Number of natural killer cells and cytokine levels in peripheral blood at various degrees of severity

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Abstract

This study aims to investigate NK cell number and cytokines level in various degrees of severity in COVID-19 cases. A total of 63 COVID-19 patient aged \textgreater 18 y were divided into mild-moderate and severe-critical groups. Patient characteristics and peripheral blood count were obtained from medical records. NK cells number, levels of IFN-\(\gamma\), IL-10, and IL-12 in peripheral blood were examined by means of flow cytometry. The severe-critical group had leukocytosis, neutrophilia, lymphopenia, higher Neutrophil Lymphocyte Ratio, lower NK cell number and higher level of IL-10. In severe-critical group, those aged \textgreater 60 years had higher IL-10. In both groups, patients with diabetes comorbidities had a higher number of NK cells (p<0.05). NK cell number and IL-10 in peripheral blood have potential as a predictor of severe COVID-19 patients.

Keywords: COVID-19; severity; NK cells; cytokines

1. Introduction

COVID-19 has caused a pandemic in which data from the WHO at the beginning of June 2022 showed 530 million cases with 6.3 million deaths [1]. In Indonesia, 6 million patients were diagnosed with COVID-19 and there were 159 thousand death cases up to June 2022 [2]. COVID-19 is caused by the SARS-CoV-2 virus, which ranges from asymptomatic to symptomatic. The common symptoms include fever, fatigue, cough, shortness of breath and muscle pain. In the advanced stages of the disease, acute respiratory distress syndrome [ARDS] or other complications might occur [3]. WHO divides COVID-19 into several degrees of disease, namely mild, moderate, severe, and critical [4].

Immune response contributes to the disease severity. During viral infection, innate immune system recognizes the virus and induces proinflammatory cytokines. Adaptive immune system further eliminates the virus by specifically killing the virus-infected cells and producing the neutralizing antibodies. One type of cell in the immune system is the NK cell [5] that has two important functions in defense against infection of the SARS-CoV-2. First, NK cells kill virus-infected cells to prevent the spread of the virus to healthy cells and second, NK cells maintain an inflammatory state to be infected by secreting IFN-\(\gamma\) and TNF-\(\alpha\) [6,7,8]. IFN-\(\gamma\) increases antigen presentation through both MHC classes 1 and 2, increases CD4 T cell differentiation, and increases macrophage activity [9]. Therefore, IFN-\(\gamma\) is seen as an important cytokine in overcoming SARS CoV-2 infection.

Several studies reported the lower number of NK cells in peripheral blood of patients with COVID-19. The number of NK cell counts is lower in patients with severe symptom [10–12]. The reduction in the number of NK cells is consistent with their impaired function [7,8]. Mazzoni et al. [11] and Osman et al. [13] found that the number of IFN-\(\gamma\)-producing NK cells in hospitalized COVID-19 patients became lower after ionomycin-stimulated cells compared to controls. Han et al. [14] found the increasing IFN-\(\gamma\) levels in patients with SARS-CoV-2 infection.

NK cell function is determined by the microenvironment, particularly cytokine signaling [15]. IL-10 is a negative accessory regulator that inhibits NK cell activation and proliferation [16,17]. IL-10 levels are elevated in COVID-19 patients, especially in severe cases [12,14]. The severity of COVID-19 in patients with hypertension can be estimated by testing IL-10 levels [18]. IL-12, an NK cell activator, has been shown to be reduced in COVID-19 patients [13]. A study by Tjan et al. in Japan [19] found that IL-12 levels were higher in mild cases compared to the severe cases of COVID-19.

In this study, the number of NK cells in the peripheral blood were evaluated by the levels of three relevant cytokines and the white blood cell count. To better understand the potential for NK cells and cytokine levels to be used as the biomarkers of COVID-19 severity, we also considered a number of
characteristics such as age and gender of men with different diseases that could affect NK cell levels and related cytokines. We analyzed gender differences in baseline characteristics for all our subjects. Based on previous research by Jin et al. (2020), gender influences the severity of COVID-19 illness, and men are more likely to develop severe illness [20]. The relationship between NK cell numbers and cytokine levels was investigated as well.

2. Materials and Methods

This cross-sectional study using observational analytic design is a part of the study "Analysis of TBNK Cells in Patients with COVID-19". It has received the ethical approval number KE-FK-0062-EC-2022 from the Ethics Committee of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada.

2.1. Subject recruitment

Patients aged minimum 18 years, diagnosed with COVID-19 from polymerase chain reaction (PCR) assay using a nasopharyngeal swab sample at the Dr. Sardjito Hospital, Yogyakarta in the period of May-August 2021 were selected by consecutive sampling method. COVID-19 patients with pregnancy, HIV, autoimmune diseases, and incomplete first-day NK cell data were excluded from this study.

WHO divides COVID-19 into several degrees of severity, namely mild, moderate, severe, and critical [4]. In this study, we divided the patients into two groups i.e. mild-moderate and severe-critical due to the similarity of pathophysiology and consideration of sample size for analysis.

The patients were divided into two groups, i.e. mild-moderate and severe-critical COVID-19. Severity was assessed based on WHO criteria at the admission. The mild-moderate group was defined as patients with symptoms without evidence of viral pneumonia or with pneumonia but no signs of respiratory distress. Whereas, the severe-critical group consisted of patients with the clinical signs of severe pneumonia, Acute Respiratory Distress Syndrome (ARDS), sepsis, and septic shock [4].

The selected patients were recruited to take part in this study by the doctor in charge upon receiving an explanation regarding the study process and signing informed consent.

2.2. Blood sampling

Blood samples for the study were taken on the first day of hospitalization. A total of 3 ml of peripheral blood was taken from each patient and put into EDTA tubes prior to be immediately sent to the laboratory.

2.3. Peripheral blood cell count and flowcytometry assay

Peripheral blood cell count and flow cytometry assay were performed immediately after the sample was received at the Laboratory of Clinical Pathology and Laboratory Medicine. The peripheral blood count was performed using an automatic hematology analyzer. The Neutrophil-Lymphocyte Ratio (NLR) was calculated as a percentage of the absolute neutrophil count divided by the absolute lymphocyte count.

NK cell number were counted with FACSCanto flow cytometer using BD Multitest™ 6-Color TBNK reagent (BD Bioscience). The assay and preparation procedures were performed by the guidelines from the manufacturer of flow cytometry machine and reagents. The results of the NK cell count (positive for the CD16 and CD56 markers) were presented in units of cells/μL.

The remaining blood sample were separated with centrifugation, and the serum samples were frozen and stored at -80°C. The measurement of the cytokine levels was performed using Human Enhanced Sensitivity Cytometric Bead Array Reagent for IL-10, IL-12 and IFN-γ by flow cytometry. Here, the amount of cytokines was expressed in pg/mL. The examination steps were based upon the guidelines from BD Bioscience.

2.4. Data analysis

Variable data with a categorical scale was displayed with frequency and proportion. Continuously scaled variable data shown in mean ± SD or median (min-max) relied on the data distribution. Baseline characteristic data or routine blood count test finding with categorical scales were analyzed by Chi-Square test. Meanwhile, continuous data that were normally distributed were analyzed by independent t test. While, the continuous data that were not normally distributed was analyzed using the Mann-Whitney test. Stratification analysis were performed using gender, age and comorbidities as the additional layers of analysis. The Mann-Whitney test was used to analyze the differences in the number of NK cells and cytokine levels of IFN-γ, IL-10, IL, and 12 between groups because the data distribution was not normal. The correlation between the number of NK cells and levels of cytokines IFN-γ, IL-10, IL, and 12 were analyzed by means of the Spearman correlation test because the data distribution was not normal. Test for normality was performed using the Kolmogorov-Smirnov test. The analysis of statistical test results was considered to be significant if p <0.05. Graph Pad Prism version 9 was used for statistical analysis.

3. Results

3.1. Baseline characteristic of the subject

A total of 90 COVID patients at Dr. Sardjito Hospital agreed to be the subject of this study, however, 27 patients were excluded due to incomplete first-day NK cell data. The total subjects of this study were 63 subjects, divided into two groups with 53.4% of subjects entering mild-moderate degrees. Subjects had a mean age of 52.67 ± 16.33 years with a domination of male subjects (55.6%) (Table 1). The age of the subjects in the mild-moderate group was younger than the severe-critical group (45.39 vs. 60.67 years; p=0.000). There was no significant difference in the proportion of sex between the two groups (p=0.735). A total of 26.7% of the subjects in the severe-critical had comorbid cardiovascular disease (p=0.007).
The results of the peripheral blood cell count showed that the leukocyte count was normal in both groups (Table 2). Although the leukocyte counts were still in the normal range, the median leukocyte count in the severe-critical group was higher than the median leukocyte count in the mild-moderate group (p=0.018). In the severe-critical group, the median neutrophil count was higher (p=0.001); while, the mean lymphocyte count and median eosinophil count were lower (p=0.000 and 0.001) compared to the median in the mild-moderate group. The number of monocytes and basophils did not differ in the two groups (Table 2).

We calculated the NLR in both groups. The median NLR in the mild-moderate group (2.9640 (0.98-27.19)) was lower than the NLR of the severe-critical group (9.17 (2.52-59.64); p=0.002).

3.3. Flow cytometry finding of NK cells and related cytokines

The distribution of the data of the number of NK cells and the cytokine level was not normal. Therefore, we presented the median (min-max) in the result. The median number of NK cells in the severe-critical group (208.55 cells/μL) was lower than in the mild-moderate group (251.85 cells/μL; p=0.048) (Figure 1). The median level of IFN-γ was not significantly higher in the severe-critical group (300.75pg/ml) than in the mild-moderate group (288.75pg/ml; p=0.735). The median IL-12 levels in the two groups were not significantly different (312.39 vs. 196.91pg/ml, p=0.752). The median level of IL-10 in the severe-critical group (1481.18pg/ml) was higher than in the mild-moderate group (926.71pg/ml, p=0.049) (Figure 1).
3.4. Number of NK cells and cytokines levels based on sex at various degrees of severity

The proportion of male subjects was 57.57% in the mild-moderate group and 53.33% in the severe-critical group. The median number of NK cells as well as the levels of IFN-γ, IL-10, and IL-12 did not differ between the sexes in both groups (p>0.05).

3.5. Number of NK cells and cytokines levels based on age at various degrees of severity

Subjects aged over 60 years were higher in the critical-severe group (63.33%). While, the mild-moderate group dominated with subjects less than 60 years old (75.57%).

The number of NK cells and cytokine levels in the mild-moderate group did not differ between age groups (p>0.05). Patients over 60 years of age had higher median IL-10 levels in the severe-critical group (1959.31 pg/μL vs. 1168.89 pg/μL; p=0.031) (Figure 2).

3.6. Number of NK cells and cytokines levels based on comorbid hypertension at various degrees of severity

Hypertension was a common comorbid found in both in the mild-moderate group (99.67%) and in the severe-critical groups (53.33%). The number of NK cells and cytokine levels in both groups were not significantly different between the patients with and without hypertension.

3.7. Number of NK cells and cytokine levels based on comorbid cardiovascular disease at various degrees of severity

More patients with comorbid cardiovascular disease fell in the severe-critical group (26.67%) compared to only 2.35% in the mild-to-moderate group. The median of NK cell number and cytokine levels did not differ between groups in the mild-moderate or the severe-critical group (p>0.05).

3.8. Number of NK cells and cytokine levels based on comorbid diabetes mellitus at various degrees of severity

The proportion of subjects with comorbid diabetes mellitus was slightly higher in the severe-critical group than that of in the mild-moderate group (43.33 vs. 30.30%). The median number of NK cells in subjects with comorbid diabetes mellitus was lower than those without diabetes mellitus in both the mild-moderate group and the severe-critical group. (p<0.05) (Figure 3).

3.9. Correlation between the number of NK cells and levels of IFN-γ, IL-10, and IL-12

The correlation between the number of NK cells and cytokines was analyzed by the Spearman correlation test because the data distribution was not normal (Figure 4). The results of the correlation test showed that the number of NK cells was not correlated with plasma IFN-γ levels (r = -0.018; p = 0.890), plasma IL-10 levels (r = -0.128; p = -0.128.) and IL-12 levels (r = 0.121; p = 0.346.).

3.10. Correlation between cytokines (IFN-γ, IL-10, and IL12)

The correlation between the levels of cytokines was analyzed by the Spearman correlation test because the data distribution was not normal. The results of the correlation test showed that IFN-γ levels were correlated with IL-12 levels (r = 0.286; p = 0.023). However, other cytokines did not show any significant correlation (Table 3).

Table 3. Correlation between cytokines

<table>
<thead>
<tr>
<th></th>
<th>IL-10 vs IFN</th>
<th>IL10 vs -IL12</th>
<th>IFN- vs IL12</th>
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<tbody>
<tr>
<td>R</td>
<td>0.242</td>
<td>0.034</td>
<td>0.286</td>
</tr>
<tr>
<td>P value</td>
<td>0.046</td>
<td>0.793</td>
<td>0.023</td>
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4. Discussions

In this study, 47.6% of the subjects were clinically diagnosed as severe-critical COVID-19 cases. The higher number of leukocytes in patients with severe-critical symptoms is supporting previous report [21]. In this study, the number of patients was limited and the number of leukocytes was still in a normal range. Other study reported that about 25% of 1099 severe COVID-19 had leukocytosis [22]. High leukocyte number is a risk factor for patients falling into critical conditions and even death [23]. Leukocytosis can occur due to coinfection and medications such as prednisone, or immune system variability [21]. Patients who have an increase in leukocytes have the characteristics of old age and comorbidities [23].

A high NLR can be a poor outcome predictor in COVID-19
patients [24]. Our results of higher NLR in the severe-critical group support previous reports [25, 26], indicating that the COVID-19 patients in the severe-critical group have higher number of neutrophils and lower number of lymphocytes in their peripheral blood.

Neutrophilia in the severe-critical group of COVID-19 patients is also associated with higher mortality [10]. It is in contrast to other viruses such as hepatitis viruses, EBV virus, CMV virus, and measles that cause neutropenia due to bone marrow suppression or peripheral destruction [27]. In COVID-19, the type I IFN response is decreased followed by an increase in chemokine signaling that increases neutrophil recruitment to the periphery and subsequent activation [28, 29]. Increasing neutrophil activators such as IL-8 (chemotaxis stimulator) and Granulocyte-Colony Stimulating Factor (hematopoiesis stimulator) have been found in severe COVID-19 patients [30].

**Figure 2.** Number of NK cells and cytokine levels based on age at various degrees (A) NK cells count, (B) IFN-γ level, (C) IL-10 level and (D) IL-12 level. *p* significant < 0.05 with Mann Whitney test except IL-12 with independent t test.

**Figure 3.** Number of NK cells and cytokine levels based on comorbid diabetes mellitus at various degrees of severity (A) NK cells count, (B) IFN-γ level, (C) IL-10 level and (D) IL-12 level. *p* significant < 0.05 with Mann Whitney test except IL-12 with independent t test.
In COVID-19 patients, neutrophils also form Neutrophil Extracellular Traps (NETs) - a structure containing DNA with histones and granule proteins and cytoskeletal proteins. The SARS-CoV-2 virus modulates neutrophils forming NETs through the ACE-2 receptor, activation of the serine protease TMPRSS2, and viral replication. NETs functions to immobilize viruses, prevent the spread, and kill them with antimicrobial proteins. However, it can damage endothelial cells, secrete granule proteins that damage tissues, and activate platelets, resulting in organ thrombosis. This process plays a role in organ damage in severe COVID-19 [28,31].

COVID-19 subjects experienced a decrease in lymphocyte numbers compared to the healthy controls. This reduction was greater in severe-critical patients [10, 32, and 23]. Lymphocytes are responsible in eliminating viral infections. Several mechanisms lead to a decrease in the number of lymphocytes in COVID-19 patients. The SARS-CoV-2 virus can infect lymphocytes via the ACE receptor causing cell death. Inflammatory and proinflammatory cytokines can cause impaired function and number of lymphocytes. A virus can also damage secondary lymphoid tissue causing lymphopenia. Another mechanism that may affect the number of lymphocytes is the administration of anti-inflammatory drugs such as steroids [33].

One type of lymphocyte cell is the NK cell that belongs to innate immune system. It kills viral-infected cells and secrete IFN-γ as well as regulate macrophage function and transmit negative feedback to suppress macrophage reactivity [34]. Our result of lower NK cell count in the severe-critical group supported previous reports showing consistency with the result from different countries, severity definition, variation in blood sampling time, laboratory examination techniques, and patient handling [35,36]. The decreasing number of NK cells, especially in severely symptomatic patients, is due to several mechanisms that are similar to the mechanisms of lymphopenia. The SARS-CoV-2 virus also induces NK cell apoptosis through an increase in active caspase-3 and CD95 [33,37].

NK cells are associated with several cytokines in carrying out their functions. We assessed three important cytokines: IFN-γ, IL-12, and IL-10. IFN-γ is produced by NK cells and it can improve immune system function during infection, increase an antigen presentation through both MHC class 1 and 2, increase CD4 cell differentiation, activate macrophage antimicrobial effector effects, induce antiviral enzymes in macrophages, increase antibody isotype change to IgG, and help leukocytes to travel to inflammatory sites [9]. IFN-γ levels were not found to differ between mild and severe symptomatic patients in the two meta-analyses. [36,38]. This study also showed the same results. The results of the flow cytometry examination showed no significant difference in median IFN-γ levels between groups.

This study found that the median IL-10 level increased significantly in the severe-critical group compared to the mild-moderate group. This result is the same as several previous studies by Han et al. [14] and Qin, et al. [12]. COVID-19 patients experienced the increasing IL-10 levels, especially in patients with severe symptoms. IL-10 can also be a predictor of disease severity [14, 19]. The increase in IL-10 levels in severe-critical degree patients is due to IL-10 as a compensation to prevent inflammation and continued organ damage [17]. Cytokine secretion (TNF-α, IL-1, IL-6, IFN-γ, and IL-12) and antigen presentation, released by macrophages and dendritic cells, are inhibited by IL-10. IL-10 can also induce Treg formation [33]. Further analysis of the relationship between elevated levels of IL-10 and disease severity led to the hypothesis that IL-10 failed to suppress the inflammatory process or that IL-10 had other functions other than as an anti-inflammatory molecule [17].

IL-10 may also function as a proinflammatory cytokine activated by two pathways. The high levels of IL-10 cause excessive IFN-γ production by CD8+ T cells. IFN-γ stimulates macrophages to produce proinflammatory cytokines. Another pathway is macrophage hypo-responsiveness to IL-10 anti-inflammatory stimuli. Macrophage hypo-responsiveness produces excess pro-inflammatory factors. This mechanism is related to the failure of STAT-3 phosphorylation in hyperglycemic conditions [17].

Some evidence have supported the function of IL-10 as a proinflammatory cytokine in the severe cases of COVID-19. First, the increase in proinflammatory cytokine levels (such as...
IL-4, IL-7, IL-18, IFN-γ, and TNF-α) was in line with IL-10 levels [17]. Second, the increase in bacterial lipopolysaccharide in severe COVID-19 cases could induce IL-10 to stimulate macrophages [17]. Third, the increase in IL-10 levels was followed by an increase in the percentage of CD8+ and CD4+ T cells producing IFN-γ in severe patients [32].

IL-12 is responsible for stopping viral replication through the induction of IFN-γ production from Th1 and NK cells [33]. It will increase NKG2D receptor expression in NK cells. The increase in this receptor will then stimulate the cytotoxic effector molecule expression, perforin, and TRAIL. Phosphorylation of ERK1/2, STAT1, and STAT4 will also occur leading to the increase the cytotoxic ability of NK cells [39]. The results of the assay showed that the median IL-12 levels did not differ between groups. A study by Huang et al. showed IL-12 levels were the same between COVID-19 patients and healthy subjects [40]. Studies in Japan showed a significant increase of IL-12 levels in mild cases of COVID-19 [20]. IL-12 was reported to be decreased in hospitalized COVID-19 patients [13]. Another study showed that IL-12 [p70] levels elevated in severe COVID-19 cases with hypertension [19]. This difference might be due to the different subject criteria.

The increase in IL-10 levels without differences in IL-12 and IFN-γ levels may be related to the time of sampling since symptoms appeared. A study conducted longitudinally showed that IL-10 increased in severe symptoms in the first two weeks after symptoms appeared. Meanwhile, proinflammatory cytokines such as IL-6, IFN-γ, IL-17, IL-1β, IL-27, and IL-12 increase in severe symptoms in the late phase of the disease [41]. Correlation results between cytokines support this possibility. The levels of IL-12 and IFN-γ are correlated with each other but not with IL-10.

When related to the number of NK cells, the median levels of IL-10, IL-12, and IFN-γ was not correlated with the number of NK cells. In peripheral blood, NK cells are dominated by dim CD56 NK cells. NK cells have high cytotoxic capacity but low IFN-γ production [6, 15]. The total number of NK cells in peripheral blood has not been able to describe its ability to produce IFN-γ. NK cell activation is determined by several cytokines such as IL-12, IL-18, IL-12, IL-21, IL-10, and type I IFN. NK cell activation in COVID-19 may be more influenced by other cytokines. The examination of other cytokines that activate NK cells is necessary to clarify the results.

The results showed that there were outliers in each variable. Further analysis of each subject showed that the subjects at >60 years old had one or a combination of comorbid hypertension, diabetes mellitus, and obesity.

Aging reduces the immune system. Elderly patients often have comorbidities and physical inactivity [42]. They are at risk for serious symptoms [12]. The study found that IL-10 levels elevated in people over 60 with severe symptoms. Previous research showed that COVID-19 patients above 65 years old had the higher levels of IL-10.

IL-10 is associated with advanced age and disease severity [43]. This may be due to the function of IL-10 as a proinflammatory cytokine. The concept of IL-10 acting as a protective cytokine requires further study.

The analysis of other variables showed that comorbid diabetes affected NK cell counts in mild-moderate and severe-moderate groups. Previous studies in patients with diabetes without COVID-19 showed the reducing NK cell counts.

Excess glucose causes an imbalance in the expression of activator and inhibitor receptors on the NK cell surface [44]. The results of this study suggest that several variables should be considered in COVID-19 patients. This study has several limitations. Cytokines were examined using the stored serum and their degradation may occur in stored serum. The levels of IFN-γ, IL-12, and IL-10 can be degraded up to 50% of initial levels for 2-3 years of storage [45]. Degradation has been tried to be minimized by the immediate separation of blood components, administration of anticoagulant EDTA, and storage at -80°C. Blood samples in this study were not taken at the same disease onset. Different disease onset allowed patients already in different clinical conditions. Since this study is observational, it cannot be definitively concluded regarding the relationship between variables. A longitudinal study is needed for the accuracy of results.

NK cells have an important role in the pathophysiology of COVID-19. Based on our study, NK cells are associated with the severity of the disease. The number of NK cells and IL-10 level are potential biomarkers for the severity of COVID-19.

5. Conclusion

The number of NK cells was higher and IL-10 levels were lower in mild-moderate COVID-19 patients than in severe-critical patients. Age affected IL-10 levels, while comorbid diabetes mellitus affected the number of NK. There was no correlation between the number of NK cells and the levels of IFN-γ, IL-10, and IL-12.

Suggestion

The cytokine assay in this investigation was performed using the stored serum. Cytokines in stored serum may deteriorate, influencing the test results. For more accurate results, more study with fresh blood samples is required. This study only looked at the yield of NK cells and cytokines at one point in time. Longitudinal research is required to determine the kinetic pattern of both. This study included blood samples taken on the first day of COVID-19 diagnosis, not the first day of symptoms. Treatment may already be affecting the immunological response. For more accurate findings, blood must be drawn on the first day of symptoms. Because this study evaluated cytokine levels in serum, the cytokines detected may be produced by cells other than NK cells.

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