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Increased Perforin- and IL-21-Expressing NK Cells in Patients with Early-Stage Chronic Lymphocytic Leukemia

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ABSTRACT

Objective: $CD5^+CD19^+$ cells have a low proliferation capacity and elevated expression of anti-apoptotic protein BCL-2, mostly in the G0/G1 cell phase and accumulate in the pathogenesis of chronic lymphocytic leukemia (CLL). Natural killer (NK) cells have the ability to kill the intracellular pathogen-infected or cancer cells and secrete cytotoxic enzymes. This study evaluated the frequency, expression of cytotoxic enzymes, and intracellular cytokine levels of NK cells in CLL patients.

Materials and Methods: In this study, peripheral blood mononuclear cells were isolated from peripheral blood samples of CLL patients (n=29) and healthy controls (n=16) by density gradient centrifugation method. The frequency of the total NK cells and the intracellular levels of perforin, granzyme, interferon (IFN)-γ, interleukin (IL)-21, IL-4 and IL-17 in NK cells were investigated.

Results: Elevated total NK cell frequency were found in the CLL patients compared to the healthy controls, and negatively correlated with $CD5^+CD19^+$ malign B cell frequency. Increased perforin expression was observed in patients' total NK cells. Additionally, increased levels of IL-17 and IL-21 in total NK cell of CLL patients were obtained compared to healthy subjects.

Conclusion: The findings suggest that in the early stage of CLL, increased total NK cell frequency, and elevated perforin and IL-21 levels in NK cells might have a protective impact against the progression of the disease for CLL patients.

Keywords: Chronic lymphocytic leukemia, cytokines, natural killer cells, perforin

INTRODUCTION

The incidence of chronic lymphocytic leukemia (CLL) is high in elderly individuals, and CLL is the most common type of leukemia in Western countries (1). CLL involves an increase of $CD5^+CD19^+$ cells, which have a low proliferation capacity, increased expression of BCL-2, and accumulated in the bone marrow and peripheral blood (1, 2). Most patients are 60-65 years at the time of first diagnosed, with only 10-15% of patients being under 55 years of age (3). The clinical outcome of CLL is variable, and treatment is delayed until the disease progresses (4). CD38 expression (30% ≥ CD38 positive and 30% < CD38 negative) identifies two subgroups of CLL patients with different clinical outcomes, and CD38 positive patients have poor clinical outcome (5). Recently Rai and Binet staging systems have been used to predict progression and develop an appropriate treatment plan (4).

Natural killer (NK) cells are large granular lymphocytes and, despite being lymphoid in origin, are a member of the

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innate immune system (6). CD3 is not expressed in NK cells, while CD16 and CD56 are expressed on the surface of NK cells (6, 7). The cells that do not express major histocompatibility complex (MHC) class I are recognized by NK cells, and cytotoxic enzymes such as perforin and granzyme are secreted by NK cells in order to target cell lysis (8). Additionally, NK cells secrete various cytokines to regulate immune responses (6) and have an important role in eliminating virus-infected cells and cancer cells. The studies have shown that individuals with low NK cell function have an increased risk of cancer early in life and that NK cells are critical in preventing tumor metastasis (9).

In this study, the total NK cell frequency and the intracellular expression of perforin, granzyme, and IFN-γ, IL-4, IL-17, IL-21 levels in NK cells were analyzed to evaluate the role of NK cells in CLL.

MATERIALS AND METHODS

Patient Groups

The study was evaluated with 9 female and 20 male CLL patients being monitored in the Hematology Department of the Istanbul Bakirkoy Sadi Konuk Training and Research Hospital. All patients were chosen according to the International Workshop on Chronic Lymphocytic Leukemia (iwCLL) 2008 diagnostic criteria who have a lymphocyte count greater than 5 x 10^3/mm^3. According to the Rai/Binet staging system, CD5 and CD19 expression levels were also detected in all patients who were at an early stage and had not received any treatment yet (Table 1). The study also included 4 females and 12 males as healthy controls, matched to the patients' age and sex, without a history of cancer, autoimmunity, infection, immunosuppressive medication, or smoking. This study was approved by the Istanbul University Faculty of Medicine Local Committee with the decision number; 587348 and dated September 24, 2021.

Cell Preparation and Sorting

Peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll-Hypaque solution from peripheral blood samples obtained from the patient and healthy subjects. PBMCs were washed in a phosphate buffered saline (PBS) solution twice and suspended in the RPMI 1640 medium (Gibco, USA) which was consisted of L-glutamine (Gibco, USA) and anti-mycotic/antibiotic (Gibco USA; at respective concentrations of 10% and

Table 1. Clinical data of the patients.

		CLL	Healthy Controls
N		29	16
Male (n)		20	12
Female (n)		9	4
Age (Mean (min - max))		65 (49 - 76)	58 (50 - 78)
WBC (10^6/mL) (mean (min - max))		40.34 (13 - 147)	7.18 (5.2 - 9.3)
Lymphocytes (10^6/mL) (mean (min - max))		34.78 (6.9 - 140)	2.90 (2.1 - 3.6)
$CD5^+CD19^+$% Mean (min - max)		89.10 (14.7 - 99.8)	-
Binet Stage	**A**	23	-
	B	6	-
	C	-	-
Rai Stage	**0**	17	-
	1	5	-
	2	5	-
	3	2	-
	4	-	-
CD38	**Positive**	10	-
	Negative	19	-
Infection		No	No
Treatment		No	No

1%,). PBMCs (5x10^6 cells/mL) were labeled with the anti-CD19 APC (BD-Biosciences, USA) and incubated for 20 minutes in the dark at room temperature. Lymphocytes were separated by gating on SSC versus FSC plot, and then CD19 negative cells in lymphocyte population were gated on SSC versus CD19 plot. CD19 negative cell population were sorted using the BD FACSAria II cell sorter (BD-Biosciences, USA). Sorted cells were labeled with anti-CD3 FITC, -CD56 PE and -CD16 PE monoclonal antibodies to detect the CD3 and CD19 negative cells. (All antibodies from BD-Biosciences, USA). After incubation, samples were analyzed by Novocyte flow cytometry (Agilent, USA), and CD3$^-$CD19$^-$ cell population considered as NK cells.

Detection of Intracellular Cytokines and Cytotoxic Enzymes in Total NK Cells

Sorted cells (1x10^6 cells) were stimulated using the cell activation cocktail with Brefeldin A (Biolegend, USA) for 5 hours. Cells were then washed and labeled with anti-CD3 FITC (BD-Biosciences, USA). Fixation and permeabilization were performed using the Fix-Perm kit according to manufacturer's instructions (Nordic MUbio, Netherlands), and the anti-IFN-γ BV510, -IL-4 PECY7, -IL-17 AlxF700, -IL-21 PE, -perforin BV510, and -granzyme AlxF700 monoclonal antibodies were added (All antibodies from BD-Biosciences, USA). Cells were then washed and analyzed by using the NovoCyte flow cytometry (Agilent, USA).

Statistical Analyses

Mann-Whitney U test was performed for the analysis of the data. Pearson's correlation test was used for the correlation analysis. GraphPad 5.3 program was used for statistical analysis and graphical designs, with $p < 0.05$ being considered statistically significant.

RESULTS

Higher Total NK Cells in CLL patients

The lymphocyte population consisted of CD3$^+$ T, CD19$^+$ B, NKT, and NK cells. The cells that did not express CD3 and CD19 considered as NK cells in order to evaluate NK cell functions in CLL patients in this study. To confirm this, CD19 negative cells were sorted from PBMCs and stained with anti-CD16 and -CD56. The purity of CD3$^-$CD56$^+$CD16$^+$ NK cells were 93-96% (Figure 1A-B).

The total NK cell frequency was higher in CLL patients compared with healthy controls (p = 0.0006; Figure 1C). No significant differences were obtained in NK cell frequency in the patient groups according to CD38 status and Rai/Binet stages. However, the total NK cell frequency in CLL patients was negatively correlated to the CD5$^+$CD19$^+$ malign B cell frequency (p = 0.03, R = -0.414).

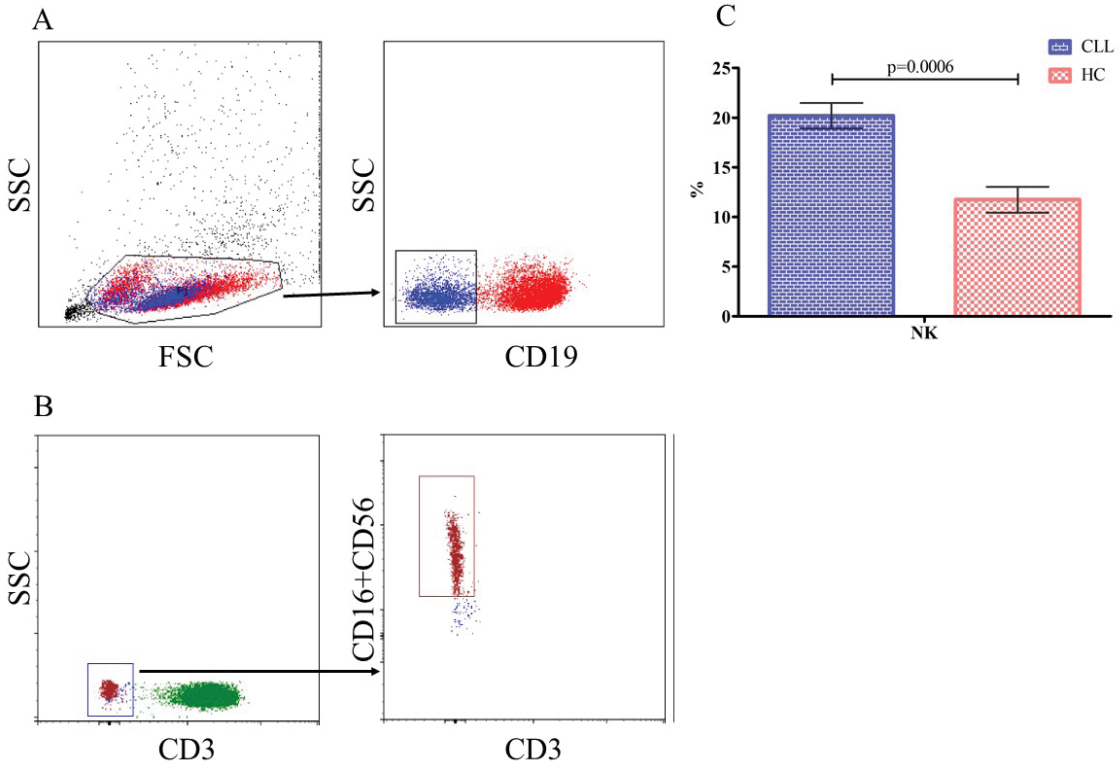

Figure 1. Gating strategy of total NK cells. CD19$^-$ cells were gated in lymphocyte gate and sorted (A). NK cell purity was analyzed according to CD16 and CD56 expression of CD3$^-$ cell population of sorting cells (B). Total NK cells in CLL patients (n=29) and healthy controls (n=16) was showed (C). NK: Natural killer, CLL: Chronic lymphocytic leukemia, HC: Healthy control.

Elevated Perforin Expression of NK Cells in CLL Patients

To evaluate the cytotoxic properties of the NK cells, intracellular perforin and granzyme expressions were analyzed using by flow cytometry (Figure 2A). Compared to the healthy controls, high perforin expressions were observed in NK cells of CLL patients ($p < 0.0001$), while granzyme expression was unchanged (Figure 2B). No significant differences were seen on the cytotoxic properties of NK cells in the patient groups in terms of CD38 expression and Rai/Binet stages. However, a positive correlation was found between the $CD5^+CD19^+$ malign B cell frequency and perforin expression of NK cells ($p = 0.03$, $R = 0.403$; Figure 2C).

Increased Intracellular IL-17 and IL-21 Levels in NK Cells of CLL Patients

When the intracellular cytokine levels of NK cells were analyzed, no significant difference was found between the IFN-γ and IL-4 levels of NK cells in patients and healthy controls (Figure 3A-B). On the other hand, IL-17 and IL-21 levels in total NK cells of patients were higher than healthy controls ($p < 0.0001$ and $p < 0.0001$, respectively; Figure 3B). No significant differences were found in the IFN-γ, IL-4, IL-17 and IL-21 levels of NK cells in the patient groups according to CD38 expression and Rai/Binet stages. IL-17 levels of NK cells were positively correlated with white blood cell count, lymphocyte count, and the $CD5^+CD19^+$ malign B cell frequency ($p = 0.04$, $R = 0.428$; $p = 0.02$, $R = 0.475$; $p = 0.05$, $R = 0.313$, respectively; Figure 3C).

DISCUSSION

Cancer cells can escape from adaptive immune responses through the various mechanisms they have developed (10). In particular, the most well-known mechanism is to reduce the MHC class I expression on their surface (10). NK cells recognize and lyse the non-MHC I expressing target cells. Because of these features, NK cells have a primary role in anti-tumor responses (6). Therefore, NK cell-based cancer immunotherapies have recently been designed (9).

The incidence of CLL is high in the northern hemisphere (11), with CLL patients having an accumulation of malignant cells of the $CD5^+CD19^+CD23^+$ phenotype (12). Unlike other types of leukemia, most patients with CLL are only monitored clinically without any treatment (13). T cell frequency, especially the $CD8^+$ T cell frequency, is known to increase in patients with CLL. However, data regarding the disease development and progression of increased T cell levels have been contradictory (14). Many studies regarding T cells and their role in CLL are present, but studies on NK cells and their role in CLL pathogenesis are few and conflicting (9).

In a study focused on small lymphocytic lymphoma (SLL) and CLL patients and reported the NK cell counts to have increase in CLL patients and be positively correlated with CD19 cell counts (15). On the other hand, it was detected that NK cell frequency were increased and had a negative correlation

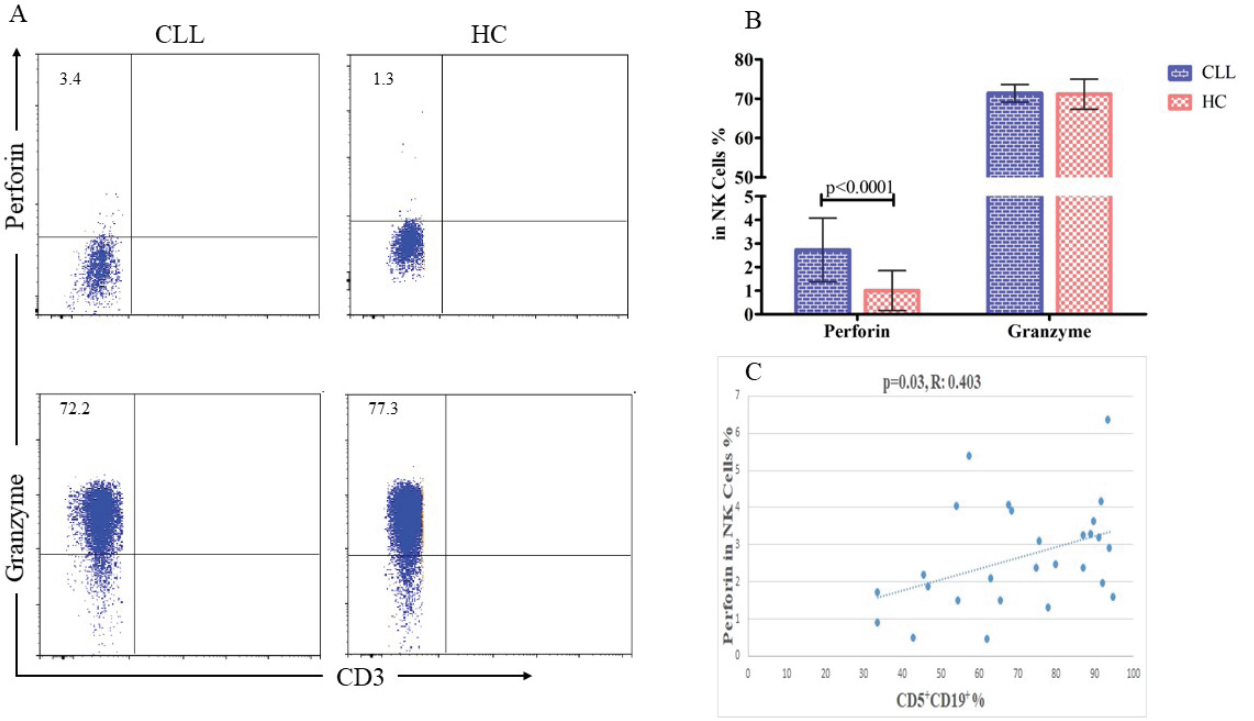

Figure 2. Representative dot-plots of perforin and granzyme expression on total NK cells in CLL patients and healthy controls (A). Perforin and granzyme expression on total NK cells in patients and healthy controls (B). Correlation graphs between perforin expression and $CD5^+CD19^+$ cell frequency (C). NK: Natural killer, CLL: Chronic lymphocytic leukemia, HC: Healthy control.

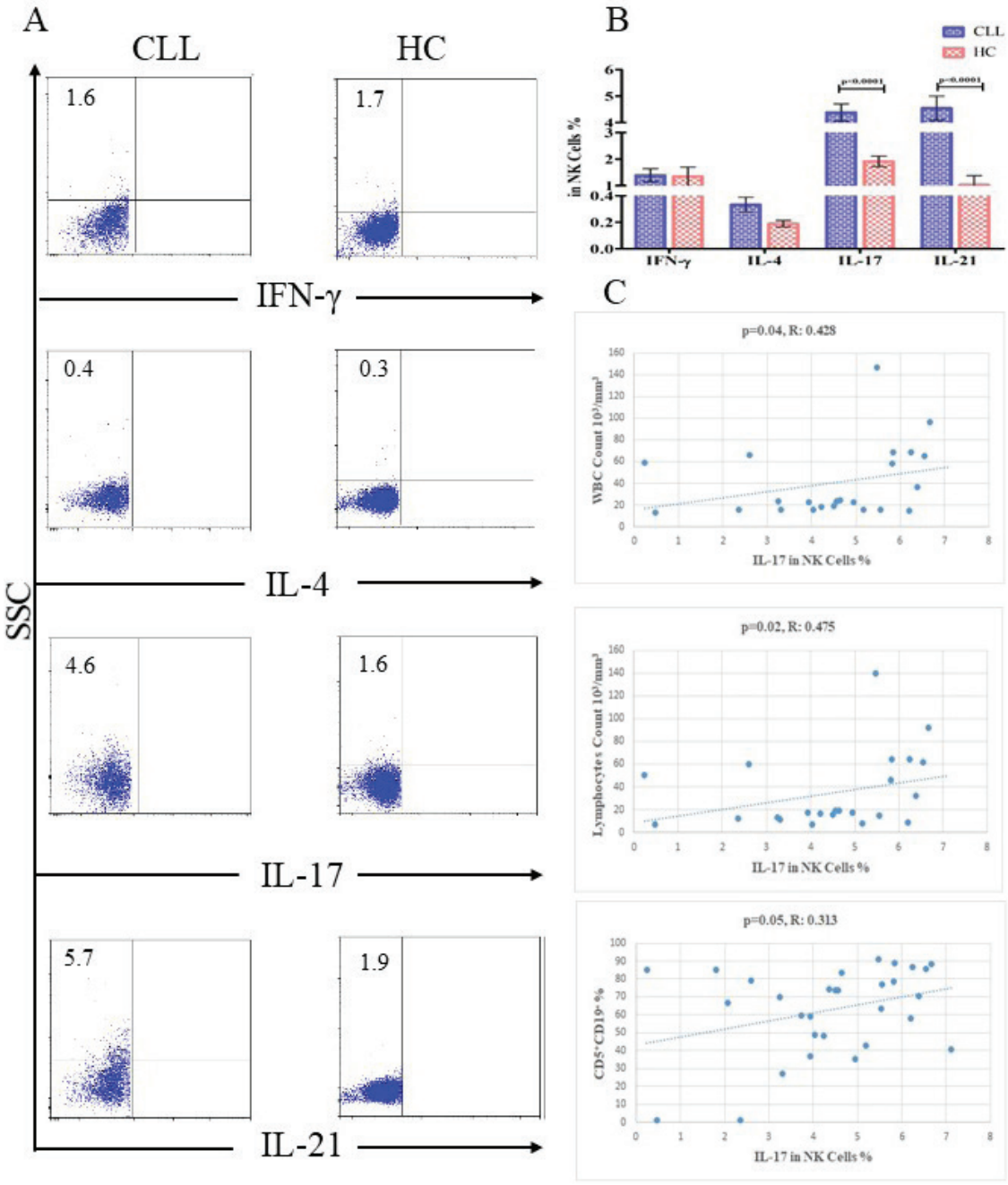

Figure 3. Representative flow graphs of IFN-γ, IL-4, IL-17 and IL-21 levels of total NK cells in patients and healthy controls (A). The statistical graph represents percentages of IFN-γ, IL-4, IL-17 and IL-21 levels of total NK cells in patients and healthy controls (B). Correlation graphs between IL-17 expression and white blood cells (WBS), lymphocytes, $CD5^+CD19^+$ cells (C). NK: Natural killer, CLL: Chronic lymphocytic leukemia, HC: Healthy control.

with the $CD5^+CD19^+$ malign B cell frequency in untreated CLL patients with the early stage. This difference might be due to the evaluation of total NK frequency in lymphocytes after $CD19^+$ cells were excluded in our study. For instance, NK cell frequency was evaluated within total PBMC in MacFarlane et al. study, and NK cell dysfunction was obtained in CLL patients (15). On the other hand, it can be thought that high NK cell frequency might be protective against the malignant B cells or disease progression.

The tumor microenvironment is very important for malignant B cells in CLL, as with many cancer types (16). Various cytokines such as IL-6 are known to be important for this tumor

environment (16), and many immune system cells, especially Th17, are known to secrete IL-17, which in turn is known to stimulate IL-6 (17, 18). Recent studies have shown CLL patients to have increased sera and plasma IL-17 levels, especially patients in the early stage (16, 19). In addition, the Th17 cell frequency has been found to be higher in patients compared to healthy controls (20). NK cells are known to mainly secrete pro-inflammatory cytokines such as IFN-γ, but recent studies have indicated IL-17 to also be secreted by NK cells (21, 22). The current study observed IL-17 expression in the total NK cell to be higher in CLL patients. According to the study's data, NK cells might contribute to possible IL-17 increases in the sera or plasma of CLL patients. Additionally, the study found a positive correlation for IL-17 expression in the total NK cell with the $CD5^+CD19^+$ cell frequency. Previous studies have shown CLL patients to have higher IL-6 levels compared to healthy subjects, as well as higher IL-6 levels to be correlated with adverse disease features and low survival (16, 23). Meanwhile, recent studies have indicated the IL-17/IL-6 axis to have an important role in CLL and to be possible therapeutic targets. When considering that IL-17 triggers IL-6 production, IL-17 expression in NK cells might be associated with a poor clinical outcome.

Recent studies have also indicated that IL-21 to be able to affect the gene expression profile of malign B cells in CLL (24). Additionally, IL-15 has been shown to increase the survival and proliferation of CLL cells, with IL-21 having the opposite effect (25). IL-21 expressing total NK cells of CLL patients were increased in our study. Furthermore, IL-21 is known to increase perforin expression, cytotoxicity, and anti-tumoral responses in NK cells (26). Consistent with this finding, the current study showed higher perforin and IL-21 levels in total NK cells. Our findings suggest that IL-21 levels of NK cells might have an autologous effect and to trigger the expression of perforin in these cells. However, these needs to be confirmed through *in vitro* studies.

NK cells are an attractive source for new immunotherapeutic strategies, including chimeric antigen receptor (CAR) therapy (9). Therefore, the role of NK cells in CLL is very important. Our findings suggest the total NK cell frequency and increased perforin and IL-21 levels of these cells might have a protective effect against the progression of early stage of CLL patients. In addition, the negative correlation observed between the malign $CD5^+CD19^+$ cell and total NK cell frequency seems to support this hypothesis. However, analyzing NK cell numbers and functions in advanced or treated CLL patients will allow us to better understand the role of NK cells have in the pathogenesis of CLL.

Ethics Committee Approval: This study was approved by the Clinical Research Ethics Committee of Istanbul University Medical Faculty (04/11/2021-587348).

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