared to the Exposed group that was not infected (Fig. 1). The severe convalescent patients (S) had a higher proportion of individuals with an absolute lymphocyte count (14.5%) higher than the normal reference value compared to the A (3.0%, $p = 0.0184$) and M (5.7%, $p = 0.0464$) groups (Fig. 2).

In the S group compared to the non-severe convalescent groups (A and M) and to those Exposed, we observed a lower relative and absolute frequency of CD19+, a lower median of relative frequency of CD3 + CD4 + and of the ratio CD4+/CD8+, as well as an increase of the median of the relative frequency of CD3 + CD8 + and NK (Fig. 1). The S group also showed (compared to A and M) an increase of the median of the absolute frequency of CD3 + CD8 + and NK (Figura1). The A and M groups had lower median of the absolute frequency of total lymphocytes, CD3+, CD3 + CD4 + and CD3 + CD8 + compared to those exposed (Fig. 1).

In another analysis we observed a higher proportion of S (21.0%) that showed values below the minimum normal of the median of the relative frequency of CD19 + compared to those of A (4.5%, $p = 0.0047$), M (5.7%, $p = 0.0018$) and the exposed group (Exposed: 5.7%, $p = 0.0047$). We also observed that the S group had a higher proportion of individuals (24.0%) with a median of the absolute frequency of CD19+, lower than the median reference value compared to the M (11.5%, $p = 0.0257$) and to the exposed (5.7%, $p = 0.0047$) groups. We also identified a higher proportion of S with a median of the relative frequency (8.1%, $p = 0.0182$) and absolute frequency (6.5%, $p = 0.0345$) of CD3 + CD4+, lower than the median minimum normal reference value compared to the A (0%) group. At the same time, the median of the absolute frequencies of CD3 + and CD3 + CD4 + were lower than the median minimum reference value in a larger proportion of the S group (8.1%, $p = 0.0047$) compared to the exposed persons (0%), with the same percentages in both sub-populations (Fig. 2).

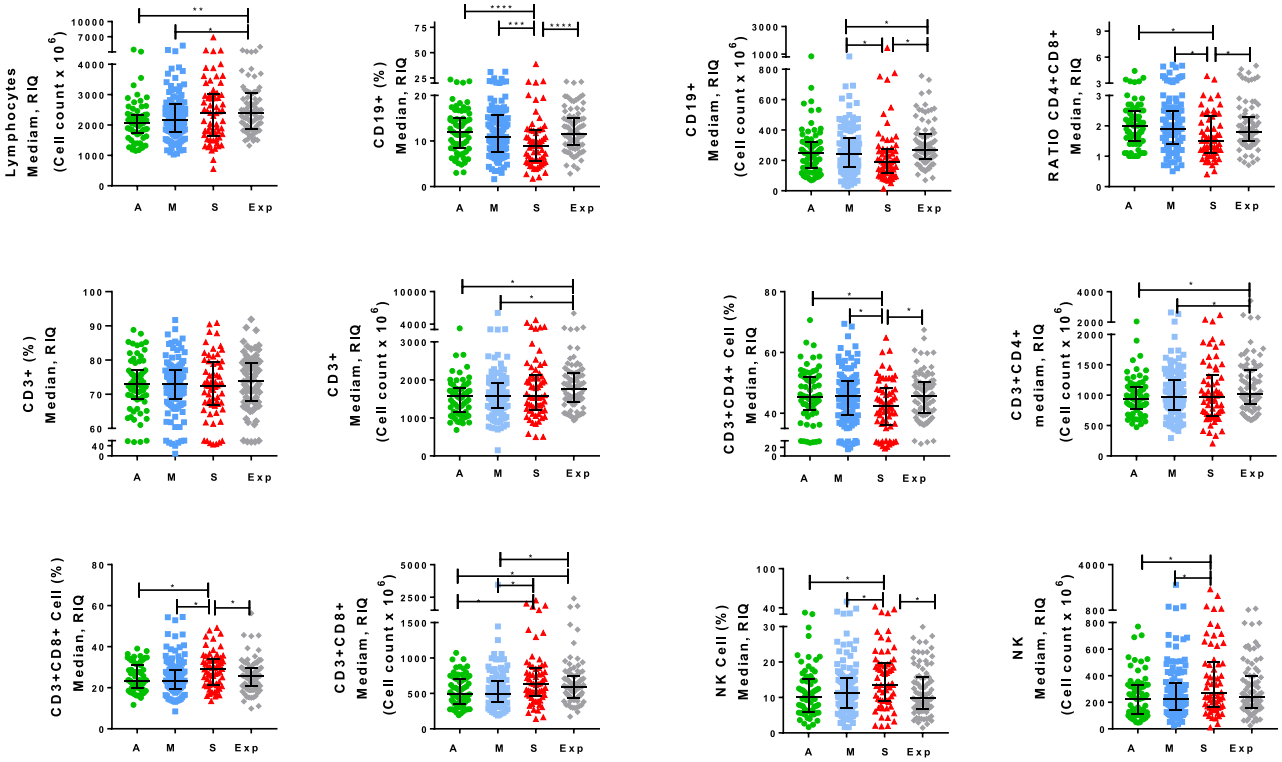

Fig. 1. Cellular immunotypes through flow cytometry in Cuban patients who suffered from SARS-CoV-2 infection according to clinical severity and in the controls. The multi-parametric analysis (relative and absolute frequencies) is represented through the flow cytometry of the cellular sub-populations, CD19+, CD3+, CD3 + CD4+, CD3 + CD8+, the ratio CD4+/CD8 + and the NK cells of COVID-19 convalescent individuals of non-infected exposed persons. Each point represents an individual, the asymptomatic patients (A, green, n = 67), those with moderate symptoms (M, blue, n = 122), and the severely ill patients (S, red, n = 62), as well as those exposed who did not become sick (Exp, gray, n = 88). The analyses between the groups were made using the Mann-Whitney test with a level of significance of $p < 0.05$. The p values and their significance are: ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. IQR: inter-quartile range.

We also observed in group S, compared to those Exposed who were not infected, a higher percentage of individuals with values higher than that of the normal established value of the median absolute frequency (severe: 21.0% vs exposed: 6.82%, p = 0.0106) and the relative frequency of NK+ (severe: 8.1% vs Exposed: 1.14%, p = 0.0325) (Fig. 2).

Interestingly, the S group had a higher proportion of individuals with values above the maximum range established as the normal value of the absolute frequency of CD3+ (12.90%), CD3 + CD8+ (12.9%) and of NK cells (21.%), compared to the A (CD3+: 1.49%, p = 0.0109; CD3 + CD8+: 0%, p = 0.0025; NK+: 3,0p = 0.0014. Similarly, the M group had a higher proportion of individuals (compared to the S group) with absolute frequencies higher than the normal maximum value of CD3+: 3.3% p = 0.0127; CD3 + CD8+: 3.3%, p = 0.0127 and NK+: 5.7%, p = 0.0018 (Fig. 2).

3.3. T Memory cells in convalescents from SARS-CoV-2 infection according to the clinical forms of COVID-19

We analyzed T CD4 + and CD8 + memory cells (TEMRA: CD45RA + CD27+, CM: CD45RA − CD27+, EM: CD45RA − CD27-) and naive (virgin) cells from 85 (convalescent) at an average of 82 days (42 days as the minimum and 107 days as the maximum) after viral clearance for SARS-CoV-2 identified by RT- PCR, who had clinical and epidemiological discharge. We also studied 29 exposed individuals.

The median of the relative frequency of total CD8 + memory cells (CM, EM, TEMRA) was significantly greater compared to the median of the T CD4 + memory cells (CM, EM, TEMRA) in all the convalescents studied ($p < 0.0001$) (Fig. 3A), and this occurred in a similar manner in each one of the groups according to the clinical forms (asymptomatic: $p < 0.0001$, moderate: p = 0.0213 and severe: $p < 0.0001$) of COVID-19 (Fig. 3A).

In the analysis of total memory cells in all convalescents (p = 0.0397) and severe cases (p = 0.0030) studied, we identified a higher median of the relative frequency of total T CD8 + memory cells in comparison to the Exposed cases (Fig. 3A).

Nonetheless, we observed that in the entire group of convalescents studied, the medians of the frequencies of CM, EM, TEMRA and naive cells were similar between convalescents and exposed for T CD4 + and CD8 + memory cells (Fig. 3B).

Within the subtypes of T CD8 + memory cells, the highest median of the relative frequency corresponded to TEMRA CD8+ (34.0%), followed by memory cells CM T CD8+ (24.8%), although no statistical differences were found between the median of the frequencies (p = 0.1889, 95% CI: −4.47 to 22.4) (Fig. 3). No statistical differences were observed between the median of the relative frequencies in the subpopulations of T CD8 + memory cells (TEMRA: CD45RA + CD27+, CM: CD45RA − CD27+, EM: CD45RA-CD27-) from the convalescent and Exposed groups (Fig. 3). Nevertheless, the median of the frequency of virgin cells (CD45RA + CD27 +) in the S group was lower compared to that of the Exposed group (9.5% vs 11.6%; p = 0.0379) (Fig. 3A).

The median of the relative frequency of the total T CD4 + memory cells in relation to the Exposed group, was similar in the total convalescent group (p = 0.3263) and in those presenting asymptomatic (p = 0.7282), moderate (p = 0.2666) and severe (p = 0.4101) forms of the disease (Fig. 3B). The median of the relative frequency of the total T CD4 + memory cells were also similar between the individuals with different degrees of COVID-19 severity (Fig. 3A).

The phenotype of T CD4 + memory cells that prevailed were the cells specialized in central memory (51.1 %), followed by the EM T CD4+ (21.3%) ($p < 0.0001$, 95% CI: 16.6 to 43.6504) (Fig. 3A).

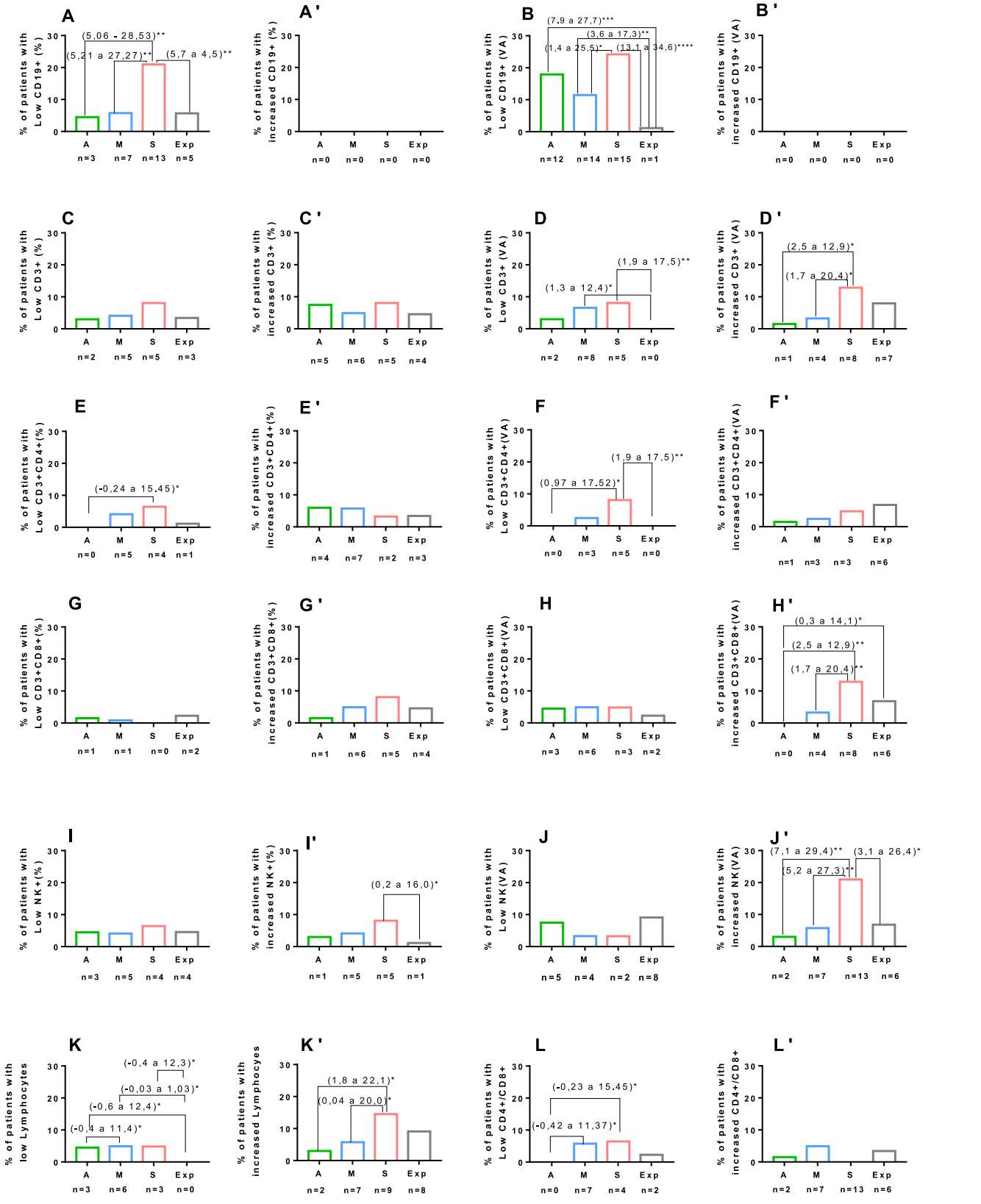

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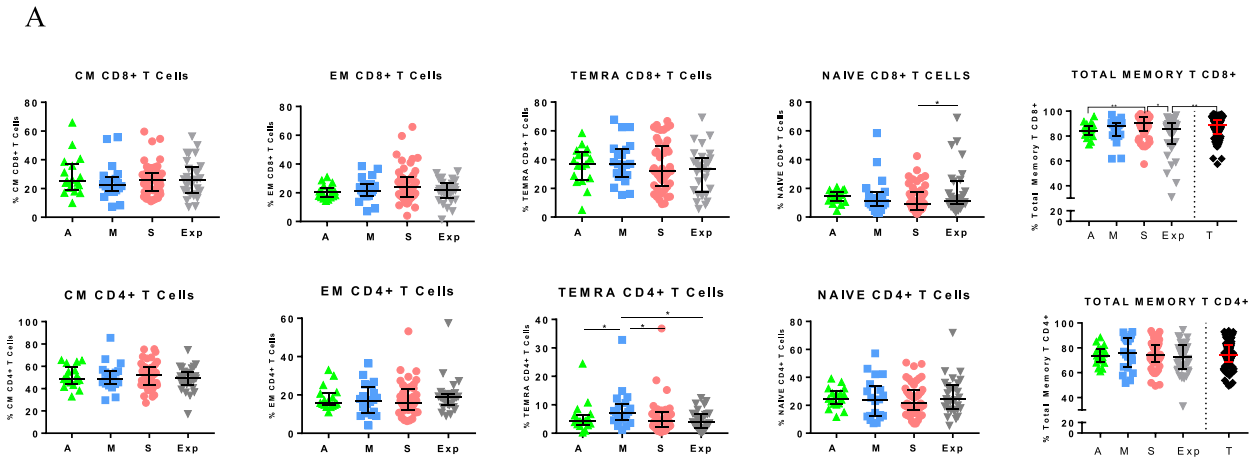
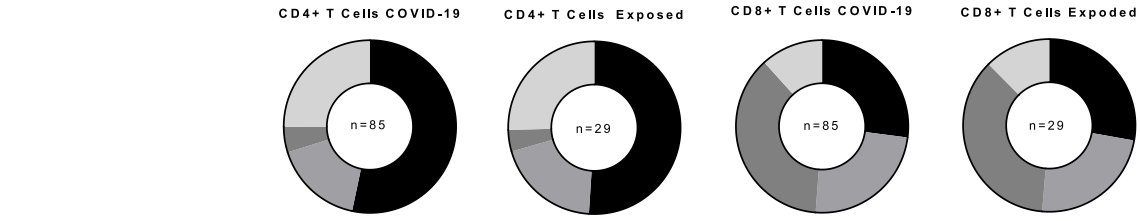
Fig. 2. Proportion of convalescents (n = 251) and exposed persons (n = 88) with cellular sub-populations that are lower and higher than the normal reference range of the Cuban population. The proportion of individuals with cellular sub-populations having lower values than the minimum normal reference was found as follows: A: CD19+ (%), B: CD19+ (VA), C: CD3+ (%), D: CD3+ (VA),E: CD3 + CD4+ (%),F: CD3 + CD4+ (VA), G: CD3 + CD8+ (%), H: CD3 + CD8+ (VA), I: NK+ (%),J: NK+ (VA), K: Total lymphocytes (VA), L:) CD4+/CD8 + Ratio. The proportion of individuals with cellular sub-populations having higher values than the normal maximum reference was found as follows: A': CD19+(%), B': CD19+ (VA), C': CD3+ (%), D': CD3+ (VA), E': CD3 + CD4+ (%), F': CD3 + CD4+(VA), G': CD3 + CD8+(%), H': CD3 + CD8+(VA), I': NK+(%), J': NK+(VA), K': Total lymphocytes (VA), L': CD4+/CD8 + Ratio. The green bars represent the proportion of convalescents with asymptomatic clinical forms: (A), moderate clinical forms (M) (blue bars); the proportion of convalescents with severe clinical forms (S) (the red bars); those exposed (Exp) (gray bars). Where n is the total number of convalescents and those exposed with alterations in comparison to the total of each group (total number of convalescents with asymptomatic disease forms, 67; total number of convalescents with moderate disease forms, 122; total number of convalescents with severe disease forms, 62; total number of exposed individuals, 88). The cellular sub-populations are identified through flow cytometry. The 95% confidence intervals are shown in parenthesis, with which we identified the statistical significance. The between-groups significance was calculated through the proportions comparison; the statistical significance was established for $p < 0.05$ and it was represented as: ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.





CM	**51,08 %**	**49,19 %**	**24,81 %**	**25,83 %**
EM	**16,15 %**	**18,83 %**	**22,15 %**	**21,86%**
TEMRA	**4,58 %**	**3,9 %**	**34,01%**	**33.65 %**
NAIVE	**24 %**	**24,49 %**	**10,73%**	**11.57 %**

Fig. 3. Phenotype of T CD4 + and T CD8 + memory and naive cells in COVID-19 convalescents and controls. A: We analyzed the T CD4 and CD8 + memory cells (central memory cells, MC, CD45RA-CD27+; effector memory cells, ME, CD45RA-CD27−; terminally differentiated T effector cells, TEMRA, CD45RA + CD27 −), total memory (MC + EM + TEMRA) and naïve cells (virgin cells) (CD45RA + CD27 +) according to clinical severity. Each point represents an individual, the asymptomatic individuals (A, green, n = 17); those with moderate symptoms (M, blue, n = 20); the severe cases (S, red, n = 48); the total number of convalescents studied (T, black, n = 85); and the controls (Exp, gray, n = 29). B: We analyzed the T CD4 and CD8 + memory cells in all convalescent and exposed. The medians and inter-quartile ranges of each group are represented. The analyses between groups were carried out using the Mann-Whitney test with the significance level of $p < 0.05$. The p values and their significance were: ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

The medians of the relative frequencies of T CD4 + CM, T CD4 + EM memory cells and naive cells from convalescent individuals of all the clinical forms analyzed in this study, were similar to those exposed. However, the median of the relative frequency of the TEMRA T CD4 + cells was higher in the M group (6.9%) compared to the Exposed group (Exposed: 3.9%, p = 0.0158) and to the S group (4.3%, p = 0.0182) (Fig. 3A).

3.4. Correlation of B, T, NK cells, memory and naive cells with clinical epidemiological variables in convalescents from SARS-CoV-2 infection according to the clinical forms of COVID-19

Using multivariate analysis, we detected that the risk of presenting an increase in the absolute frequency of NK + in convalescents was 4.0 times greater in those presenting the severe forms of the disease (severe) (adjusted OR: 34.0; 95% IC: 1,47–10,8; p = 0.007). Furthermore, it was 1.06 times higher for each day the acute disease was extended (duration

of the disease) (adjusted OR: 1.06; 95% IC: 1.0–1.11; p = 0.036). We also found that the convalescents that progressed with severe forms of the disease showed a trend towards an increase in the relative frequency of the NK + cells (adjusted OR: 2.32; 95% IC: 0.58–9.28; p = 0.2320) and a rise in the relative frequency (adjusted OR: 1.49; 95% IC: 0.37–6.08; p = 0.5790), and absolute frequency of CD3 + CD8+ (adjusted OR: 3.23; 95% IC: 0.95–11.0; p = 0.0610) (Fig. 4, Table 1S).

Convalescents, of 60 or more years of age, had a higher risk of decreasing the relative frequency (adjusted OR: 2.70; 95% IC: 1.07–6.78; p = 0.0390) and absolute frequency (adjusted OR: 2.84; 95% IC: 1.34–6.03; p = 0.007) of CD19+ (Fig. 4, Table 1S).

Age was positively correlated to the total number of CD8 + memory cells in convalescent patients (r = 0.5748. p < 0.0001, Spearman correlation) and a similar behavior was found in the exposed persons (r = -0.5688, p = 0.0013, Spearman correlation). Furthermore, age was negatively correlated with T CD8 + naive cells in the S and M groups (r = -0.5213, p < 0.0001, Spearman correlation, data not shown) and in those exposed +(r = -0.5491, p = 0.0020, Spearman correlation, data not shown).

The duration of the disease was found to be positively correlated with the T CD8 + EM cells (r = 0.3480, p = 0.0192 Spearman correlation, data not shown).

3.5. *Correlation of B, T, NK, memory and naive cells with the response of specific antibodies to SARS-CoV-2 in convalescent cubans according to the clinical forms of COVID-19*

IgG/RBD titers are positively correlated with the relative frequency of the CD8 + CM memory cells (r = 0.3132, p = 0.0320) in the S group (Fig. 5-A). TEMRA CD8 + showed a tendency to correlate negatively, but there were no statistical differences (r = -0.2405, p = 0.1035) (Fig. 5-B).

Those of the S group with antibody titers IgG/RBD > 1/200 compared to those with titers ≤ 1/200, showed lower relative frequency of TEMRA CD8 + cells (IgG/RBD > 1/200: 28.7% vs IgG/RBD ≤ 1/200:

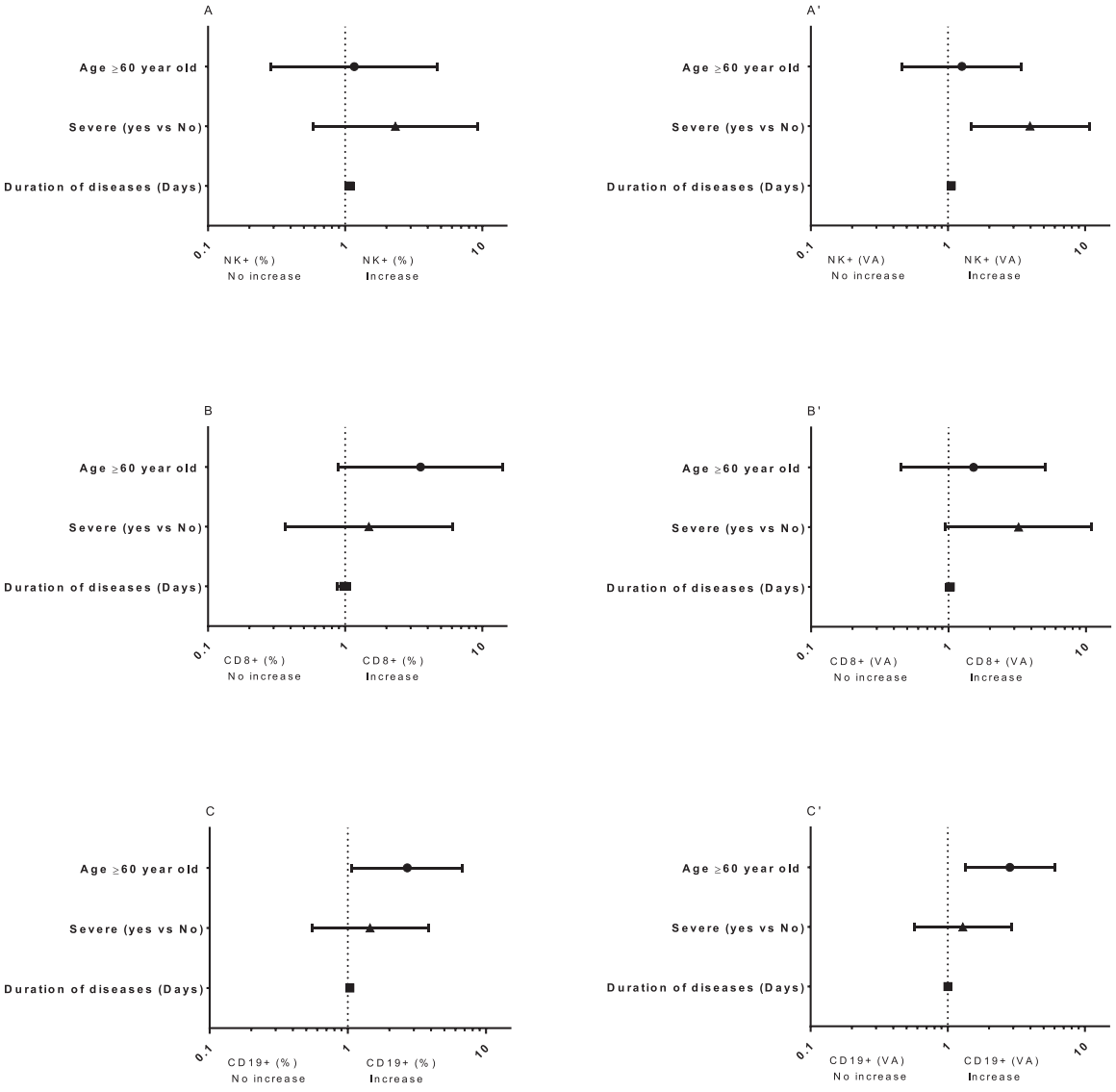


Fig. 4. Relationship between age ≥ 60 years old, severity and duration of the disease, on the main alterations of the cellular sub-populations in COVID-19 convalescent patients. We carried out a logistic regression analysis in 233 SARS-CoV-2 convalescent patients using for the analysis the information that was complete. We evaluated the influence of age ≥ 60 years old, severity (the characteristic of the severe disease against the non-severe case) and the duration of the disease (time lapse of the disease) on the increase of NK+ (%) [A], the increase of NK+ (VA) [A'], the increase of CD8+ (%) [B], the increase of CD8+ (VA) [B'], the decrease of CD19+ (%) [C] and of CD19+ (VA) [C']. VA: Absolute value. In the Forest plot we show the 95% confidence intervals.

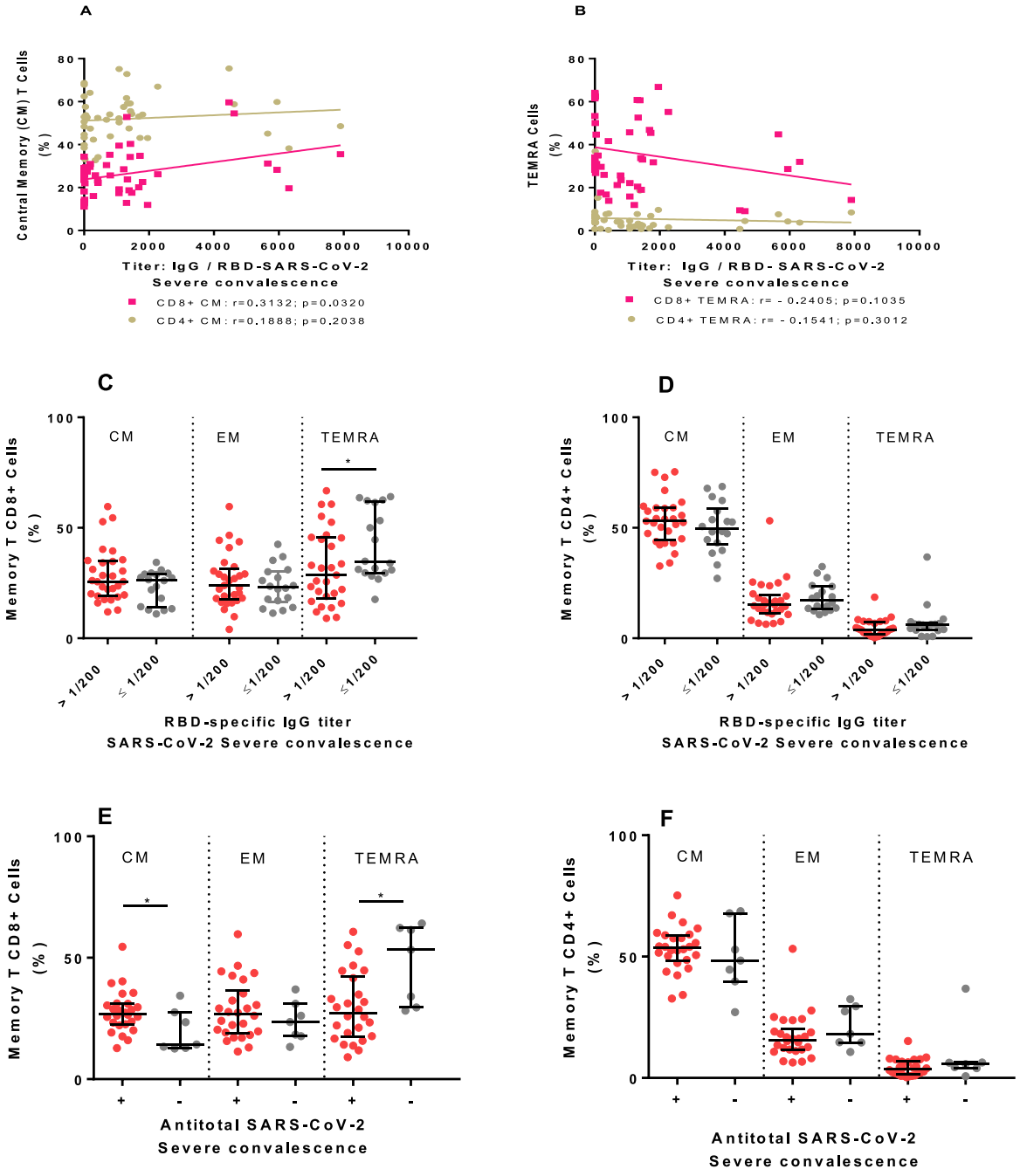


Fig. 5. Relationship of the memory cells with the specific response to SARS-CoV-2 in convalescents that progressed to severe form of COVID-19. We show the analysis of the correlation between the antibody titer IgG/RBD and the central memory cells (CM) (Fig. 1-A) and with the terminally differentiated memory cells (TEMRA) (Fig. 1-B) in convalescents that progressed to severe forms of the disease (n = 45). A non-parametric Spearman's correlation test was used for both correlations. We show the comparison of the medians of the memory cells (central memory: CM, effector memory: EM, terminally differentiated: TEMRA) T CD4+ (Fig. 1-C) and T CD8+ (Fig. 1-D) between the severe convalescent patients with titers of $< 1/200$ (n = 18) and $\geq 1/200$ (n = 28). This comparison was made using the Mann-Whitney test. Figures E and F show the comparison of the medians of the memory cells T CD4+.

34.58%, p = 0,0357) (Fig. 5-C and D).

In line with the previous results of IgG/RBD, we identified a lower frequency of TEMRA T CD8 + cells (53.3% vs 27,3 %, p = 0.0137) in the peripheral blood of the positive S group for total antibodies against fragments of the N and S protein of SARS-CoV-2 (compared to those that were negative to this antitotal antibody) (Fig. 5- E).

Among the individuals with a presence or absence of antitotal antibodies to SARS-CoV-2, we identified similar relative frequencies of CD19+, CD3+, CD3 + CD4+, CD3 + CD8+, NK + and the ratio CD4/CD8 + in all ranges of severity studied (results not shown).

4. Discussion

The alterations of the B, T and NK cells, as well as the more and more frequent presence of signs and symptoms, have been reported in COVID-19 convalescents, (Greenhalgh et al.,2020; Shuwa et al., 2021). There

are, however, discrepancies in the magnitude and the protective or pathogenic role of the following immune response to SARS-CoV-2 infection (Kuri-Cervantes et al., 2020; Chen and John Wherry, 2020; Mathew et al., 2020).

As observed by other authors, in this study we did not observe lymphopenia in COVID-19 convalescents (Grifoni et al., 2020; Rodriguez et al., 2020). A higher proportion of severe convalescents with values above the reference range of total lymphocytes could be influenced by the larger number of T CD8 + lymphocytes identified as a response to clear the persistence and the greater antigenic magnitude of SARS-CoV-2 and because of the use of immunomodulators according to the national protocols (Ministry of Public Health, 2021a; Hernández Cedeño et al., 2021), since it has been reported that the administration of biological therapies in COVID-19 patients, produces an increase of circulating lymphocytes (Giamarellos-Bourboulis et al., 2020; Hernández Cedeño et al., 2021).

The normalization of CD19 + cells in COVID-19 convalescents is reported in the literature (Mathew et al., 2020; Sherina Sherina). A decrease of CD19 + cells has also been observed in patients with severe forms of the disease, (Grifoni et al., 2020; Deng et al., 2020) as those found in this study. The decrease of CD19 + lymphocytes could suggest that sub-populations of this compartment, such as B regulator cells with anti-inflammatory functions, are low, and as a consequence the convalescents show a delay in their complete recovery, and are vulnerable to auto-immune, auto-inflammatory and infectious processes (Mauri and Menon, 2017).

Several mechanisms may explain the lymphopenia of CD19 + and CD3 + CD4 + observed in this study, such as the presence of a greater viral load and exposure time to SARS-CoV-2 in more severely ill patients. This leads to an increase in the direct action of SARS-CoV-2 on the cells, and the damage mediated by the immune system, the sequester of cells from the lung or peripheral lymphoid organs induced by the cytokine storm, apoptosis and the suppression of the bone marrow and the thymus (Wen et al., 2020). The literature also reports, however, that convalescent individuals have similar values of CD3 + CD4 + to those of individuals who did not become ill (Shuwa et al., 2021; Townsend et al., 2021; Wen et al., 2020).

The absence of asymptomatic convalescent patients with a decrease of the T CD4 + lymphocyte values and a higher frequency of these cells in relation to the convalescents with symptoms (moderate and severe), may correspond with the evidence that asymptomatic individuals have a higher secretion of INF -γ e IL-12, as well as a proportional secretion of IL-10 and of pro-inflammatory cytokine (IL-6, TNF-α e IL-1β). This fact suggested that the asymptomatic patients have the ability of developing a less intense inflammatory process, but their antiviral response is protective, efficient, balanced and specific, so that it protects the host and does not produce any apparent pathology (Le Bert et al., 2021).

The discrete increase in T CD3 + CD8 + lymphocytes in convalescents from the severe illness supports the role of these cells when facing a greater antigenic exposure and an exaggerated immune response to achieve effective viral clearance (Wen et al., 2020; Thieme et al., 2020). These results agree with the expansion of T CD8 + lymphocytes in convalescents reported by other authors (Shuwa et al., 2021; Wen et al., 2020). However, the recovery from lymphopenia of T CD8 + characteristic of the acute phase of the disease is also reported. This has led to the idea that the virus produces this alteration and that the effective anti-viral therapy leads to the recovery of T CD8 + cells (Zheng et al., 2020). The increase of T CD8 + lymphocytes in convalescents may have implications in the development of later infections or the perpetuation of inflammatory processes, depending on the capacity of the cytokine secretion of these cells (Shuwa et al., 2021).

Similarly, other studies have reported an increase in NK cells during the convalescent stages (Rodriguez et al., 2020; Wen et al., 2020). It has been reported that an effective therapy for SARS-CoV2 is accompanied by an increase of NK cells (Zheng et al., 2020). The increase in NK + cells is considered to be a valuable biomarker for monitoring the progression of the acute phase of the disease toward recovery stages in severe patients (Rodríguez et al., 2020). In contrast, values of NK + cells are also reported to be similar between convalescents and persons who are not infected by SARS-CoV-2 (Townsend et al., 2021; Liu et al., 2021). This research also showed that an increase in the frequency of these cells is associated to the severity and duration of the disease. This supports the antiviral role and the participation in the immunopathology of these cells on the severity of the disease and on the stages of inflammation that may persist (Townsend et al., 2021; Fox et al., 2012; Market et al., 2020).

The immunologic memory is considered to be of great importance in preventing the recurrence of severe forms of COVID-19 in individuals who are seronegative to SARS-CoV-2, whether they are exposed or not (Sekine et al., 2020). It is possible that a small part of the population infected with SARS-CoV-2, having a poor immunological memory, will be susceptible to reinfection shortly after recovering from the acute process (Dan et al., 2020).

The similarity of the memory and naive cells among all convalescents and uninfected persons identified in this study has been reported in the literature (Mathew et al., 2020).

The prevalence of central memory cells in the compartment of T CD4 + memory in the present study, as well as the identification in severe convalescents of a positive correlation between the T CD4 + CM cells and the RBD titers, corresponds to the capacity of these cells of extravasation and migration to secondary tissues. These show a high proliferative capacity and a low dependence on co-stimulators, thus favoring the formation of specific antibodies against SARS-CoV-2, as observed (Wen et al., 2020; Peng et al., 2020; Weiskopf et al., 2020; Neidleman et al., 2020; Mahnke et al., 2013).

Consistent with other reports from the literature, (Grifoni et al., 2020; Wen et al., 2020; Dan et al., 2020; Peng et al., 2020; Weiskopf et al., 2020; Neidleman et al., 2020; Yang et al., 2007), in this study there was a predominance of TEMRA cells in the sub-set of T CD8 + memory cells, which agrees with their function as potent producers of interferon -γ and perforins that mediate in the specific cytotoxicity of the antigen. This makes them highly important in viral infections (Sallusto et al.,2004).

The high frequency of TEMRA memory cells in convalescent individuals presenting moderate forms of the disease (compared to the severe forms), supports the protective role of these cells in the development of severe forms of COVID-19 and endorses the substantial role of T-cell immunity in SARS-CoV-2, (Sekine et al., 2020; Dan et al., 2020; Peng et al., 2020; Le Bert et al., 2020), and other viral infections (Sridhar et al., 2013). Hence, the formation of T memory cells has been associated to recovery from COVID-19, and it was reported that this response could predict severity and it could become a marker associated to the loss of the effectiveness of the anti-viral response (Odak et al., 2020).

Previous studies have demonstrated that the severity of the disease is inversely correlated with the immunity of T-cells (Ni et al., 2020), and that the deficient T-cell response prevents the positive action of the immune system against SARS-CoV-2 (Odak et al., 2020; Wang et al., 2020). Consistent with these reports, we observed that the convalescents who became ill with severe COVID-19 showed a lower frequency of TEMRA cells associated to higher titers of IgG/RBD and to the presence of total antibodies against fragments of the N and S proteins of SARS-CoV-2 (TEMRA CD8 +). We also found a negative correlation between TEMRA CD4 + and the IgG/RBD titers. On the other hand, the similarity in the immune response (T CD3 + CD4+, CD3 + CD8+, NK and CD19 +) in individuals who were seropositive or not to SARS-CoV-2, suggests that the state of protection evaluated through the detection of antibodies against SARS-CoV-2, may be underrated (Sekine et al., 2020).

Although we did not determine the specific SARS-CoV-2 T cells, we did observe that the frequency of the memory and naive cells in all convalescents was similar to that of the exposed individuals. It has been found a response of specific SARS-CoV-2 T cells in persons having close

contact with COVID-19 patients, in which no positive RT-PCR was detected, nor the presence of antibodies anti-SARS-CoV-2; therefore, it suggested that there were no infections due to the limited exposure of the persons to viral particles, or the short exposure time (Sekine et al., 2020; Wang et al., 2021).

The decrease of T CD8 + naive cells in patients having severe forms of COVID-19 may be the result of the mobilization of effector cells, as the cytotoxic lymphocytes, in order to eradicate viral infection (Odak et al., 2020; Thieme et al., 2020). Other authors observed a decrease in T CD8 + naive cells in individuals having a mild or moderate SARS-CoV-2 infection (Odak et al., 2020). It should be mentioned that the group of severely ill patients were older, and that the decrease of naive T cells has been associated to immunosenescence, as a consequence of aging ((García Verdecia et al., 2013; Saavedra et al., 2017).

It must be considered that older age could produce a hyper-inflammatory state, starting with the fact that during aging and immunosenescence there are changes occurring in the immune system (Sauce and Appay, 2011). These include the effect on several cellular compartments, (García Verdecia et al., 2013) deregulation of cytokine secretion and association to a state of "inflammaging" (low-degree chronic and sterile inflammation during aging), producing an increase in the frequency of infectious, neurodegenerative, and cardiovascular diseases and cancer (Fulop et al., 2018).

One limitation of this study was the fact that we did not analyze any specific cells for SARS-CoV-2. Also, nor we did not carry out a longitudinal study, with patients through the time (samples were collected between 14- and 106-days post-infection). The evaluation of specific responses to SARS-CoV-2 in a longitudinal study could have provided a more comprehensive view of the dynamics of the immune response during COVID-19 and in the convalescent study time, as reported by other authors (Rodriguez et al, 2020; Liu et al, 2021; Wen et al., 2020). It would have been interesting to have included a group of patients experiencing acute COVID-19, as well as to correlate the results of all groups with the clinical condition present in the convalescence period. (Shuwa et al., 2021; Sekine et al., 2020; Peng et al, 2020). Despite these limitations, it was observed that the identified alterations in the immune response in convalescents is influenced by the severity of the disease, similar to what other authors affirm (Peng et al, 2020; Shuwa et al., 2021; Sekine et al., 2020; Wen et al., 2021). Besides, these patients are susceptible to subsequent complications mediated by the immune system (Shuwa et al., 2021). The study of T cells specific to SARS-CoV-2 would have allowed the identification of the presence of cross reaction T cells against SARS-CoV-2 and other coronaviruses, which could have possibly been an element to explain the fact that uninfected exposed individual did not get sick (Grifoni et al., 2020; Le Bert et al., 2021, Wang et al, 2021).

5. Conclusion

The immune status of COVID-19 convalescents is influenced by the severity of the disease. The alterations of the lymphocytes CD19+, CD8+, NK cells and of the specific antibodies against SARS-CoV-2 in severe convalescents suggest that these patients could be vulnerable to infectious, autoimmune or autotinflammatory processes. These findings could be associated with a more unfavorable recovery and the instauration of new sequels of the disease, thereby needing close medical supervision. The alterations in the effector memory cells may be related to the evolution towards severe forms of the disease.

CRediT authorship contribution statement

Bárbara Torres Rives: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Yaíma Zúñiga Rosales:** Conceptualization, Methodology, Investigation. **Minerva Mataran Valdés:** Formal analysis, Visualization. **Hilda Roblejo Balbuena:** Supervision, Conceptualization, Investigation, Writing – review & editing. **Goitybell Martínez Téllez:** . **Jacqueline Rodríguez Pérez:** Investigation. **Lilia Caridad Marín Padrón:** Investigation. **Cira Rodríguez Pelier:** Resources, Investigation. **Francisco Sotomayor Lugo:** Resources, Methodology, Investigation. **Anet Valdés Zayas:** Methodology. **Tania Carmenate Portilla:** Methodology, Investigation. **Belinda Sánchez Ramírez:** Methodology, Resources, Investigation. **Luis Carlos Silva Aycaguer:** Methodology, Investigation, Writing – review & editing. **José Ángel Portal-Miranda:** Resources, Investigation. **Beatriz Marcheco Teruel:** .

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.imbio.2022.152216.

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