Immunology Letters

Immune, inflammatory and prothrombotic parameters in COVID-19 patients treated with an anti EGFR antibody

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ARTICLE INFO

ABSTRACT

SARS-CoV-2 infection causes a range of clinical presentations and induces changes in both innate and adaptive branches of the immune system. Furthermore, direct viral action to the cells of the lung promotes over-expression of the epidermal growth factor receptor (EGFR) which triggers pro-inflammatory response, contributes to coagulopathy and intravascular thrombi as well as lung fibrosis. Based on the role of this signaling pathway in the pathophysiology of the disease, nimotuzumab, an anti-EGFR monoclonal antibody, was used to treat patients with COVID-19. The aim of this study was to determine IL-6 and PAI-1 concentrations and lymphocyte subpopulations profiles in moderately and severely ill COVID-19 patients diagnosed during the B.1.617.2 variant wave in Cuba and included in a phase I/II trial to evaluate the safety and preliminary effect of nimotuzumab in COVID-19 disease. We observed high serum levels of IL-6, elevated plasma concentration of PAI-1, mean values of neutrophils to lymphocytes ratio (NLR) above three and CD4+ lymphopenia in both groups of patients. PAI-1 and IL-6 circulating levels decreased in patients treated with nimotuzumab. More than 95% of patients in which IL-6 decreased or increased slightly, were alive within 14 days after the monoclonal antibody administration. Patients with moderate and severe disease, were no different regarding the studied parameters, addressing the idea that several immune alterations could be present before the infection becomes clinically relevant. These findings suggest that nimotuzumab could be an attractive therapeutic option to interfere with the negative relationship between cytokines and procoagulant mediators in the inflammatory and prothrombotic phases of the disease.

1. Introduction

Coronavirus disease 2019 (COVID-19) causes a range of clinical presentations, from mild symptoms, to severe disease characterized by respiratory dysfunction and/or multiple organ damage that causes disease severity and even death [1]. Not only clinical symptoms are characteristic of COVID-19 disease, but SARS-CoV-2 infection also induces changes in both innate and adaptive branches of the immune system [2, 3]. Pathogenesis of COVID-19 is complex and given by a variety of mechanisms. On one side, there is lung damage caused by direct viral action, and on the other, a hyper-reactive immune response contributing to cytokine release syndrome that leads to a coagulopathy characterized by intravascular thrombi [4].

Patients with severe COVID-19 usually exhibit an increase in circulating neutrophils as well as appearance of circulating neutrophil precursors, elevated neutrophil to lymphocyte ratio (NLR) and changes in the adaptive immune system that include lymphopenia with reduction in CD4+ and CD8+ T cells activation [5,6].

Healthcare providers typically stratify COVID-19 patients based on clinical presentations. These clinical assessments are essential but

sometimes subjective, and could only manifest at late stages of the disease. To identify at-risk patients early, some laboratory biomarkers can provide objective information that could enable the clinicians for more efficient classification of the patients and treatment [3].

More recently, evidence of the role of the Epidermal Growth Factor Receptor (EGFR) in the pathogenesis of the disease has emerged. Acute lung injury, together with STAT-1 downregulation and STAT-3 upregulation, induced by SARS-CoV2, are thought to be responsible for EGFR overexpression found in alveolar epithelial cells of patients affected by COVID-19. This EGFR upregulation triggers pro-inflammatory response, via NF-kB pathway and further STAT-3 activation. A positive response circle between STAT-3 and PAI-1 is established. In consequence, there is an increased PAI-1 secretion that contributes to coagulopathy and intravascular thrombi [7–9]. In a recent publication, our group demonstrated the expression of EGFR in cells morphologically resembling type I and II pneumocytes, alveolar macrophages and fibroblasts in the lung of patients who died from COVID-19 [8].

Nimotuzumab is a humanized IgG1 isotype monoclonal antibody (Mab) targeting EGFR developed at the Center of Molecular Immunology in Cuba. It binds to the extracellular domain of EGFR, preventing tyrosine kinase activation, and thus inhibiting, signaling pathways involved in proliferation, survival, angiogenesis and inflammation [10, 11]. Nimotuzumab has been widely used and is indicated for the treatment of epithelial tumors, such as glioblastoma, squamous cell carcinoma of the head and neck, and esophageal, nasopharyngeal, pancreatic and non-small-cell lung cancers. Its efficacy and safety profile is backed up by several clinical trials [10,11]. Given the crucial regulatory role of EGFR in lung fibrosis, inflammation and immune thrombosis, a clinical trial to evaluate for the first time the effect of using nimotuzumab in the COVID-19 was performed. The use of this EGFR antagonist accompanied by the standard of care treatment proved to be safe and effective, with a recovery rate of over 80% [8].

This work describes the immune response of moderately and severely ill COVID-19 patients treated with nimotuzumab. Additionally, we will report here, the effect of this Mab on circulating IL-6 and PAI-1 levels in patients with severe and moderate illness.

2. Methods

2.1. Patients and treatment

Thirty-two laboratory-confirmed COVID-19 patients, classified as severely ill and moderately ill were included in a prospective, non-controlled, open, multicenter phase I/II trial carried out to evaluate the safety and preliminary effect of nimotuzumab monoclonal antibody. Nimotuzumab was administered together with the standard of care: low-molecular-weight heparin, steroids and antibiotics. Some patients could also receive CIGB-258, a peptide with immunoregulatory properties which obtained Emergency Use Authorization from the Cuban regulatory agency, according to the national COVID-19 guideline [8].

Patients with disease severity should had one of the following conditions: oxygen saturation (SpO2) <94% on room air at sea level or need for oxygen therapy to maintain SpO2 >93%, a pressure of arterial oxygen to fractional inspired oxygen (PaO2/FiO2) <300 mm Hg, a respiratory rate >30 breaths/min or lung infiltrates >50% of both lung fields. Moderately ill patients were those with evidence of lower respiratory disease according to the clinical assessment or imaging, persisting fever for 48 hours, polypnea and who had a SpO2 ≥94% on room air.

The patients were treated in the Clinic and Surgery Hospital Dr. Salvador Allende Hospital and in the Clinic and Surgery Hospital Julio Trigo, both in Havana. The protocol was approved by the ethics committees of the participating hospitals and by the National Regulatory Agency, CECMED. All patients signed the informed consent. All patients who entered the trial with a severe condition were treated at the ICU, while patients with moderate illness received nimotuzumab at the hospital conventional ward. The time lag between the onset of

symptoms and nimotuzumab treatment was 8.5 days, while for the severe patients the time interval between ICU admission and nimotuzumab was 3 days. The protocol was listed in the public registry of clinical trials (https://rpcec.sld.cu/ensayos/RPCEC00000369-En).

2.2. Peripheral blood samples

Blood samples were collected the same day of recruitment and before the first dose of nimotuzumab. A second peripheral blood sample were collected from all patients seven days after the first nimotuzumab administration. Hematological parameters and leukocyte formula were measured on a hematology analyzer Spincell 3 (SPINREACT, Girona, Spain).

Venous blood samples were collected into serum tubes. All samples were centrifuged at 3500 rpm for 10 min, and the serum was stored at −80℃. Serum IL-6 concentrations were measured by sandwich enzyme-linked immunosorbent assay (ELISA) using the R&D Systems human ELISA kit (Minneapolis, USA; Human IL-6 Quantikine ELISA Kit).

PAI-1 levels were determined on citrated plasma samples using a commercially available enzyme immunoassay (Asserachrom PAI-1; Diagnostica Stago, Asnières sur seine, France). All assays were performed according to the manufacture's protocols.

2.3. Flow cytometry

Red cells were isolated from whole blood with lysing solution (NH4Cl, EDTA (tetrasodium), KHCO3). White blood cells (WBCs) were washed twice with Citometry solution (PBS, BSA, Azide 20%).

Antibodies were initially titrated to determine the optimal conditions for flow cytometry analysis before staining. The anti-human antibodies used were anti-CD3 (RPE-Cy5, clone UCHT1, MCA463C, Bio-Rad and PE, clone UCTH1, MCA463G, BioRad), anti-CD4 (AF700, clone RPA-T4, 300526 Biolegend), anti-CD8 (FITC, clone RPA-T8, 396580, BD Biosciences), anti-CCR7 (Alexa-fluor 647, clone 150,503,560,816, BD Pharmingen), anti-CD45RA (PE-CF594, clone H100, 5,622,978, BD Horizon), anti-CD28 (PE clone CD28.2, 302908, Biolegend), anti-CD19 (APC/Cyanine7, clone SJ25C1, 363009, Biolegend), anti-CD27 (PE, clone M-T271,555,441, BD Pharmingen), anti-IgD (FITC, clone IA6-2, 555,778, BD Pharmingen), CD56 (APC, clone NCAM16.2, BD Biosciences), CD16 (PerCP/Cyanine5.5, clone 3G8, 302028, BioLegend), CD127 (FITC, clone A019D5, 351312, BioLegend), CD25 (PerCP-Cyanine5.5, clone BC96, 45-0259-42 eBioscience),FOXP3 (PE-Cyanine7, clone PCH101, 25-4776-42, eBioscience)

The monoclonal antibodies were used for staining in the following panels:

CD3 RPE-Cy5/ CD4 AF700/ CD8 FITC/ CD45RA PE-CF594/ CCR7 AF647/CD28 PE
CD3 PE/ CD56 FITC/ CD16 PerCP-Cyanine5.5
CD3 PE/ CD4 AF700/ CD127FITC/ CD25 PerCP-Cyanine5.5/FOXP3 PE-Cyanine7
CD19 APC-Cyanine7/CD27 PE/IgD FITC

For surface staining, WBCs (1×10^5 cells in 100 μL citometry solution) were incubated with the antibodies in the dark at 4°C for 20 min. Subsequently, cells were washed twice and fixed. For Foxp3 intracellular staining, the fixed cells were then permeabilized with FoxP3 staining buffer set (BD Bioscience). Data acquisition was performed with a Gallios flow cytometer (Beckman Coulter, 3-laser configuration). The data were processed with FlowJo software (Tree Star Inc., v10(2)) and data exported as tabulated results for statistical analyses. All the data generated was obtained from fresh samples.

2.4. Statistical analysis

The Shapiro-Wilk and Kolmogorov-Smirnov normality test were used

to determine the normal distribution of variables. Statistical significance between groups was evaluated using t tests and Wilcoxon, for paired values tests, when data passed or failed normality test, respectively. These statistical analyses were performed using GraphPad 7 (San Diego, CA, USA). The statistical data were considered significant if p < 0.05. To evaluate the clinical relevance of IL-6 decrease as predictor of mortality and its discrimination threshold, a receiver operator characteristic (ROC) curves was built. After dichotomizing the IL-6 decrease values by the found threshold, a contingency table and Fisher exact test were performed.

3. Results

3.1. Characteristics of COVID-19 patients

Thirty-two laboratory-confirmed COVID-19 patients were included in a prospective, non-controlled, open, multicenter phase I/II trial carried out to evaluate the safety and preliminary effect of nimotuzumab monoclonal antibody. The patients, 12 females and 20 males were categorized into two groups regarding the severity of illness: moderately ill, n=8 and severely ill, n=24. The mean age was 60.54 years old, 59.83 for severely ill and 62. 63 for moderately ill (Table 1).

3.2. IL-6 serum concentrations in moderately and severely ill COVID-19 patients treated with nimotuzumab

IL-6 is one of the key mediators of inflammation and viral cytokine storm in COVID-19 patients [12]. At admission, the mean serum IL-6 levels were 64.44 pg/mL (median 47.11 pg/mL; interquartile range (IQR): 15.75- 87.39) for severely ill and 66.56 pg/mL (median 37.25pg/mL; IQR: 7.88- 117) for moderately ill patients. Surprisingly, the serum concentration in moderately ill patients was as high as in severely ill (p>0.05, Mann Whitney test, Fig. 1a).

Analyzing together all the patients before and seven days after the administration of nimotuzumab, the median of the baseline of circulating IL-6 decreased, although no statistical significance was reached (Fig. 1b, p=0.0934). The median values reduced from 43.52 pg/mL to 14.51 pg/mL, near to the normal limit [13].

Looking for the clinical relevance of IL-6 decrease, we focused on the difference between IL-6 serum levels at admission, before nimotuzumab treatment and seven days after. The cutoff selected by ROC curve to stablish the association between IL-6 concentration difference and mortality was 14.27 pg/mL with a sensitivity of 80% and specificity of 85.2% (Fig. 1c). Performing a contingency table, 95.8% of patients in which circulating IL-6 decreased or slightly increased (less than 14.27

pg/mL), were alive within 14 days after receiving the first monoclonal antibody infusion (n=23). Contrarily, only 50% of patients that augmented more than 14.27 pg/mL (n=4) remained alive during the same period (p=0.009, Fisher exact test).

3.3. Neutrophil to lymphocyte ratio (NLR) in moderately and severely ill COVID-19 patients

The NLR has been suggested as a good predictive marker of disease severity and mortality in COVID-19 patients [14]. The NLR was evaluated on admission and seven days after the administration of nimotuzumab. There were no significant differences between these values in moderately (median: 3.16, IQR: 1.42–5.43) and severely ill patients (median: 5.39, IQR: 2.81–9.21) (p>0.05, Mann Whitney test). There were no changes in the median values of each group after the administration of nimotuzumab.

3.4. Plasma PAI-1 concentrations among patients with COVID-19

Plasma levels of PAI-1 were evaluated in 14 patients (8 moderately and 6 severely ill) before and after the administration of nimotuzumab. Regarding the reference range of the circulating levels, all patients had elevated values before the treatment (reference range 4-43 ng/mL). The mean PAI-1 at baseline was 80.11 ng/mL (median 60.9 pg/mL; IQR: 51.05-105.5) and it decreased to 69.26ng/mL (median 53.82 pg/mL IQR: 41.5-94.33) seven days after the first dose of nimotuzumab (p=0.06, Wilcoxon test). The circulating levels showed a decreasing trend in 12 patients (85.7%), while only 2 patients (14.3%) showed increased plasma concentrations (Fig. 2).

3.5. Immune subpopulations in COVID-19 patients

Among the hallmarks of severe COVID-19 it has been suggested the presence of elevated inflammatory cytokines, coagulopathy and lymphopenia [3]. T cell lymphopenia, driven by CD4+ T cells were detected in severe and moderately ill COVID-19 patients (Fig. 3a, b). Interestingly, no differences were found between both groups of patients. Severely ill patients had a median of 851.6 cells/μL CD3+ T cells, whereas moderately ill had 816.7 cells/μL. Medians of CD4+ T cells were below 500 cells/μL in both groups of patients (474.7 cells/μL in severely ill and 396.5 cells/μL in moderately ill patients). Absolute count of CD8+ T cells were in the normal range [15] (median of 292.3 cells/μL in severely ill and 309.3 cells/μL in moderately ill) (Fig. 3b). Although there were no differences in CD4/CD8 ratio between moderately and severely ill patients, 50% of moderates had an inverted ratio (CD4/CD8 ratio <1), while for the severe group was 13.6% (Fig. 3c). When evaluating the variations in T cell numbers (CD3+, CD4+, CD8+) at baseline and seven days after treatment, no differences were found.

Based on the expression of CD45RA and CCR7, we performed the analysis of differentiation subsets inside CD4+ and CD8+ T cells among patients classified as severe, before and seven days after the treatment with nimotuzumab. There was a high frequency of terminally differentiated effector T cells reexpressing CD45RA lymphocytes (EMRA, CD45RA+CCR7-) within CD8+ T cells, meanwhile in CD4+ T cells, effector memory (EM) cells were the most prominent (Fig. 3d, e). The analysis showed a significant decrease in the percentage of naive (p=0.0269; Wilcoxon test; Fig. 3d) and central memory CD4+ T cells (p=0.0269; Wilcoxon test; Fig. 3d) one week after the administration of the first dose of nimotuzumab. Regarding CD8+ T cells, a significant increase of central memory was observed seven days after the first administration of nimotuzumab (p=0.0024, Wilcoxon test; Fig. 3d).

We also explored regulatory T cells. When comparing severe and moderate patients at baseline, no differences were identified. Similarly, no changes occurred seven days after the first dose of nimotuzumab, either, when evaluating the whole population or independently according severity (data not show).

Table 1
Demographics of moderately and severely ill COVID-19 patients treated with nimotuzumab. IQR: Interquartile range.

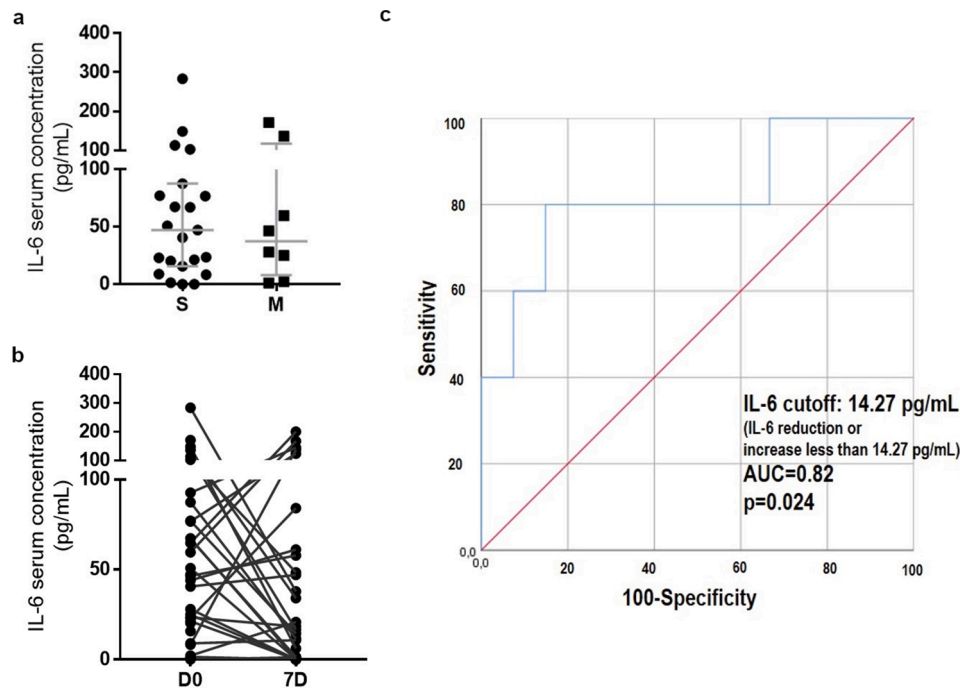|	Moderately ill n=8	Severely ill n=24	Total n=32
Age (median, IQR)	65 (53.75-70.5)	62 (46-74)	63 (52-72)
Sex (%)			
• Female	4 (12.5)	8 (25)	12 (37.5)
• Male	4 (12.5)	16 (50)	20 (62.5)
Comorbidities (%)			
• Hypertension	4 (50)	13 (54.1)	17 (53.13)
• Diabetes	3 (37.5)	10 (41.7)	13 (40.63)
• Asthma	1 (12.5)	6 (25)	7 (21.88)
• Cardiovascular disease	1 (12.5)	4 (16.7)	5 (15.63)
• Other	-	3 (12.5)	3 (9.38)
Concurrent treatments (%)			
• Antibiotics	21 (87.5)	7 (87.5)	28 (87.5)
• Anticoagulants	21 (87.5)	2 (25)	23 (71.86)
• Steroids	21 (87.5)	3 (37.5)	24 (75)
• CIGB-258	1 (12.5)	12 (50)	13 (40.63)

Fig. 1. IL-6 concentration in the sera of COVID-19 patients. a Median IL-6 levels in the two studied groups of COVID-19 patients (severely ill patients n=24, moderately ill patients n=8). b Kinetic of the median of IL-6 levels in COVID-19 patients before and 7 days after the treatment with nimotuzumab. c ROC curve to stablish the association between IL-6 difference and mortality. AUC = 0,822 (CI 0,590-1,00) ROC: receiver operator characteristic; AUC: area under curve.
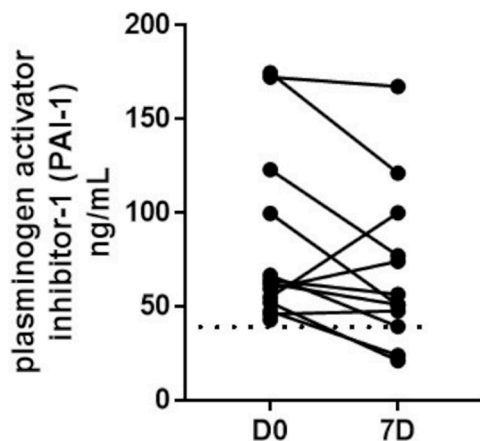

Fig. 2. Levels of PAI-1 among patients with COVID-19. Plasma levels of PAI-1 were measured at baseline (D0) and seven days after the first dose of nimotuzumab. Dot line represents the upper normal limit (reference range: 4-43 ng/mL).

Peripheral B and NK cells were also evaluated in moderately and severely ill COVID-19 patients in the moment of their inclusion in the clinical trial and seven days after the treatment with nimotuzumab. Total B cell count was significantly lower among moderately ill patients than in severely ill patients (p=0.0317, Wilcoxon test; Fig. 4a, b). Additionally, we evaluated the stages of differentiation of B cells according to the expression of IgD and CD27, at baseline and seven days after the administration of nimotuzumab in the severe group. No variations in total B cell counts nor B cell subpopulations was observed comparing baseline and seven days after the administration of this Mab (Fig. 4c).

Within NK cells no differences were found regarding disease severity (p>0.05, Wilcoxon test, Fig. 4b), nor between baseline and seven days after treatment with nimotuzumab.

4. Discussion

Not only the clinical symptoms such as fever, cough and dyspnea are characteristic features of COVID-19 disease. Some immunological parameters and high incidence of alterations in inflammatory and coagulation-related biomarkers may also indicate this infection and have been associated with a poor prognosis [16]. We described here the immunological, inflammatory and prothrombotic evaluation of moderately and severely ill COVID-19 patients included in a clinical trial to evaluate for the first time, the effect of using an anti-EGFR monoclonal antibody in the COVID-19 scenario during the B.1.617.2 (Delta) variant wave in Cuba.

The rapid deterioration of severely affected COVID patients may be attributable to an over-reacting immune system defined as cytokine release syndrome (CRS) [16]. This manifestation is characterized by a pro-inflammatory response with increase of inflammatory cytokines such as IL-6 [12]. Surprisingly, the serum IL-6 concentration of moderately and severely ill patients in the present study, was similar. Previous results of our group reported during the first wave of COVID-19 in Cuba, showed higher levels of IL-6 in severe patients than in moderately ill [17]. Additionally, it is interesting to remark that the median serum levels of IL-6 in our cohort of Delta-variant-infected patients are higher than those reported in our previous work with D614G variant-infected patients [17]. The delta variant is characterized by mutations in the spike proteins. These mutations have led the virus to become more transmissible than previous variants. This variant also causes more severe disease, has a significant higher risk of hospitalization, intensive care unit admission, pneumonia developing and about 137% greater risk of death compared to non-delta SARS-CoV-2 [18].

In COVID-19 patients treated with nimotuzumab IL-6 serum levels decreased near to the normal limit. Additionally, more than 95% of patients in which circulating IL-6 decreased or increased less than 14.27 pg/mL recovered or were discharged 14 days after receiving the first monoclonal antibody infusion. In a recent publication showing the preliminary results of nimotuzumab clinical trial in COVID-19 moderately and severe patients, our group reported that the antibody was safe;
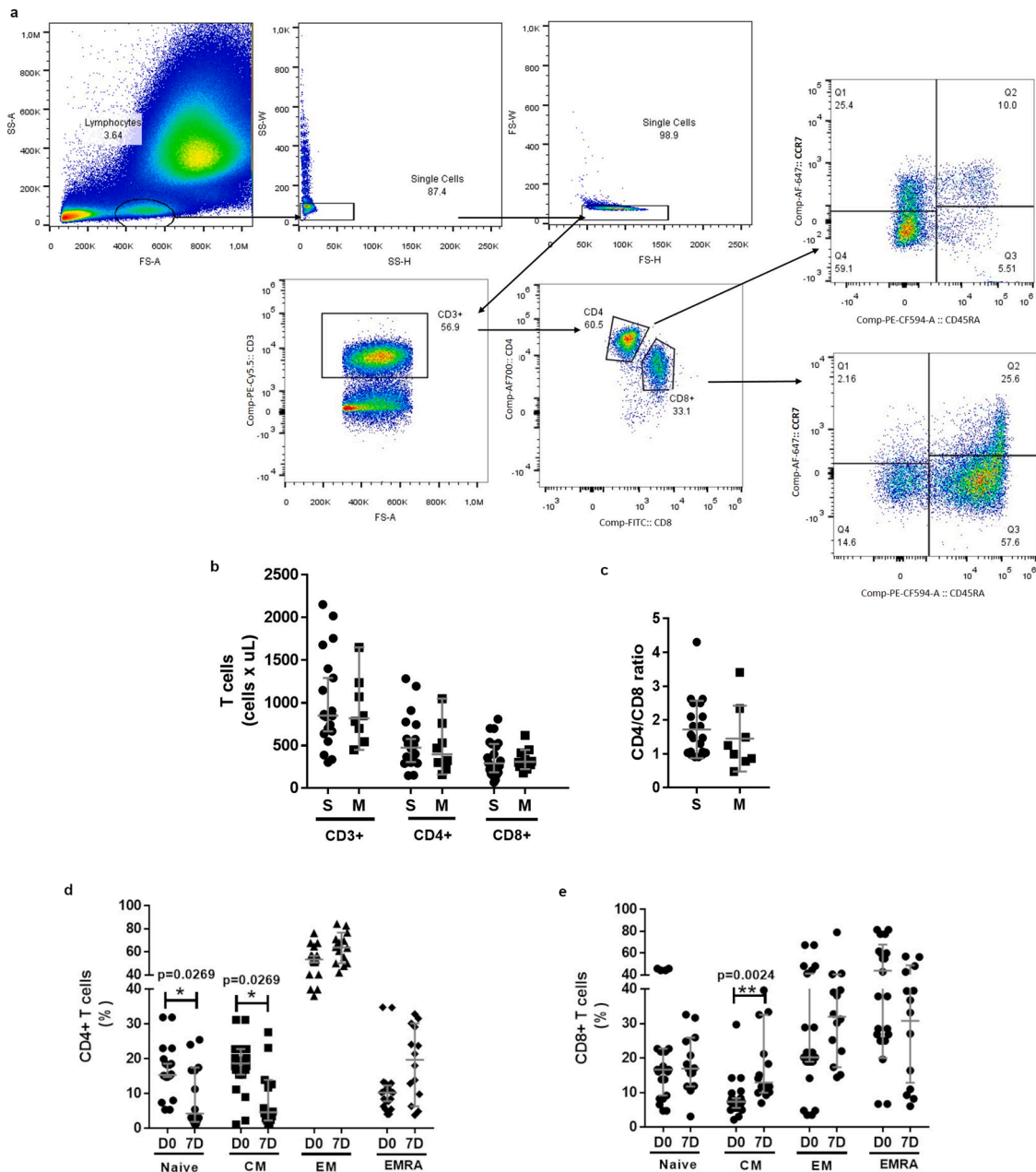
Fig. 3. T cell subpopulations in moderately and severely ill patients. a Gating strategy for single CD3+ T cells, then gated on CD4+ and CD8+ T cells and subsets: naïve (CD45RA+CCR7+), central memory (CM, CD45RA-CCR7+), effector memory (EM, CD45RA-CCR7−) and terminally differentiated effector T cells reexpressing CD45RA (EMRA, CD45RA+CCR7-) CD4+ and CD8 T+ cell subsets. b CD3+, CD4+, CD8+ T cells in severe (n=24) and moderate (n=8) groups. c CD4/CD8 ratio in severe (n=24) and moderate (n=8) groups. d Stages of cell differentiation in CD4+ T lymphocytes in severe patients (n=24). e Stages of cell differentiation in CD8+ T lymphocytes in severe patients (n=24). The asterisks indicate statistically significant differences among the groups (*p<0.05; **p<0.01); using paired t test or Wilcoxon test.

the recovery rate was above 80% for severe patients and none of the patients had signs of fibrosis at the follow-up evaluation [14].

NLR has been recognized as a very useful independent predictive factor for identifying COVID-19 patients developing severe disease [3]. Data from our group in a study conducted in Villa Clara suggested that NLR higher than three was a predictive biomarker of COVID-19 severity (personal communication). The median value of NLR in our cohort of moderately and severely ill patients was higher than 3. Interestingly, we did not find differences between moderate and severe groups concerning this biomarker of systematic inflammation.

Plasma levels of PAI-1 were elevated at baseline in all evaluated patients. It has been proposed that IL-6 plays a central role in the production of other cytokines and PAI-1 during COVID-19 disease [16].

Endothelial cells and also senescent alveolar type II cells secrete PAI-1, which promotes a profibrotic phenotype [19]. The vascular endothelium has emerged as a leading player in COVID-19. The endothelitis manifested during this disease could explain the systemic impaired microcirculatory function in different vascular beds, their clinical sequelae and the increased severity of the disease found in patients with pre-existing endothelial dysfunction, which is associated with hypertension, diabetes, obesity and previous cardiovascular disease, all of them associated with poor prognosis in COVID-19 [20]. In moderately and severely ill COVID-19 patients treated with nimotuzumab, a tendency towards a decreased plasma levels of PAI-1 after the first two doses of the monoclonal antibody, was observed.

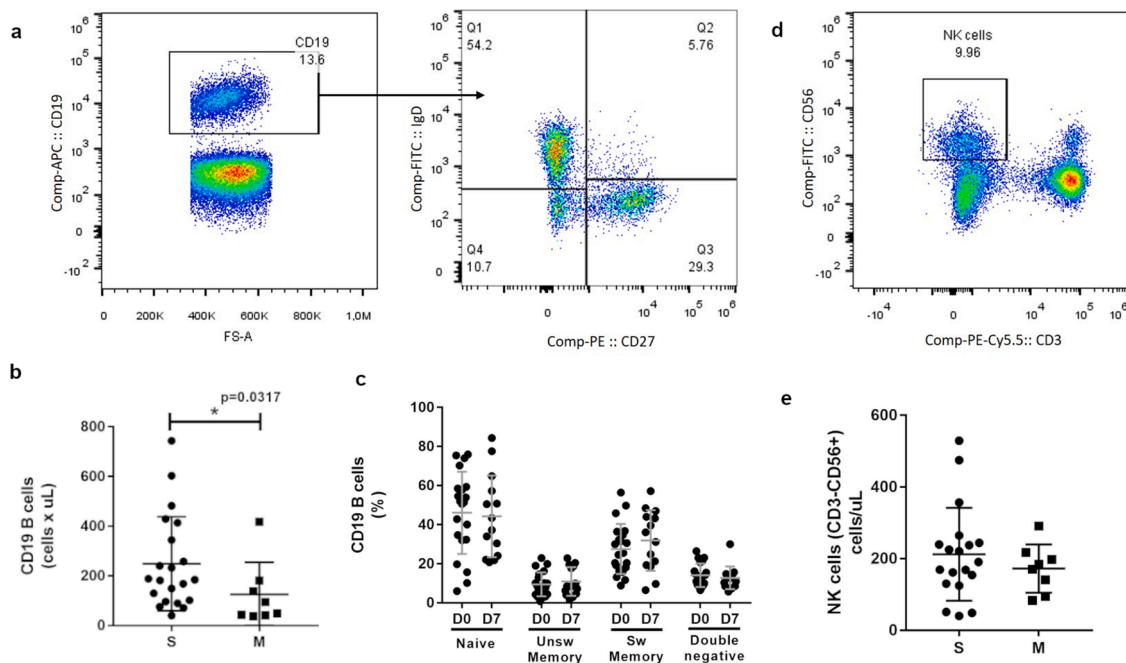Sars-CoV-2 infection is characterized by a severe lymphopenia, with

Fig. 4. B cell (CD19+) subsets in moderately and severely ill COVID-19 patients. a Gating strategy for single CD19+ T cells, then gated for CD19+ subsets: naive (IgD+CD27-), unswitched memory (Unsw Memory, IgD+CD27+), switched memory (Sw Memory, IgD-CD27+) and double negative (IgD-CD27-). b CD19+ B cells in moderately (n=8) and severely ill (n=24) COVID-19 patients. c Stages of cell differentiation in CD19+ B lymphocytes in severe patients (n=24). d Gating strategy for single NK cells. e NK cells in moderately (n=8) and severely (n=24) ill COVID-19 patients. The asterisks indicate statistically significant differences among the groups (*p<0.05); using paired t test or Wilcoxon test.

reduction of both CD4+ and CD8+ T cells [1]. Our results also showed CD4+ T cell lymphopenia. Nevertheless, absolute count of CD8+ T cells were in the normal range, when we compared our data with a previous work regarding normal lymphocyte subpopulations in healthy Cuban adults [15]. The mechanisms of lymphopenia during COVID-19 infection have been deeply hypothesized. Some of them include immune dysregulation due to cytokine accumulation, which affects lymphocyte apoptosis, migration of immune cells into the lungs, and impairment of lymphoid organs [1]. Vedder et al. evaluated the cellular profile in bronchoalveolar lavage in COVID-19 patients and observed increased CD8+ T-cell values compared to other Corona virus types. Consequently, they suggested the migration of lymphocytes to the site of infection as an explanation for peripheral blood lymphopenia [21].

Regarding B cell compartment, conflicting results have been reported. In the present study, CD19+ cells were significantly lower in moderate patients compared to severely ill. A higher frequency of CD19+ B cells in severe COVID-19 patients has been previously described by Sosa-Hernández and colleagues. [22]. Contrarily, Çölkesen et. al. found that a lower number of B cells was associated with severe disease and was linked to poor outcomes in COVID-19 patients [23]. SARS-CoV-2 infection has also been proposed to affect both the number of circulating NK cells and their phenotype, leading to impairment, exhaustion and hyporesponsiveness. These alterations are more evident in patients with more severe clinical presentations [24]. However, in our study, no differences in NK cells were found between severe and moderate patients.

There was higher frequency of EMRA within CD8+ T cells whereas in CD4+ T cells, the EM population were the most prominent at baseline. After one week of the first evaluation, less differentiated CD4+ T cells significantly decreased, while, CD8+ CM T cells increased. T cells differentiate from naive into memory T cell subpopulations in response to viral antigen exposure. Consequently, they guarantee a much faster and stronger response when the same pathogen is encountered again. In the acute phase of antiviral immune response against SARS-CoV-2, there is a vast majority of effector and EM cell populations. Some of these

memory subpopulations might be functionally exhausted during acute COVID-19 [25]. Kalpacsi and colleagues have reported that CD4+ CM T cells and CD4+ naive T cells are critically reduced in the course of severe disease [26]. Regarding CD8+ T cells it has been described an increase in stem cell-like memory as well as in EM subpopulations during the viral phase [27]. Therefore, the shift towards a memory phenotype found in these patients, could be attributed to a physiologic immune response to SARS-CoV2 infection, rather than an effect of the treatment with nimotuzumab.

It was unexpected the similitude found among our severely ill and moderately ill patients regarding some parameters such as IL-6 serum concentration, NLR and frequency of several lymphocyte subpopulations. The definition of disease severity consists mainly in clinical characteristics like respiratory rate ≥ 30 breaths/min, oxygen saturation (SpO2) <94% at rest, a pressure of arterial oxygen to fractional inspired oxygen (PaO2/FiO2) < 300. Patients with moderate illness are those with evidence of lower respiratory disease according to the clinical assessment or imaging, able to maintain SpO2 >92% at rest (or above 90% for patients with chronic lung disease), fever >38°C or persistent cough [8]. Our data thus indicate that several immune alterations are present when the infection becomes clinically relevant, before the clinical manifestations suggest the presence of a severe disease.

In summary, our results suggest that moderately and severely ill COVID-19 patients, diagnosed during the B.1.617.2 variant wave in Cuba, had high serum levels of IL-6, elevated plasma concentration of PAI-1, median NLR above three and lymphopenia mainly related with CD4+ T cells. Additionally, our work suggests that treatment with nimotuzumab might reduce IL-6 and PAI-1 in these patients.

5. Conclusions

The results from this work show that both moderately and severely ill COVID-19 patients diagnosed during the Delta variant wave in Cuba, exhibit evidence of hyperinflammatory and prothrombotic status, as well as CD4+ lymphopenia. Surprisingly, patients with moderate and

severe disease, were not different regarding the studied parameters, addressing the idea that several immune alterations could be present before the infection becomes clinically relevant and highlighting the necessity of the use of biomarkers to promptly identify individuals at risk and prevent severity. Further, the decrease in IL-6 and PAI-1 levels in patients treated with nimotuzumab, suggests that this therapy could be able to interfere with the close relationship between cytokines and procoagulant mediators that has been related to the hypercoagulable state, associated with enhanced risk for thrombotic events and mortality in the course of COVID-19.

Author contributions

Conceptualization, Project asministration, Supervision and Validation: D Saavedra, AL Añé-Kourí, T Crombet Ramos and M Ramos Suzarte . Investigation: H Diaz Londres, J Jiménez Armada, A Hernández Martínez, Y Hernández Sánchez (administered the experimental drug and followed COVID-19 patients at the hospital ICU or conventional wards), EM Llanez Gregorich, J Mena, D Saavedra and A AL Añé-Kourí (contributed with sample collection), EM Llanez Gregorich and J Mena (performed the hemochemical studies), D Saavedra and AL Añé-Kourí (performed the immunological studies), Y Zamora González (did the determinations of PAI-1). Data curation: M Troche Concepción and L Medel Pérez were responsible for monitoring. Formal analysis: D Saavedra, AL Añé-Kourí, T Crombet Ramos and P Lorenzo-Luaces . Writing -original draft: D Saavedra and AL Añé-Kourí . Writing - review & editing: T Crombet Ramos, M Ramos Suzarte, AL Añé-Kourí and D Saavedra . All authors contributed to manuscript revision, read, and approved the submitted version.

Ethical conduct of research

The protocol was approved by the ethic committees of the participating hospitals and by the National Regulatory Agency, CECMED. All patients signed the informed consent before trial inclusion. The protocol was listed in the public registry of clinical trials (https://rpcec.sld. cu/ensayos/RPCEC00000369-En).

Declaration of Competing Interest

This study was funded by the Cuban Ministry of Health and the Center of Molecular Immunology. D Saavedra, AL Añé-Kourí, P Lorenzo-Luaces, L Medel Pérez, M Troche Concepción, M Ramos Suzarte and T Crombet Ramos currently work for the Center of Molecular Immunology, the institution that generated and originally patented nimotuzumab. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Acknowledgements

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