

# The phenotype of CD3–CD56<sup>bright</sup> and CD3–CD56<sup>dim</sup> natural killer cells in systemic lupus erythematosus patients and its relation to disease activity

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## Abstract

**Introduction:** Systemic lupus erythematosus (SLE) patients have decreased natural killer (NK) cell counts. The decrease in the number of NK cells has implications for a decrease in the function of NK cells which can affect the progression of SLE disease. The study aim was to determine profiles of CD3–CD56<sup>bright</sup> and CD3–CD56<sup>dim</sup> NK cells in SLE patients and their relation to disease activity.

**Material and methods:** This study included 36 patients of SLE who fulfilled the ACR 1997/SLICC 2012 criteria, women aged 18–49 years. Disease activity was assessed by the Mex-SLEDAI. Peripheral blood samples from SLE patients were analyzed by flow cytometry to evaluate NK cell subsets, according to differential expression of the main subset of NK cells, which is CD3–CD56<sup>bright</sup> and CD3–CD56<sup>dim</sup>.

**Results:** The mean percentage of regulatory NK cell count (CD3–CD56<sup>bright</sup>) in active SLE patients was significantly lower ( $p = 0.000$ ) than in inactive SLE patients. The mean percentage of cytotoxic NK cell count (CD3–CD56<sup>dim</sup>) in active SLE patients was significantly ( $p = 0.000$ ) higher than in inactive SLE patients. A correlation was observed between two subsets of NK cells with disease activity ( $p = 0.00$ ). The percentage of CD3–CD56<sup>bright</sup> NK cells was negatively correlated with disease activity ( $r = -0.766$ ), whereas the percentage of CD3–CD56<sup>dim</sup> NK cells positively correlated with disease activity ( $r = 0.761$ ).

**Conclusions:** There is a difference in the mean percentage of the number of NK cells (CD3–CD56+) in both a subset of regulatory NK cells (CD3–CD56<sup>bright</sup>) and cytotoxic NK cells (CD3–CD56<sup>dim</sup>) in active and inactive SLE patients and it is closely related to SLE disease activity.

**Key words:** systemic lupus erythematosus, natural killer cells, disease activity.

## Introduction

Immune system dysregulation causes chronic inflammation in systemic lupus erythematosus (SLE). The dysfunction of the immune system results in the hyperactivity of macrophage and natural killer (NK) cells, reduced phagocytosis, and accumulation of apoptotic debris.

The adaptive immune system mediated by the B cells and T cells becomes autoreactive by producing autoanti-

bodies. The accumulation of autoantibodies and apoptotic debris form an immune complex that will stimulate chronic inflammation causing damage to various organs [1, 2].

Acute exacerbations and remissions characterize the disease course of SLE. To obtain a better therapeutic outcome of SLE, physicians should closely monitor the patients' disease activity. Evaluation of disease activity is helpful as a guide for therapy monitoring. Several tools are widely used for the disease monitoring of SLE.

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Submitted: 29.03.2022; Accepted: 07.08.2022

One of the most common examinations to assess the SLE disease activity is the SLEDAI (Systemic Lupus Erythematosus Disease Activity Index) score. There are several versions of SLEDAI; one of them is the Mexican (Mex)-SLEDAI.

The Mexican SLEDAI is more practical for health centers with limited screening tools because it does not include immunological measurements such as anti-double-stranded DNA (anti-dsDNA) and complement levels [3, 4].

Natural killer (NK) cells are essential for eliminating viruses and tumor cells and have a vital role in some autoimmune diseases. Natural killer cells in humans are defined as CD3<sup>+</sup>CD56<sup>+</sup>.

Based on the density of the CD56 molecules, NK cells are divided into two main subsets, namely CD3<sup>+</sup>CD56<sup>bright</sup> (regulator) and CD3<sup>+</sup>CD56<sup>dim</sup> (cytotoxic). Regulatory NK cells can produce cytokines such as interferon  $\gamma$  (IFN- $\gamma$ ) which are more than cytotoxic NK cells. In contrast, cytotoxic NK cells have more remarkable cytolytic ability than the regulatory NK cells [5–7].

Patients with SLE have reduced NK cell counts [8]. The decrease in the number will lead to lower NK cell function. Studies on the subset and activity of NK cells will provide a better picture of the pathophysiology of NK cells in SLE patients. Based on this background, it is necessary to determine the profile of CD3<sup>+</sup>CD56<sup>bright</sup> and CD3<sup>+</sup>CD56<sup>dim</sup> NK cells in SLE patients and their relationship with SLE disease activity scores.

## Material and methods

### Design and study subjects

This research was a cross-sectional study performed in Saiful Anwar General Hospital, Malang, Indonesia. The subjects of this study were 18–49-year-old female SLE patients who fulfilled the American College of Rheumatology (ACR) 1997 or Systemic Lupus International Collaborating Clinics (SLICC) 2012 criteria and were referred to the Rheumatology Clinic in Saiful Anwar General Hospital, Malang, Indonesia [9].

Patients who were pregnant, breastfeeding, had a previous history of cancer, trauma, or severe infections were excluded from the study. All SLE patients in the clinic were assessed for eligibility by the attending physicians and rheumatologists.

All patients were evaluated for the disease activity using the Mex-SLEDAI score as the routine examination. Patients were classified as active if the Mex-SLEDAI score was  $\geq 5$  and inactive if the Mex-SLEDAI score was  $< 5$  [10].

In patients who fulfilled the criteria, 15 ml of peripheral venous blood was taken and put in a vacutainer with ethylenediaminetetraacetic acid (EDTA). The mini-

mal number of the subjects was calculated by the following formula:  $n = 2 [(Z_{\alpha} + Z_{\beta}) S / ((x_1 - x_2))]^2$  [11].

This study used 95% as the confidence interval (CI), with the power of the study being 80%. According to the previous study, the calculation of  $S$ ,  $x_1$ , and  $x_2$  was 40.8, 14, and 7 [8].

Thus, the minimal number of participants from each group was 18 SLE patients.

Patients were recruited by a simple random sampling method to minimize bias. In addition, to minimize the information bias, assessment of the disease activity and eligibility of the subjects was carried out by at least two doctors from the clinic.

### Peripheral blood mononuclear cell isolation and sorting natural killer cells by flow cytometry

Isolation of PBMC was performed for the determination of NK cell subsets and NK cell surface markers. Peripheral blood mononuclear cells were obtained from 18 mm of blood samples with EDTA from all SLE patients by centrifugation of density Lymphoprep 1.07 (Axis-Shield, Norway), then separated and washed.

The purity of the isolation results was confirmed by FACSMelody (BD Bioscience) by staining PerCP-conjugated anti-CD3 antibody (BioLegend, San Diego, CA) and FITC-conjugated anti-CD56 antibody (BioLegend, San Diego, CA).

The number of cells was analyzed by BD Cell Quest software. The analysis resulted in the percentage (%) of cells. According to Lin et al. [12], the population of lymphocytes was gated to confirm CD3<sup>+</sup> and CD3<sup>+</sup> lymphocytes.

Then, the population of CD3<sup>+</sup> lymphocytes was gated for the next analysis of CD56 expression. The results are expressed as the percentage of isolated NK cells (CD3<sup>+</sup>CD56<sup>+</sup>). The isolated NK cells were further sorted as CD56<sup>dim</sup> and CD56<sup>bright</sup>.

### Statistical analysis

The Mann-Whitney  $U$  test was used to analyze differences in the number and expression of NK cell phenotypes between active and inactive SLE patients. Spearman's test was used to assess the correlation between variables. Data were described as mean  $\pm$  standard deviation (SD) and considered significant if the  $p$ -value  $< 0.05$ . Statistical analysis was performed using SPSS version 21.

### Bioethical standards

The Ethics Committee of Saiful Anwar Malang Hospital approved this study (No. 400/231/K.3/302/2019), and informed consent was obtained from all SLE patients.

**Table I.** Characteristics of active and inactive patients with systemic lupus erythematosus

Characteristic	Active (n = 18) (mean ±SD)	Inactive (n = 18) (mean ±SD)	p-value
Age, mean (range)	31.5 ±7.3 (21–44)	29.3 ±6.3 (19–47)	0.485
Average disease duration [months]	34.6 ±30.8	36.0 ±35.4	0.849
Mex-SLEDAI	8.8 ±2.8	3.2 ±1.0	0.000*
Hemoglobin [g/dl]	10.3 ±2.5	11.1 ±1.5	0.296
Leukocytes [ $\times 10^3/\mu\text{l}$ ]	6.523 ±4.055	6.567 ±2.362	0.506
Lymphocytes [ $\times 10^3/\mu\text{l}$ ]	0.962 ±0.529	1.401 ±0.638	0.058
Thrombocytes [ $\times 10^3/\mu\text{l}$ ]	226.388 ±83.457	317.216 ±71.400	0.003*
Medications [frequency (%)]			
GC	18 (100)	11 (61.1)	0.003*
Mean GC dose (range) Methylprednisolone [mg/day]	8.7 ±10.5 (4–48)	2.4 ±2.0 (0–4)	0.001*
Hydroxychloroquine	10 (55.5)	11 (61.1)	0.735
Azathioprine	8 (44.4)	4 (22.2)	0.157
Mycophenolate mofetil	2 (11.1)	1 (5.5)	0.546

\* Shows statistical significance with  $p < 0.05$ .

GC – glucocorticosteroid, Mex-SLEDAI – Mexican Systemic Lupus Erythematosus Disease Activity Index, SD – standard deviation.

## Results

### Patients' characteristics

The flowchart of the eligibility for the participants in this study is shown in Figure 1. There were 67 SLE patients from the Rheumatology Clinic who were assessed for the study's eligibility. Twenty-nine (43.2%) participants were excluded from the study because they did not fulfill the inclusion criteria. There were 38 patients enrolled in this study.

However, two (2.9%) were excluded from the analysis because of incomplete data. In the end, 36 patients were included in the analysis (18 active SLE and 18 inactive SLE patients). The characteristics of the patients are shown in Table I.

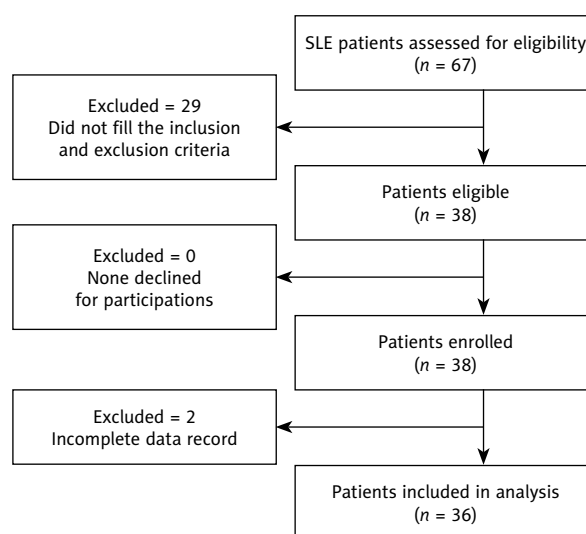
Mexican SLEDAI in active SLE patients was significantly higher than inactive ( $8.8 \pm 2.8$  vs.  $3.2 \pm 1.0$ ;  $p = 0.000$ ). The Mex-SLEDAI score was used to differentiate the status of SLE disease activity between active and inactive patients. Patients with active disease also had a significantly lower mean platelet count compared to the inactive SLE patients ( $226,388.9 \pm 83,457.7/\mu\text{l}$  vs.  $317,216.7 \pm 71,400.6/\mu\text{l}$ ;  $p = 0.003$ ). The distribution of medication that was given to the subjects is shown in Table I.

The frequency of glucocorticosteroids (GCs) given in active SLE patients was higher in active patients compared to inactive SLE patients ( $p = 0.003$ ). The mean dosage of GCs given was also significantly higher in active patients compared to inactive patients ( $p = 0.001$ ).

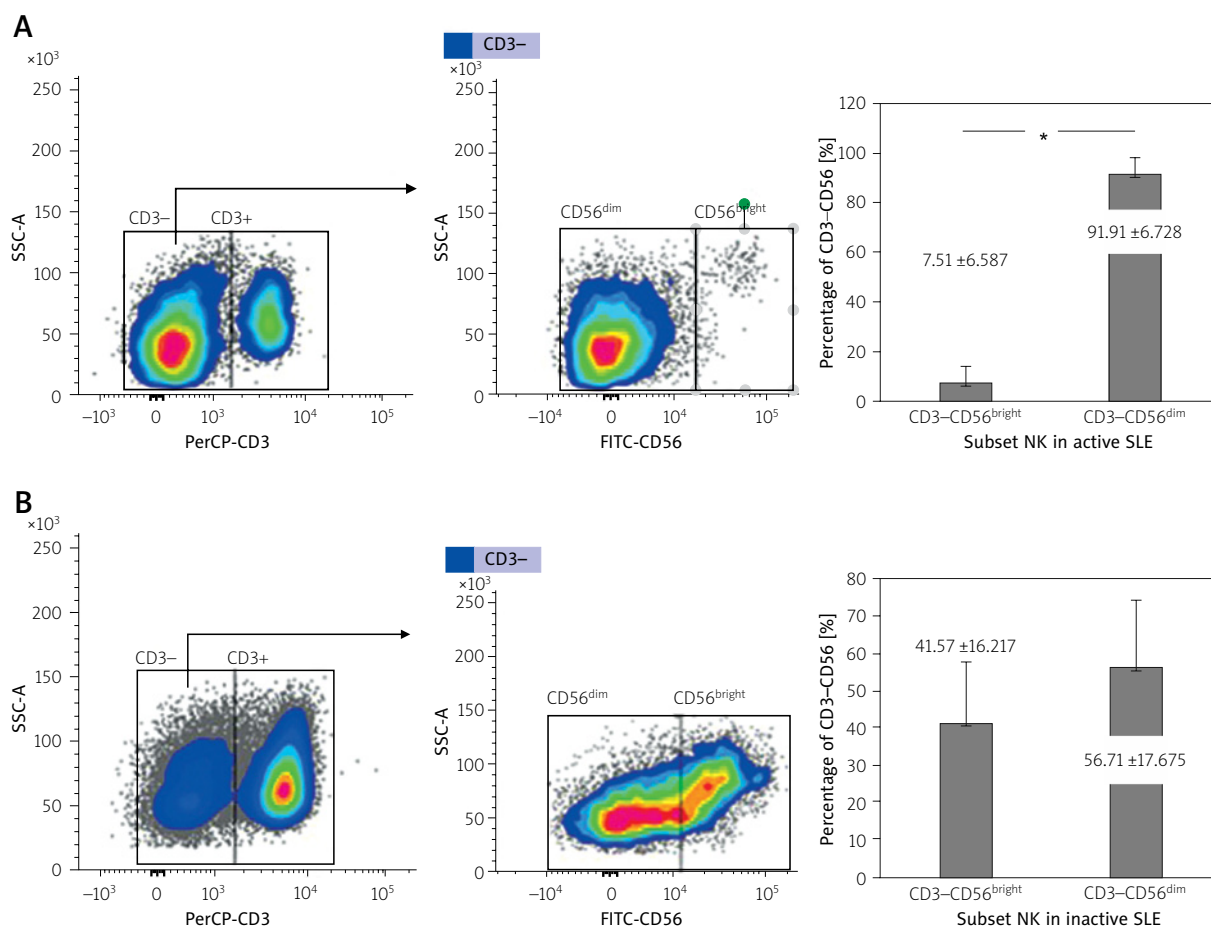
On the other hand, there was no significant difference in the frequencies for the other treatments given by both groups.

### Percentages of CD3–CD56<sup>bright</sup> and CD3–CD56<sup>dim</sup> of systemic lupus erythematosus patients

Natural killer cells expressed as regulatory CD3–CD56+ were sorted into two main subsets, regulatory CD3–CD56<sup>bright</sup> and cytotoxic CD3–CD56<sup>dim</sup> NK cells (Fig. 2). There was a difference between the percentages of the regulatory NK cell subset (CD3–CD56<sup>bright</sup>) and the cytotoxic NK cell subset (CD3–CD56<sup>dim</sup>).



**Fig. 1.** Flowchart of participation of patients included in the study.



**Fig. 2.** Percentages of CD3-CD56<sup>bright</sup> and CD3-CD56<sup>dim</sup> of systemic lupus erythematosus patients. Natural killer cells obtained from PBMC isolation of systemic lupus erythematosus patients, stained with PerCP anti-human CD3 and FITC anti-human CD56, then analyzed with FACSMelody. The lymphocyte population was gated to identify CD3- and CD3+. The CD3-negative lymphocytes were gated for further analysis of CD56 expression. Then CD3-CD56+ was sorted into 2 subsets of CD56<sup>dim</sup> and CD56<sup>bright</sup> of NK cells. The CD3-CD56<sup>bright</sup> subset was significantly lower than CD3-CD56<sup>dim</sup> in active SLE (A); CD3-CD56<sup>bright</sup> was lower than CD3-CD56<sup>dim</sup> in inactive systemic lupus erythematosus, but without significance (B).

In active SLE patients, the percentage of CD3-CD56<sup>bright</sup> was lower than CD3-CD56<sup>dim</sup> (7.5 ± 6.6 vs. 91.9 ± 6.7;  $p = 0.000$ ). No significant difference was found between the percentage of CD3-CD56<sup>bright</sup> and CD3-CD56<sup>dim</sup> in inactive patients (41.6 ± 16.2 vs. 56.7 ± 17.7;  $p = 0.085$ ).

Moreover, our data showed some significant differences between the mean percentage of the NK cells in both subsets between active and inactive SLE patients, as shown in Figure 3.

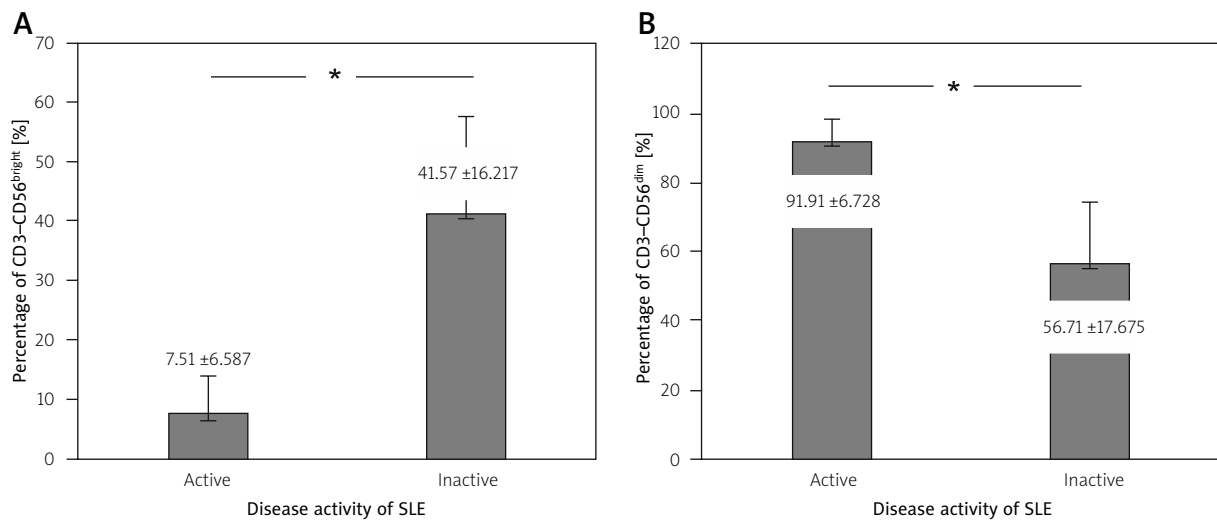
In active SLE patients, the percentage of regulatory CD3-CD56<sup>bright</sup> was significantly lower than in inactive patients (7.5 ± 6.6 vs. 41.6 ± 16.2;  $p = 0.000$ ). The percentage of cytotoxic NK CD3-CD56<sup>dim</sup> in active SLE patients

was significantly higher compared to inactive patients (91.9 ± 6.8 vs. 56.7 ± 17.7;  $p = 0.000$ ).

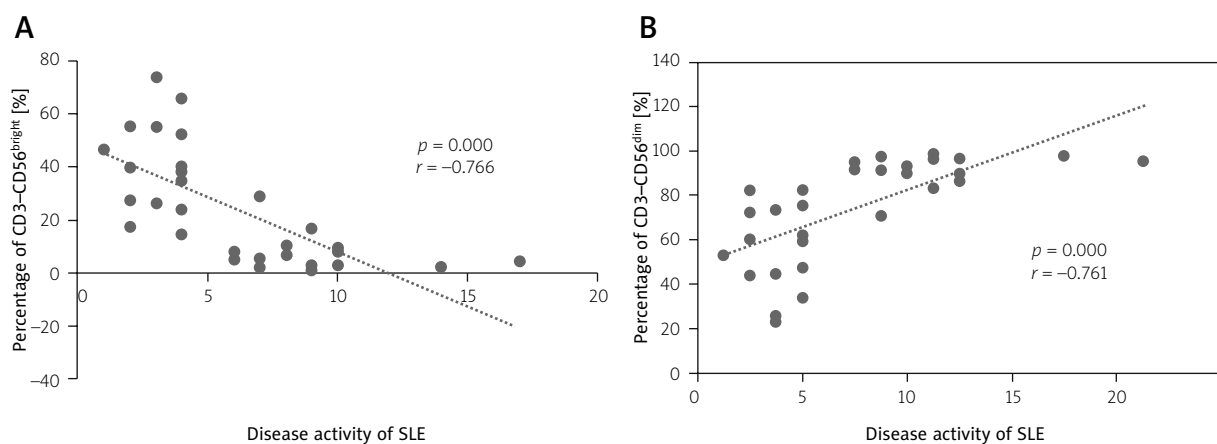
### Correlation between the number of CD3-CD56<sup>bright</sup> and CD3-CD56<sup>dim</sup> natural killer cell subsets in systemic lupus erythematosus patients with systemic lupus erythematosus disease activity

The correlation of the percentages from subsets of regulatory NK cells (CD3-CD56<sup>bright</sup>) and cytotoxic NK cells (CD3-CD56<sup>dim</sup>) with SLE disease activity is shown in Figure 4.

The results demonstrated that there was an inverse correlation between the mean percentage of regula-



**Fig. 3.** The difference of NK subsets in active and inactive SLE. The mean percentage of CD3-CD56<sup>bright</sup> in active was lower than in inactive SLE. The mean percentage of CD3-CD56<sup>dim</sup> in active was higher than in inactive SLE patients.



**Fig. 4.** Correlation between SLE disease activity with subset of NK cells. (A) Inverse correlation between disease activity with percentage of CD3-CD56<sup>bright</sup>; (B) Positive correlation between disease activity with CD3-CD56<sup>dim</sup>.

tory NK cells (CD3-CD56<sup>bright</sup>) and SLE disease activity ( $p = 0.000$ ;  $r = 0.766$ ) (Fig. 4 A). There was also a positive correlation between the mean percentage of the cytotoxic NK cell subset (CD3-CD56<sup>dim</sup>) and SLE disease activity ( $p = 0.000$ ;  $r = 0.761$ ; Fig. 4 B).

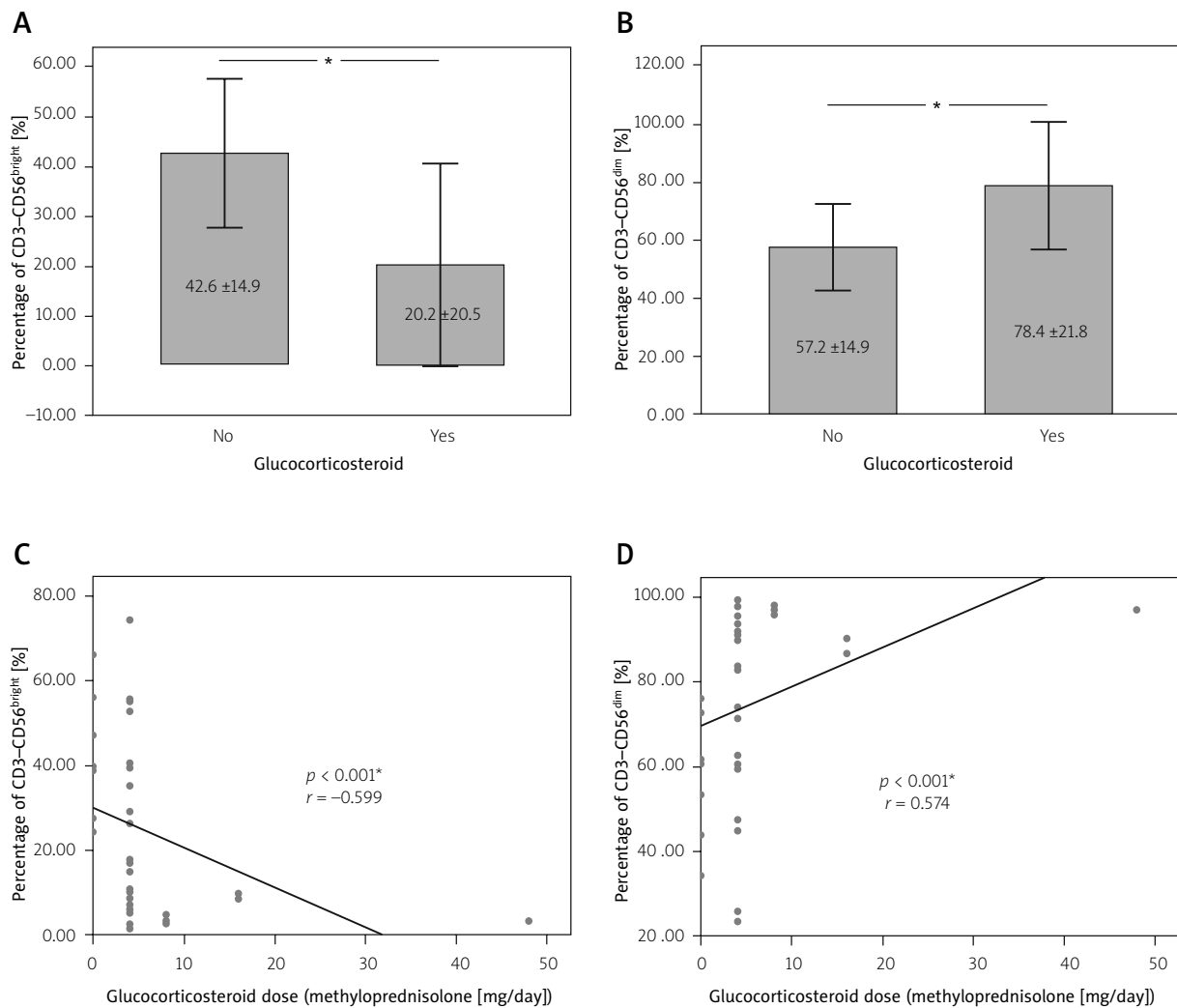
#### Percentages of CD3-CD56<sup>bright</sup> and CD3-CD56<sup>dim</sup> according to the medication given in systemic lupus erythematosus patients

Figure 5 A, B shows the comparison between the percentages of CD3-CD56<sup>bright</sup> and CD3-CD56<sup>dim</sup> according to the GCs given to the subjects. Systemic lupus erythe-

matusus patients who received GCs had significantly lower CD3-CD56<sup>bright</sup> percentages (20.2 ± 20.5% vs. 42.6 ± 14.9%,  $p = 0.009$ ) and higher CD3-CD56<sup>dim</sup> percentages (78.4 ± 21.8% vs. 57.2 ± 14.9%,  $p = 0.014$ ) compared to the subjects who did not receive the GCs.

On the other hand, the dose of GCs given to the SLE patients was significantly correlated with the CD3-CD56<sup>bright</sup> ( $p < 0.001$ ,  $r = -0.599$ ) and CD3-CD56<sup>dim</sup> percentages ( $p < 0.001$ ,  $r = 0.574$ ), as shown in Figure 5 (C-D).

However, the CD3-CD56<sup>bright</sup> and CD3-CD56<sup>dim</sup> percentages did not significantly differ in patients who obtained hydroxychloroquine ( $p = 0.776$  and



**Fig. 5.** Correlation between systemic lupus erythematosus disease activity and GC therapy. Comparison of CD3-CD56<sup>bright</sup> according to the use of GC (A); comparison of CD3-CD56<sup>dim</sup> according to the use of GC (B); inverse correlation of CD3-CD56<sup>bright</sup> with the GC dose, positive correlation of CD3-CD56<sup>dim</sup> with the GC dose (C).

$p = 0.950$ ), azathioprine ( $p = 0.156$  and  $p = 0.128$ ), or mycophenolate mofetil ( $p = 0.872$  and  $p = 0.872$ ) compared to the patients who did not receive these medications.

### Discussion

Our study demonstrated that the profile of NK cells, including regulatory NK cells (CD3-CD56<sup>bright</sup>) and cytotoxic NK cells (CD3-CD56<sup>dim</sup>), was different between patients with active and inactive disease activity. The average percentage of regulatory NK cell counts (CD3-CD56<sup>bright</sup>) in active SLE patients was lower than in inactive SLE patients.

In contrast, the mean percentage of cytotoxic NK cell counts (CD3-CD56<sup>dim</sup>) in active SLE patients was higher than in inactive SLE patients. The low percentage of the number of the two major subsets of NK cells, especially in active SLE patients, suggests that there might be a decrease in the total number of NK cells in autoimmune disease conditions such as SLE.

The lower NK cell count in SLE patients is similar to the previous studies' findings [5, 8, 13].

In autoimmune conditions, the induction of apoptosis due to excessive antibody production causes the dysregulation and destruction of immune cells, leading to a decreasing number of blood cells, including NK cells.

The decreasing number of NK cells can also later be associated with disease activity.

The condition of active SLE indeed contains more antibodies and pro-inflammatory cytokines, which will affect the activity of immune cells, including NK cells, causing a change in the proportion of the NK cell subset [14]. With a reduction of total NK cells in patients with active SLE, the ability of NK cells to proliferate is also lower.

This study demonstrated a significant relationship between the mean percentage of the subset of regulatory NK cells (CD3–CD56<sup>bright</sup>) and cytotoxic NK cells (CD3–CD56<sup>dim</sup>) with SLE disease activity. Natural killer cell of CD56<sup>bright</sup> was reported to be a regulatory NK cell that could suppress the T cell responses through many mechanisms [15].

A previous study also showed that CD56<sup>bright</sup> was impaired in patients with SLE and the administration of IL-2 was associated with the attenuation of disease activity and improvement of CD56<sup>bright</sup> populations in the peripheral blood [16].

In contrast to the CD56<sup>bright</sup> NK cells, CD56<sup>dim</sup> NK cells were associated with the cytotoxic activity of NK cells. CD56<sup>dim</sup> was able to secrete pro-inflammatory cytokines, such as IFN- $\gamma$  [17].

Research by Huang et al. [18] demonstrated that NK cells participated in the pathogenesis of SLE by activation and production of IFN- $\gamma$  in an amyloid-induced experimental lupus-like syndrome model.

We found that the medications for the SLE patients were correlated with the CD56<sup>bright</sup> or CD56<sup>dim</sup> NK cells, especially the GCs. However, it is still not known whether the correlation was directly caused by GCs or another factor, since the dosage of GCs itself was also positively correlated with disease activity.

The role of GCs in the physiology of NK cells remains elusive. Systemic lupus erythematosus patients were documented as having impaired cytotoxicity in NK cell function, showed by decreased CD56<sup>dim</sup> NK cells.

A previous study showed that the therapy of SLE would increase the number of NK cells [19]. Ni et al. [20] also reported that the administration of a GC or immunosuppressant agent was able to reshape the CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells, changing them from the cytotoxic subset into the regulatory subset.

## Study limitations

Our study described the role of NK cells in patients with SLE. However, there were some limitations of this study. The number of subjects was relatively small. This research was carried out during the COVID-19 pandemic, so it was quite difficult to obtain patients according to the inclusion criteria.

This preliminary study also only showed the number of NK cell subsets and did not demonstrate the function of these subsets in SLE patients, for example, the ability to secrete pro-inflammatory cytokines. This study did not use the EULAR/ACR 2019 criteria because we collected the subjects before establishing the EULAR/ACR 2019 criteria. Thus, we used the previous ACR1997/SLICC 2012 criteria during the study.

In addition, to assess the disease activity in this study, the Mex-SLEDAI score was used instead of the SLEDAI-2K because of limitations in the clinical settings. However, a previous study showed that the Mex-SLEDAI score was strongly correlated with the SLEDAI-2K score [10] and could be applied in several settings when complement or anti-dsDNA could not be observed.

Despite several limitations in this study, there is still an open opportunity to develop the study about the role of NK cells in the pathogenesis and treatment of SLE with a bigger sample size and more parameters.

## Conclusions

The results of the present study indicate a difference in the mean percentage of the number of NK cells (CD3–CD56+), both subsets of regulatory NK cells (CD3–CD56<sup>bright</sup>) and cytotoxic NK cells (CD3–CD56<sup>dim</sup>) in active and inactive SLE patients and a relationship between these NK subsets and SLE activity.

## Acknowledgements

The authors would like to thank all patients who participated in this study. Also, they would like to express their gratitude to dr. Rifa'i, dr. Putra, dr. Singgih, dr. Mirza, dr. Ramadi, Mr. Yudha, Ns. Elvira, Mrs. Lilis for their support and technical assistance in the hospital and the laboratory.

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*The authors declared no conflict of interest.*

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