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DIFFERENTIATION OF $\gamma\delta$ LYMPHOCYTE CELLS EXPRESSING INTERLEUKIN-17 ON HEALTHY PERSONS AND ADULT ACUTE MYELOID LEUKEMIA PATIENTS

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ABSTRACT

Acute Myeloid Leukemia (AML) is a hematological cancer causing deaths of 1.2%. It is a relatively rare disease but by the end of the decade there is an increase in the number of new cases. The immune system in AML disturbance is caused by gene mutations giving immunosuppressive effects so that the immune system will be inhibited in eliminating leukemia cells. The immune response of tumors is important to determine the prognosis and development of new cancer immunotherapy as well. One of the subset of lymphocytes T is $\gamma\delta$ T lymphocyte cell with innate nature, but until now, no information is obtained about the $\gamma\delta$ T cell profile in AML patients. The delta T gamma cells have properties as antitumors are resulted by Interferon production γ (INF γ), and the nature of protumor by interleukin 17 (IL-17). The percentage of lymphocyte T (CD3⁺) of AML patients and healthy persons do not differ ($p = 0.528$), indicating, it is not being activated for proliferation. The delta T gamma Lymphocyte cells percentage in healthy persons are determined by race, genetic and exposure to the surrounding environment such as infection. Percentage of $\gamma\delta$ T lymphocyte of AML patients and healthy persons is not different from ($p = 0.694$), as showed as an immune response by $\gamma\delta$ T cells uneffected to proliferate. The percentage of $\gamma\delta$ T lymphocytes expressing the interleukin 17 ($\gamma\delta$ T17 cells) in AML patients and healthy persons do not differ significantly ($p = 0.436$), this indicates an inhibited proliferation.

Key words: Acute myeloid leukemia, T lymphocytes, $\gamma\delta$ T cells, gd17T cells, IL-17

INTRODUCTION

Acute Myeloid Leukemia (AML) is a hematological malignancy that causes cancer and then leading to death as much as 1.2%. Even though it covers a relatively rare disease, but at the end of the decade there has been an increase in the number of new cases. Acute myeloid leukemia is also considered as a life-threatening disorder, which requires a precise and fast diagnosis to choose the most appropriate therapeutic approach. The diagnosis for this disorder can be established based on morphology, immunophenotype, cytogenetics, and molecular analysis in order to distinguish various types of leukemia.¹

The prognosis for AML, moreover, is determined by disease-related factors and factors associated with its patients. Old age, blast percentage, and cytogenetic results are considered as the most important risk factors for AML. Besides, gene

mutations, comorbidity, and low tolerance for intensive chemotherapy are known as markers of additional risk.² The current new strategy for management of its therapy is by utilizing the immune system to eliminate leukemia cells.² The immune system in AML is mostly resulted by T lymphocytes and dominated by immunosuppressive properties. Gene mutations in AML can enhance immunosuppressive properties so that the immune system will be inhibited in removing leukemia cells.^{3,4} Thus, knowledge of tumor immune responses is now growing rapidly, especially in determining prognosis, developing a new cancer immunotherapy, and evaluating therapies.^{5,6}

Furthermore, many researches on tumor immune responses have largely concentrated on CD4 T cell lymphocytes. One subset of T lymphocytes, gamma delta T cells with innate traits and without the need for Major Histocompatibility Complex (MHC) are actually considered as potential immunotherapy

candidates in the treatment of AML patients. Unfortunately, there is not enough information about the profile of delta T gamma cells in AML patients. The delta T gamma cells are known to have both antitumor properties triggered by its production dominated by Interferon γ (INF γ), as well as protumor properties dominated by interleukin 17 (IL-17). As a result, this research aimed to determine T $\gamma\delta$ cells expressing IL-17 profiles in AML patients compared to those in healthy persons.

METHODS

A total of 31 samples were collected, comprising of 20 samples of AML patients and 11 samples of healthy persons. Inclusion criteria for the samples of AML patients were patients aged over 18 years old, newly diagnosed AML based on FAB criteria, and willing to participate in this research by signing the consent form. Meanwhile, inclusion criteria for the samples of healthy persons were people aged from 18 years old to 64 years old and signing the consent form.

This research was conducted at the Department of Internal Medicine and the Department of Clinical Pathology of the Dr. Soetomo General Hospital in Surabaya from October 2016 to December 2017. This research used a cross-sectional design in which samples were taken sequentially.

Next, venous blood samples were collected, and aspiration of bone marrow was performed according to the following procedure. Firstly, whole blood as much as 3 mL was stored in vacutainer EDTA tubes, and then processed in one hour after collection in order to examine characteristics of AML patients as well as T $\gamma\delta$ cells expressing IL-17. Secondly, the diagnosis of AML subtype was established based on hematological examination results, morphology of

peripheral blood smear and bone marrow according to FAB criteria, as well as immunophenotyping if needed obtained from secondary data.

Subsequently, the percentage of T $\gamma\delta$ cells expressing IL-17 was examined with flow cytometry method using whole blood samples that had been lysicated with BD FACS Lysing Solution (catalog no. 349202) mixed with specific antibody BD reagents conjugated with fluorochrome, such as 3 PerCP (catalog no. 340417), anti- $\gamma\delta$ TCR-1 FITC (catalog no. 347903), and anti-Human PE Mouse IL-17A on a cocktail basis. For intracellular staining, BD cytofix/cytoperm was added, and then read with BD FACS Calibur using a laser wave length of 488 nm.

Afterwards, flow cytometry method and cell detection with fluorocromium-labeled monoclonal antibodies resulted in cell populations to be examined in both samples of adult AML and healthy persons. The percentage of cells then was determined using gating strategy in order to determine the population of CD3⁺ (the number of CD3+ T $\gamma\delta$ cells⁺ and CD3+ T $\gamma\delta$ ⁻ cells), T $\gamma\delta$ cells (CD3+ T $\gamma\delta$ ⁺ cells), and T $\gamma\delta$ cells expressing IL-17 (T $\gamma\delta$ 17 cells) as shown in Figure 1.

Statistical analysis was then performed using SPSS ver 2.1. The samples were considered to be normally distributed after analyzing using Kolmogorov-Smirnov test. After that, differences in proportions between categorical variables were analyzed using independent t test with PT. A p-value of <0.05 was considered to be statistically significant with a 95% confidence interval.

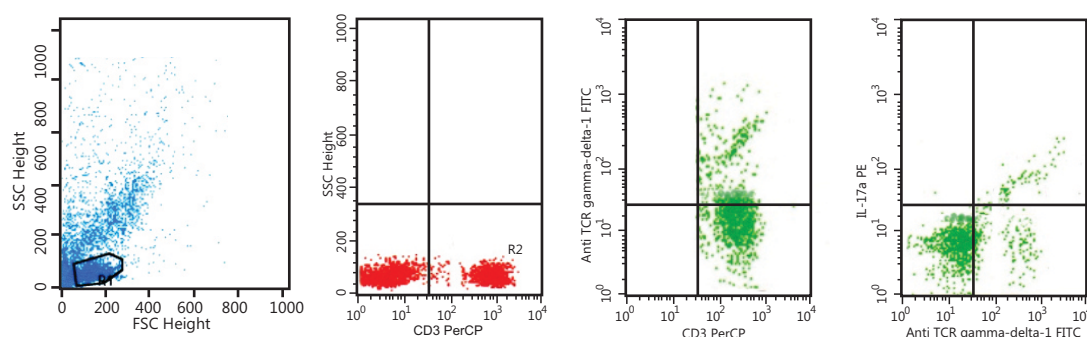


Figure 1. Analysis of research variables using the flow cytometry method

- FSC and SSC showing areas of "gating" lymphocytes from peripheral blood,
- Gating CD3⁺ areas,
- CD3⁺ expressions and T $\gamma\delta$ ⁺ cells demonstrating the percentage of T cells expressed by CD3⁺,
- The expressions of T $\gamma\delta$ ⁺ cells and IL-17⁺ cells indicating the percentage of T $\gamma\delta$ ⁺ cells expressing IL-17. FSC: Forward Scatter; SSC: Side Scatter; CD3: cluster of differentiation 3; T $\gamma\delta$, cells: T $\gamma\delta$ lymphocyte cells; and IL-17: interleukin 17.

Table 1. Characteristics of research subjects

Characteristics of samples	AML patients n=20	Healthy persons n=11
Sex:		
Male (total number (%))	7 (35%)	3 (46%)
Female (total number (%))	13 (65%)	8 (54%)
Age:		
Median (min – max)	35 (23 – 75)	31 (26 – 40)
Mean (standard deviation)		
WBC ($\times 10^3/\mu\text{L}$)	45.3 (33.6)	-
Hb (g/dL)	9.6 (1.65)	-
Plt ($\times 10^3/\mu\text{L}$)	85.1 (90.4)	-
AML subtypes:		
AML-M2	2 (10%)	
AML-M4	10 (50%)	
AML-M5	8 (40%)	

Table 2. Percentages of T cells and T17 cells in both groups of AML patients and healthy persons

Variables	Groups	N	Mean (SD)	P-values
T $\gamma\delta$ cells (%)	AML patients	20	20.18 (9.54)	0.694
	Healthy persons	11	19.27 (2.75)	
T $\gamma\delta 17$ cells (%)	AML patients	20	4.24 (3.09)	0.436
	Healthy persons	11	3.39 (2.43)	

range of 23-75 years old. Those patients were newly diagnosed with AML and had no chemotherapy. The diagnosis then was established based on FAB criteria applied on the evaluation results of peripheral blood smear and bone marrow. If immunophenotyping had been required, AML-M4 subtype would have been obtained as much as 50%.

Unlike in the samples of AML patients, the age range of healthy persons was 26-40 years old. The mean level of leukocytes (SD) was 45,262 (33,600)/ μL , while the mean level of hemoglobin (SD) was 9.6 (1.65) g/dL and 85,071 (90,400)/ μL for the mean level of platelets. The number of AML patients with AML-M4 subtype was 10 patients (50%), while the number of AML-M5 patients was 8 patients and 2 patients with AML-M2 subtype as shown in Table 1.

In addition, Table 2 illustrated the percentage of T $\gamma\delta$ cells expressing IL-17 (T $\gamma\delta 17$ cells). Table 2 also depicted the differences in the percentages of T $\gamma\delta$ cells expressing IL-17 (T $\gamma\delta 17$ cells) between the group of AML patients and the group of healthy persons.

The average percentage of T cells in healthy people in this research was 19.27% (2.75), while the average percentage of T cells in AML patients was 20.18% (9.54). It meant that there was no significant difference in the average percentage of T cells between the groups with a p-value of more than

0.05. As shown in the box and Whisker diagrams, it seemed clearly that there was an overlap in the average percentage of T cells between healthy people and AML patients.

Besides, the mean percentage (SD) of T17 cells in Cd3 + T $\gamma\delta^+$ cells in the group of AML patients was 4.24 (3.09), while 3.39 (2.43) in the group of healthy persons. Although it appeared to be different in nominals, there was no significant difference ($p > 0.05$) as shown in Table 2, indicated by examining the minimum and maximum standard deviation results from the box and whisker diagrams (see Figure 2).

The characteristics of AML patients in this research were dominated by the AML-M4 subtype, excluding the AML-M3 subtype. As a result, the immune response expected to occur in AML patients was a process of recognition of blast cells with myeloid series.

Moreover, the samples used for the examination in this research were whole blood analyzed by lysis solution. The whole blood was selected rather than Peripheral Blood Mononuclear Cell (PBMC) due to its easy processing and little blood volume in order mainly to avoid the loss of blood components due to PBMC manufacturing process.⁷

Interleukin 17, on the other hand, is produced by groups of T lymphocytes, such as T $\gamma\delta$ cells (T $\gamma\delta 17$ cells), CD4⁺ (Th17) T cells, CD8 T cells, and

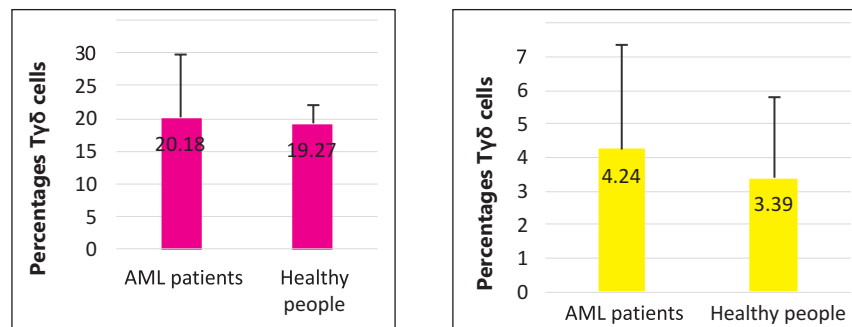


Figure 2. Box and whisker diagrams illustrating the percentages a. Tγδ cells and b. Tγδ17 cells in AML patients and healthy persons

HCV cells. IL-17 can also be produced by neutrophils and macrophages. Besides, IL-17 in cancer has been known to have an ability to induce angiogenesis and immunosuppressive which helps cancer cells to proliferate and metastasize.^{8,9}

In addition, Tγδ cell is one of the subsets of T lymphocyte cells that are innate. In its activities, Tγδ cell does not require MHC as an effector. Besides, the response activities of Tγδ cell occur at the same time with a process of direct recognition of cancer cells. Tγδ cells are also known to have antitumor properties triggered by interferon γ , perforin, and granzim. Unfortunately, Tγδ cell also has a protumor effect triggered by IL-17.¹⁰ Thus, this research was conducted to determine the percentage of Tγδ and Tγδ17 cells as Tγδ cell subsets in both patients newly diagnosed with AML, but having no chemotherapy, as well as healthy persons.

In this research, the mean (SD) percentage of Tγδ cells to the total T lymphocyte cells in the healthy group was 19.27 % (2.75), higher than that in Tγδ cells in samples of PBMC in a previous study about 1% -10%. This difference occurs because the samples in this research were whole blood. The use of whole blood can give higher results than the use of PBMC since the use of whole blood can avoid the loss of blood components to be examined in the PBMC process.⁷

Moreover, Esin examining the percentage of Tγδ cells in healthy individuals of certain ethnic groups argued that the median percentage of Tγδ cells in samples of non-Japanese Asian individuals was 9.2% with a minimum percentage of 1.9% and a maximum of 26.2%, different from other ethnic groups. This finding was related to environmental factors, such as exposure to subclinical infections and allergic diseases that can increase the percentage of T cells, as well as genetic differences.¹¹ The percentage of Tγδ cells in AML patients, furthermore, showed a non-significant difference ($p > 0.05$) when compared with the

percentage of Tγδ cells in healthy persons. It indicated that Tγδ cells in AML patients did not expand or proliferate. Aswald examining patients newly diagnosed with AML, but having no chemotherapy yet, found a lower percentage of Tγδ cells in AML patients than those in healthy persons. This was because of the weakening of T lymphocytes and those expressing T receptor cells thereby decreasing the normal immune defense response and also showing failure of immune surveillance. Meanwhile, in solid tumors, those conditions indicated advanced malignancy with a short survival period. This was because of the different phenotypes of each tumor and the variability of various and multilevel immune responses in each type of cancer.¹²

The increase in Tγδ cell immune response in AML patients is probably due to leukemia cells unable not only to provoke the immune response of Tγδ cells, but also to express innate immune responses, or to respond to a stress-associated antigen related to the amount insufficient to stimulate that response. It can be proven in-vitro that the absence of a leukemic cell ligand against T cell receptors does not affect the ability of leukemic blast cells to provoke the immune response of Tγδ cells.^{13,14}

Meeth also argued that the dominance of one of the Tγδ cell subtypes did not reflect a process of expansion or proliferation of Tγδ cells themselves. But, this dominance indicated more the tendency of Tγδ cell differentiation in one of its subtypes in response to stimulation received. In other words an increase in the percentage of Tγδ cell subtype would be followed by a decrease in other Tγδ cell subtypes.^{13,14}

In addition, the results of the analysis in this research showed that there was no significant difference in the percentage of Tγδ cells expressing IL-17 (Tγδ17 cells) between AML patients and healthy persons ($p > 0.05$). The mean (SD) of Tγδ cells in

healthy persons in this research was 3.39% (2.43). The percentage of T $\gamma\delta$ cells indicated the number of T $\gamma\delta$ cells expressing IL-17 divided by the total number of peripheral blood T $\gamma\delta$ cells. It also indicated the presence of an innate body response that will produce T $\gamma\delta$ cells in small amounts without going through a stimulation process. The insignificant difference in T $\gamma\delta$ 17 cells between AML patients and healthy persons may be due to lack of stimulation of T $\gamma\delta$ cells to differentiate into T $\gamma\delta$ 17 cells.

Finally, the results of this research indicated that the stimulation received by T $\gamma\delta$ cells in AML patients at the time of diagnosis, but still not yet treated, was not able to increase its differentiation into T $\gamma\delta$ cells producing IL-17 (T $\gamma\delta$ 17 cells). Another previous research conducted by Ismail also revealed that other cells producing IL-17 (T-helper 17) in AML patients significantly increased compared to those in healthy persons ($p < 0.05$).¹⁵

Nevertheless, this research still has some weaknesses. Firstly, the level of IL-17 produced by T $\gamma\delta$ 17 cells in this research was not examined to determine T $\gamma\delta$ 17 activities. Secondly, this research also did not examine cytokines and other effectors stimulating differentiation of T $\gamma\delta$ cells into T $\gamma\delta$ cells expressing IL-17. Therefore, types of stimulus needed for differentiation into the subset of T $\gamma\delta$ cells expressing IL-17.

CONCLUSION AND SUGGESTION

In conclusion, there was no significant difference in T $\gamma\delta$ 17 cells between AML patients and healthy persons. Nevertheless, further researches should be focused on why the immune system does not respond to leukemia cells.

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