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ORIGINAL RESEARCH

Residual Bone Marrow T & NK-Cells at Diagnosis in Pediatric Pre-B-ALL: A Case–Control Study

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Background: Mature bone marrow T lymphocytes and NK may have a special relevance in the control of the malignant growth.

Objective: We aimed to assess the percentage of the residual BM T-cells, (T-helper –T-cytotoxic- NKT) and the NK cells of childhood precursor B-lymphoblastic leukemia (B-ALL) as an indicator of innate and adaptive immunity in these patients.

Subjects and Methods: This study was conducted on 40 B-ALL patients, and 40 apparently healthy matched children served as a control group. The flow cytometry was used to assess the percentage of the residual BM T-cells (T-helper, T-cytotoxic and NKT), and the NK cells.

Results: Compared with the control group, the percentage of the residual BM T-cells, its subtypes (T-helper, T-cytotoxic), and NKT cells in addition to the NK cells was significantly decreased in Group IA, and Group IB, but there was no significant difference between Group IA and Group IB in all studied parameters. In terms of the CD4/CD8 ratio, there was a significant increase in Group IA as compared to the control group ($P < 0.026$), but there were no significant statistical differences in CD4/CD8 ratio between Groups IB, and the control. Likewise, in CD4/CD8 ratio between groups IA, and Groups IB ($P > 0.05$). The percentage of NK, and NKT cells shows a significant increase in Hepatomegaly and Splenomegaly, as compared to non-Hepatomegaly and non-Splenomegaly patients of Groups IB ($P < 0.05$). However, there was a significant increase in statistical differences in the percentage of NKT cell between non-Splenomegaly, as compared to Splenomegaly patients of Group IA ($P < 0.05$). Additionally, there is a negative correlation between B.M Blast% to CD4/CD8 ratio and NK%, but there is no significant correlation between B.M Blast% to NK T% in the group 1 A.

Keywords: CD3, CD56, CD4, precursor B-lymphoblastic leukemia, B-ALL

Introduction

Globally, acute lymphoblastic leukemia (ALL) is the most frequent childhood cancer, accounting[11] for one-third of all childhood cancers in Egypt. In children, ALL therapy outcomes tend to improve over time.[8] The five-year survival rate is currently above 80%, and it is largely dependent on the risk factors measured at the time of diagnosis, even in the early stages of therapy.[18]

ALL, like other malignancies, appears to be caused by a combination of hereditary and environmental factors. The immune response has been shown to be critical in the detection and elimination of cancerous cells in the last decade.[14] T lymphocytes, together with NK cells, appear to be the immune cells that dominate the immunological response to leukemia. Multiple discrepancies in the relationship between NK and ALL have been reported in studies.[3]

Natural killer (NK) cells are categorized as Group 1 innate lymphoid cells because they grow from the hematopoietic stem cells and mature outside the bone marrow (BM) compartment.[20] They have the capability to migrate to several tissues to launch immune responses to infections and cancer.[2]

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TNK cells are a distinct type of T-cell, which are characterized by the combined expression of T cells and NK-cell markers. It expresses the surface CD3 and T-cell receptor (TCR) receptors. However, the TCR is of limited variability compared to the conventional T cells. It is either CD4, CD8, or the double-negative population; this heterogeneity is location-based, signifying the different functions of different organs.[6] Different subtypes secrete different lymphokines, bearing the NK markers, CD56, CD16, CD161, CD122, and CD38, and produces the NK characteristic cytokines, such as interleukin (IL)-4 and interferon (IFN)-α.[1]

Subjects and Methods

Study Design and Subjects

In this case–control research, 40 pediatric patients that suffered from the precursor B-cell lymphoblastic leukemia (B-ALL) were included and selected from Al-Zahraa hospital, El-Hussein hospital, Nasser Institute, and Ain Shams University Hospital during the period between March 2019 and October 2021. The subjects were divided into two categories: Group IA (n = 28 newly diagnostic B-ALL cases), and Group IB (n = 12 relapsed B-ALL cases). Forty apparently healthy individuals were enrolled as the control group.

Inclusion Criteria

Patients were fulfilling the 2016 World Health Organization (WHO) criteria of the pediatric precursor B lymphoblastic leukemia (B-ALL).

Exclusion Criteria

Children with any other malignancies, autoimmune disease, infection and allergies were excluded from this study.

Ethical Consideration

The study was conducted according to the guidelines of the World Medical Association Declaration of Helsinki for studies on human subjects. It was approved by the Institutional Review Board (IRB) of our university hospital (Al-Zahraa Hospital), and written informed consent was obtained from the children's guardians.

Procedures and Variable Assessments

The diagnosis of the B-ALL was based on the morphological examination of the peripheral smears, the bone marrow aspirates, the Immunophenotyping, cytogenetics, and the molecular genetics.

All involved participants were subjected to the following:

(I) A complete blood count (CBC): was performed with the automated hematology analyzer HORIBA YUMIZEN (France), and the chemical analyses (kidney and liver functions) were performed by the Cobas c311 clinical chemistry auto analyzer (Germany).

(II) Morphological examination of the peripheral blood smear and bone marrow aspiration.

(III) The ALL diagnosis was established using the multicolor flow cytometric analysis using the basic panel of acute leukemia including the following: CD34, CD13, CD33, CD14, CD117, CD10, CD19, CD22, CD2, CD3, CD5, CD7, CD4, CD8, MPO, TDT and HLA-DR. Samples were considered to be positive for a marker of ≥20% of the gated blast cells, except for the MPO and CD34 (positivity was considered ≥10%).

(IV) Molecular studies included the fluorescence in situ hybridization (FISH) t (1:19), t (9:22), t (12:21), t (4:11), and karyotyping.

(V) Flow cytometric analysis of the residual BM T Cells and the NK cells was performed at the time of diagnosis of the pediatric B-ALL patients. BM samples were diluted with the phosphate buffered saline (PBS) to a concentration of $10X10^9$/L, and then 50 μ of each dilution was mixed with the following Monoclonal antibodies; FITC-conjugated CD3, PE-conjugated CD4, and APC-conjugated CD56; the samples were incubated

for 20 mins in the dark at room temperature, then the samples were washed with the PBS, then incubated with lysing solution for 8 mins in the dark, then centrifuged, washed again, and suspended in the sheath fluid.

Flow cytometry was conducted in our university hospital using the four-color flow cytometry FACS Calibur (BD Biosciences, San Jose, USA). Cell Quest Pro software was used for the data analysis. Compensation parameters were established using the color calibrate beads (BD, Biosciences, San Jose, USA, and lot no. 8192516). After adjusting the sample count for acquisition (50,000 events), unstained samples were obtained to identify the sample auto-fluorescence.

Gating of T Cells, T Subtypes and the NK Cells

The percentages of the residual BM T Cells and the NK cells were identified using CD3 FITC, CD4 PE, and CD56 APC using the dot plot characteristics (Figure 1).

Statistical Analysis

The SPSS Version 23 was used to code and enter the data (Chicago, Illinois, USA). Moreover, the mean, median, standard deviation, minimum, and maximum values were used to represent quantitative data, while the frequency (count), and the relative frequency (%) were used to summarize categorical data. Non-parametric comparisons of the quantitative variables were made using the Mann–Whitney tests. To compare the categorical data, the chi-square ($\chi 2$) test was utilized. When the anticipated frequency is less than 5, the exact test is utilized instead. The correlations between the

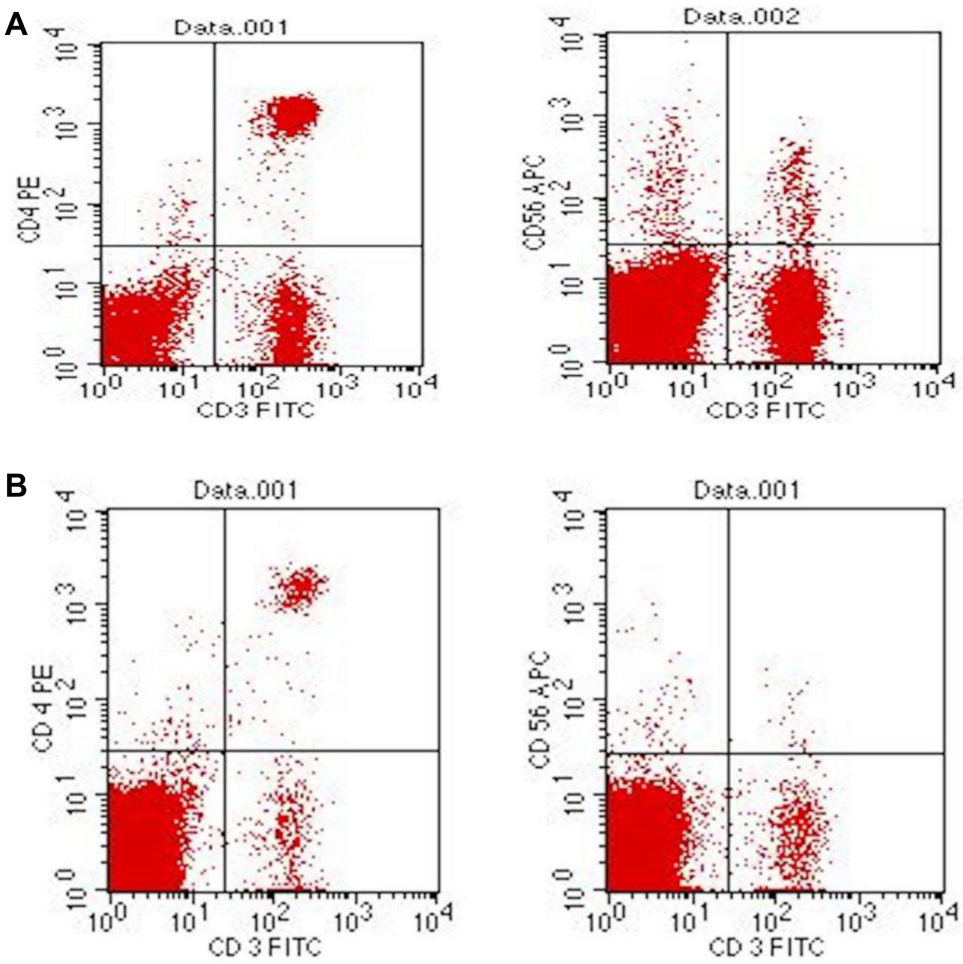

Figure 1 Representative dot plots demonstrating the percentage of bone marrow T-cells, T-helper, T-cytotoxic, NKT and NK cells in: (**A**) control, (**B**) Group IA (newly diagnosed B-ALL case), (**C**) Group IB (relapsed B-ALL case).

quantitative variables were determined using the Spearman correlation coefficient. P-values less than 0.05 were considered statistically significant, while 0.01 was considered as highly significant.

Results

Forty patients were enrolled, including 30 males and ten females (age 1–12 years, with a mean age of 6.90 ± 3.26 years). Patients were categorized into Group IA (n = 28, newly diagnosed B-ALL cases) and Group IB (n = 12, relapsed B-ALL cases). Table 1 summarizes the clinical characteristics of the cases examined. Control samples were 40 individuals, 30 males and 10 females, of age 2–12 years, with a mean age of 8.00 ± 2.70 years.

In Table 2, there was one positive case for BCR/ABL fusion gene, and 2 positive cases for the t (9:22) P190 gene in group IA, while we saw one positive case for BCR/ABL fusion, t (9:22) P190 genes and 2 positive cases for T (1:19) and for t (12:21) genes in group IB.

In Table 3, the percentage of the residual BM T cells, their subtypes (T-helper, T-cytotoxic, and NKT), and NK cells shows a highly significant decrease in Groups IA and IB as compared to that of the control group ($P < 0.001$), but there were no significant statistical differences between Groups IA and IB in all the studied parameters. Regarding the CD4/CD8 ratio, there were no significant statistical differences in the CD4/CD8 ratio between Groups IA and IB ($P > 0.05$). Alternatively, there was a significant increase in the CD4/CD8 ratio of Group IA as compared to that of the control group ($P < 0.026$) and no significant statistical differences in CD4/CD8 ratio between Groups IB and the control ($P > 0.05$).

In Table 4, there was no significant statistical difference in the percentage of NK, NKT cells, and CD4\CD8 ratio in hepatomegaly patients compared with that in non-hepatomegaly patients in Group IA ($P > 0.05$). The percentage of NK and NKT cells showed a significant increase in the hepatomegaly patients as compared to that in the non-hepatomegaly patients in Group IB ($P < 0.05$), but there was no significant statistical difference in the CD4\CD8 ratio between hepatomegaly patients and non-hepatomegaly patients in Group IB ($P > 0.05$).

From Table 5, a significant increase in statistical differences existed in the percentage of NKT cells between non-splenomegaly patients and splenomegaly patients in Group IA ($P < 0.05$), but there were no significant statistical differences in the percentage of NK cells and CD4\CD8 ratio between non-splenomegaly patients and splenomegaly patients in Group IA ($P > 0.05$). Also, the percentage of NK and NKT cells showed a significant increase in

Table 1 Clinical Characteristics of B-ALL Cases

		Group I A (n = 28, Newly Diagnosed B-ALL)		**Group I B (n = 12, Relapsed B-ALL)**	
		Count	**%**	**Count**	**%**
Age (years) Mean ± standard deviation (SD)		7.14 ± 3.37		6.33 ± 3.06	
Range		2–12		1–11	
Gender	M	18	65.5	12	100%
	F	10	34.5	0	0%
Fever	P	15	51.7%	6	50.0%
Lymphadenopathy	P	15	51.7%	3	25.0%
Hepatomegaly	P	13	44.8%	3	25.0%
Splenomegaly	P	13	44.8%	4	33.3%
Bone ache	P	13	44.8%	3	25.0%
CNS infiltration	P	1	3.4%	1	8.3%

Table 2 Descriptive Cytogenetics and Molecular Study of the Patient Groups

	Newly Diagnosed B-ALL Group IA (n = 28)	Relapsed B-ALL Group IB (n = 12)
BCR/ABL fusion gene		
Negative	27 (96.4%)	11 (91.7%)
Positive	1 (3.6%)	1 (8.3%)
MLL rearrangement		
Negative	28 (100.0%)	12 (100.0%)
T (1:19)		
Negative	28 (100.0%)	10 (83.3%)
Positive	0 (0.0%)	2 (16.7%)
t (12:21)		
Negative	28 (100.0%)	10 (83.3%)
Positive	0 (0.0%)	2 (16.7%)
t (4:11)		
Negative	28 (100.0%)	12 (100.0%)
t (9:22) P190		
Negative	26 (92.9%)	11 (91.7%)
Positive	2 (7.1%)	1 (8.3%)

splenomegaly patients as compared to that in non-splenomegaly patients in Group IB ($P < 0.05$), but there was no significant statistical difference in the CD4\CD8 ratio between splenomegaly patients and non-splenomegaly patients in Group IB ($P > 0.05$).

From Table 6, there was a negative correlation between B.M Blast% to CD4/CD8 ratio and NK%, but no significant correlation existed between the B.M Blast% and NKT% in Group 1A. There was no significant correlation between the B.M Blast% to CD4/CD8 ratio and NK%, and NKT% in Group 1B.

The BM blast% of the newly diagnosed B-ALL Group IA mean was 90.29 ± 6.31, with a range of 80–98, while in the relapsed B-ALL Group IB, the mean was 86.25 ± 19.46, with a range of 31–99.

No significant correlation existed between the percentage of NK–NKT cells in patients with leukocyte counts of >20,000/mm^3 or with lower counts. WBCs mean was 60.37 ± 79.13 and its Range 1.21–302 in Group 1A, while in Group 1B the Mean was 45.61 ± 98.22 and the Range 1.44–348.28.

Discussion

The global incidence of childhood cancer is estimated at 152.8 million people annually in children aged 0–19 years, with a slightly higher incidence in children aged 0–4 and 15–19 years.[21] Childhood leukemia accounts for 30% of all childhood cancer cases, with ALL accounting for 80% and acute myeloid leukemia for 15–20% of the cases.[17] There are only a few cases of chronic myeloid leukemia in children.[15] Mature BMT lymphocytes and NK cells may play

Table 3 Comparative Study Between the Percentage of T-, T-Helper, T-Cytotoxic, CD4/CD8 Ratio, NKT, and NK Cells in the Newly Diagnosed, Relapsed, and Control Groups

		Newly Diagnosed B-ALL Group IA (n = 28)	Relapsed B-ALL Group IB (n = 12)	Control Group (n = 40)	P1	P2	P3
The T-cells %	Mean ± SD	3.53 ± 4.23	3.19 ± 4.73	31.46 ± 1.19	0.352	<0.001**	<0.001**
	Range	0–13.81	0.01–13.5	28–33			
T-helper cell %	Mean ± SD	1.97 ± 2.06	1.46 ± 2.09	15.53 ± 2.96	0.170	<0.001**	<0.001**
	Range	0–6	0–5.52	10.5–22			
T-cytotoxic%	Mean ± SD	1.91 ± 2.17	1.75 ± 2.71	17.70 ± 2.99	0.225	<0.001**	<0.001**
	Range	0.02–7.81	0.02–8.08	10–22			
CD4/CD8 ratio	Mean ± SD	1.22 ± 0.78	1.22 ± 1.05	0.89 ± 0.19	0.497	0.026*	0.957
	Range	0–4	0–3.46	0.6–1.45			
NK%	Mean ± SD	0.40 ± 0.52	0.11 ± 0.15	4.50 ± 0.86	0.074	<0.001**	<0.001**
	Range	0.01–1.79	0.01–0.56	2.8–6			
NK T%	Mean ± SD	0.35 ± 0.62	0.20 ± 0.34	1.30 ± 0.64	0.584	<0.001**	<0.001**
	Range	0–2.92	0–0.98	0.4–2.6			

Notes: Using: z-Mann–Whitney test; $P > 0.05$ NS; $*P < 0.05$ S; $**P < 0.001$ HS. P1 = *P*-value between Groups IA and IB; P2 = *P*-value between Groups IA and control; P3 = *P*-value between Groups IB and control.

Table 4 Comparative Study Between NK%, NKT%, and CD4/CD8 Ratio Between Hepatomegaly and Non-Hepatomegaly Patients in Both Groups

Newly Diagnosed B-ALL Group IA (n = 28)		Non-Hepatomegaly	Hepatomegaly	*U*-test	p-value
NK%	Mean ± SD	0.39±0.52	0.42±0.55	−0.256	0.798
	Range	0.01–1.51	0.01–1.79		
NKT%	Mean ± SD	0.50±0.77	0.16±0.27	−1.540	0.124
	Range	0.01–2.92	0–0.94		
CD4/CD8 ratio	Mean±SD	1.40±0.90	0.98±0.53	−1.022	0.307
	Range	0.61–4	0–2		
Relapsed B-ALL Group IB (n=12)					
NK%	Mean ± SD	0.05±0.05	0.28±0.24	−2.138	0.033*
	Range	0.01–0.18	0.14–0.56		
NKT%	Mean±SD	0.06±0.07	0.64±0.48	−2.130	0.033*
	Range	0–0.22	0.09–0.98		
CD4/CD8 ratio	Mean ± SD	1.38±1.17	0.76±0.36	−0.647	0.518
	Range	0–3.46	0.42–1.14		

Note: $P > 0.05$ not significant (NS) $*P < 0.05$ significant (S).

Table 5 Comparative Study Between NK%, NKT%, and CD4/CD8 Ratio in Splenomegaly and Non-Splenomegaly Patients in Both Groups

Newly Diagnosed B-ALL Group IA (n = 28)		**Non-Splenomegaly**	**Splenomegaly**	**U-test**	**p-value**
NK%	Mean ± SD	0.58±0.61	0.20±0.30	−1.730	0.084
	Range	0.02–1.79	0.01–1		
NKT%	Mean ± SD	0.54±0.77	0.14±0.30	−2.500	0.012*
	Range	0.01–2.92	0–1.09		
CD4/CD8 ratio	Mean ± SD	1.28±0.84	1.14±0.72	−0.138	0.890
	Range	0.61–4	0–2.88		
Relapsed B-ALL Group IB (n=12)					
NK%	Mean ± SD	0.05±0.06	0.23±0.23	−2.220	0.026*
	Range	0.01–0.18	0.06–0.56		
NKT%	Mean ± SD	0.06±0.07	0.50±0.49	−2.042	0.041*
	Range	0–0.22	0.06–0.98		
CD4/CD8 ratio	Mean ± SD	1.48±1.21	0.72±0.31	−1.019	0.308
	Range	0–3.46	0.42–1.14		

Note: *P* > 0.05 NS; **P* < 0.05 S.

Table 6 Correlation Between B.M BlasT% to CD4/CD8 Ratio, NK%, and NK T%

B.M Blast%	**CD4/CD8 Ratio**		**NK%**		**NK T%**	
	Rs	**P – value**	**Rs**	**P - value**	**Rs**	**P - value**
Newly diagnosed B-ALL Group IA (n = 28)	−0.412*	0.030	−0.416*	0.028	−0.171	0.383
Relapsed B-ALL Group IB (n = 12)	−0.046	0.887	0.887	0.126	−0.389	0.212

Note: Using: Spearman's rank correlation coefficient *P* > 0.05 NS; **P*-value.

a special role in the control of malignant growth. In acute leukemia, immune cells in the BM are in direct proximity to the disease as it evolves. Moreover, T cells circulating in the peripheral blood may be recruited into the leukemic BM.[4]

This study indicated that the percentage of the residual BM T-cells, their subtypes (T-helper, T-cytotoxic, and NKT), and NK cells of the patients showed a highly significant decrease in Group IA and IB compared with that of the control group (P < 0.001), but there was no significant difference between Groups IA and IB in all studied parameters.

Valenzuela-Vazquez et al discovered that patients with ALL reported a reduction in the percentage of NK cells in the peripheral blood of patients upon diagnosis and the impaired cytotoxicity in patients with B- and T-ALL. Furthermore, in B-ALL patients with leukocyte counts of >50,000/mm3, NK cell-mediated cytotoxicity was discovered to be impaired compared with those with lower counts. These data suggest that the abnormal effector function of the NK cells is not equally observed in all pediatric patients with B-ALL and that there are other contributing factors to the NK cell-mediated cytotoxicity.[22]

Duault et al discovered that NK-cell activity in B- and T-ALL patients significantly decreased in both the BM and peripheral blood of the B-ALL. NK cells were shown to have significantly reduced cytotoxic capabilities than those from

healthy donors when killing assays were employed with the ALL-sensitive leukemia cells, indicating an immunosuppressed phenotype.[5]

Ismail et al discovered that children at the time of their ALL diagnosis have higher proportions and absolute cell numbers of the bulk CD4+ and CD8+ T-cells in their peripheral blood.[9,16]

Regarding the CD4/CD8 ratio, among the residual lymphocytes, we found a significant increase in the newly diagnosed group than in the control group ($P < 0.026$). There were no significant statistical differences in the CD4/CD8 ratio between Groups IB and the control. There were no significant statistical differences in the CD4/CD8 ratio between Group IA and IB ($P > 0.05$). CD4+ effector T-cells have previously been attributed to play an active role in antitumor immunity, including ALL.[7] Lymphocytes may actively participate in local immunosuppression within the BM niche as part of the protective immune responses. The accumulation of T-cells with a regulatory phenotype and suppressive function is one of the best-defined mechanisms of local tumor immune evasion.[13]

Lustfeld et al investigated the phenotype of (nonmalignant) T-cells present in the BM at diagnosis in 39 children with ALL. Data showed a higher variable CD4:CD8, with a median of 1.22, slightly above that reported for normal BM.[12]

Herein, it was observed that the percentage of NK and NKT cells significantly increased in splenomegaly and hepatomegaly patients than in the non-splenomegaly and non-hepatomegaly relapsed B-ALL Group IB. Therefore, we suggest that splenic enlargement may reflect an immunological process contributing to the control of leukemia. There was a significant increase in the statistical difference in the percentage of NKT cells between non-splenomegaly than splenomegaly in newly diagnosed B-ALL Group IA ($P < 0.05$). Chemotherapeutics against leukemia have been shown to reduce the number and activity of NK cells during treatment. However, upon treatment discontinuation, normal NK-cell levels may slowly recover, even if their cytotoxicity remains unchanged.[10]

There was a negative correlation between BM Blast% to CD4/CD8 ratio and NK%, but there was no correlation between BM Blast% and NKT% in Group 1A.

There was no significant correlation between the percentage of NK–NKT cells in patients with leukocyte counts of >20,000/mm3 or with lower counts. The leukocyte count is considered a highly significant prognostic variable. Patients with counts higher than 10,000/μL have a worse prognosis. The cutoff at which a good prognosis is defined in children varies in different centers, but leukocyte counts higher than 50,000/μL are clearly associated with a poor outcome. This value has been proposed by a National Cancer Institute (NCI)-sponsored workshop as the value with which to identify pediatric patients with a poor prognosis.[19]

Conclusion

In this study, it was observed that the percentage of the residual BM T-cells, their subtypes (T-helper, T-cytotoxic, NKT), and NK cells demonstrated a significantly significant drop in B-ALL patients, indicating that children's immunity was lowered during this illness.

Disclosure

The authors report no conflicts of interest in this work.

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