Assessment of NKT Cells in CMV-Infected and Non-Infected Leukemic Children in Menoufia University Hospital

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Abstract: Natural killer T (NKT) cells constitute a unique subset among mature lymphocytes cells. As a part of the innate immune system, NKT cells play a critical early role in host defense in response to cytomegalovirus infection and leukemia. The potential importance of NKT cells in leukemic children was clear in this study. CMV infection has been associated with a number of hematological malignancies including leukemia. This may be due to the fact that patients suffering from haematopoietic malignancies are under immunosuppressive conditions induced by increased tumor cells and administration of chemotherapeutics. Although, NKT cell numbers significantly decreased in patients with leukemia, it remains unclear how NKT cells are involved in anti-tumor immunity because NKT cell numbers are extremely small.

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Key wards: CMV, NKT, leukemia

1. Introduction

The innate immune system plays a critical early role in host defense in response to viruses, bacteria, and tumor cells. It has been proven that a natural function of the immune system is to seek out and eradicate transformed cells [(1)]. Among innate lymphocytes, Natural Killer cells have an important number of effecter functions, including recognition and lyses of infected, stressed, or transformed cells and production of immuno-regulatory cytokines particularly IFN-δ.[(2)]. NKT (natural killer-like T) cells were originally described as a unique population of T cells with the co-expression of NK cell markers. [(3)]. It constitute a unique subset among mature lymphocytes represent an important cell lineage that do not require prior sensitization for effecter function and are vital both for initially combating infection and for subsequently activating the adaptive immune system by physical interaction with dendritic cells and by the secretion of immuno-regulatory cytokines[(4)].

Acute leukemias represent a clonal expansion and arrest at a specific stage of normal myeloid or lymphoid hematopoiesis. Leukemias are the most common malignant neoplasm in childhood, accounting for about 25-30% of all malignancies that occur in children younger than 15 years of age [(5)].

Children being treated for leukemia are rendered significantly immuno-compramised by their underlying cancer and through the use of chemotherapy. Infection with and reactivation of human cytomegalovirus (CMV), Epstein-Barr virus (EBV), and adenovirus (ADV) are frequent on those patients [(6)].

CMV infection is widespread in the human population, it is easily transmitted, usually through contact with body fluids, blood transfusion, venereal, oral, respiratory routes or by placental transfer [(7,8)]. Sero-prevalence rates vary by socio-economic class and geographic location, but the overall sero-prevalence is estimated to be in the range of 30–70%. This virus is a member of the *Herpesviridae* family which is icosahedral, double-stranded DNA virus. Cytomegalovirus reactivation and infection remains one of the major complications in the immuno-compromised patients as leukemic patients specially following allogeneic stem cell transplantation causing many critical infections as pneumonitis, hepatitis, and colitis[(9)]. NK cells mediate anti-viral protection, in particular against cytomegalovirus [(10,11)].

2. Methodology:

2.1. Patients

Two groups of 100 patients were included in this study:

Group1. It included 80 children, 31 females and 49 males, suffering from leukemia, aged from 2 - 13 years (5.1 ± 2.7). The patients of this study were from Pediatric department, Hematology/ oncology unit, Faculty of Medicine, Menoufia University

Group2. It included 20 apparently healthy children; 11 females and 9 males, aged from 3 -10 years (4.0 ± 2.1).

The Ethics Committee of Medical Research had approved the study and informed consents were obtained from the caregivers of each patient and healthy child before enrolment in the study.

2.2. Specimen collection:

From each child 8 ml blood was collected and it was divided as following:

- Two ml in an EDTA-contained sterile tube for complete blood counts
- Two ml in an EDTA-contained sterile tube for flow cytometric analysis. It freshly processed and analyzed within 24 hours
- Two ml in an EDTA-contained sterile tube and used for separation of peripheral mononuclear cells and stored in -20°C for PCR analysis to detect latent cytomegalovirus infection.
- Two ml in a plan sterile tube the allowed to coagulate then the serum transferred to another sterile tube and stored in -20°C for PCR analysis to detect active cytomegalovirus infection.
- Blood samples were collected form patient's group at diagnosis and before starting the treatment protocols
- The leukemic patients were diagnosed by CBC, BM examination and immunophenotype (by specific monoclonal panels to determine the type of leukemia)

2.3. Routine investigations for diagnosis of leukemia these included:

• Complete Blood Count (CBC):

CBC was measured by Pentra-80 automated blood cell counter (ABX-France).

• Blood films: stained with Leishman stain for differential leucocytic count and assessment of peripheral blood malignant cells.

• Bone marrow smears examination.

• Immunophenotyping of peripheral blood specimen done by BD immune cytometry systems, (San Jose, USA). The instrument set up was checked weekly using QC windows beads (Flow cytometry standard, San Juan, PR). Forward scatter and side scatter measurements were made using linear amplifiers, whereas fluorescence measurements were made with logarithmic amplifiers. Then flow cytometric two parameters dot plots and quadrant statistics were generated by cell quest software (Becton Dickinson immune-cytometry systems).

2.3. Nested PCR Analysis for CMV

Nested PCR has extremely high sensitivity because of the dual amplification process. The DNA product from the first round of amplification contains the hybridization sites for the second primer pair. The amplification by the second primer set, therefore, verifies the specificity of the first-round product [12]

Procedure of Nested PCR for CMV

The nested PCR was used for detection of CMV DNA in cell free serum samples, that indicates active disease as well as in PBMCs detect latent virus [12]**.** This involved four main steps: preparation of PBMCS, DNA extraction, amplification of CMV DNA, and detection of the amplified products.

1. Preparation of PBMCs: By Buffy coat method

Buffy coat is a leukocyte-enriched fraction of whole blood. Preparing a buffy-coat fraction from whole blood is simple and yields approximately 5–10 times more DNA than an equivalent volume of whole blood. Buffy coat was prepared by centrifuging whole blood at 2500 x *g* for 10 min at room temperature. After centrifugation, three different fractions were distinguishable: the upper clear layer was plasma; the intermediate layer is buffy coat, containing concentrated leukocytes; and the bottom layer contained concentrated erythrocytes [13].

2. DNA Extraction

CMV DNA was extracted by using QIAamp DNA Mini Kit **(Qiagen, Germany)**, according to spin column protocol [14]**.**

*** Principle of the procedure (QIAamp Spin column Procedure):**

Cell lysis is done by incubating the sample with special lyses buffer in the presence of protease. Subsequently; the lysed sample is applied to the QIAamp Spin Column where the nucleic acids bind specifically to the QIAamp silica-gel membrane during a brief centrifugation step. The lysate buffering conditions (salt and pH) are adjusted to allow optimal binding or adsorption of the nucleic acids to the QIAamp membrane whereas the protein and other contaminants, which can inhibit PCR, are not retained on the membrane. Then, DNA bound to the QIAamp membrane is washed in two centrifugation steps using two different wash buffers. Wash conditions ensure complete removal of any residual contaminants or impurities without affecting DNA binding. Finally, the purified DNA is eluted from the QIAamp Spin Column in concentrated form in elution buffer.

3. DNA Amplification:

A. Taq PCR Master Mix (Qiagen, Germany):

Taq PCR Master Mix is a premixed ready-to-use 2X solution containing Taq DNA polymerase (1.25 unit in the final 50 µl reaction volume), dNTPs, MgCl2 and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR.

B. Primers, (Qiagen, Germany):

- Four oligonucleotide primers were used:

The outer primers were consisted of the upstream primer

(1): (5′-AGAGTCTGCTCTCCTAGTGT-3′) and downstream primer

(2):(5′-CTATCTCAGACACTGGCTCA-3′);

The inner primers were consisted of the upstream primer
(3):(5′-CCACCCGTGGTGCCAGCTCC-3′) and downstream primer
(4):(5′-CCCGCTCCTCCTGAGCACCC-3′)
The primers were supplied as lyophilized agents to be reconstituted upon use (according to manufacture's instructions):
- The concentrated and working primer solutions were then stored in aliquots at -20°C to be subsequently used.

C. Components of final reaction mixture

50 µl reaction mixture, which contained:-

(a) For the first round: 10 mM Tris–HCl (pH 8.3), 2 mM MgCl2, dATP, dGTP, dCTP and dTTP (400 µM each), 1.25 U of Taq polymerase, 1 µM each primer and distilled water to complete the final volume.

(b) For the *nested* PCR, a reaction volume of 50 µl contained 5 µl of the previously amplified product from the first round used as a template, deoxynucleoside triphosphates at 200 µM each, 1 µM each of the inner primers and 1.25 U of Taq DNA polymerase and the reaction buffer

D. Cycling conditions

Amplification was carried out in a thermal cycler **(Biometra, Germany)**.

- **For the first round**, denaturation was by one 10 min cycle at 94 °C followed by 35 amplification cycles 2 min at 94 °C, 1 min 30 s at 65 °C and 1 min at 72 °C. This was followed by one cycle of 7 min 30 s at 72 °C.
- **For the second round** PCR, the 35 cycles were performed for 1 min at 94 °C, 30 s at 62 °C and 1 min at 72 °C

4. Detection of the amplification products:

The amplified PCR products were detected by agarose-gel electrophoresis

*** Procedure steps:**

1. The agarose gel (2%) was prepared by adding 2 gm agarose to 100 ml of 1X TAE buffer. The agarose was dissolved and boiled on heater for 4 minutes until the color become transparent.
2. The agarose solution was allowed to cool to 50°C, and then 5µl of ethidium bromide (its concentration is 10 mg /ml) were added to the gel for staining.
3. The gel was poured in an electrophoresis tray and allowed to be solid then the tray was flooded with 1X (TAE) buffer just enough to cover the gel to depth of about 1mm.
4. 10 µl of the amplification products were slowly loaded into the slits of the submerged gel using a micropipette. A DNA molecular weight marker was run in parallel.
5. The electrical leads were attached so that the DNA will migrate toward the anode. The voltage applied was 100 volts for about 2hour.
6. The electric current was turned off and the leads and lid were removed from the gel tank once the loading dye has migrated for an appropriate distance through the gel.
7. The DNA bands were visualized on UV trans-illuminator and photographed.

2.4. Flowcytometric analysis for NKT

Patients' peripheral blood samples were collected in heparinized green top vacationers (Becton Dickinson, San Jose, CA) containing anticoagulant (10 units/ml) and then were adjusted to a final concentration of 10^6/ml by PBS. To block Fc receptors, 34 ul of a 3 mg/ml solution of normal mouse IgG (Caltag /Burlingame, Ca) was added to different tubes and incubated on ice for 10 minutes. Samples were then liquated into different tubes of 100 ul each and stained monoclonal antibody antiCD3 (FITC) and antiCD56 (PE) (10 ul of each monoclonal antibodies). After gentle mixing, cells were incubated for 15 minutes on ice. Erythrocytes were lysed using three ml of pre-warmed lysing reagent into each tube, inverted twice and centrifuged at 1500 G for three minutes at room temperature. After discarding the supernatant and re-suspending the cells in residual buffer the cells were fixed in 200 ul of 2% ultra-pure formaldehyde [15]

- Data were acquired on a FACS caliber flowcytometer (BD immune cytometry systems, San Jose, CA).
- The instrument set up was checked weekly using QC windows beads (Flowcytometry standard, San Juan, PR).
- Forward scatter and side scatter measurements were made using linear amplifiers, where as fluorescence measurements were made with logarithmic amplifiers and flowcytometric two parameters dot plots and quadrant statistics were generated by cell quest software (Becton Dickinson immune-cytometry systems).
- Analysis was performed after manual gating around a lymphocyte population on a forward scatter versus side scatter dot-plot.
- Results were expressed as percentages of cells positive for CD3, CD56 or both markers.

Statistical Analysis

Results were collected, tabulated, statistically analyzed by IBM personal computer and statistical package SPSS version 11.

Two types of statistics were done:

Descriptive statistics: e.g. percentage (%), mean (x) and standard deviation (SD).

Analytic statistics:

- Chi-square test ($\chi 2$): was used to study association between two qualitative variables.
- Student t-test: is a test of significance used for comparison

3. Results

Patients diagnosed as having leukemia and then subdivided ***according to:***

- The type of leukemia either acute lymphoid leukemia (ALL), 71 patients or acute myeloid leukemia (AML) (9 patients)

The cytomegalovirus (CMV) infection status based on PCR results:- Patients with positive serum samples were considered to have active CMV infection. Patients with negative serum samples while having positive cellular samples (PCR analysis of peripheral mononuclear cells) were considered to have latent CMV disease. – Patients with negative serum and cellular PCR sample are considered not infected. So patients could be divided into patients with active CMV disease, patient with latent CMV disease and non infected patients.

Table (1) Age and sex distribution among the studied groups

	Groups				Test of significance	*P* value
	Patients (n = 80)		Control (n = 20)			
	No	%	No	%		
Sex • Male • Female	 49 31	 61.25 38.75	 12 8	 60.0 40.0	X^2 0.01	 >0.05 NS
Age / years Mean ± SD	5.1 ± 2.7		4.0 ± 2.1		t-test 1.7	>0.05 NS

Table (2): Comparison between patients and control groups as regards different clinical laboratory indices and NKT cells count.

Clinical lab. index	Patients (n = 80)		Control (n = 20)		t-test	*P* value
	Mean	±SD	Mean	±SD		
1- Hb (g/dl)	6.9	1.35	10.5	1.3	11.94	<0.001 HS
2- TLC ($x10^9$/l)	13.17	13.65	7.09	3.02	0.92	>0.05 NS
3- platelets ($X10^9$/l)	90	12	220	35	29.78	<0.001 HS
4-NKT ($X10^{4)}$	53.35	17.24	91.73	9.52	13.35	<0.001 HS

Table (3): Prevalence of CMV infection among the studied groups

CMV infection	Patients n = 80		Control n = 20		Test of significance	*P* value
	No	%	No	%		
1- active infection	17	21.25	0	0.0	6.83	<0.05 S
2- latent infection	13	16.25	2	10.00		
3- no infection	50	62.50	18	90.00		

Table (4): Level of TLC in different CMV infection status among patients and controls.

Groups	TLC ($x10^9$/l)	CMV infection state			Kruskal Wallis test	*P* value
		Active infection	Latent infection	No infection		
Patients	Mean ± SD	22.87 ± 22.85	11.57 ± 9.65	11.63 ± 11.29	3.4	<0.05 **S**
control	Mean ± SD	---------	6.96 ± 1.66	7.11 ± 2.13	0.013	>0.05 **NS**

Table (5): NKT cell count in different CMV infection status among patients and controls.

Group	NKT ($x10^4$/l)	CMV infection state			ANOVA test	*P* value
		Active infection	Latent infection	No infection		
Patients (n=80)	Mean ± SD	42.4 ± 12.47	69.38 ± 17.6	53.22 ± 16.52	6.46	< 0.01 HS
Control (n=20)	Mean ± SD	-------	93.5 ± 8.86	91.42 ± 9.86	0.116	> 0.05 NS

Table (6): NKT percent and absolute count among the studied groups.

Groups	NKT %		NKT absolute count ($x10^4$/l)		ANOVA test	*P* value
	Mean	±SD	Mean	±SD		
ALL (n=71)	0.048	0.017	51.90	16.15	50.5	<0.001 HS
AML(n=9)	0.063	0.035	64.77	22.08		
Control (n=20)	0.080	0.032	91.73	9.52		

Table (7): Pearson's correlation between NKT status and TLC among leukemic children.

Parameters	**TLC**	
	Correlation coefficient (r)	***P* value**
NKT	**- 0.35**	**<0.001 HS (-ve correlation)**

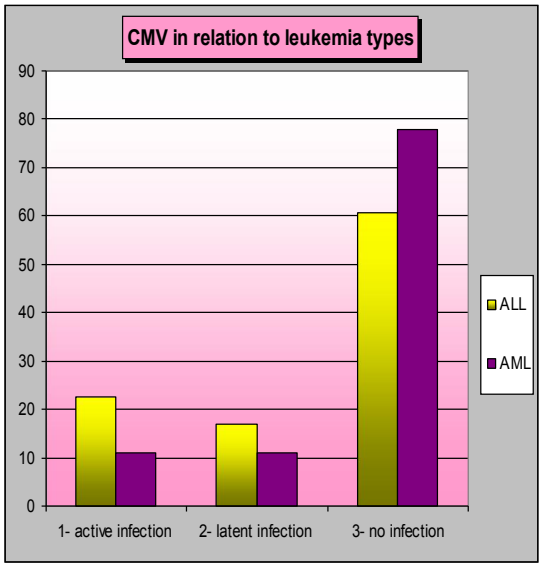

Figure (1): Correlation between CMV infection status and type of leukemia.

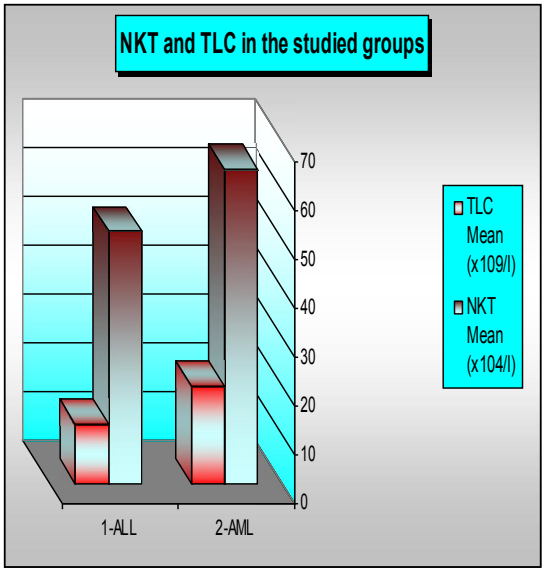

Figure (2): NKT and TLC in the studied groups

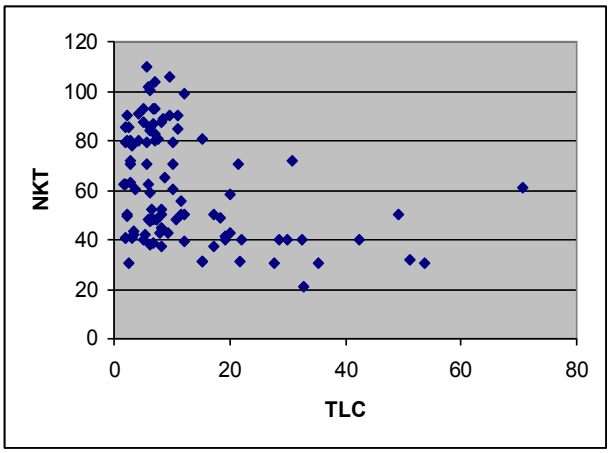

Figure (3): Correlation between TLC & NKT cell count.

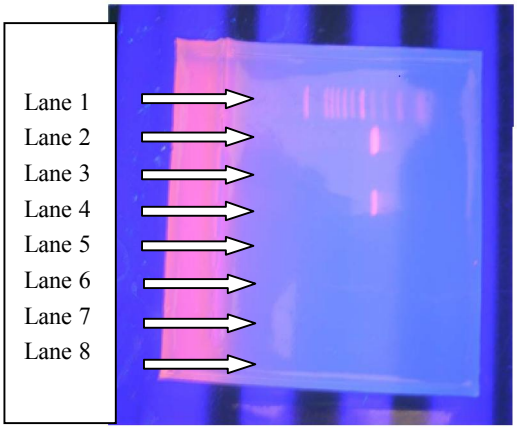

Figure (4): Results of positive PCR analysis of patients with CMV infections.

This is a picture of agarose gel electrophoresis of PCR product after amplification. Lane 1 shows the DNA marker ladder), lane 2 and lane 4 show positive case at the between 200 and 300 bp of the ladder) while other lanes show negative

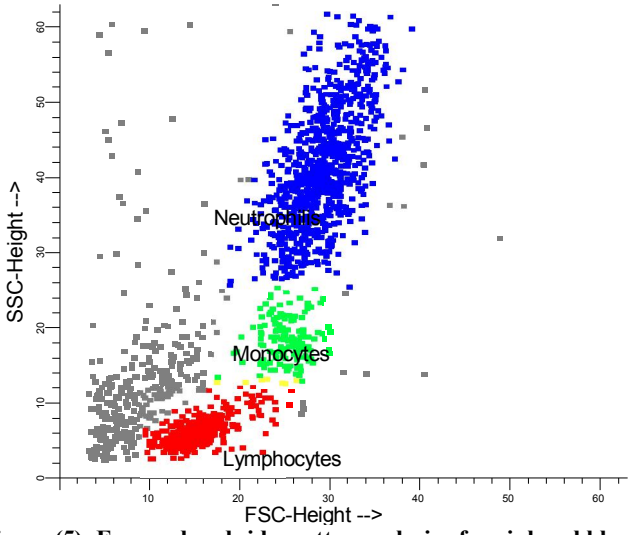

Figure (5): Forward and side scatter analysis of peripheral blood.

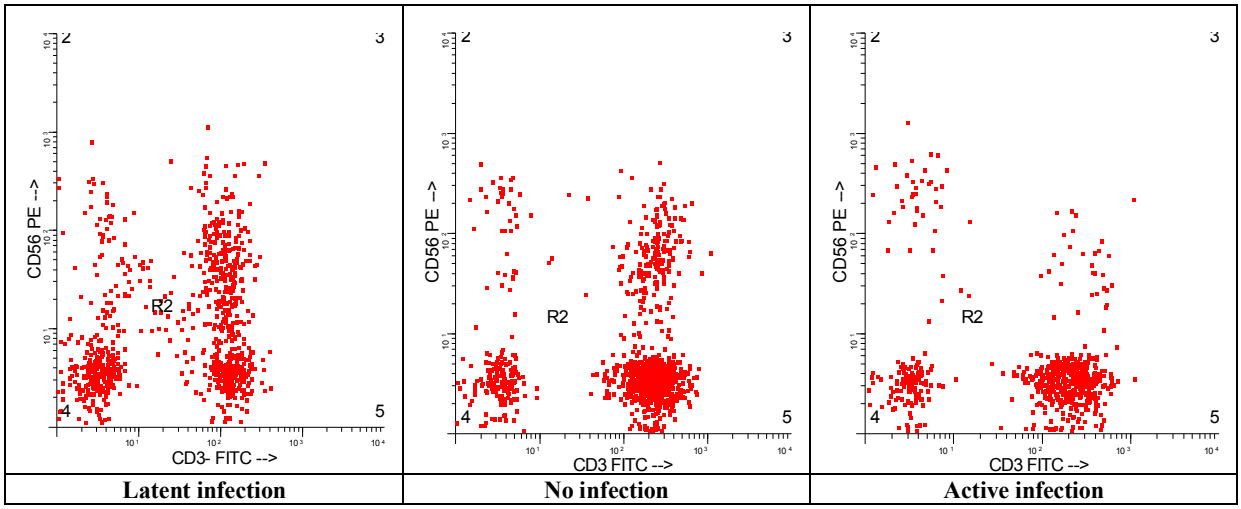

Figure (6): NKT cells among the studied group

4. Discussion:

There was statistical significant difference (*P* value < 0.001) regarding sex distribution of leukemia (both ALL and AML) being more common in males (ALL, 44 and AML, 5 cases) than females (ALL, 27 and AML, 4 cases). While, there was no statistical significant difference (*P* value > 0.05) between patient group and control group regarding the sex or the age.

On studying the CMV epidemiology (by serum and cellular PCR), it was found that active CMV infection was detected in 17 patients (21.25%), while there was 13 patients (16.25) with latent infection. On the other hand, the control group showed no active infection and only 2 had latent infections. The statistical analysis revealed that the difference in active infection is significant (*P* value < 0.05) while the difference in latent infection is not significant, this may be explained by the immune suppressed status in the leukemic patients that encourage conversion of latent infection into active one while, this did not occur in control group being healthy and immuno-competent. This result is matched with ***Mariana et al.,*** [16] **and Emery** [8]**,** They reported that CMV was higher in immuno-compromised patients including leukemic patients

George et al., [17] reported a high incidence of CMV infection in patients with hematologic malignancies especially in patients with severe neutropenia**.** Moreover, ***Kostareli et al.,*** [18] provided the molecular evidence for CMV persistence in patients with lymphocytic leukemia**.**

On statistical correlation between CMV infection and the type of leukemia (ALL or AML) the results revealed significant difference between ALL and AML (*P* value < 0.05), although there was no significant difference in the total leucocytic count (TLC) between the types of leukemia studied. However, the high standard deviation (Mean ± SD = 13.17 ± 13.65) in the TLC may explain this insignificance regarding the TLC count between the leukemia types .

This significant difference in CMV infection between types of leukemia (ALL or AML) in our study is not matching with ***George et al.,*** [17]

NKT level (absolute count and percentage) was significantly (*P* value < 0.001) decreased in both AML (Mean ± SD was 51.90±16.15) and ALL (Mean ± SD was 64.77±22.08) than the control group (Mean ± SD was 91.73±9.52). However the statistical difference between the two types of leukemia was not significant (*P* value > 0.05). These results suggest the importance of the NKT cell functions in patients with haematopoietic malignancy. This is in agreement with ***Ken-ichirou et al.,*** [15] who reported statistical significant decrease of the absolute numbers of NKT cells in the patients with hematologic malignancy, in comparison with normal healthy controls**.** Moreover, ***Madhav et al.*** [19] mentioned data which support the hypothesis that NKT effecter function may play a role in the control of malignant growth of hematological malignancy and suggested that measurement of NKT cell count and assessment of its function may be a useful predictor of clinical outcome in these patients**.**

Edward et al., [20] reported that NKT cells were found to play important role in leukemia eradication, in allogeneic hemopoietic stem cell transplantation (SCT) which is the definitive therapy for variety of hematological malignancies. Following allogeneic SCT, the beneficial immunological graft-versus-leukemia (GVL) occurs, although the definitive mechanism remains to be determined, NKT cells are believed to augment this reaction [20]

The result of NKT analysis of the present work is matched with what was reported in other types of malignancy, where ***Johan et al.,*** [21] studied circulating NKT cell levels in a 120 patients with various epithelial cancers (melanoma, breast-colorectal, renal cell-cancer and head and neck squamous cell carcinoma) in comparison with 69 healthy controls, after correction for the influence of both age and gender, cancer patients had a selective numeric NKT cell deficiency within the circulating cell pool (average 47% reduction compared to healthy controls; P value = 0.013, linear regression analysis) [21]**.** *Also,* ***Tahir et al.,*** [22] repotted that NKT had diminished IFN-γ production and a striking decrease in their IFN-γ:IL-4 ratio. The IFN-γ deficit was specific to the NKT cells, as T cells from prostate cancer patients produced normal levels of IFN-γ and IL-4. These findings support an immunoregulatory function of NK T cells in humans mediated by differential production of Th1 versus Th2 cytokines.[22]

Although some controversy exists not with the fact of NKT decrease in count but with regard to the capacity of the residual NKT cells of cancer patients to respond to immunotherapy [21].

Statistical correlation between NKT count in the peripheral circulation and CMV infection status revealed significant difference where NKT cell were significantly decreased in patient with active infection compared with those with latent or no infection (*P* value < 0.01) as well as when compared with control group.

Also, on statistical correlation between NKT count in the peripheral circulation and CMV with the type of leukemia, it was found that in patients with acute lymphocytic leukemia, the difference in NKT count was highly significant (*P* value < 0.001) between the patients and controls with latent CMV infection. Also, the difference was highly significant between the patient and controls without CMV infection. The same results were found in patients with acute myelocytic leukemia. Where, the difference in NKT count was highly significant (*P* value < 0.001) between the patient and controls with latent or no infection CMV infection.

These results indicate the role of NKT in CMV infection outcome and immune response. This result may be explained and also emphasize what mentioned above regarding the immune suppressive effect of CMV, ***Hal et al.,*** [23] reported that Infection with CMV impacts hematopoiesis, in the form of suppression of hematopoietic progenitor cell proliferation *in vitro* and myelo-suppression *in vivo*. But exactly how CMV mediates its effects is not clear [23]

Moreover, while efforts to learn how to control CMV infection are ongoing, it is important to better understand the cellular component of the immune system involved in these interactions in order to design better protocols to treat CMV infection and its consequences [24]**.**

Our results magnified the role of NKT cells in protection of progenitor cells from CMV-induced suppression and suggest that NKT cells may be of value in an adoptive transfer setting to treat CMV-induced suppression of hematopoiesis and immune response in immuno-compromised individuals, and emphasized that natural killer T cells would play a protective role in CMV infection, and that NKT cells would be useful for counteracting the hematopoietic progenitor cell–modulating effects of CMV infection and accelerates viral clearance

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