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Analysis of the Blood Levels of NK and NKT Cells in Patients with Severe SARS-CoV-2 Infection

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ABSTRACT

Background: Clinical features of SARS-CoV-2 infection vary, ranging from asymptomatic cases to pneumonia, and other serious complications. Some populations have been observed to be at higher risk for severe disease and death compared to other ethnical groups.

Objective: To evaluate two parameters of the innate immune system, that play a significant role in viral immunity.

Methods: In samples of peripheral blood from sixteen patients with severe COVID-19, ten with asymptomatic to mild illness, and fifteen healthy subjects, the percentage of NK and NKT cells, the expression of different NK cell receptors and the blood levels of pro-inflammatory cytokines were tested.

Results: We observed that patients with severe COVID-19 showed significantly lower frequencies of both $CD56^{dim}$ and $CD56^{bright}$ NK cells compared to patients with mild illness or healthy controls. Furthermore, patients with severe manifestation of COVID-19 exhibited an aberrant expression of the natural cytotoxicity receptors NKp30, NKp44 and NKp46. Similarly, NK cells from these patients showed statistically significant differences in the expression of various killer immunoglobulin-like receptors (KIRs) in the two main cell subsets ($CD56^{bright}$, $CD56^{dim}$) compared to controls or patients with mild disease. Moreover, patients with severe illness displayed decreased frequency of NKT cells (defined as $CD3^{+}CD56^{+}$) and elevated blood levels of the cytokines IL-6 and IL-8.

Conclusion: This study suggests that the abnormal features of NK and NKT cells observed in patients with severe SARS-CoV-2 infection may play an important role in the outcome of this infectious disease in various population groups.

Keywords: COVID-19, NK Cells, NKT Cells, SARS-CoV-2, Membrane Receptors

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INTRODUCTION

Clinical signs of SARS-CoV-2 infection (COVID-19) vary and can range from asymptomatic cases to pneumonia. This may be accompanied by serious complications such as an acute respiratory syndrome, multiple organ failure and thromboembolic events (1). Although the pathogenesis of COVID-19 is complex, it is evident that severe manifestations are closely related to a defective regulation of the immune system. This plays a role in the delayed elimination of the virus and an uncontrolled inflammatory phenomenon (2, 3). In this regard, patients with severe manifestations of SARS-CoV-2 infection show decreased levels of T, B, and NK lymphocytes, while neutrophils are increased (4-6). Likewise, increased blood levels of different pro-inflammatory mediators such as IL-6, IL-1 and TNF-α have been reported (7-10).

The causative agent of COVID-19 can infect lung cells, vascular endothelial cells and alveolar macrophages (11-14). Once the viral infection is established, innate immune cells recognize pathogen-associated molecular patterns (PAMP's), such as viral ssRNA. Moreover, the lysis of infected cells leads to the release of danger-associated molecular patterns (DAMP's), which are also identified by immune cells. This prompts the activation of signaling pathways that stimulate the production of type 1 interferons and proinflammatory cytokines (15).

NK cells are able to recognize target cells, which induces their activation, increasing their cytotoxic activity (16). Accordingly, NK cells are an effective mechanism of defense in viral infections and against neoplastic cells (16, 17). However, NK cells may also have an important pro-inflammatory effect, mainly through the synthesis of cytokines (17). The recognition of target cells by NK cells is mediated by various membrane molecules, including the natural cytotoxic receptors, (NCRs) such as NKp46, NKp44 and NKp30 as well as killer cell Ig-like receptors (KIRs) like CD158, ILTs/LILRs (CD85) and CD94/NKG2 molecules (18, 19). Different subpopulations of NK cells have been characterized based on the expression of these and other receptors (e.g., CD56) (17). It has been estimated that, a large number of NK cell subsets can be identified based on the presence or absence and the abundance of these receptors, (17, 20).

NKT lymphocytes are characterized by the expression of cell receptors found in NK cells as well as by bearing the T cell receptor (TCR) that primarily detects glycolipids (21). Although these cells are rare, they likely play an important role in various physiological and pathological conditions (22). Several data suggest that NKT cells play an important role as the first line of defense against various pathogens, including viruses (23, 24). It has been proposed that glycolipids and cytokines (mainly IL-12) induce the activation of these lymphocytes, which in turn, through various mechanisms, promotes the activation of cytotoxic $CD8^+$ T cells (21).

Levels of various subsets of NK cells in the peripheral blood from patients with SARS-CoV-2 infection have been analyzed in several studies, (10, 15, 19, 25, 26). Similarly, the levels of NKT cells in COVID-19 patients have also been reported (24). However, since various data indicate that some patients with SARS-CoV-2 infection are at high risk of developing severe disease and death (27-29), the aim of this study was to conduct a comparative analysis of NK and NKT cell levels in the peripheral blood of patients with mild and severe SARS-CoV-2 infection, as well as healthy subjects. Our findings suggest that patients with severe disease exhibit distinct abnormalities in the frequency of NK and NKT cells, which may be linked to a compromised innate immune response against SARS-CoV-2.

MATERIALS AND METHODS

Subjects

In a pilot observational and comparative study, hospitalized patients with COVID-19

(confirmed by an RT-qPCR test) were included (n=16) as along with patients with mild or asymptomatic disease (n=10), and healthy subjects without clinical signs and symptoms of COVID-19 (and a negative RT-qPCR test, n=15). In this pilot study, sample sizes were not calculated, and the number of individuals included was determined by their availability and the estimated time needed to carry out the study. A 6.0 ml peripheral blood sample was obtained from all individuals included in this study, and the number of PBMC isolated in each group (arithmetic mean and SD) was as follows: 7.2 x 10^6 in healthy controls, 6.7 x 10^6 in patients with mild illness and 5.4 x 10^6 in patients with severe manifestation of SARS-CoV-2 infection, with no apparent differences among them (p>0.05, ANOVA test). Patients were classified into two groups, mild or severe disease, accordaning to standard criteria and evaluated by an experienced clinician. Patients with a breathing frequency of ≥30/min, oxygen saturation <94% and lung infiltrates >50% were considered to have severe COVID-19. Patients with a normal pulmonary CT scan, normal oxygen saturation and no evidence of organ involvement (pulmonary, renal, CNS, etc.) were classified as having mild COVID-19. All patients with severe disease required supplemental oxygen and the mean number of days of hospitalization was 7.0±5.0 (arithmetic mean±SD). Seven of these patients required assisted mechanical ventilation (AMV). Table 1 shows the clinical features and demographic characteristics of the patients included in the study. All patients and controls signed an informed consent for their participation in this study, which was conducted in accordance with the Declaration of Helsinki. The Bioethical Committee of our State (San Luis Potosí, México) approved this study.

Flow Cytometry Analysis

The peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Ficoll-Hypaque, GE Healthcare Life Sciences, Issaquah, WA), and cellular viability was evaluated by trypan blue staining and conventional microscopy. The cells were then washed and stained with the following monoclonal antibodies (mAbs): CD3/PercP, clone UCHT1, (BioLegend, San Diego, CA), CD56/APC, clone NCAM16.2, (BD Biosciences, San Jose, CA), NKp30/FITC, clone CLH9, (eBioscience, Waltham, MA), NKp44/FITC, clone P44-8, (BioLegend), NKp46/FITC, clone 9E2, (eBioscience), CD158a/h/g/PE (KIR2DKL1/KIR2DS1/KIR2DS5), clone HP-MA4, (Invitrogen Life Technologies Corp, Carlsbad, CA) and CD158e/k/PE (KIR3DL1/KIR2DL2), clone 5.133 (Miltenyi Biotec, San José, CA) for 30 minutes on ice in darkness. Then, the PBMC's were washed twice and analyzed in a flow cytometer (FACSCanto II, Becton Dickinson). The data was processed using the software FlowJo v10.0 (Tree Star Inc, Ashland, OR). Gate setting was carried out using fluorescence minus one (FMO) as the negative control. Our flow cytometry analyses did not include the use of the CD16 marker, mainly because in peripheral blood NK cells there is a strong association between the lack of CD16 and the high expression of CD56 ($CD56^{bright}CD16^{-}$ cells) as well as between the presence of this Fc receptor and the low-to-medium expression of CD56 ($CD56^{low/medium}CD16^{+}$ cells) (17).

Quantification of Pro-inflammatory Cytokines

Serum levels of the cytokines IFN-γ, IL-17A, IL-2, IL-4, IL-6, IL-8, IL-10, and TNF-α, were analyzed using a flow cytometer (Accuri C6, Becton Dickinson) and a Cytometric Bead Array (CBA) (BD Biosciences), following the manufacturer's instructions. The data were analyzed with FCAP Array Software v3.0 (BD Biosciences).

Statistical Analysis

Data are presented based on their distribution (Gaussian or non-Gaussian) as either the mean and standard deviation (SD), or the median and interquartile range. Analysis of two groups was conducted using either the Mann-Whitney U test or the t test,

Table 1. Clinical data from patients with asymptomatic and/or mild and severe COVID−19

	Patients from asymptomatic and/or mild COVID−19	Patients from severe COVID−19
n	10	16
Age (years)	41.7±18.4	57.18±19.8
Sex (%)		
Female	40	45
Male	60	55
Days of hospitalization	Not applicable	7±5.5
Therapy/treatment		
Supplemental oxygen (%)		100
Assisted mechanical ventilation (%)		25
Antibiotic therapy (%)		95
Azithromycin	Not applicable	58
Ceftriaxone		53
Linezodil		10.5
Meropenem		10.5
Cefotaxime		16
Ceftaroline		10.5
Signs and symptoms (%)		50
Fever		61.1
Cough		78
Headache	20	39
Myalgia	10	28
Arthralgia		78
Dyspnea		10.5
Anosmia/dysgeusia	10	22.2
Odynophagia		5.5
Exanthema		11.1
Runny nose		53
Tiredness	30	42.1
Nausea/vomiting/diarrhea		
Intensive care unit (%)	Not applicable	42
Percentage of deceased	Not applicable	30
Without comorbidities (%)		0
One comorbidity (%)	Not applicable	6
Two or more comorbidities (%)		94
Comorbidities (%)		
Diabetes		76.5
Arterial hypertension		83
Cardiovascular disease		23.5
Hypothyroidism	Not applicable	30
Asthma		6
Epilepsy		6
Chronic kidney disease		23.5
Breast cancer		6
Depressive disorder		6
Body mass index	22.5±1.3	27.8±3.2
Normal weight (%)	90	21
Overweight (%)	10	79
Smokers (%)	10	12
Allergies (%)		35
Drugs	Not applicable	43
Foods		72
Flu vaccine (%)	50	40
Severe acute respiratory infections in year (%)	10	10

while tests involving three groups or more were performed using ANOVA or the non-parametric Kruskal-Wallis H test, with *post hoc* analysis, as needed. For association analysis, either the Spearman or Pearson tests were employed. All data were analyzed using GraphPad Prism software v.8.0.1 (GraphPad Inc, Boston, MA) and values of $p<0.05$ were considered statistically significant.

RESULTS

Blood Levels of NK Cells in Patients with COVID-19

The strategy of flow cytometry analysis of NK cell subsets is shown in Fig. 1A. We found that patients with severe COVID-19 exhibited a significantly lower percentage of NK cells ($CD3^-CD56^+$) compared to healthy individuals ($p<0.001$, Fig. 1B). Conversely, comparable levels were observed in healthy individuals and patients with mild illness ($p>0.05$, Fig. 1B). Similarly, the levels of $CD56^{bright}$ NK cells were significantly decreased in severe cases of COVID-19 ($p<0.05$, compared to controls, Figs. 1C), with similar numbers in patients with mild infection and controls (Fig. 1C). In addition, the percentage of another major subset of NK cells ($CD56^{dim}$) was significantly lower in patients with severe COVID-19, compared to those with mild disease or healthy controls ($p<0.05$, $p<0.01$, respectively, Fig. 1D). Moreover, the proportion of NK cells expressing NKp30 was lower in severe COVID-19 compared to healthy controls ($p<0.05$, Fig 1E). However, the frequency of $NKp44^+$ NK cells was higher in patients with severe disease ($p<0.05$, Fig. 1F). Similarly, the percentage of $CD56^{dim}$ NK cells that express the receptors NKp44 and NKp46 was significantly higher in patients with severe COVID-19 ($p<0.05$, Figs. 1H and 1I), and similar results were observed for the $CD56^{bright}$ NK cells expressing the NKp46 receptor ($p<0.05$, Fig. 1J).

When patients with severe SARS-CoV-2 infection were classified, based on whether or not they were admitted to the intensive care unit (ICU), we observed similar percentages of $CD3^-CD56^+$ cells in both groups ($p>0.05$, Fig. 2A). Nevertheless, the proportion of NK cells bearing the $NKp44^+$ receptor was increased in patients admitted to the ICU ($p<0.01$, Fig. 2B). An additional correlation analysis revealed a positive association between the levels of NK cells and the body mass index (BMI) of the patients with severe disease ($r=0.57$, $p=0.02$, Fig. 2C). Furthermore, the percentages of $NKp30^+CD3^-CD56^{dim}$ NK cells were higher in obese patients (BMI>30) compared to patients with overweight (BMI 25-29; $p<0.001$, Fig. 2D). However, no significant associations were observed between BMI and other parameters ($p>0.05$, data not shown).

Analysis of potential differences in the expression of certain KIRs by NK cells revealed that patients with severe SARS-CoV-2 infection exhibited a significantly lower ratio of cells expressing the receptors $KIR3DL1^+/KIR2DL2^+$ ($CD158e^+/k^+$) (Figs. 3A and 3B). However, similar levels of $KIR3DL1^+/KIR2DL2^+$ NK cells were observed in controls and patients with mild SARS-CoV-2 infection (Fig. 3B). Furthermore, the levels of $KIR2DKL1^+/KIR2DS1^+/KIR2DS5^+$ ($CD158a^+/h^+/g^+$) NK cells were also diminished in patients with severe COVID-19, compared to controls or those with mild illness, with no differences between the latter groups (Fig. 3C). An association analysis between BMI and KIR expression, revealed a nearly significant correlation in the case of the KIR3DL1/KIR2DL2 receptors ($r=0.47$, $p=0.06$, Fig. 3D). In addition, no significant correlation was observed between the age of patients and the expression of the KIR´s studied (data not shown).

Blood Levels of NKT Cells in Patients with COVID-19

When we analyzed the levels of NKT cells (defined as $CD3^+CD56^+$), a significantly lower percentage of these NKT cells was observed

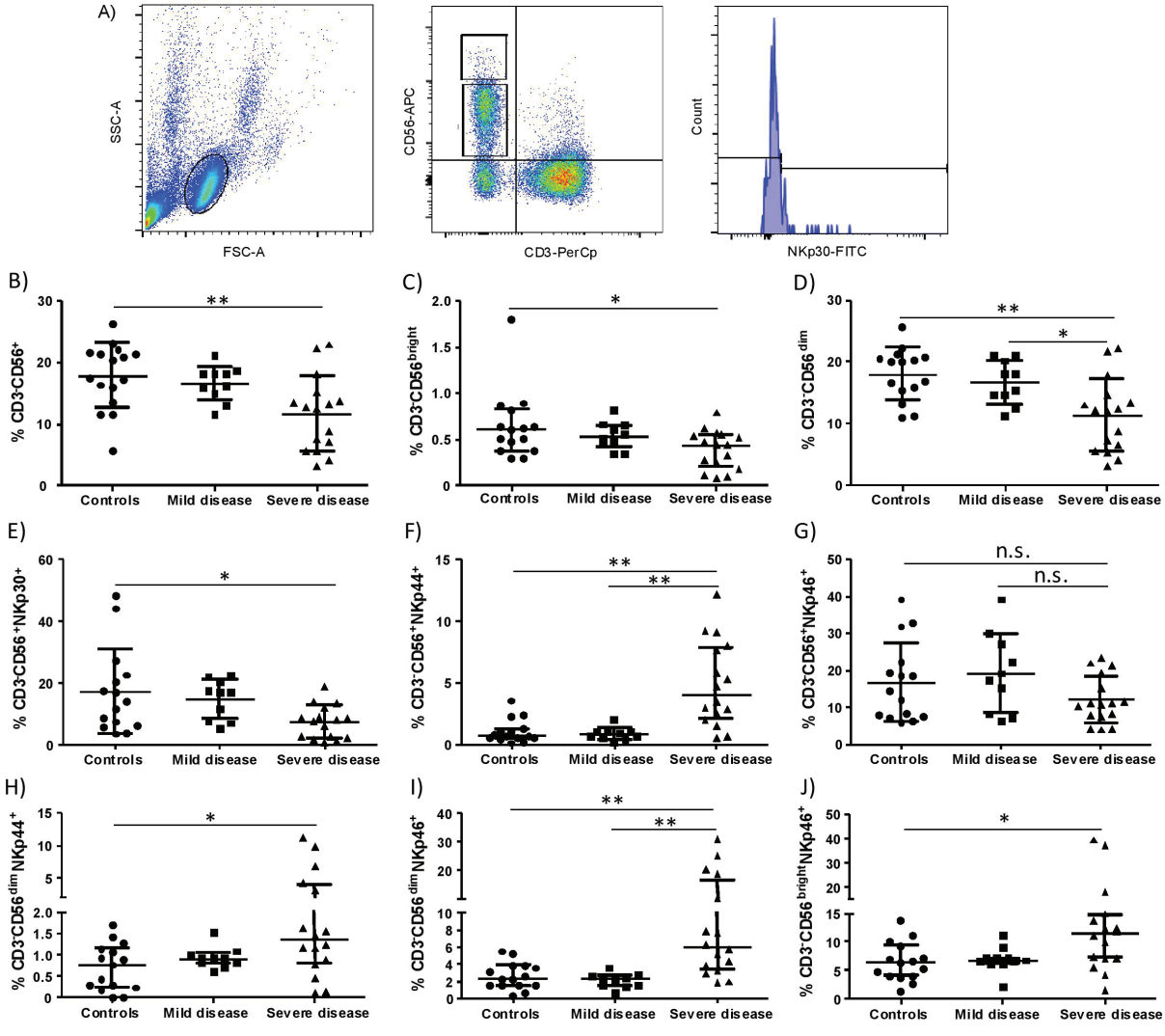

Fig. 1. Flow cytometry analysis of NK cells in patients with COVID-19. Peripheral blood mononuclear cells were isolated from healthy controls and patients with mild and severe COVID-19, and analyzed by flow cytometry, as stated in Materials and Methods. A) Flow cytometry strategy for the analysis of NK cells and various cell subsets. Percentages of cells in the corresponding gates are indicated. B) Comparison of the frequency of NK cells (defined as $CD3^{-}CD56^{+}$ cells, relative to the total count of lymphocytes) in the three study groups. C) Comparison of the percentages of the $CD56^{bright}$ NK cell subset in the three study groups. D) Comparison of the frequency of the $CD56^{dim}$ NK cell subset in the three study groups. E) Comparison of the frequency of the $NKp30^{+}$ NK cells in the three study groups. F) Comparison of the frequency of the $NKp44^{+}$ NK cells in the three study groups. G) Comparison of the frequency of the $NKp46^{+}$ NK cells in the three study groups. H) Comparison of the frequency of the $CD56^{dim}NKp44^{+}$ NK cells in the three study groups. I) Comparison of the frequency of the $CD56^{dim}NKp46^{+}$ NK cells in the three study groups. J) Comparison of the frequency of the $CD56^{bright}NKp46^{+}$ NK cells in the three study groups. *p<0.05; **p<0.01; n.s., non-significant difference. Horizontal lines correspond to the arithmetic mean and SD (Figs. B-E, H, J) or median and interquartile range (Figs. F, G, I). Values of NK cells are relative to the total count of lymphocytes.

in patients with severe SARS-CoV-2 infection compared to healthy individuals and those with mild infection (p<0.05 in both cases, Fig. 4A). However, the percentage of NKT cells that express $NKp44^{+}$ was significantly higher in patients with severe COVID-19, compared to controls and patients with mild illness (p<0.05 in both groups, Fig. 4B). Nevertheless, comparable levels of $NKp30^{+}$ NKT cells were observed in all three groups studied (Fig. 4C). Likewise, there were no statistically significant differences when analyzing the NK: NKT cell ratio in the three groups included in this study (Fig. 4D).

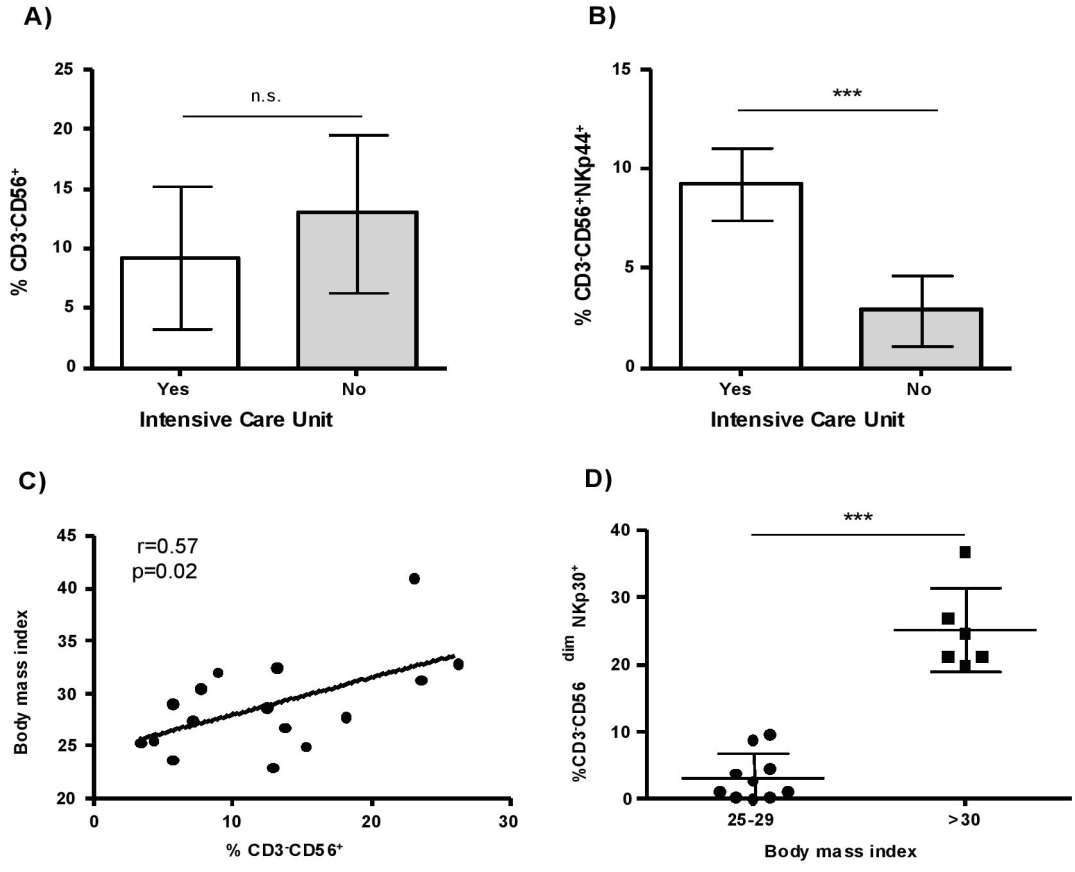

Fig. 2. Analysis of the association between the levels of NK cells and clinical parameters in patients with COVID-19. A) The percentages of NK cells ($CD3^-CD56^+$) in patients with severe COVID-19 who were admitted to the intensive care unit versus those who were not. B) The percentages of $NKp44^+$ NK cells in patients with severe COVID-19 who were admitted to the intensive care unit versus those who were not. C) Correlation analysis between the levels of NK cells and body mass index in patients with severe COVID-19 with r and p values are provided. D) The percentages of $CD3^-CD56^{dim}NKp30^+$ cells in patients with COVID-19 based on their body mass index (25-29, overweight; ≥30, obesity). Horizontal lines represent the arithmetic mean and SD. ***p<0.001; n.s., non-significant difference. Values of NK cells are relative to the total count of lymphocytes.

Serum Levels of Different Cytokines in Patients with COVID-19

Finally, when the serum concentrations of different cytokines were evaluated, significantly increased concentrations of the cytokines IL-6 and IL-8 were observed in patients with severe SARS-CoV-2 infection (healthy controls, 4.85±10.54 pg/ml; mild disease 9.15±21.03 pg/ml, severe disease 76.13±304.8 pg/ml; and healthy controls, 3.7±0.43 pg/ml, mild severe, 4.08±1.12 pg/ml; severe disease, 6.04±4.82 pg/ml, respectively, mean ± standard deviation, p<0.05 in both groups of study, Figs. 5A and 5B). However, the levels of IFN-γ were similar between the different groups in the study, including controls and patients (p>0.05, ANOVA test, Fig. 5C). Additionally, similar concentrations of the two pro-inflammatory cytokines IL-6 and IL-8 were found in healthy subjects and patients with mild COVID-19. Furthermore, when patients with severe COVID-19 were classified into those admitted to the ICU or not, significantly increased concentrations of the cytokines IL-6 and IL-8 were detected in the former group (p<0.05 in both groups, Kruskal-Wallis test with *post hoc* analysis, Figs. 5D and 5E). Finally, the levels of the other cytokines tested (IL-2, IL-4, IL-10, TNF-α and IL-17A), were similar in patients with COVID-19 (admitted or not to the ICU) and healthy controls or patients with mild SARS-CoV-2 infection (p>0.05 in all comparisons, data not shown).

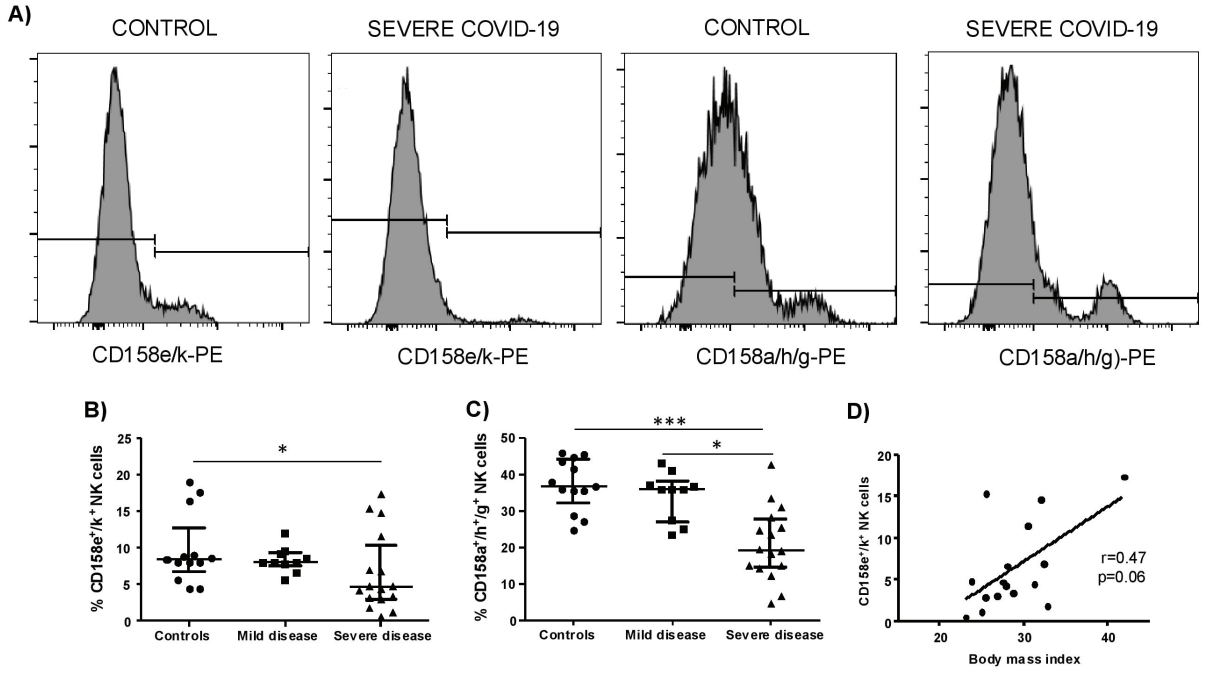

Fig. 3. Analysis of the expression of several KIR receptors by NK cells from patients with COVID-19. Peripheral blood mononuclear cells were isolated from both healthy controls and patients with mild and severe COVID-19 and analyzed using flow cytometry to determine the expression of the KIR3DL1/2DL2 (CD158e/k) or the KIR2DL1/2DS5/2DS1 (CD158a/h/g) killer Ig-like receptors by NK cells. A) Representative flow cytometry histograms of the levels of $CD158e^+/k^+$ and $CD158a^+/h^+/g^+$ NK cells in healthy controls and COVID-19 patients. The percentages of positive cells in the corresponding gates are indicated and referred to as the NK cell population ($CD3^-CD56^+$ cells). B) The levels of $CD158e^+/k^+$ NK cells in healthy controls and patients with mild and severe COVID-19. C) The levels of $CD158a^+/h^+/g^+$ NK cells in healthy controls and patients with mild and severe COVID-19. D) Correlation analysis between the levels of $CD158e^+/k^+$ NK cells and the body mass index in COVID-19 patients. The r and p values are provided. *p<0.05; ***p<0.001. Horizontal lines in B and C represent the median and interquartile range. Values of NK cells (Figs. B-D) are relative to the total count of lymphocytes.

DISCUSSION

Since some individuals show an increased risk of severe COVID-19 and death due to this infection (27, 29), we decided to conduct a comparative analysis of NK and NKT cells levels in the peripheral blood of patients with mild and severe COVID-19. In this regard, the role of NK cells in lysing of cells infected by viruses, as well as their regulatory role in triggering immune responses mediated by cells against the virus have been characterized (30). It has been proposed that a defective innate immune response directed towards SARS-CoV-2 virus is a significant risk factor for severe COVID-19 infection and death. Various data suggest that a defective innate immune response to SARS-CoV-2 allows for increased replication of the virus, leading to the activation of other immune cells and the generation of a severe inflammatory responses, causing extensive tissue damage (19, 30, 31).

Our results indicate that patients with severe SARS-CoV-2 infection show low levels of both $CD56^{dim}$ and $CD56^{bright}$ NK cells, suggesting that this viral infection does not have a preferential effect on the two main subsets of this immune cell population. It is worth noting that $CD56^{dim}$ NK cells exhibit a high cytotoxic activity, while $CD56^{bright}$ NK lymphocytes are considered immature cells and have a great capacity to synthesize different cytokines (e.g., TNF-α and IFN-γ), in response to IL-12 or IL-18, among others (19, 25). In addition, we found that a severe form of COVID-19 infection is associated with abnormal expression of various KIR receptors by NK cells, as well as increased

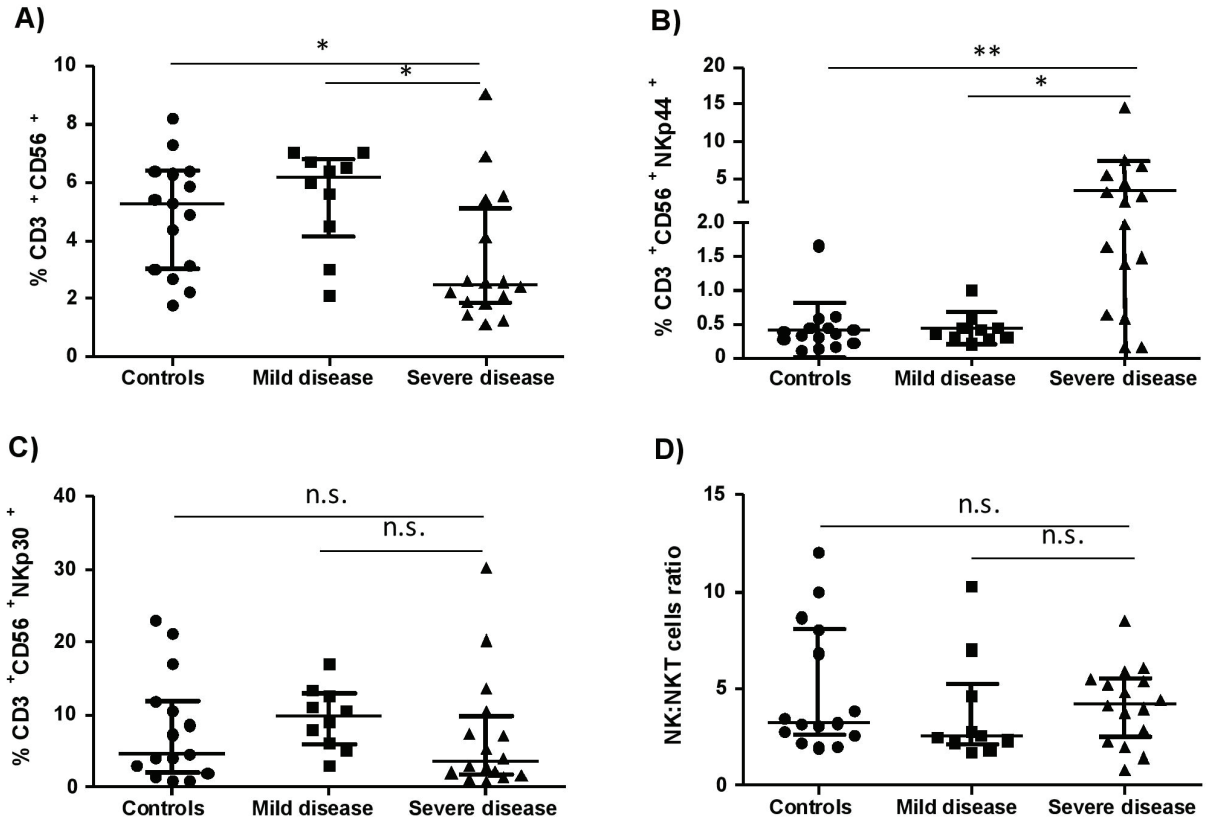

Fig. 4. Flow cytometry analysis of NKT cells in patients with COVID-19. Peripheral blood mononuclear cells were isolated from healthy controls and patients with mild and severe COVID-19, and analyzed using flow cytometry, as described in the Materials and Methods section. A) The levels of NKT cells (defined as $CD3^+CD56^+$) in healthy controls and COVID-19 patients. B) The levels of $NKp44^+$ NKT cells in healthy controls and COVID-19 patients. C) The levels of $NKp30^+$ NKT cells in healthy controls and COVID-19 patients. D) The NK: NKT cell ratio in the peripheral blood of healthy controls and COVID-19 patients. *p<0.05; ** p<0.01; n.s., non-significant difference. Horizontal lines represent the median and interquartile range. Values of NKT cells (Figs. A-C) are reported as a percentage of total lymphocytes.

blood levels of inflammatory cytokines such as IL-6 and IL-8. Furthermore, patients with severe COVID-19 were found to have significantly lower levels of NKT cells. . We believe that the quantitative abnormalities in NK/NKT cells detected in patients with severe COVID-19 may be associated with a defective innate immune response towards the SARS-CoV-2 virus, thus promoting the development of a hyperinflammatory condition. In this regard, we found it interesting that there is a close to significant correlation between the expression of the KIR3DL1/KIR2DL2 receptors and BMI (r=0.47, p=0.06). This is important because there is a well-known association between being overweight with systemic chronic inflammation, as well as other immune abnormalities, which include the number and functional capability of several subsets of regulatory lymphocytes, which also play a role in the pathogenesis of severe COVID-19 illness (32). However, it is well known that the KIR3DL1/KIR2DL2 molecules are inhibitory receptors that suppress the cytotoxic capability of NK cells. The possible explanation and clinical significance of this putative association are important points that still need to be revealed. It is likely that the defects we detected in NK/NKT cells are causally associated with severe COVID-19 infection, another interesting possibility to be explored in future studies. In any case, we believe it is important to determine whether these innate immune characteristics could be used as risk indicators for severe SARS-CoV-2 infection and death.

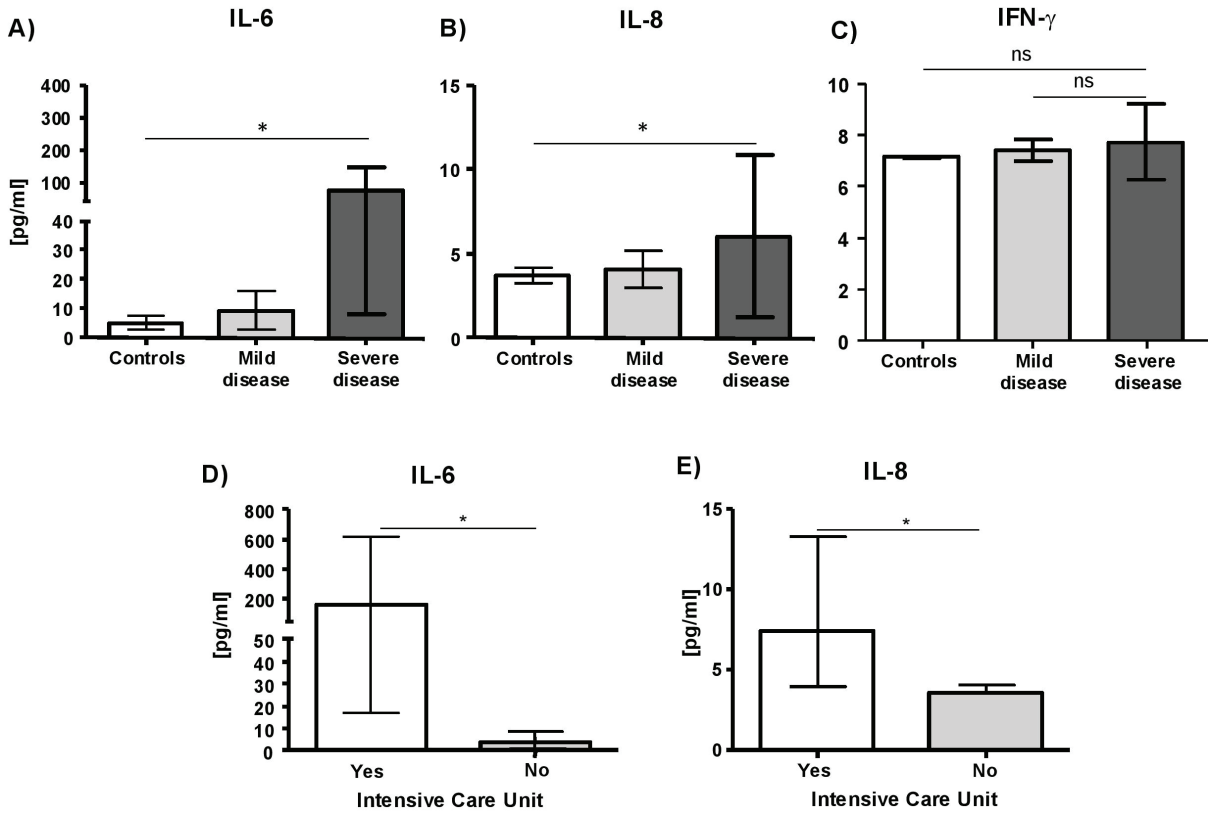

Fig. 5. Serum levels of cytokines in patients with COVID-19. Concentrations of IL-6, IL-8 and IFN-γ were analyzed in serum samples from healthy controls and patients with mild and severe COVID-19 using flow cytometry A) Serum concentrations of IL-6 in healthy controls and COVID-19 patients. B) Serum concentrations of IL-8 in healthy controls and COVID-19 patients. C) Serum concentrations of IFN-γ in healthy controls and COVID-19 patients. D) Serum concentrations of IL-6 in patients with severe COVID-19 admitted to the intensive care unit versus those who are not. E) Serum concentrations of IL-8 in patients with severe COVID-19 admitted to the intensive care unit versus those who are not. *p<0.05. ns=non-significant. Data represent the median and interquartile range. *P* values in A-C panels were calculated using the Kruskal-Wallis test and *post hoc* analysis, while panels D and E were analyzed using the Mann-Whitney U sum rank test.

There have been various reports on the levels of different subsets of peripheral blood NK cells in patients with COVID-19. Low levels and dysfunction of NK cells have been described in patients with severe infection by the SARS-CoV-2 virus in various population groups (4, 33-36). Similarly, abnormalities in the levels of several membrane receptors of NK cells have been detected in peripheral blood from patients with SARS-CoV-2 infection in several studies, including the inhibitory receptors KIR3DL1/CD158e (as detected by us) and NKG2D/CD314 as well as the activating molecule KIR2DS4/CD158i (37, 38). Furthermore, it has been proposed that a decreased expression of inhibitory KIR receptors along with an increased proportion of NK cells expressing activating receptors is associated with severe COVID-19 (38). However, it is worth mentioning that the detailed analysis of the presence of different membrane receptors by NK cells in healthy or diseased individuals is a very complex matter. This complexity arises because these cells simultaneously express a wide array of receptors, both inhibitory and stimulatory, at varying degrees. This is reflected in their mean fluorescence intensities in flow cytometry analysis, and they also exhibit different combinations of these receptors (17, 20). A large number of NK cell subsets can be identified in both, healthy and diseased individuals (17, 20). An increased proportion of activated NK cells (expressing perforin and

producing higher levels of proinflammatory cytokines) has been observed in patients with severe SARS-CoV-2 infection (38). This suggests that various complex abnormalities in the number and NK cell membrane receptor repertoire are associated with SARS-CoV-2 infection and severe COVID-19. In any case, it is important to keep in mind the protective role of NK cells against viral infections. This protection is achieved through different mechanisms, such as the lysis of infected cells, participation in antibody-dependent cellular cytotoxicity (ADCC), synthesis of proinflammatory cytokines and inducing the generation of Th1 lymphocytes and the cellular immune response including the activation of cytotoxic CD8+ T lymphocytes (20).

NKT lymphocytes are innate-like immune cells that also appear to be involved in defending against virus infected cells, among other functions (39). It has been described that the main and most abundant subset of these cells (iNKT cells) releases large amounts of various cytokines and chemokines upon activation. These molecules modulate the activation of different cells belonging to the innate and adaptive immune response (21, 40). Therefore, we have hypothesized in this study that abnormal numbers of NKT cells could be related to an increased risk for the severe form of COVID-19 and death. Accordingly, we have found that patients with severe SARS-CoV-2 infection exhibit significant a significant decrease in the percentage of NKT lymphocytes in their peripheral blood. However, these patients also showed an increased frequency of NKT cells expressing NKp44, a molecule that is observed upon cell activation (18, 24, 41). Thus, the data from this study suggest that although patients with severe COVID-19 have a lower level of NKT cells, a higher proportion of them are activated. As mentioned above, these activated cells can synthesize large amounts of cytokines, which might contribute to the hyperinflammatory phenomenon observed in severe COVID-19.

Although it is evident that the abnormal numbers of NK and NKT cells and the percentage of these cells expressing different membrane receptors observed in severe COVID-19 may be due to various factors, it seems feasible that these characteristics could be used as laboratory markers to detect individuals at higher risk for severe disease and death. Nevertheless, it is evident that additional and sequential studies are needed, involving a larger number of individuals.

Although the ultimate cause(s) of the abnormal numbers of several immune cell subpopulations observed in the patients with severe COVID-19 remains as a relevant point to be disclosed, it seems evident that this is a complex issue. Thus, our results along with many others already published further indicate that future studies on the involvement of NK cells in the pathogenesis of COVID-19 (and other viral infections) should be performed with a prospective design, involving a large number of individuals and utilizing complex flow cytometry analyses, including many different cell markers.

CONCLUSION

The quantitative defects in NK and NKT cells detected in the blood of individuals with severe SARS-CoV-2 infection further support the idea that the outcome of patients with COVID-19 is linked to various and intricate abnormalities in the frequency, immunophenotype and function (including cytotoxic and cytokine synthesis activities) of innate immune cells. Our data also suggests that additional studies on this topic are necessary, involving a large number of patients and utilizing the advanced cytometric tools that are currently available.

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AUTHORS' CONTRIBUTION

MV-N conducted and designed the experiments, analyzed the data, and wrote the original draft. DLA-H contributed to the flow cytometry analyses and blood sample processing. RS-G performed the assays of cytokine quantification and contributed to data analyses. BH-C contributed to the design of flow cytometry analyses, data processing and figures design. LG-B performed the nutritional evaluation of healthy individuals and patients and contributed to obtaining the blood samples and their processing. SB-S and AC-G performed the clinical and laboratory evaluation and selection of patients and healthy controls and reviewed the final manuscript. CS-T and RG-A conducted and designed the research, analyzed the findings, and edited/refined the manuscript with a focus on critical intellectual contributions.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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