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RESEARCH ARTICLE

Investigation of Treg in Pediatric Acute Lymphocytic Leukemia Patients during Chemotherapy Stages and Relapse

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ABSTRACT

A cross-sectional case-control study has been carried out on Treg cells in pediatric acute lymphoblastic leukemia (ALL) patients admitted to Al-Basrah Children Teaching Specialty Hospital from November 2022 to May 2023. A total number of 70 patients (25 newly diagnosed, 12 relapse, 21 during induction, and 12 during consolidation chemotherapy) were enrolled, aged 2 to 14 years, along with 54 healthy controls who were the same age and gender as the study. Blood samples were collected from all participants for flow cytometer applied to study Treg cell markers. The results of the current study showed that the highest percentage of ALL patients was in the age group of 2 to 5 years 54.3% followed by age group 6 to 12 years 41.4%, whereas the lowest percentage was in patients older than 12 year 4.3% (*p-value* 0.802). The *p-value* was considered significant if it is ≤ 0.05 and highly significant if ≤ 0.001. Regarding the flow cytometry analysis results for CD3, CD4 and CD25, ALL patients had a significantly higher mean of these markers than the control group (*p-value* ≤ 0.005). A slightly higher level CD4 was noted in new cases compared to relapse cases, and induction and consolidation (p = 0.080). The results of Treg levels cells in relapse, induction, consolidation and new diagnosis cases. On the other hand, demonstrate higher levels in the relapse group, with highly significant differences for the two parameters: CD3–CD25 (p ≤ 0.005). The level of CD25 displayed a highly significant difference when comparing induction with consolidation (p ≤ 0.005). From this study, we conclude that the immunological marker (CD 25) specifically provided the most highly significant value as immunosuppression parameters in all patients, whereas chemotherapy represents the key risk factor for immunosuppression in all patients varies significantly among chemotherapy stages, with consolidation having the most impact. This effect may be due to the high dose of MTX.

Background: T regulatory cells (Tregs) are immunosuppressive cells that can be divided into numerous subsets. Tregs comprise a small but heterogeneous population, which the phenotype may identify as CD3+CD4+CD25+. They play a crucial role in the preservation

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of immunological homeostasis and self-tolerance. They also play important roles In the control of cancer immunity. Tregs may also be important in acute leukemia.

Patients and Methods: A7 0 blood samples of both sexes with the age range 2 to 14 years old were collected from patients with ALL. Additionally, this investigation included 54 healthy controls included in CD3, CD4 and CD25 expression on leukemic blast cells were assessed using flow cytometry.

Results: Increased values of CD3+CD25+ T cells were observed in children with all in comparison to healthy controls with significant differences in the markers for Tregs (mean ± SD, 14.971 ± 11.06 *vs.* 5.680 ± 2.96 pg/mL) ($p < 0.05$).

Following these findings, significant differences in the levels of CD25 was higher in consolidation than in the induction chemotherapy stage (mean ± SD, 17.657 ± 13.890 *vs.* 5.100 ± 5.1438) pg/mL ($p < 0.05$), children with all were also found to have significantly higher levels of CD4 in the current study when compared to healthy controls (mean ± SD, 29.261 ± 13.828 *vs.* 28.3465 ± 11.17146) pg/mL ($p < 0.05$), Table 2.

According to the chemotherapy stage there were significant significant differences in the levels of CD25 in the relapse state, which was higher than the chemotherapy stage for new diagnosis (mean ± SD, 22.185 ± 15.148 *vs.* 13.649 ± 5. 83) pg/mL ($p < 0.05$). A further finding was that the frequency of lymphoblast that expresses CD25 was considerably higher in the high-risk group compared to the standard risk group (mean ± SD, 15.75 ± 4.74 *vs.* 7.92 ± 1.012) pg/mL ($p < 0.05$) Table 3.

Conclusion: Chemotherapy represents the interested immunosuppression risk factor in ALL patients. There is a significant variation among chemotherapy stages in ALL patient immunosuppression. Consolidation represent the high influence on ALL patient in immune suppression may be related to the high dose of MTX.

Keywords: Treg, Acute lymphoblastic leukemia, relapse.

INTRODUCTION

Leukemia is a diverse category of hematologic cancers that emerge when leukocytes in the process of growing proliferate abnormally. It is divided into two categories: acute and chronic, as well as myelocytic and lymphocytic.[1] Acute lymphoblastic leukemia (ALL) is a moderately frequent clinical malignancy of the hematological system characterized by an aberrant clone of bone marrow lymphoid group cells. The aberrant growth of disease lymphocytes is not functional, and the invasion of malignant cells prevents the normal hematopoietic function of the bone marrow, which results in the accompanying symptoms (Coccaro *et al*., 2019).[2] A healthy immune system must be able to tolerate itself while producing powerful reactions to outside antigens. Tregs are crucial players in this type of immunological control.[18] Treg cells, which can suppress the immune response in many unique ways, include thymic Treg cells and peripheral Treg cells. Many energy metabolism processes influence Treg cell differentiation, growth, suppressive action, and survival.[19] Tregs use a variety of mechanisms to carry out their immunosuppressive activity. The first suppressive mechanism is associated with cytokines and involves Tregs that have high levels of CD25 expression expending IL-2, and cytokine inhibition (such as TGF-, IL-10, and IL-35).[3] Recent years have seen the recognition of exhaustion as a mechanism of T-cell malfunction in cancer.[4,5] It's significant to note that the development of tumor resistance to checkpoint blockade therapy has been linked to the overexpression of exhaustion-demarcating immunological checkpoints by T-cells.[6]

Patients and samples

Blood samples were collected from 70 patients in three groups of children who were hospitalized between July 2022 and December 2023, as follows:

Group 1 (G1): Included 25 samples of newly diagnosed children with ALL before initiating therapy (chemotherapy or steroid).
Group 2 (G2): Included 21 samples of patients with ALL during induction chemotherapy
Group 3 (G3): Included 11 samples of patients with ALL during consolidation chemotherapy
Group 4 (G4): A total of 12 patients who were relapsed after achieving complete remission. The current study included 54 healthy boys and girls aged 2 to 14 years and served as a contract study.

MATERIALS AND METHODS

A sample of 2 mL blood from each patient was collected by vein puncture and put in EDTA tube for flow cytometer. For CD25 and CD4 and CD3 analysis, three steps were used in the processing/lyse/wash technique. Briefly :

- First step (processing): One in a single tube, 5 µL of the CD25-APC and 5 µL of CD4 -FITC, and 5 µL of CD3 per CP were added to 100 µL of ethylene diaminetetraacetic acid (EDTA) whole blood in a 12X 75-mm tube.
- Vortex gently and incubate for 15 to 30 minutes in the dark at room temperature.
- Added 2 mL of 1x BD FACS lysing solution mixed, and left for 15 minutes in the dark, centrifuge at 300 g for 5 minutes; remove the supernatant.
- Vortex gently and incubated for 10 minutes in dark at room temperature.
- Centrifuge at 300 g for 5 minutes. Remove the supernatant.
- Added 2 to 3 mL of BD cell WASH solution and centrifuge at 200 g for 5 minutes to remove the supernatant.

Tow step (lauded) three step then Data analysis was performed using FlowJo X 10.0.7 software (FlowJo, Ashland, OR, USA) and analyzed using a flow cytometer (FACSCanto flow cytometer with CellQuest software; Becton Dickinson).

RESULTS

Age and Sex Distribution of Study Groups

The studied group was divided into ages 2 to 5 years, 6 to 12 years, and < 12 years. No significant difference exists between cases and the control group (p = 0.802). Also, no significant difference is noticed between the two groups in regard to sex distribution (p = 0.041) Table 1.

Table 1: General characteristics of the study groups

Character	*Patient*	*Control*	*p-value*
Age	*No(%)*	*No(%)*	
2-5	54.3%	53.7%	0.802
6-12	41.4%	44.4%	
>12	4.3%	1.9%	
Total	100.0%	100.0%	
Sex	*Patient*	*Control*	*p-value*
	No(%)	*No(%)*	
Male	62.9%	44.4%	0.041
Female	37.1%	55.6%	
Total	100.0%	100.0%	

Chi-square p ≤ 0.05

Flow Cytometer Study

Increased value of CD3+CD25+ T cells were observed in children with ALL in comparison to healthy controls with significant differences in Tregs markers (mean ± SD, 14.971 ± 11.06 *vs.* 5.680 ± 2.96 pg/mL (p <0.05)

Following these findings, significant differences in the levels of CD25 was higher in consolidation than induction chemotherapy stage (mean ± SD, 17.657 ± 13.890 *vs.* 5.100 ± 5.1438) pg/mL (p <0.05), in the present study were observed level of CD4 significantly increased in children with ALL in comparison to healthy controls (mean ± SD, 29.261 ± 13.828 *vs.* 28.3465 ± 11.17146) pg/mL (p <0.05), Table 2 in the relapse state, there was significant differences in the levels of CD25 which was higher than new diagnosis chemotherapy stage (mean ± SD, 22.185 ± 15.148 *vs.* 13.649 ± 5. 83) pg/mL (p <0.05). Another observation was that the frequency of CD25 expressing lymphoblasts was found to be significantly higher in high-risk than in standard risk group (mean ± SD, 15.75 ± 4.74 *vs.* 7.92 ± 1.012) pg/mL (p <0.05) Table 3.

Statistical Analysis

Statistical Package of Social Science (SPSS) version 26 was used to analyze the data. Qualitative data were expressed as numbers and percentages, while quantitative data were expressed as mean ± standard deviation and median with minimum and maximum values. Kolmogorov Smirnoff and Shapiro-Will tests were used to assess the normality of quantitative data distribution. The chi-square test was used to investigate associations between two or more qualitative variables. Mann Whitney U and Kruskal Wallis tests were used to investigate differences between quantitative data. A statistical relationship with *p-value* less than 0.05 was considered as significant.

DISCUSSION

Strong immunological suppressive properties of regulatory T cells prevent the antitumor immune response in tumor-bearing hosts. An unfavorable prognosis is associated with high Treg cell infiltration in the TME in patients with various forms of cancer.[8]

Some research looked at whether patients with ALL, particularly B-ALL, have different levels of Treg cells in their peripheral blood mononuclear cells than do healthy controls (Idris *et al*., 2015; Bhattacharya *et al*., 2014; Liu *et al*.,2018 and Wu *et al*., 2010; Alhosseini *et al*., 2023)

In line with Liu *et al*.'s (2018) findings, the current study found that the mean CD25 level was higher in B-ALL patients (14.971 pg/mL) compared to healthy controls (5.680 pg/mL). The results of the current investigation, which other researchers supported (Owaidah *et al*., 2008; Zahran *et al.,* 2021) showed that the frequency of CD25-expressing lymphoblasts was significantly greater in the high-risk group than in the standard-risk group. The mean high-risk 14.98 pg/mL was higher than the standard risk group›s mean (7.9243).

In our study, the new diagnosis cases were found to have significantly high levels of CD25+ cells, with a mean level of CD25 (13.649) pg/mL. This finding is agreed with Wu *et al*. (2011), Idris *et al*. (2015), and Salem *et al.* (2018).

Yang and Xu, showed that When cancer is diagnosed, the number of Tregs present in blood and cancer tissue is a subliminal indicator of the illness's severity and aggressiveness (Yang and Xu, 2013).

According to our findings, significant differences in the levels of CD25 were found to be higher in induction than consolidation. The mean levels of CD25 in induction were 5.100 pg/mL and in consolation were 17.65 pg/mL, both with *p-value* ≤0.05 (Figures 1 and 2).

Salem *et al*. observed CD25 decreases and increases, respectively, therefore employed T regas as a biomarker to assess the prognosis for AL, predict chemotherapy sensitivity, and participate in the immune response to infection (Salem *et al*.,2018)

In addition, we observed a significant decrease with the occurrence of induction, and this result was agreed with (Niedźwiecki *et al*.,2019; Wu *et al*., 2010; Aref *et al*., 2020). We showed the increased value of regulatory T cells in pediatric ALL patients in the consolidation stage.so there was a conflict of T reg value according to chemotherapy type and stage. This could be impairing the cellular immune response against leukemia. and this result was agreed with (Rosales *et al*., 2020).

Methotrexate (MTX) is used widely as a chemotherapy treatment for malignant tumors. High-dose methotrexate (HD-MTX) is systemic consolidation in children with acute lymphoblastic leukemia (ALL) an effective treatment for extramedullary infiltration and, it can increase the long-term survival rate in children (Sajith *et al*., 2020). The dose, duration, genetic susceptibility, and risk factors all play a role in MTX-related toxicity (Mandal *et al*., 2020). MTX is a

Table 2: Level of (CD3-CD4-CD25) in patient, control, new, relapse, induction and consolidation groups

Marker	*Control (n=54)*				*Patient (n=70)*				*p-value*
	Mean	*SD*	*Minimum*	*Maximum*	*Mean*	*SD*	*Minimum*	*Maximum*	
CD3	53.46	16.34	15.56	74.39	61.90	22.85	3.94	96.82	0.0001
CD4	22.36	2.01	6.57	47.45	33.35	12.66	1.15	56.32	0.0001
CD25	5.68	2.961	0.2	13.42	14.97	11.06	.28	65.29	0.0001
	New (n=26)				Relapse(N=12)				
CD3	52.0	25.8	3.94	88.12	57.719	17.705	17.47	80.97	0.0001
CD4	30.2	15.6	1.15	56.32	21.480	10.508	4.98	35.08	0.080
CD25	13.6	5.38	6.60	29.62	22.185	15.148	10.51	65.29	0.0001
	induction				Consolidation				
CD3	76.37	15.58	44.15	95.38	68.98	19.09	9.65	96.82	0.0001
CD4	36.44	10.01	22.52	54.79	28.69	13.26	2.99	51	0.184
CD25	5.1	5.14	.28	17.45	17.65	12.34	5.55	65.02	0.0001

*Kruskal wallis test $p \leq 0.05$

Table 3: The level of (CD3-CD4-CD25) in risk groups

Risk group	*N*	*CD3*				*CD4*				*CD25*			
		Mean	*SD*	*Minimum*	*Maximum*	*Mean*	*SD*	*Minimum*	*Maximum*	*Mean*	*SD*	*Minimum*	*Maximum*
High RG	19	45.64	27.68	3.94	88.12	27.9	1.7	1.15	56.3	15.75	4.74	8.41	29.62
StanderRG	6	69.24	4.48	62.47	74.84	21.28	12.87	33.4	40.3	7.924	1.02	6.60	9.27
p-value	0.030					0.348				0.0001			

*Mann-whitney U test $p \leq 0.05$

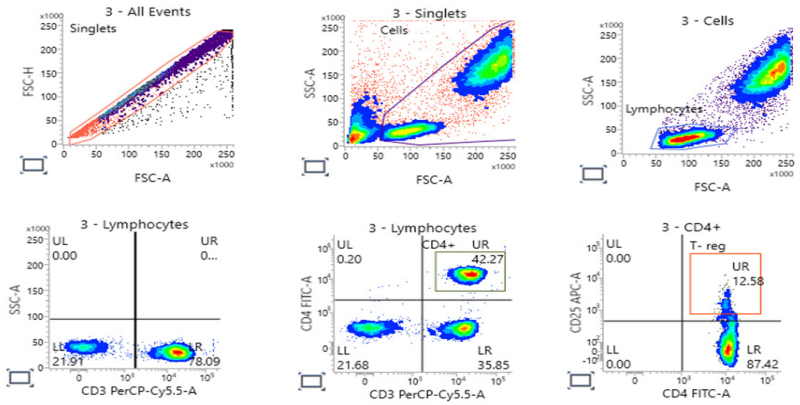

Figure 1: Treg (CD25), CD4 and CD3 in induction group

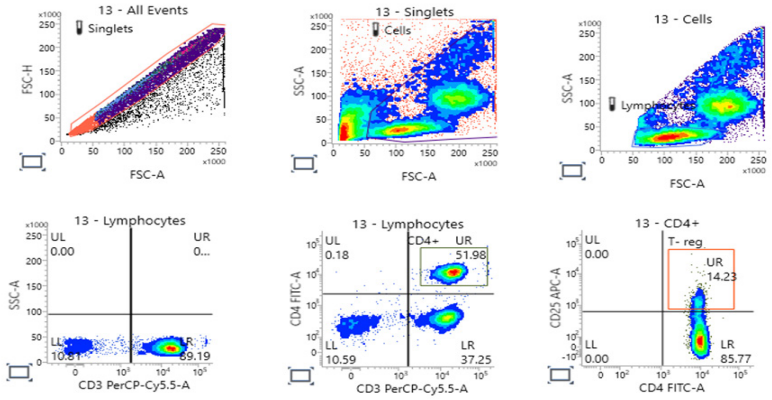

Figure 2: T reg (CD25),CD4 and CD3 in consolidation

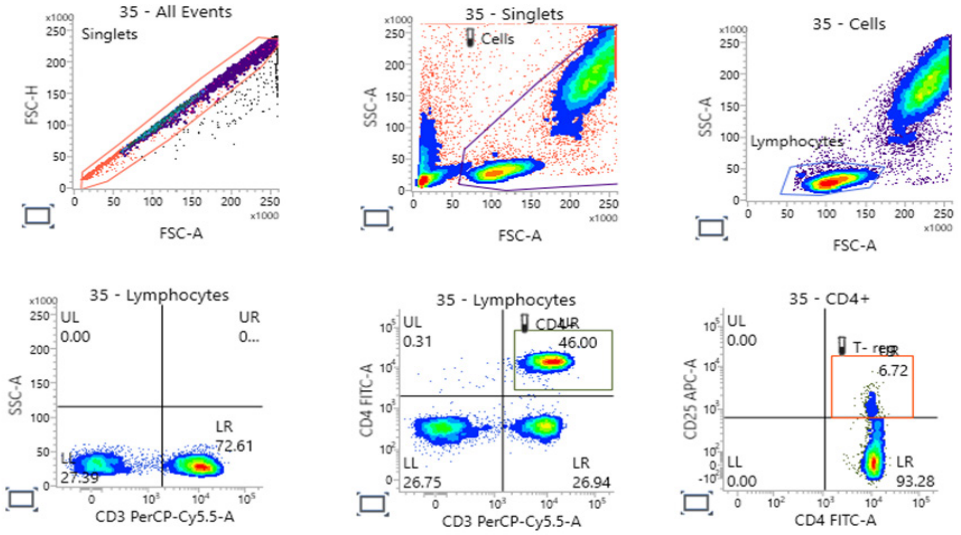

Figure 3: Treg (CD25), CD4 and CD3 in control group

Folic acid blocker that reduces DNA synthesis, repair, and cell proliferation by inhibiting multiple related enzymes in MTX metabolism engaged in synthesizing nucleotides, eventually resulting in cell death (Abdi *et al*., 2021).

Yet 75% of patients will experience HD-MTX treatment-related side effects, and even 1 to 3% of patients will die from drug-induced toxicity (Pavlovic *et al*., 2019 We suggested the increased number of CD25 at consolidation may due to the effects of MTX chemotherapy drug.

Following these findings, there were significant differences in the levels of CD25, which was higher in relapes patients with B-ALL compared with healthy controls. This result was agreed with (Duell *et al.,* 2017).

The present study observed a significant reduction in CD4 + T-cell levels in Treg. The mean was 33.35 pg/mL in patients than in control 22.36 pg/mL and this result agreed with Lustfeld *et al.* (2014).

Lustfeld *et al.* (2014). suggested that early remission of the illness may be conditioned by CD4+ non-Treg cells in leukemic bone marrow upon diagnosis, but they did not discover any impact of Tregs on the prognosis for children ALL. Conversely, CD4 + T-cell increase in Terg (Zahran *et al*., 2021).

Other studies have reported significantly decreased CD4 + compared with in healthy controls(Bhattacharya *et al*., 2014). The present study showed a significant reduction in Treg in relapse compared to the control group. This result was agreed with (Jingjing *et al*., 2021) (Figure 3).

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