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Is IFN expression by NK cells a hallmark of severe COVID-19?

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ABSTRACT

Natural Killer cells (NK) are crucial in host defense against viruses. There are many unanswered questions about the immune system in COVID-19, especially the mechanisms that contribute to the development of mild or severe forms of the disease. Although NK cells may have an essential role in the pathogenesis of COVID-19, the mechanisms involved in this process are not yet fully elucidated. Here, we demonstrate that $CD3^{-}CD56^{+}$ NK cells frequency in the volunteers who recovered from mild COVID-19 (Mild CoV) presented a significant increase compared to the healthy control (HC) and individuals recovering from severe COVID-19 (Severe CoV) groups. Furthermore, distinct IFN profiles in recovered COVID-19 patients with mild or severe clinical forms of the disease were observed in the total NK cells ($CD3^{-}CD56^{+}$). In the first group, NK cells express increased levels of IFN-α compared to the severe CoV, while higher production of IFN-γ in severe CoV was found. Moreover, NK cells in mild CoV express more cytolytic granules depicted by granzyme B and perforin. Compared to HC, PBMCs from mild CoV presented higher Ki-67 and TIM-3 production after Pool CoV-2 and Pool Spike CoV-2 peptides stimulus. In addition, non-stimulated PBMCs in the mild CoV group had higher NK TIM-3+ frequency than severe CoV. In the mild CoV group, Pool Spike CoV-2 and Pool CoV-2 peptides stimuli elicited higher granzyme B and perforin coexpression and IFN-α production by PBMCs. However, in severe CoV, Pool Spike CoV-2 reduced the coexpression of granzyme B, perforin, and CD107a suggesting a decrease in the cytotoxic activity of NK cells. Therefore, our study shows that NK cells may have a crucial role in COVID-19 with the involvement of IFN-α and cytotoxic properties that aid in developing qualified immune responses. Furthermore, the data suggest that higher amounts of IFN-γ may be linked to the severity of this disease.

1. Introduction

In late 2019, an outbreak of pneumonia from an unknown infectious agent was reported in Wuhan, China. After a month, the World Health Organization (WHO) named the condition "Coronavirus Disease 2019" (COVID-19). At the same time, the International Committee on Taxonomy of Viruses (ICTV) named the virus "Severe Acute Respiratory Syndrome Coronavirus 2" (SARS-CoV-2). Shortly after that, COVID-19 was recognized as a "Public Health Emergency of International Concern" (PHEIC) and announced as a pandemic situation [1,2].

Abbreviations: [1]Mild CoV, volunteers recovered from mild COVID-19; HC, healthy control; Severe CoV, individuals recovered from severe COVID-19; WHO, World Health Organization; ICTV, International Committee on Taxonomy of Viruses; PHEIC, Public Health Emergency of International Concern; SEB, Staphylococcal enterotoxin B from *Staphylococcus aureus*; SSC-A, Side Scatter; FSC-A, Forward Scatter.

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About 500 million people have been diagnosed with COVID-19, with more than 6 million deaths worldwide [3]. Despite the most extensive efforts of vaccination, an average of 584.360 new cases are observed every day until the paper's submission [3]. In late 2020 and early 2021, most new infections were caused by the Delta variant (B.1.617), considered the most infectious variant of the coronavirus at that moment compared to the other Variants of Concern (VOC) Alpha (B.1.1.7), Gamma (B.1.1) .28.1) and Beta (B.1.351). However, the Omicron variant (B.1.1.529) changed this scenario in 2022, with more than 32 mutation changes in its Spike protein. This main protein guarantees the entry of the coronavirus into cells. These mutations made the Omicron variant better at adhering to human cells and evading immunity against infections, forcing the need to administer a third booster dose of the existing vaccines worldwide [4–6].

The innate immune system develops a pivotal role during SARS-CoV-2 infection, which could contribute to distinct courses observed in COVID-19 [7]. Interferons (IFNs) are natural antiviral and immunomodulatory agents that initially react to viral infections and determine the subsequent course of the immune response to the infection. It has been shown that in the course of infection by Severe Acute Respiratory syndrome-associated coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) viruses, the expression and subsequently the functions of Type I IFNs are markedly suppressed, and the administration of exogenous Type I IFNs have been shown to reduce the severity of symptoms of these diseases [8–10].

Natural Killer (NK) cells exert antiviral activity against SARS-CoV-2 infected cells, providing evidence of their ability to contribute to the host's antiviral defense [11]. Furthermore, blood NK cells are hyperactivated in COVID-19 patients, as measured by elevated fractions of $CD56^{bright}$ cells that express the cytotoxic molecules granzyme B and perforin [12]. However, they also exhibit markers of functional exhaustion and downregulation of genes in patients with the severe form of the disease [13,14]. Thus, it is essential to develop strategies for the treatment with regulators and modulators of the innate immune response [8,9].

However, the actual role of NK cells during SARS-CoV-2 infections remains unclear. For this reason, our objective in this study was to evaluate the expression of cytotoxic, inhibitory, and proliferation markers and cytokine expression by total NK cells from convalescent patients who presented mild or severe COVID-19.

2. Materials and methods

2.1. Ethics statement

The National Commission approved this study of Ethics in Research (Certificate CAAE: 31354720.0.0000.5188). All experiments were performed in compliance with relevant regulations and institutional guidelines and by the ethical standards of the Declaration of Helsinki. Informed consent was obtained from all enrolled patients and healthy individuals.

2.2. Patients recruitment

This study was carried out from May 2020 to May 2021 in Brazil, when the original lineage of SARS-CoV-2 was present until December 2020, with the alpha and gamma variants appearing in the same month and becoming more frequent until April 2021. Subsequently, these studies ended in May 2021, a few days after the delta variant being identified for the first time in the country (26 April 2021) [15,16]. Eighteen non-vaccinated patients who recovered from COVID-19 were recruited, with symptoms observed between May 2020 and May 2021. The SARS-CoV-2 diagnosis was based on RT qPCR for COVID-19 during the acute phase and serological tests to IgG (Euroimmun Anti-SARS-CoV-2 assay Perkin Elmer Company) after cure. The COVID-19 patients were assigned either mild (without pulmonary symptoms and no need for hospitalization, called Mild CoV group, n = 11 patients) or severe (with pulmonary symptoms requiring hospitalization, called Severe CoV group, n = 7 patients) group. In addition, a healthy control (HC, n = 9) group was included. Individuals in the HC group were not previously vaccinated to COVID-19, were reportedly asymptomatic for the last 10 weeks, negative by certified SARS-CoV-2 antibody test (Euroimmun Anti-SARS-CoV-2 assay Perkin Elmer Company), and had a negative RT qPCR test for SARS-CoV-2. Whole blood for flow cytometry analysis was collected simultaneously in sodium heparin-coated vacutainers and kept on gentle agitation until processing. Blood samples were processed on the day of collection. For epidemiologic purposes, COVID-19 was defined as severe dyspnea, a respiratory rate of 30 or more breaths per minute, a blood oxygen saturation of 93% or less, and infiltrates in more than 50% of the Lung were observed [17].

2.3. RT qPCR assay

During blood collection for flow cytometry experiments, quantitative real-time PCR (RT qPCR) RT qPCR was performed as described previously [18]. RNA was isolated from nasopharyngeal or oropharyngeal swabs, and RNA was extracted (QIAprep&amp Viral RNA UM Kit QIAGEN, USA) and amplified by one-step RT qPCR (SARS-CoV-2N1 + N2 Assay Kits - QIAGEN, USA). This procedure is essential to ensure no SARS-CoV-2 infection or reinfection in the HC and recovered patients, respectively.

2.4. Design and preparation of SARS-CoV-2 epitope pools (EP)

Our analyses searched for the most frequent HLA-class I/II alleles in the Brazilian population, excluding the alleles from isolated populations. Then we performed MHC-I and II binding affinity and antigenicity prediction and peptides molecular dynamics of the best-fitted MHC-I and II/protein of SARS-CoV-2 complexes. SARS-CoV-2 virus-specific CD4 and CD8 peptides (Table 1) **(**patent number: BR 10 2022 005518 1**),** resuspended in H_2O (MiliQ), pooled (Pool CoV-2, which contained peptides from the spike protein and non-spike proteins, and Pool Spike Cov-2, which contained peptides from the spike protein only, Table 1) were synthesized (GenOne Biotechnologies, Rio de Janeiro, Brazil) lyophilized and stored at −20 °C until use.

SARS-CoV-2 epitopes were predicted using the protein sequences derived from the Brazilian SARS-CoV-2 sequences deposited in GenBank and IEDB analysis resources [20]. CD4 SARS-CoV-2 epitope prediction was performed as previously reported, using the NetMHCIIpan 4.0 algorithm and CD4 Immunogenicity prediction (IEDB), HLA-DRB*1:03:01; HLA-DRB*1:07:01; HLA-DRB*1:15:01; HLA-DRB*3:01:01; HLA-DRB*3:02:02; HLA-DR*4:01:01 and HLA-DRB*5:01:01. Since the Brazilian population is extremely heterogeneous, we were careful to add frequent HLA I and II found in the Brazilian population and the most frequent HLA populations in the world. CD8 SARS-CoV-2 epitope prediction was performed as previously reported, using the NetMHCpan4.0 algorithm (HLA-A* 01: 01; HLA-A* 02: 01; HLA-A* 11: 01; HLA-A* 24: 02; HLA-A* 68: 01; HLA-A* 23: 01; HLA-A* 26: 01; HLA-A* 30: 02; HLA-A* 31: 01; HLA-B* 07: 02; HLA-B* 51: 01; HLA-B* 35: 01; HLA-B* 44: 02; HLA-B* 35: 03; HLA-C* 05: 01; HLA-C* 07: 01 e HLA-C* 15: 02). Despite inferring cellular populations of T lymphocytes, this strategy extrapolates to lymphocyte-like cells such as NK cells because a specific prediction program for NK cell populations is not yet available.

2.5. Isolation of plasma

Plasma samples were collected after centrifugation of whole blood at 400*g* for 10 min at room temperature (RT). The undiluted plasma was then transferred to cryotubes, aliquoted, and stored at −80 °C for subsequent analysis.

Table 1
List of peptides chosen in this study, through prediction studies and Spike peptide pool.

	Name	Peptide	a.a. position	Source
Pool CoV-2	ORF1a	ILASFSASTSAFVET	476–490	This study
	ORF1a	FLHFLPRVFSAVGNICYTP	2880–2890	This study
	ORF1b	FVDGVPFVVSTGYHFR	4726–4741	This study
Pool Spike CoV-2	SPIKE_NTD	NIDGYFKIYSKHTPINLV	196–210	[19]
	SPIKE_RBD	ATRFASVYAWNRKRI	344–358	[19]
	SPIKE_S2	ALQIPFAMQMAYRFN	893–907	[19]
	ORF3	KKRWQLALSKGVHFV	66–80	This study
	E	FYVYSRVKNLNSSRV	56–70	This study
	M	LYLYALVYFLQSINFVRIIM	114–123	This study
	M	KEITVATSRTLSYYK	166–180	This study
	ORF8	FYSKWYIRVGARKSA	41–55	This study

2.6. Isolation of PBMCs

PBMC from HC and recovered COVID-19 patients were obtained from heparinized venous blood by density gradient centrifugation (Ficoll-Paque ^{TM} Plus, GE Healthcare, Life Sciences, Pittsburgh, PA, USA). PBMC have centrifuged for 40 min at 2000 rpm and washed three times with PBS before counting. PBMC was maintained on RPMI-1640 (Sigma-Aldrich) supplemented with antibiotics (penicillin, 200 U/mL and streptomycin, 0.1 mg/mL), 1 mM L-glutamine (1 mM), and 10% heat-inactivated AB Rh + human serum (Sigma-Aldrich), from now on referred to as medium. Cultures were set up using a concentration of 2.5 $\times$ 10^5 cells in 96-well plates in the presence or absence of SARS-CoV-2 antigens and Staphylococcal enterotoxin B from *Staphylococcus aureus* (SEB, Sigma-Aldrich). PBMC were submitted and incubated to four different conditions: non-stimulated (medium), stimulated by SARS-CoV-2 antigens (Pool Spike Cov-2 and Pool Cov-2, each at 1ug/well), and stimulated by SEB (1ug/well) at 5% CO_2 in an incubator at 37 °C for 16 h. Next, brefeldin-A (1 mg/mL, Sigma-Aldrich) was added, and the samples were incubated at 5% CO_2 in an incubator at 37 °C for 4 h.

2.7. Flow cytometry assay

In brief, freshly isolated PBMCs were plated in a concentration of 2.5 $\times$ 10^5 cells per well in a 96-well U-bottom plate. Cocktails of antibodies for extracellular staining were added and were incubated for 30 min at 4 °C. Isotype antibodies were used as a negative control. For extracellular staining, the following antibodies from BD Biosciences were used: APC conjugated anti-CD3 (clone UCHT1, mouse BALB/c IgG_1, κ, cat. 555335), PE-Cy5 conjugated anti-CD56 (clone B159, mouse IgG_1, κ, cat. 555517), IgG isotypes control antibodies IgG3-FITC (clone J606, mouse BALB/c IgG_3, κ, cat. 555578) and IgG1-PE-Cy-7 (clone MOPC-21, mouse IgG_1, κ, cat. 557872). PE CD366 (TIM3) (Thermo Fischer Scientific, clone F38-2E2, mouse IgG_1, κ, cat. 12–3109-42) was also used for extracellular staining. After incubation with antibodies for 30 min at 4 °C, cells were washed with 150 μL of PBS. The plate was centrifuged (8 min, 244 $\times$ *g*, 4 °C), the supernatant was removed, and 100 μL of 4% formaldehyde and 100 μL of PBS were added to the wells. The plate was incubated at room temperature (RT) for 20 min to fix the extracellular staining. After centrifugation (8 min, 244 $\times$ *g*, 4 °C), the supernatant was discarded, and the samples were washed with 150 μL of PBS. Again, the plate was centrifuged (8 min, 244*g*, 4 °C), and the supernatant was discarded. For intracellular staining, the cells were permeabilized with 150 μL of permeabilization buffer (0.5% BSA, w/v, and 0.5% saponin, w/v, in PBS) for 10 min at room temperature. After centrifugation (8 min, 244*g*, 4 °C), the supernatant was removed, and the intracellular staining was added. The following conjugated antibodies from BD Biosciences were used to detect intracellular molecules: PE-conjugated anti-perforin (clone δG9, mouse BALB/c IgG_{2b}, κ, cat. 556437), FITC conjugated anti-granzyme B (clone GB11, mouse BALB/c IgG_1, κ, cat. 560211), PE anti-human IFN-α[2b] (clone 7N4-1, mouse BALB/c IgG_1, κ, cat. 560097), PE/Cy^{TM}7 anti-human IFN-γ (clone B27, mouse BALB/c IgG_1, κ, cat. 557643). The following antibodies from Thermo Fisher Scientific were also used for intracellular staining: PE/Cyanine7 anti-human CD107a (LAMP-1) (clone eBioH4A3, Mouse / IgG1, κ, cat. 25-1079-42) and FITC conjugated anti-Ki-67 (clone 7B11, mouse BALB/c IgG_1, κ, cat. MHKI6701). All antibodies were used according to the manufacturer's instructions. Then, the plate was incubated for 45 min at RT, and 150 μL of permeabilization buffer was added. After centrifugation, the supernatant was removed (8 min, 244*g*, 4 °C). Finally, 200 μL of Wash B (PBS/BSA) was added, and the samples were transferred to FACS tubes and kept at 4 °C. At least 70,000 gated events were acquired using FACS CANTO II (BD Biosciences) and analyzed using the FlowJo v.10.8 software (BD, Ashland - USA).

2.8. Flow cytometry data analysis

All parameters evaluated on NK cells were analyzed using FlowJo software v.10.8 (BD, Ashland - USA). Limits for the quadrant markers were set based on negative populations (cells) and isotype controls. Five different fluorochromes were analyzed in each analysis. Two fluorochromes, anti-CD3 APC and anti-CD56 PE-Cy5 were used to identify the NK cells ($CD3^-CD56^+$). We first selected the total lymphocyte gate through size-FSC-A and granularity-SSC-A profiles, followed by singlets separation through FSC-A $\times$ FSC-H parameters. Next, we selected the $CD3^-CD56^+$ cells. This analysis strategy is shown in Figs. 1 and 2.

2.9. Statistical analysis

Shapiro-Wilk normality test of all parameters evaluated by flow cytometry was performed. If normally distributed, multiple comparisons were performed using two-way ANOVA and Tukey's posthoc test or one-way ANOVA and Tukey's *posthoc* test. If not normally distributed, Kruskal-Wallis with Dunn's multiple comparisons test was performed. Differences were considered significant at $P < 0.05$. Statistical analysis was performed using GraphPad Prism 7. The heat map to express immunological markers of healthy control, Mild CoV, and Severe CoV was created using the Morpheus software and adjusted to Z-score [21].

3. Results

3.1. Characteristics of COVID-19 patients

Twenty-seven subjects were evaluated, comprising 11 mild COVID-19 (Mild CoV) cases, seven severe COVID-19 (Severe CoV) cases, and nine healthy controls (HC). The mean age of Mild CoV patients was 37.72 (±11.27) years, of Severe CoV patients was 37.28 (±7.60) years, and HC was 37.22 (±9.17) years. There were no statistical differences in age among the study groups. Most Mild CoV patients presented low fever, headache, arthralgia, and low fatigue as the primary clinical symptoms. All volunteers experienced low blood oxygen saturation, Lung infiltrates, and severe headaches in Severe-CoV. Moreover, almost all individuals also had tachypnea, high fever, and fatigue. The most

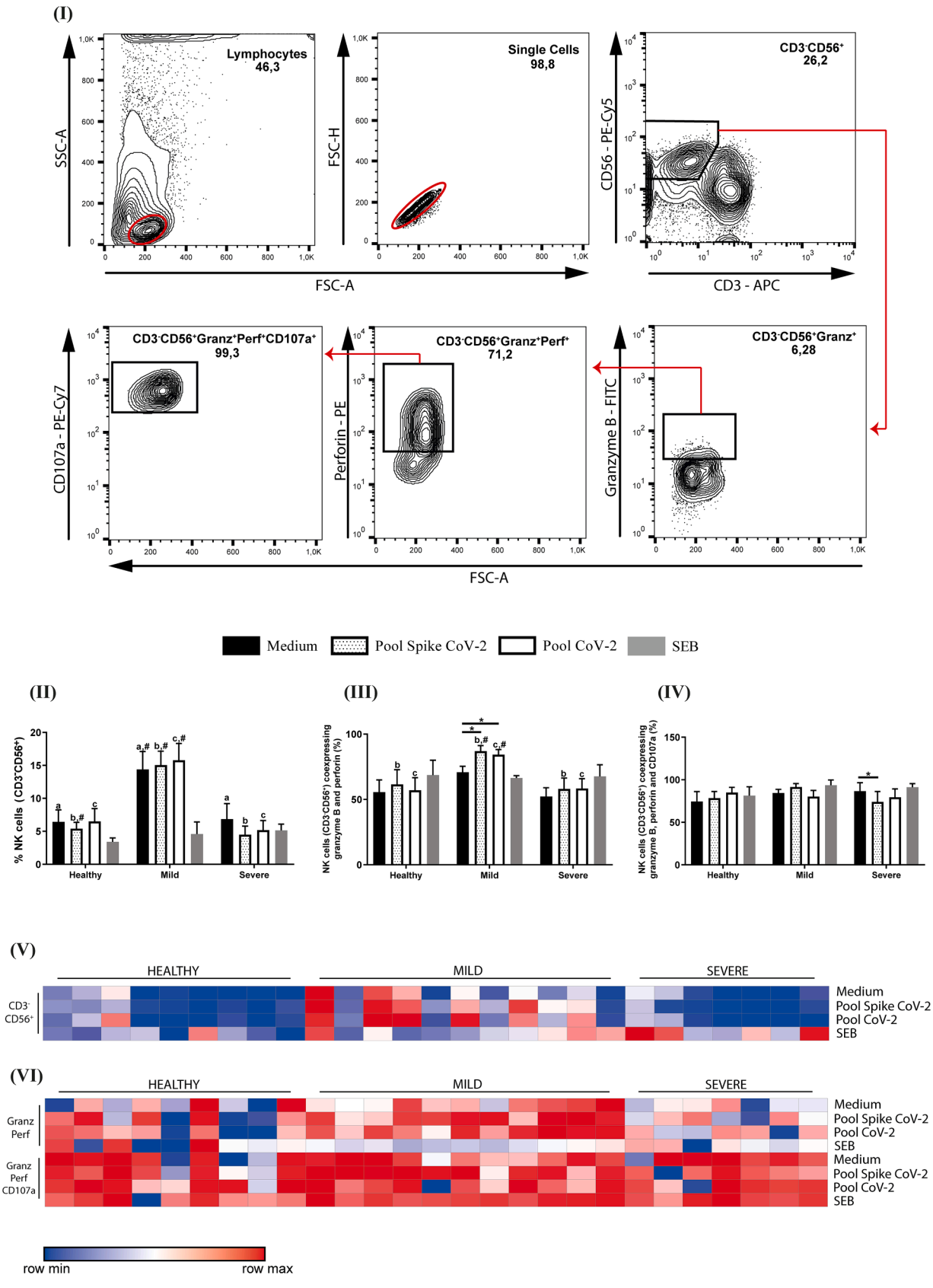

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Fig. 1. Analysis of $CD3^{-}CD56^{+}$ NK cells frequency and Granzyme B, Perforin, and CD107a coexpression by these cells in healthy individuals (HC) and mild (Mild CoV) and severe (Severe CoV) COVID-19 recovered patients. (I) Representative analysis strategy of Granzyme B, Perforin, and CD107a expression by cytotoxic $CD3^{-}CD56^{+}$ NK cells. Graphs are represented from left to right: lymphocyte gating based on Side Scatter (SSC-A) and Forward Scatter (FSC-A) parameters; single-cell isolation through FSC-A and FSC-H; $CD3^{-}CD56^{+}$ NK cells identification using CD3 and CD56 parameters; Granzyme $B^{+}Perforin^{+}$ coexpression by $CD3^{-}CD56^{+}$ NK cells using Granzyme B and Perforin with FSC-A parameters. Granzyme $B^{+}Perforin^{+}CD107a^{+}$ coexpression by $CD3^{-}CD56^{+}$ NK cells using Granzyme B, Perforin, and CD107a with FSC-A parameters. (II) $CD3^{-}CD56^{+}$ NK cells frequency. Graphs depict mean of $CD3^{-}CD56^{+}$ NK frequency ± SEM for Healthy Control (HC) (n = 9), Mild CoV (n = 11), and Severe CoV (n = 7) groups. Lowercase letters represent the significant difference of the same condition between groups as follows: (a) non-stimulated (medium) PBMC (black filled square) in HC vs. Mild CoV group and Severe CoV vs. Mild CoV group. (b) PBMCs incubated with Pool Spike CoV-2 (dotted filled square) in HC vs. Mild CoV group and Severe CoV vs. Mild CoV group. (c) PBMCs incubated with Pool COV-2 (non-filled square) in HC vs. Mild CoV group and Severe CoV vs. Mild CoV group. PBMCs incubated with SEB inoculum control (gray filled square) were non-significant in any group. The HC vs. Severe CoV group was non-significant for any stimulus and non-stimulated. (III) Frequency of Granzyme B and Perforin coexpression by $CD3^{-}CD56^{+}$ NK cells. The graph depicts the mean of $CD3^{-}CD56^{+}GranB^{+}Perf^{+}$ NK frequency ± SEM for Healthy Control (n = 9), Mild CoV (n = 11), and Severe CoV (n = 7) groups. Lowercase letters represent the significant difference of the same condition between groups as follows: (b) PBMCs incubated with Pool Spike CoV-2 (dotted filled square) in HC vs. Mild CoV group and Severe CoV vs. Mild CoV group. (c) PBMCs incubated with Pool COV-2 (non-filled square) in HC vs. Mild CoV group and Severe CoV vs. Mild CoV group. Non-stimulated PBMCs and SEB inoculum control (gray filled square) were non-significant. The HC vs. Severe CoV group was non-significant for any stimulus and non-stimulated. (IV) Frequency of Granzyme B, Perforin, and CD107a coexpression by $CD3^{-}CD56^{+}$ NK cells. The graph depicts the mean of $CD3^{-}CD56^{+}GranB^{+}Perf^{+}CD107a^{+}$ NK frequency ± SEM for Healthy Control (n = 9), Mild CoV (n = 11), and Severe CoV (n = 7) groups. The bars represent mean values, and the error bar shows the standard error of the mean (SEM) for each group. The line above bars (– – –) identifies the two groups with significant differences within each group. * represents p-value < 0.05. # above each bar identifies significant differences compared to the SEB stimulation within each group. Multiple groups were compared by two-way ANOVA and Tukey's posthoc analysis and one-way ANOVA and Tukey's posthoc test or Kruskal-Wallis with Dunn's multiple comparisons test where appropriate. (V and VI) Heatmap of differential frequency of $CD3^{-}CD56^{+}$, $CD3^{-}CD56^{+}$Granzyme $B^{+}Perforin^{+}$, and $CD3^{-}CD56^{+}$Granzyme $B^{+}Perforin^{+}CD107a^{+}$ NK cells. This comparison was performed on the HC, Mild CoV, and Severe CoV groups based on flow cytometry analyses of PBMCs. Each row indicates the stimuli used (shown on the right), and each sequence of 4 rows indicates a marker analyzed (shown on the left). Each column indicates individual patients in their respective groups.

frequent comorbidities among the patients were obesity and hypertension.

During the questionnaire response, we also asked the volunteers to state how they describe themselves regarding ethnic-racial self-classification in white, brown (pardo), black, yellow (Asian), and indigenous (Native American). As result, the majority of individuals described themselves as brown (59.25%) and white (33.33%), followed by black (3.7%) and indigenous (3.7%). Regarding the ethnic-racial variety per group (HC, mild CoV, and severe CoV), the HC was comprised by white (55.55%), brown (33.33%), and indigenous (11.11%) individuals. On the other hand, mild CoV was composed by brown (81.81%) and white (18.18%) while severe CoV comprised brown (57.14%), white (28.57%), and black (14.28%) people. Tables 2–4 detail the demographic and clinical findings of the volunteers in the HC, Mild Cov, and Severe Cov groups.

3.2. Pool Spike and Pool CoV-2 lead to high production of cytotoxic profiles in NK cells ($CD3^{-}CD56^{+}$)

PBMC were stimulated with two pools of SARS-CoV-2 peptides to investigate NK cells with cytotoxicity against SARS-CoV-2. Our results show that $CD3^{-}CD56^{+}$ NK cells frequency in the HC and Severe CoV groups was not statistically different, regardless of treatments (non-stimulated, Pool Spike Cov-2-, Pool CoV-2-, and SEB-stimulated). In contrast, the Mild CoV group presented a significantly higher NK cell frequency than the HC and Severe CoV groups in the following conditions: medium, Pool Spike CoV-2, and Pool CoV-2 (Fig. 1II). Moreover, in HC, the frequency of NK cells in the SEB condition was lower than in Pool Spike CoV-2-stimulated samples. At the same time, in mild CoV, unstimulated and SARS-CoV-2 peptides stimulated PBMC presented more CD3-CD56 + cells than SEB stimulus (Fig. 1II).

The coexpression of granzyme B and Perforin in the Mild CoV was higher in the Pool Spike Cov2- and Pool CoV-2-stimulated PBMC than in the same conditions in the HC and severe CoV groups. Interestingly, in the mild CoV group, the SARS-CoV-2 peptide stimulus induced the coexpression of the granules. Also, in both Pool Spike CoV-2 and Pool CoV-2 conditions, there were higher levels of NK granzyme $B^{+}perforin^{+}$ compared with SEB-stimulated samples (Fig. 1III). Finally, the coexpression of granzyme B, perforin, and CD107a was also evaluated in $CD3^{-}CD56^{+}$ NK cells. Although similar expression levels were found between the studied groups, in severe CoV, Pool Spike CoV-2 leads to a reduction of NK granzyme $B^{+}perforin^{+}CD107a^{+}$ frequency (Fig. 1IV).

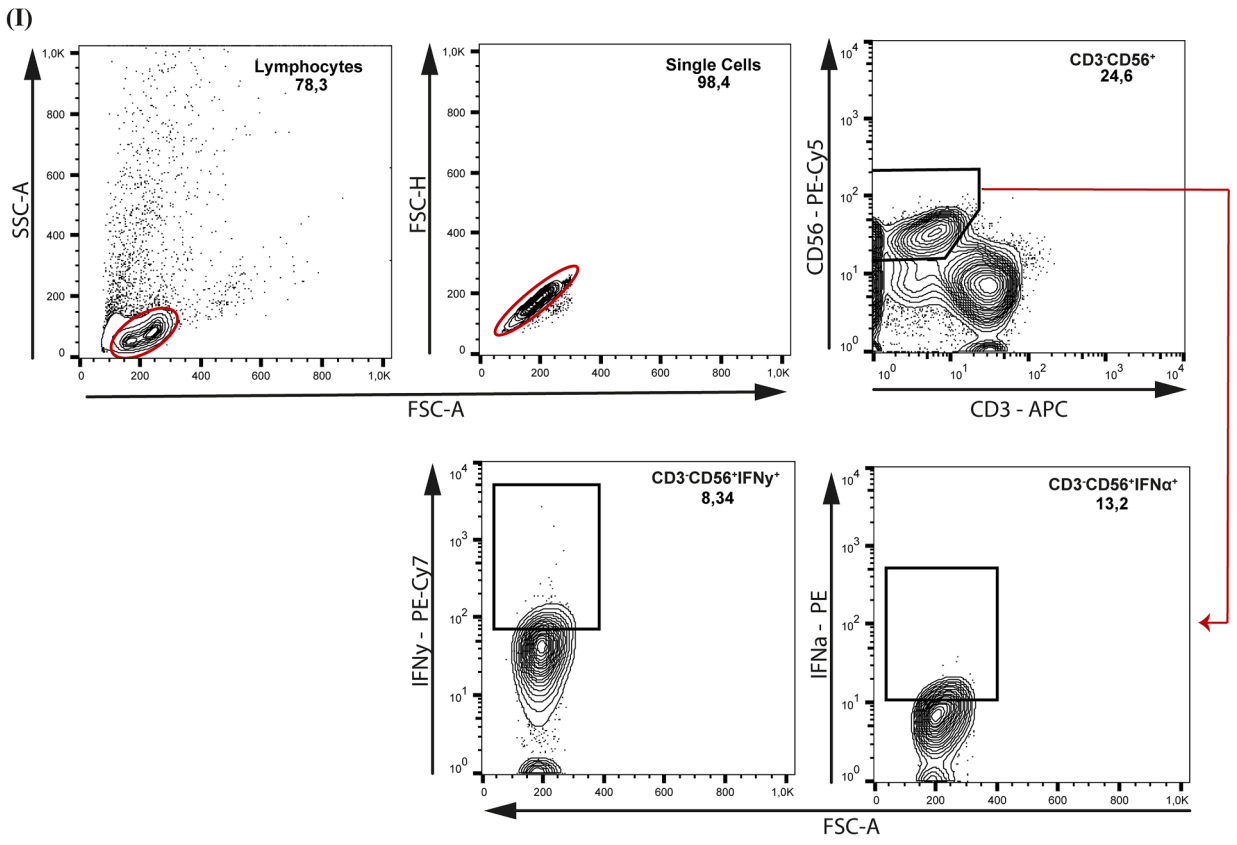
3.3. IFN-α expression by total NK cells is lower in severely recovered patients

We investigated cytokine production by total NK cells ($CD3^{-}CD56^{+}$) after stimulation with Pool Spike CoV-2, Pool CoV-2, and SEB (Fig. 2). Severe CoV volunteers were characterized by diminished production of IFNα by NK cells. In all severe CoV treatments (non-stimulated, Pool Spike Cov2-, Pool CoV-2-, and SEB-stimulated), the frequency of $CD3^{-}CD56^{+}IFN\alpha^{+}$ significantly reduced higher than HC group. Also, in the Mild CoV group, both SARS-CoV-2 peptide stimuli elicited elevated levels of NK IFNα + compared to the severe group. NK IFNα + in the non-stimulated condition was lower than SEB stimulus in HC and Mild CoV groups. On the other hand, in Mild CoV, the SARS-CoV-2 peptide stimulus led to higher IFNα + expression by NK cells compared to the non-stimulated conditions (Fig. 2 II).

On the other hand, expression of IFNγ by $CD3^{-}CD56^{+}$ NK cells was more prevalent in the severe group. NK cells of PBMCs stimulated with Pool Spike Cov-2, and Pool CoV-2 in the Mild CoV group produced more IFNγ than in the HC group. Compared with the HC group, Severe CoV was marked by higher levels of $CD3^{-}CD56^{+}IFN\gamma^{+}$ in the medium, Pool Spike CoV-2, and Pool CoV-2 conditions. Finally, NK leukocytes of Severe CoV individuals expressed more IFNγ than the Mild CoV in non-stimulated and Pool Spike CoV-2-stimulated PBMCs. In the HC group, the Pool CoV-2 stimulus induced a higher expression of IFNγ compared to stimulation by Pool Spike CoV-2. In the same group, stimulation of PBMCs with SEB induced higher $IFN\gamma^{+}$ expression than all groups. Moreover, in the Mild CoV and Severe CoV groups, SEB stimulus elicited more $CD3^{-}CD56^{+}IFN\gamma^{+}$ frequency than non-stimulated, and Pool CoV-2 stimulated samples, respectively (Fig. 2III).

3.4. NK cell activation profile

Finally, we analyze activation and proliferation marker (Ki-67) and inhibitory receptor (TIM-3) expression in $CD3^{-}CD56^{+}$ NK cells. Regarding TIM-3 expression, statistically, significant differences were observed between the Mild CoV and Severe CoV groups in non-stimulated PBMC and between the HC and Mild CoV groups with Pool Spike CoV-2 stimulus (Table 5). Concerning $CD3^{-}CD56^{+}Ki\text{-}67^{+}$ NK cells, we found higher cell frequency in the Mild CoV compared to the HC group after Pool CoV-2 stimulation (Table 5).



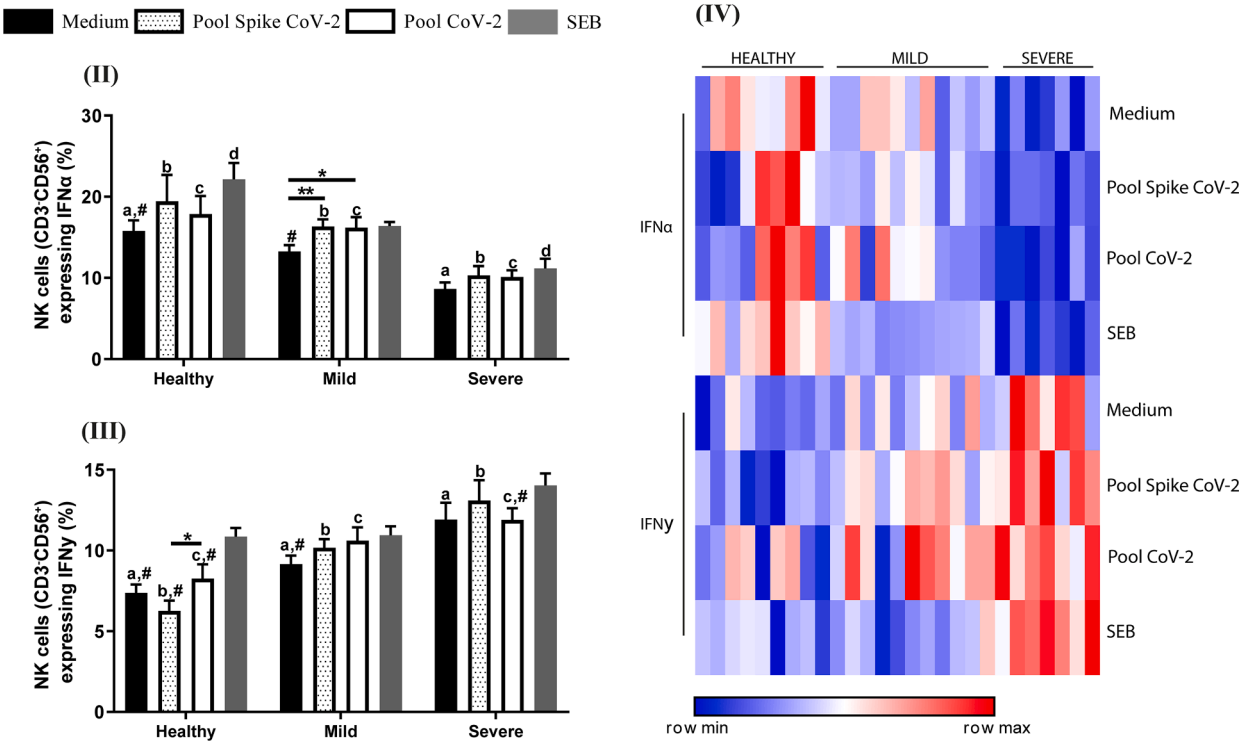


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Fig. 2. Cytokines IFN-α and IFN-γ expression by CD3$^-$CD56$^+$ NK cells in healthy individuals (HC), mild (Mild CoV), and severe (Severe CoV) COVID-19 recovered patients. (I) Representative analysis strategy of cytokine expression by CD3$^-$CD56$^+$ NK cells. Graphs represented from left to right: lymphocyte gating based on Side Scatter (SSC-A) and Forward Scatter (FSC-A) parameters; single-cells isolation through FSC-A and FSC-H. CD3$^-$CD56$^+$ NK cells identification using CD3 and CD56 parameters; IFN-α expression by CD3$^-$CD56$^+$ NK cells using FSC-A and IFN-α. IFN-γ expression by CD3$^-$CD56$^+$ NK cells using FSC-A and IFN-γ parameters. **(II) Frequency of the CD3$^-$CD56$^+$ NK cells expressing IFN-α.** Graphs depict mean of the CD3$^-$CD56$^+$ IFN-α$^+$ NK frequency ± SEM for Healthy Control (HC) (n = 9), Mild CoV (n = 11), Severe CoV (n = 7) groups. Lowercase letters represent the significant difference of the same condition between groups as follows: **(a)** non-stimulated PBMC (black filled square) in HC vs. Severe CoV groups **(b)** PBMCs incubated with Pool Spike CoV-2 (dotted filled square) in HC vs. Severe CoV groups and Mild CoV vs. Severe CoV groups **(c)** PBMCs incubated with Pool COV-2 (non-filled square) in HC vs. Severe CoV groups and Mild CoV vs. Severe CoV groups. **(d)** PBMCs incubated with SEB inoculum (gray filled square) in the HC vs. Severe CoV groups. Non-stimulated and stimulated PBMCs were non-significant in group HC vs. Mild CoV groups. **(III) Frequency of the CD3$^-$CD56$^+$ NK cells expressing IFN-γ.** Graphs depict mean of the CD3$^-$CD56$^+$ IFN-γ$^+$ NK frequency ± SEM for Healthy Control (HC) (n = 9), Mild CoV (n = 11), Severe CoV (n = 7) groups. Lowercase letters represent the significant difference of the same condition between groups as follows: **(a)** non-stimulated PBMC (black filled square) in HC vs. Severe CoV groups and Mild CoV vs. Severe CoV groups. **(b)** PBMCs incubated with Pool Spike CoV-2 (dotted filled square) between all groups **(c)** PBMCs incubated with Pool COV-2 (non-filled square) in HC vs. Mild CoV group and HC vs. Severe CoV group. PBMCs cultured with SEB inoculum (gray filled square) were non-significant in any group. The bars represent mean values, and the error bar shows the standard error of the mean (SEM) for each group. The line above bars (– – –) identifies the two groups with significant differences within each group. * represents p-value < 0.05; ** represents p-value < 0.01. # above each bar identifies significant differences compared to the SEB stimulation within each group. Multiple groups were compared by two-way ANOVA and Tukey's posthoc analysis and one-way ANOVA and Tukey's posthoc test or Kruskal-Wallis with Dunn's multiple comparisons test where appropriate. **(IV) Heatmap of differential frequency of CD3$^-$CD56$^+$IFNα$^+$ and CD3$^-$CD56$^+$IFNγ$^+$ NK cells.** This comparison was performed on the HC, Mild CoV, and Severe CoV groups based on flow cytometry analyses of PBMCs. Each row indicates the stimuli used (shown on the right), and each sequence of 4 rows indicates a marker analyzed (shown on the left). Each column indicates individual patients in their respective groups.

Table 2
Gender and age of healthy control individuals (HC group).

#Code	Gender	Age	ethnoracial self-classification
CTL 1	Female	47	White
CTL 2	Female	48	Brown
CLT 3	Male	47	White
CLT 4	Female	28	White
CLT 5	Male	36	White
CLT 6	Male	22	Brown
CLT 7	Female	36	Indigenous
CLT 8	Male	40	White
CLT 9	Female	31	Brown
MEAN	**5F:4M**	**37.22(±9.17)**	

*F = Female; M = Male

4. Discussion

Our results revealed that Natural Killer (NK) cells from patients from both the Mild and Severe CoV groups and the healthy control group were stimulated by peptide antigens designed in this work. Although it is not common for NK cells to recognize protein antigens, previous studies have demonstrated the specificity of NK cell memory responses to simian immunodeficiency virus protein antigens in non-human primates, thus suggesting a way in which antigen-driven memory is induced in humans. Furthermore, epigenetic signatures in NK cells initiated by cytomegalovirus infection demonstrate how NK cells can be programmed by initial exposure to a protein antigen or vaccine potential [22–24]. Moreover, Kim et al. [25] demonstrated that Spike peptides derived from SARS-CoV-2 could interact with NKG2D receptor of NK cells resulting in enhancement of cytotoxic activity and IFNγ expression by these cells. Our data showed that SARS-CoV-2 peptides led to higher expression of IFNα and coexpression of perforin and granzyme B by NK cells in mild CoV. In addition, the fact that Pool Spike CoV-2 and Pool CoV-2 also conducted alterations of NK granzyme B + perforin + CD107a and NK IFNγ + frequency in severe CoV and HC, respectively, raises the question regarding the modulation of NK activity by SARS-CoV-2 peptides [25].

NK cells are innate components of the immune system that develop pivotal roles against viral diseases. Despite its importance in the fast viral clearance, these lymphocytes can also contribute to the immunopathology of some infections, which makes it significant to understand their participation in a pathologic or protective immune response during and after infection [26]. PBMC analyses revealed significantly higher NK cells in the Mild CoV than in the HC and Severe CoV groups. Mild COVID-19 seems to be marked by an enhanced and balanced antiviral signature since PBMCs in this condition had more cytotoxic granules and higher IFN-α and IFN-γ expression. These results suggest a more effective NK cell response to a secondary SARS-CoV-2 infection. The increased

Table 3
Gender, age, comorbidities, and symptoms of mild recovered patients (Mild COV group).

#Code	Gender	Age	ethnoracial self-classification	Comorbidities	Symptoms
COV3	Male	22	Brown	Guillain-Barré	Headache, Coryza, Cough with phlegm, Nasal obstruction, Chills, and Fatigue
COV5	Female	34	Brown	Obesity	Fever (37.5 °C), Headache, Dry cough, Myalgia, Chills, Fatigue, and Anosmia
COV6	Male	25	Brown		Fever (37.5 °C), Headache, Coryza, Sore throat, Dyspnea, Diarrhea, Fatigue, Anosmia, and Ageusia
COV7	Male	40	Brown		Fever (39.8 °C) and Dry cough
COV8	Male	33	Brown	Obesity	Runny nose, Sore throat, Nasal obstruction, Diarrhea, and Fatigue
COV9	Male	40	Brown		Headache, Coryza, and Dyspnea
COV10	Female	36	White	Obesity	Arthralgia and eye swelling
COV11	Female	39	Brown		Fever (38 °C), Headache, Coryza, sore throat, Dry cough, Dyspnea, Tachypnea, Arthralgia, Myalgia, Nausea, Vomiting, Diarrhea, Chills, Fatigue, Anosmia, and ageusia
COV12	Male	64	Brown	Hypertension	Fever (38 °C), Headache, Runny nose, Dry cough, Nasal obstruction, Chills, and Fatigue
COV13	Female	48	White	Hypertension	Headache, Dry cough, Dyspnea, Nasal obstruction, Arthralgia, Myalgia, Nausea, Vomiting, Diarrhea, Chills, Fatigue, Eye swelling, Anosmia, and Ageusia
COV15	Female	34	Brown	Obesity	Headache, Runny nose, Sore throat, Dry cough, Productive cough, Nasal obstruction, and fatigue
MEAN	**5F:6M**	**37.72 (±11.27)**			

*C = Celsius
***F = Female; M = Male.

Table 4
Gender, age, comorbidities, and symptoms of severe recovered patients (Severe COV group).

#Code	Gender	Age	ethnoracial self-classification	Comorbidities	Symptoms
COV1	Female	28	Brown		Low blood oxygen saturation, Headache, Lung infiltrate, Sore throat, Dry cough, Dyspnea, Nasal obstruction, Tachypnea, Diarrhea, and Fatigue
COV2	Male	44	Brown	Hypertension and Obesity	Fever (39 °C), Low blood oxygen saturation, Headache, Arthralgia, Myalgia, Lung infiltrate, Tachypnea, Diarrhea, Fatigue, Nausea, Eye swelling, Anosmia, and, Ageusia
COV4	Female	39	White	Hypertension and Obesity	Fever (39 °C), Low blood oxygen saturation, Headache, Dry cough, Productive cough, Dyspnea, Tachypnea, Myalgia, Vomiting, Lung infiltrate, Chills, and Fatigue
COV14	Male	25	Black	Obesity	Low blood oxygen saturation, Fever (40 °C), Headache, Lung infiltrate, Runny nose, Sore throat, Dry cough, Dyspnea, Nausea, Fatigue, Eye swelling, and Anosmia
COV16	Male	43	Brown	Asthma and Obesity	Low blood oxygen saturation, Fever (40 °C), Headache, Sore throat, Cough with blood, Lung infiltrates, dyspnea, and, Tachypnea
COV17	Female	40	Brown		Fever (39 °C), Headache, Sore throat, Low blood oxygen saturation, Arthralgia, Myalgia, Lung infiltrate, Chills, Fatigue, Anosmia, Ageusia, and Tachypnea
COV18	Male	42	White	Obesity(IMC 38.5)	Low blood oxygen saturation, Fever (39.4 °C), Headache, Runny nose, Sore throat, Lung infiltrate, Dry cough, Dyspnea, Nasal obstruction, Tachypnea, Myalgia, Diarrhea, Chills, Fatigue, Anosmia, and, Ageusia
MEAN	**3F:4M**	**37.28 (±7.60)**			

*C = Celsius
**F = Female; M = Male.

and sustained expansion of NK cells was shown by Björkström et al. [27]. In their work, the absolute number of these lymphocytes was higher up to 60 days after human hantavirus infection [27]. In addition, we demonstrated elevated Ki-67-expressing NK cells in the Mild CoV, even though this was noticed only in the comparison between the HC and Mild Cov groups after Pool Cov-2 stimulation. Ki-67 can be used as a proliferation marker [28]; thus, these data could partially explain the elevated frequency of NK lymphocytes in some patients who recovered from mild COVID-19.

Cell detection is achieved by various activating and inhibitory receptors through which the integration of signals rigorously regulates the NK cell response [29,30]. Under sufficient activation, signaling pathways can culminate with the polarized release of preformed granules containing perforin and granzymes [31,32]. These cytotoxic granules will induce target cell apoptosis, thus mediating the cytolytic killing function of NK cells. Our result shows that the Mild CoV group presented a higher frequency of NKs cells coexpressing granzyme B and perforin in samples stimulated with SARS-CoV-2 peptides. These data could point to a better capacity of NK cells in the Mild CoV group in producing these

Table 5
Ki-67 and TIM-3 expression by $CD3^{-}CD56^{+}$ NK cells in healthy individuals (HC), mild (mild CoV), and severe (severe CoV) COVID-19 recovered patients.

Ki-67	Healthy Control	Mild CoV	Severe CoV
MEDIUM	25.73 ± 3.17	41.5 ± 13.02	19.73 ± 9.92
POOL SPIKE CoV-2	21.97 ± 4.20	43.90 ± 13.44	31 ± 1.41
POOL CoV-2	23.87 ± 8.06[a]	42.33 ± 14.28[a]	26.1 ± 3.72
TIM-3	**Healthy Control**	**Mild CoV**	**Severe CoV**
MEDIUM	16.81 ± 6.90	32.01 ± 15.00[b]	20.77 ± 15.44[b]
POOL SPIKE CoV-2	19.52 ± 9.4[c]	36.98 ± 11.37[c]	15.40 ± 2.26
POOL CoV-2	21.23 ± 5.88	48.39 ± 10.86	29.8 ± 12.51

Table with a mean (±Standard Deviation) of Ki-67 and TIM-3 expression by $CD3^{-}CD56^{+}$ NK cells. Lowercase letters represent the significant difference (p-value < 0.05) of the same condition between groups as follows: **(a)** statistically significant differences in the expression of Ki-67 between the HC and Mild CoV groups after Pool CoV-2 stimulation; **(b)** statistically significant differences in the expression of TIM-3 between the Mild CoV and Severe Cov groups in non-stimulated PBMC. **(c)** statistically significant differences in the expression of TIM-3 between the HC and Mild CoV groups after Pool Spike Cov-2 stimulation. Multiple groups were compared by two-way ANOVA and Tukey's posthoc analysis and one-way ANOVA and Tukey's posthoc test or Kruskal-Wallis with Dunn's multiple comparisons test where appropriate.

cytolytic granules, which would help in faster viral control during reinfection. The coexpression of granzyme B and perforin induced by Pool Spike CoV-2 and Pool CoV-2 can corroborate the hypothesis mentioned above. However, the fact that granzyme B, perforin, and CD107 coexpression was similar between the studied groups suggests similar degranulation capacity and, indirectly, cytotoxicity in recovered COVID-19 patients [33,34]. On the other hand, since Pool Spike CoV-2 led to a decrease in NK granzyme B^{+}perforin^{+}CD107a^{+} frequency in severe CoV, it is possible that, upon reinfection, people who presented a severe form in the first COVID-19 infection may present attenuated cytotoxic capacity by NKs cells that could be compensated by CD8 T cells, for example.

In a secretion process distinct from degranulation, NK cells will additionally release potent proinflammatory cytokines, such as type 1 interferons (IFN). As a member of the type 1 IFN family, IFN-α helps create antiviral immune states, which, in turn, helps to control the spread of viral infections [35]. However, the efficiency of this IFN-mediated control mainly depends on time and IFN-α concentration levels [35,36]. Our data point to a low frequency of IFN-α in the Severe CoV group. Our results suggest that individuals with mild COVID-19 would have sufficient IFN-α expression for viral control without excessive activation in the acute disease.

A study that reported treatment with IFN-α used as an immunomodulatory agent in HIV-1 cure strategies showed that HIV + individuals unable to control the infection fail to do so due to impaired cytokine production and/or cytotoxic effectors cell function. It was possible to observe that the healing success of the IFN-α treatment made it possible to increase cytokine secretion, polyfunctionality, degranulation, and the cytotoxic potential of NK cells from healthy donors and chronic HIV-1 progressions [37]. The data mentioned above could partially explain the essential role of IFN-α in COVID-19 and the low expression of that cytokine observed in the Severe CoV group. Also, Kwaa's [37] work, along with ours, suggests a possibility of a therapeutic resource against COVID-19 through the treatment with IFN-α, which could lead to an increase of cytotoxic effector cells contributing to disease resolution. In this sense, it is crucial to investigate if diminished or impaired IFN-α expression in NK cells during COVID-19 is maintained in the convalescent phase. It is noteworthy that during reinfection, these cells would adopt different strategies against SARS-CoV-2 that better contribute to the viral clearance through a trained immunity process [38]. Moreover, the fact that SARS CoV-2 peptide stimuli have elicited the expression of IFN-α by NK cells could demonstrate the importance of this cytokine not only in primary COVID-19 but also during reinfection.

Another interferon belonging to the type II family (IFN-γ) is produced by NK cells and T lymphocytes at different stages of the immune response [39]. IFN-γ is essential for antiviral defense, activating cytokine production in T cells and increasing the killing activity of cytotoxic T lymphocytes [40]. However, persistently high levels of IFN-γ worsen systemic inflammation and increase tissue damage, triggering organ failure [41]. In PBMCs stimulated with SARS-CoV-2 peptides, NK cell producers of IFN-γ were higher in COVID-19 recovered individuals. In contrast, non-stimulated cells showed elevated NK IFN-γ^{+} only in the severe CoV group compared to HC. Although significant differences were not found in PBMCs stimulated with Pool CoV-2 between the mild and severe groups, in this group, samples not stimulated or stimulated with Pool Spike presented a higher frequency of NK IFN-γ + compared to mild CoV. This data could indicate that these cells contribute to an excessive proinflammatory response against SARS-CoV-2, leading to more severe disease. Asymptomatic individuals may experience a coordinated immune response through the proportional production of proinflammatory cytokines and IL-10, while Grau-Expósito et al. [42] showed higher expression of IFN-γ by T cells in hospitalized patients [43]. Together with our work, these studies suggest the potential of NK cells to act synergically with other immunologic components to deepen the immune imbalance observed in severe cases [44].

Next, we analyzed proliferation and activation marker Ki-67 expression in NK cells [12,28,45]. Our result showed that this marker was higher in the Mild CoV group after Pool CoV-2 stimulation, revealing a prominent activated phenotype. It is essential to highlight here that this increase in Ki-67 expression does not necessarily present a deregulated function but that many cells with this marker could indicate their responsive power. On the other hand, signs of upregulation of the inhibitory immune checkpoint receptor were also observed through the high frequency of TIM-3 in the Mild CoV group compared with the Severe CoV and HC groups in non-stimulated Pool Spike CoV-2-stimulated PBMCs, respectively [46]. The expression of TIM-3 could point to a regulatory role in NK cell response during reinfection through control of effector activity, contributing to a balanced immune response developed by these cells. Herrmann and colleagues [46] showed a correlation between activation markers and coexpression of PD-1 with LAG-3 and PD-1 with TIM-3 on CD4 + and CD8 + T cells of COVID-19 and malaria patients. However, if the higher expression of TIM-3 in the mild CoV group contributes to a beneficial or harmful effect of NK response during reinfection needs to be further studied.

5. Conclusion

Here, we show that an imbalance in IFN response could lead to severe disease once, unlike IFN-α data, the frequency of NK IFN-γ^{+} was higher in severe CoV. In addition, even with higher cytolytic granules found in the Mild CoV group, the similar NK granzyme B^{+}Perforin^{+}CD107^{+} frequency could point to similar cytotoxic activity between the evaluated groups. However, the decreased frequency of this NK subpopulation under Pool Spike CoV-2 stimulus in severe CoV can suggest an impairment of cytotoxic activity or just an attempt to control the NK responsiveness against SARS-CoV-2. Moreover, NK cells from mild CoV presented more Ki-67 and TIM-3 expression after the CoV-2 stimulus, suggesting that this group may have a better capacity for activation during the infection.

Finally, based on our observations, SARS-CoV-2 peptides induced changes in cytotoxic markers and IFN expression within HC and/or CoV groups suggesting a modulation of NK cell activities by these peptides. Many unknowns remain in elucidating the differential NK cells in the SARS-CoV-2 infection. The COVID-19 in this setting offers a window for characterization of the association between different triggers and differential patterns of disease. Additional studies are urgently needed to fully elucidate the importance of IFNs and peptides developed here in the dynamics of the pathophysiology of this disease.

This study has some limitations. The Brazilian population is marked by admixed genomes including European, sub-Saharan African, and

Native Americans [47]. Thus, we understand that the low number of patients does not necessarily reflect Brazilian people considering the diversity of age, gender, racial and ethnic profile. In this case, factors as age and genetic variability can lead to a differential response to infection [47,48]. Considering that the present study encompassed just one elderly volunteer, and understanding the high risk of severe COVID-19 in older adults, further studies are pivotal to elucidate the correlation between distinct populations group and NK responsiveness to the dynamics of COVID-19 [48].

However, although this research did not evaluate the influence of each aforementioned populational parameter and others, our results could help the scientific community understanding the participation of these cells in the immunopathology of COVID-19. Thus, even with some limitations, the present work shows interesting results and raises important questions about Natural Killer cell's response during SARS-CoV-2 infection once the role of these lymphocytes in COVID-19 is not fully understood.

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CRediT authorship contribution statement

Bárbara Guimarães Csordas: Investigation, Data curation, Formal analysis, Visualization, Writing – original draft. **Pedro Henrique de Sousa Palmeira:** Investigation, Data curation, Formal analysis, Writing – original draft. **Rephany Fonseca Peixoto:** Investigation, Data curation. **Fernando Cézar Comberlang Queiroz Davis dos Santos:** Investigation. **Isac Almeida de Medeiros:** Formal analysis, Resources. **Fátima de Lourdes Assunção Araújo de Azevedo:** Formal analysis, Resources. **Robson Cavalcante Veras:** Formal analysis. **Daniele Idalino Janebro:** Formal analysis. **Ian P.G. Amaral:** Writing – review & editing. **José Maria Barbosa-Filho:** Resources. **Tatjana Souza Lima Keesen:** Conceptualization, Methodology, Validation, Resources, Supervision, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing.

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.

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