JMALS

Journal of Medical and Life Science

https://jmals.journals.ekb.eg/

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Relationship between Interleukin 21 and Activation of Natural Killer cells in Iraqi patients with CML

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DOI: 10.21608/jmals.2025.450015

Abstract

Background: CML is a persistent hematological malignancy, distinguished by aberrant white blood cell growth. It is caused by a genetic abnormality that leads to the uncontrolled production of a specific type of white blood cell called a chronic myeloid leukemia cell. Preclinical findings reveal that IL-15 and IL-21 have the potential capacity to enhance the activity of NK cells, which are important for killing cancer cells. Additionally, IL-21 may help to regulate the immune response in CML, potentially reducing the risk of complications associated with the disease.

Materials and Methods: Sixty-five Iraqi patients with CML were recruited from the Hematology Center in Baghdad. The participants who were on tyrosine kinase inhibitors (TKIs) and those who had just received a diagnosis were split into two groups. There was also a control group of twenty-five healthy people. The study. The IL-21 estimation by Sandwich ELISA and NK subsets detection by Flowcytometry. **Results:** The study observed significant changes in natural killer cell subsets between CML patients and healthy controls with differences based on treatment status. IL-21 level showed no significant variation, suggesting it may not play a key role in CML. While some NK cells subsets correlated with IL-21, the overall link with CD56 +CD 16 + NK cells was not statistically significant.

Keywords: NK cells, CML, interleukin 21, TKIs.

Introduction

The myeloproliferative neoplasm known as chronic myeloid leukemia CML develops in pluripotent cells and is identified by the translocation of t(9,22) (q34;q11) chromosomes (1), which the resultant [BCR/ABL1] protein is the activation of multiple signal pathways, leading to a malignant transformation(2). Like other malignancies, CML also has a compromised immune system that allows cancerous cells to escape the body's defenses, which is necessary for the growth of the disease. The innate immune system's cells are malfunctioning in individuals with CML (3). The BCR-ABL protein activates downstream signaling pathways through phosphorylation and leads to disease phenotype by causing changes in cell adhesion and blocking apoptosis, while promoting differentiation and inducing proteasomal degradation of essential proteins. CML emerges as one of the cancers most responsive to treatment through immunological

Received: April 22, 2025. Accepted: August 1, 2025. Published: August 27, 2025

manipulation based on this finding(4). Cells of the "innate immunity "play an essential role in tumor immune therapy(5). NK cells are important innate immune cells that have the capacity to trigger potent antiviral and antitumor responses. NK cells' potent cytolytic action against tumors allows them to regulate tumor development and metastatic dissemination (6). Nk cells are capable of identifying and eliminating tumor cells that either have increased stress-induced ligand expression or have lost MHC Class I expression. Numerous studies have suggested that the NK cells' ability to eliminate tumor cells in CML patients is impaired both at diagnosis and throughout the use of tyrosine kinase inhibitors (TKIs). (7). The functional effects of NK cells are mediated via cytokine-induced cytotoxicity (8)."IL-15, IL-21, and IL-18" can activate NK cells, with the IL-15 and IL-21 combination playing a significant role in regulating their proliferation and cytotoxicity (9). These cytokines are essential in controlling the activities of NK and T-cells. IL-21, a T cell-derived cytokine linked to IL-2, IL-4, and IL-15, reveals the "IL-2/IL-15Rβ "subunit and the common (γc) chain with IL-15R. Despite shared receptors, IL-15 triggers STAT5, while IL-21 preferentially activates STAT3, leading to separate effects. IL-21 improves NK cell cytotoxicity and IFN-γ production but inhibits IL-15-induced proliferation of inactive NK cells. This suggests IL-21 may help modify immune responses from innate towards adaptive immunity(10,11).

Materials and Methods

The case control study has been carried out on 65 CML patients (40 males and 25 females at age range 18 - 60 year) who were diagnosed by hematologist with CML at At the Work Center for Blood Research - Al-Mustansiriya University - Baghdad - Iraq during the period from February to December 2023, and then they were diagnosed by reacting PCR by detecting the BCR-ABL gene.. Patients were categorized into two main groups; the first group included 20 patients who were newly diagnosed, while the second group included 45 patients with a history of disease from a few months to several years and under treatment with TKIs either with imatinib drug (n=25) as well as with nilotinib drug (n=20). In addition to patients, a control group of 25 healthy participants who were matched in age and gender were also included in the study

Inclusion criteria:

- CML patients their aged between 18 – 60 years
- Non-treated and Treated CML Patients with Tyrosine Kinase Inhibitors

Exclusion criteria: Subjects (patients and healthy) who were excluded from this study include:

• All subjects under the age of 17 years.

• Patients with crisis and the accelerated phase of CML

• Normal subjects with any chronic or autoimmune disease.

Samples Preparation: Centrifuged Blood to collect serum (300 x g, 10 minutes)and stored in -80 °C until used.

Estimation of IL-21 Concentration Using Snswich ELISA:

ELISA Procedures: Used a human IL-21 ELISA kit (R&D system, USA). Add standards and serum in a pre-coated 96-well plate, incubate for 2 hours at room temperature, wash, and add biotinylated detection antibody for 2 hours.

Detection: Add Streptavidin-HRP for 30 minutes, followed by TMB substrate, and stop the reaction with 1M sulfuric acid.

Analysis: Measure the Absorbance at 450 nm. Calculate IL-21 concentrations using a standard curve.

Detection NK (CD $^{56+}$ CD $^{16+}$) cells by Flowcytometry:

Identification and calculation of NK cells in blood samples for all studied subjects (patients and controls) was performed by using eight-colored flow cytometry (BD FACS CANTO II BD Biosciences/USA) to detect immunophenotyping

NK cells and their subsets. The surface receptors used for detection of NK cells are (CD45 conjugated PE/DAZZLE (Biolegand/USA), CD3 conjugated APC/FIRE (Biolegand/USA), CD56 conjugated PRILLIANT Violate 711(Biolegand/USA), and CD16 conjugated PRILLIANT Violate 421) (Biolegand/USA). The NK cells' immunophenotype was presented as ($CD45^{+}$ $CD3^{-}$ $CD56^{+}$ $CD16^{+}$)

Preparation of blood samples for flow cytometer assay

Two tubes with 100 µl of anti-coagulated blood were prepared. One tube received 5 µl of fluorochrome-conjugated antibodies; the other was an unstained control. Both were incubated in the dark for 15 minutes. After adding 2 ml of 1X RBC lysis buffer and incubating for 10 minutes, the tubes were centrifuged at 2300 rpm for 5 minutes.

Pellets were washed twice with wash buffer, centrifuged, and resuspended in 0.5 ml of 1X cell fix buffer. Samples were analyzed using a FACS Canto II flow cytometer (BD Biosciences).

Serial gating of assay: The serial gating of flowcytometry used in this study was illustrated in figure **1** in which; dot plot of all cells **(upper left)**, dot plot to choose singlets cells **(uppermiddle)**, dot plot to identify leukocyte common antigen protein tyrosine phosphatase (CD45+) **(upper right)**, dot plot to identify NK and dendritic cells (CD3-CD56+) **(lower left)**, dot plot to identify CD56+CD16+ (NK cells), as well as detection of cytokine-producing NKs ($CD56^{bright}$ $CD16^{dim}$) and cytotoxic NKs ($CD56^{dim}$ $CD16^{bright}$) **(lower middle)**

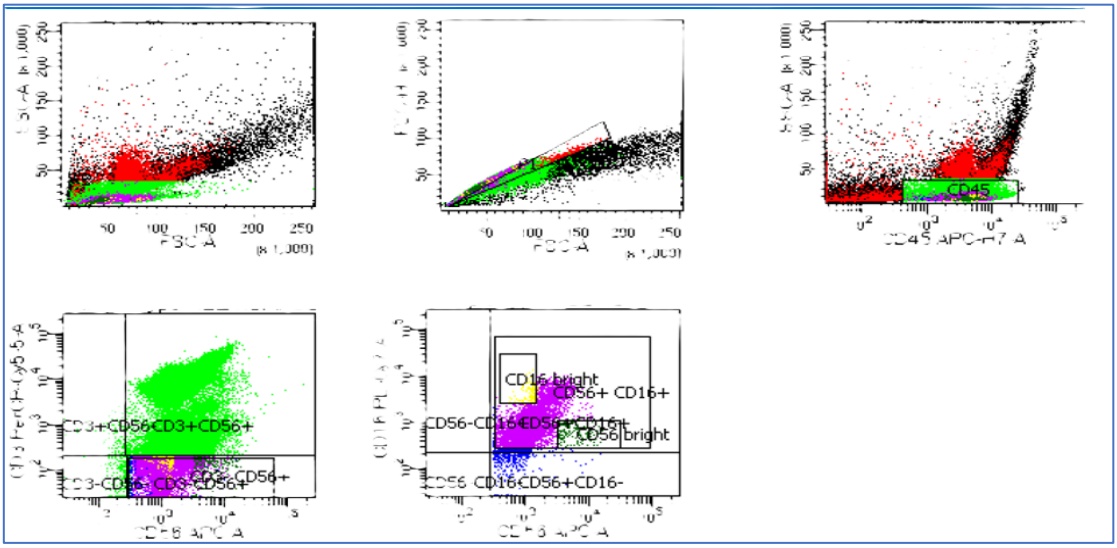

Figure 1: Dot plot for serial gating of flow cytometry analysis.

Statistical analysis

The statistical analysis was conducted using the software package SPSS, IBM Corp., Released 2021. IBM SPSS Statistics for Windows, Version 26.0. (IBM Corp., Armonk, NY). Demographic data were characterized using descriptive statistics, ANOVA test, Ratio, and Pearson correlation (r). Estimated p-values <0.05 were considered significant.

Results

This study was conducted on 65 patients with CML, 20 patients were newly diagnosed and before starting the treatment regimen, while the rest 45 patients were previously diagnosed and under treatment, 25 of them were treated with imatinib and the other were treated with nilotinib. All patients with the chronic phase of CML.

Along with those patients, 25 normal subjects were enrolled to act as a control group with matched age and gender. The age difference between the control group (39.2 ± 12.1 years) and the patients (35.6 ± 12.2 and 43.3 ± 13.9 years, respectively) is not statistically significant, according to Table 1. Additionally, this data indicates that the male-female ratio (MFR) in CML patients is 1.6:1. As a result, the control group's male-to-female ratio (1.5:1) is matched with that of the patients, with no discernible difference.

The study compared the percentage of total natural killer cells and their subsets in blood samples between control groups and patients with chronic myeloid leukemia (CML) including newly diagnosed and treated patients, in the control group n=25, the mean percentage of CD 56+CD 16 + natural killer cells was 84.5±5.2 %, this percentage was lower in newly diagnosed CML patients n=20, at 76.8 ±10.1 % but higher in treated CML patients n= 45 at 87.6 + - 6.4 p% with a significant P value <0.001. For the CD 56^{dim} subset, the control group had a mean percentage of 1.66 ±0.76% which increased significantly to 7.22±4.07% in newly diagnosed CML patients and decreased to 2.91 ±1.53% in treated patients, with a p-value < 0.001. The CD16 ^{dim} subset showed a mean percentage of 1.43 ±1.1% and control, which decreased to 0.72 ±0.39% newly diagnosed CML patients and increased to 1.58±0.82% in treated patients, also with the p-value <0.001. These results indicate a significant alteration in the natural killer subset between the control group and CML patients, with notable differences based on treatment status

Table 1: Demographic Comparison of Patient and Control Groups.

Character		Control (n=25)	CML Patients		P value
			New diagnosis (n=20)	Treated (n=45)	
Age (years)	Range	19 – 61	18 - 55	18 – 60	0.103
	M±SD	39.2 ± 12.1	35.6 ± 12.2	43.3 ± 13.9	
Sex (n, %)	Male	15 (60%)	40 (62%)		0.975
	Female	10 (40%)	25 (38%)		
	MFR	1.5	1.6		
M±SD: Mean ± standard deviation; MFR: male/female ratio					

Table 2: Percentage of Total Natural killer cells and their subsets in the blood sample of CML patients and control groups.

Parameters (%)		Controls N=25	CML patients		P value
			New diagnosis N=20	Treated N=45	
CD56+CD16+	Mean±SD*	84.5±5.2	76.8±10.1	87.6±6.4	<0.001
CD56 ^{dim} CD16^{bright}	Mean±SD*	1.66±0.76	7.22±4.07	2.91 ± 1.53	<0.001
CD56^{brigh} ^{t}CD16^{dim}	Mean±SD*	1.43 ± 1.1	0.72 ± 0.39	1.58 ± 0.92	<0.001
*Standard division					

The level of IL-21 in the control group and patients with CML, including newly diagnosed and treated patients. In the control group n= 25 the mean IL- 21 level was 26.2 ±1.5 pg/ml for CML patients, the mean IL- 21 level was slightly higher in newly diagnosed patients n=20 and 27.7 ± 1.2 pg/ml and was 26.6 ±1.3 pg/ml and treated patients n= 65 the P-value for this comparison was 0.540 indicating not statistically significant differences in IL- 21 level between the control group and either newly diagnosed or treated CML or patients.

The results in Table 4 showed the correlation between natural killer (NK) cells, their subsets, and IL-21 levels. The analysis revealed a p-value of 0.059 for the correlation between IL-21 and CD56+CD16+ NK cells, indicating that the relationship did not reach statistical significance at the 0.05 level. However, the correlation was noted to be significant at the 0.01 level for CD56b^{right} and CD16^{bright} subsets, suggesting a potential association between these NK cell subsets and IL-21 levels.

Table 3: Level of IL-21 in the CML patients' group and Control.

Parameters Pg/ml		Controls N=25	CML patients		P-value
			New diagnosis N=20	Treated N=45	
IL-21	Mean±SD*	26.2 ± 1.5	27.7 ± 1.2	26.6 ± 1.3	< 0.540
*Standard division					

Table 4: Correlation between NK cells and their subsets and IL-21

		CD56+ CD16+	CD56^{bright}	CD16^{bright}
IL-21	r	-0.219	-0.384**	0.585**
	p-value	0.059	0.001	0.001
r: Pearson Correlation **Correlation is significant at the 0.01 level.				

Discussion

CML is an aberrant pluripotent bone marrow stem cell that gives rise to clonal myeloproliferative hematological cancer. It is caused by a translocation reciprocal of t (9; 22) q (34; 11) that leads to the integration of ABL-1 gene sequences (9q 34) downstream of (BCR gene) sequences (22q 11) and is cytogenetically called tyrosine kinase that can activate numerous signal pathways, which cause a malignant alteration(2,12).

CML has an annual incidence of 1 to 2 cases per 100000 and constitutes 15% of adult leukemias. The typical age range for diagnosis is 45 – 55 years, and the condition is more common in males, with a male-to-female ratio of 3:2 (13)

NK cells, integral to the innate immune response, possess a strong and rapid cytotoxic capacity against physiologically stressed cells, such as malignant or virus-infected cells, without the necessity of prior activation or peptide antigen detection(14) In contrast to T-cells, NK cells initiate rapid anti-tumor responses that rely on signals from a variety of activation and inhibitory cell surface receptors. These receptors detect altered protein expression on target cells and modulate cytotoxic activity through the release of substances such as perforin and granzyme B (15,16). They can moreover lyse, which is used in the treatment of cancers (17). NK cells may also destroy tumor cells by producing and secreting cytokines and chemokines, among many other cytokines that are crucial for innate immunity and also affect the immunological response that follows(18,19).

NK cells are innate immunity's primary anti-tumor effectors. NKs exhibit inhibitory, limiting the immune response, or activating, causing the effector to operate (20). In this work, peripheral blood NK cells of CML patients and normal individuals have been investigated based on gating parameters forward scatter and side scatter (FSC; SSC) alongside the assessment of surface markers (CD45, CD56, CD3, and CD16), which allowed accurate identification of NK cells while excluding T-lymphocyte cells.

Since their discovery by Kärre *et al.* (21) The complexity of NK cells has been progressively elucidated over time. Initially recognized primarily for their cytotoxic functions and their ability to eliminate cancer cells and virus-infected cells without prior sensitization (22) NK cells are now known to have other functions, such as the ability to release cytokines. They can control the activity of several cell types, including immune cells, thanks to this cytokine synthesis(23). NK Activating and inhibitory receptors are the two primary classes into which cell receptors are usually divided. A fine balance between signals from these inhibitory and activating receptors is necessary for the start of cytotoxic reactions (24,25).

The results of this study demonstrated that the percentage of NK cells in newly diagnosed CML patients is significantly lower than in the control group and treated patients. However, from all NK cell population, a significant enlargement of the cytotoxic NK cells (CD16bright) subset was observed in newly diagnosed CML.

The higher expression found that the $CD56^{+bright}$ NK subset is significantly reduced in all patients with CML. Also, it has been suggested that the number of NK cells in patients with leukemia seems to gradually reduce as the disease progresses from the chronic phase to blast crisis, and their activity against tumor cells in different types of leukemia at an advanced stage of the disease shows reduced cytotoxicity(26). Although the two major subsets of NK cell surface markers CD^{56} and CD^{16} are describe in human cells, and produce different utilities in NK cells(27), Elevated number of cytotoxic NK cells ($CD16+^{bright}$) reported in this study couldn't recognize and attack the tumor cells in newly diagnosed patients with CML, to interpret this condition, NK cells in newly diagnosed CML patients may be lost their recognition capability or their cytotoxicity. Numerous studies have

demonstrated that natural killer cells may recognize tumor cells by their unique receptors or surface markers. Two main models are used in this research, causing them to become activated. According to the stress-inducing recognition paradigm, tumor cells and damaged proteins attach to NK cells' activating receptors to initiate their lethal action (28,29). NK cells additionally eradicate tumor cells directly by means of mechanisms such as ADCC, granzyme and perforin release, or death receptor-mediated apoptosis (30). Indirectly, NK cells help destroy tumor cells by releasing cytokines and chemokines that stimulate the growth and activation of other immune cells, including B cells, dendritic cells, monocytes, macrophages, and cytotoxic T lymphocytes (CTLs). Following their targeting of tumor cells, these immune cells cause necrosis or apoptosis to kill the cells(27,28,31). Additionally, Binelli *et al.,* (2021), because NK cells play a crucial role in immune responses and can target tumor cells, it has been observed that some patients have a profile of immature and less effective NK cells with reduced cytotoxicity, especially those who do not respond to first- and second-generation tyrosine kinase inhibitors (TKIs). More research is required to advise NK cell-based immunotherapy for hematological malignancies and develop strategies that enhance NK cell function in managing CML (32).

According to recent research, cytokines have a significant impact on the formation of tumors because they foster the growth and spread of cancerous cells. Because IL-21 seems to have a key role in controlling carcinogenesis, research has concentrated especially on this protein (33).

Conclusion

These results indicate significant alterations in NK cell subsets between control groups and CML patients, with notable differences based on treatment status. This implies that IL-21 may not play a critical role in the pathogenesis or treatment response of CML, at least in the context of this study. While there may be some interplay between specific NK cell subsets and IL-21, the overall correlation with CD56+CD16+ NK cells is not statistically significant. Further research is guaranteed to explore these relationships in greater detail and to understand the biological implications of these interactions.

Conflict of interest: NIL

Funding: NIL

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