Original Article

Children with acute lymphoblastic leukemia show high numbers of CD4+ and CD8+ T-cells which are reduced by conventional chemotherapy

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ABSTRACT

Background: Acute lymphoblastic leukemia (ALL) is considered as one of the most common cancer in pediatric malignancies. Among ALL, B-cell Acute Lymphoblastic Leukemia (B-ALL) represents 80% to 85% of the childhood ALL. **Problem:** Although anti B-ALL chemotherapy kill B-ALL, it associates with alteration in the numbers of $CD4^+$ and $CD8^+$ T-cells, and thus impacts the overall immunity. **Aim:** To evaluate the impact of anti B-ALL on the numbers of $CD4^+$ and $CD8^+$ T-cells in correlation to the numbers of $CD10^+$ B cells in B-ALL pediatric patients. **Materials and Methods:** Peripheral blood samples were drawn from previously diagnosed B-ALL before ($n = 10$ cases) and after ($n = 10$ cases) chemotherapy as well as from healthy controls ($n = 10$ cases). The numbers of $CD4^+$, $CD8^+$ T-cells and $CD10^+$ B cells were measured in these samples by flow cytometry. **Results:** As expected, the numbers of $CD10^+$ B-cells were increased in B-ALL patients before chemotherapy which were associated with increases in the numbers of $CD4^+$ and $CD8^+$ T-cells. Chemotherapy of B-ALL patients, during the induction phase, induced dramatic decreases in the numbers of $CD10^+$ B cells, which were associated with decreases in the numbers of $CD4^+$ and $CD8^+$ T-cells. Tin spite of this alteration, the ratio of CD4/CD8 in B-ALL patients were remained similar before and after chemotherapy as compared to those in healthy controls. **Conclusion:** Anti B-ALL chemotherapy induces alterations in the frequencies of T-cell subsets. Given the importance of these cells in anti-tumor immunity, our data may lead to further studies to investigate the different subsets of these cells, in particular regulatory T-cells.

Key words: B-acute lymphoblastic leukemia, B-ALL, B-cells, cancer, CD10, CD4, CD8, chemotherapy, T-cells, T_{regs}

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common malignancy diagnosed in children,[1,2] representing more than a quarter of all pediatric cancers.[2] Patients with ALL receive intensive courses of chemotherapy which are followed by a period of severe leukopenia,[3] even if those patients have a functional $CD4^+$ and $CD8^+$ T-cell system. The immature blasts in B-acute lymphoblastic leukemia (B-ALL) have no unique morphologic or cytochemical features.[3] Therefore, immunophenotyping of ALL has been routinely performed in the diagnostic evaluation, to determine the leukemic B-cells through specific surface markers associated with B-cell linage, including $CD10^+$.[4] CD10

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is commonly expressed on the majority of precursor B-ALL during the early stages of B-cell maturation and development.[5] Approximately three-quarters of patients express this common B-ALL precursor.[6] B-ALL cases which express CD10 have the best prognosis among B-ALL cases,[7] where absence of CD10 expression is associated with poor prognosis.[8]

Significant progress of treatment of pediatric B-cell malignancies including leukemia has been efficacious. However, many children with B-ALL still do not respond to the standard chemotherapy,[9] which is often associated with disturbance in the numbers of T-cell populations. This alteration in T-cells is likely to impact the endogenous anti B-ALL immunity as well as any trial of immunotherapy. Immunotherapy, in particular adoptive T-cell transfer, depends mainly on harvesting $CD8^+$ cytotoxic T-cells from the patient, activating them *in vitro* and infusing them back to the same patients with the main goal to attack cancer cells by the adoptively transferred T-cells.[10] Therefore, understanding how chemotherapy alters T-cell population upon chemotherapy is crucial to design effective T-cell-based immunotherapy since the alteration in T-cell numbers may associate with emergence of regulatory $CD4^+$ T (T_{reg}) cells with immunosuppressive effects. T_{reg} cells are defined as inhibitor of immune system due to their immunosuppressive mechanism with the progressiveness of malignancy.[11] Indeed, previous results reported the emergence of these T_{reg} cells in some hematological malignancies with a poor prognosis.[12]

In this study, we investigated the numbers of $CD4^+$ and $CD8^+$ cells in the peripheral blood of pediatric patient with B-ALL before and after the induction of chemotherapy. Our results showed a correlation between the impact of chemotherapy on leukemic $CD10^+$ B cells and the alteration in the numbers of $CD4^+$ and $CD8^+$ T-cells. Our results open new avenues for further studies to investigate the subsets of these cells in particular, regulatory T-cells.

MATERIALS AND METHODS

Patients

Patients included in this study were risk stratified according to risk classification system and were treated according to treatment protocols including high risk pre B-ALL protocol, standard risk pre B-ALL. The research study was approved by the Local Ethics Committee, Faculty of Medicine, Tanta University and informed consent was obtained from all patients before participation. The study was conducted among patients $n = 20$; before ($n = 10$) and after ($n = 10$) the induction of chemotherapy; mean age = 6, as compared to healthy subjects ($n = 10$); mean age = 5. Patients were recruited from Hematology/Oncology Unit, Pediatric Departments, Tanta University Hospital, Tanta Cancer Center, Tanta, Egypt.

Patient diagnosis

Bone marrow biopsy was used to confirm the presence of leukemic blasts using laboratory microscopic investigation followed by immunophenotyping using flow cytometry in order to identify the subtype of acute leukemia using the routinely diagnostic antibodies, including CD10, CD19, CD2, CD7, CD13, CD117, CD33, CD14, CD64, CD34, HLA-DR and CD45 (BD Biosciences, CA, USA).

B-acute lymphoblastic leukemia treatment protocol

Patients were treated according to treatment protocols including high risk pre B-ALL protocol, standard risk pre B-ALL. Follow-up of patients was carried out clinically and by blast count in bone marrow on day 21 after induction chemotherapy. The treatment included: Vincristine 1.5 mg/kg/m^2/week IV (days 0, 7, 14, 21, 28, 35), doxorubicin 25 mg/m^2/week IV infusion (days 0, 7, 14, 21, 28, 35), L-asparginase 6000 U/m^2 SC on alternate days for 10 doses, and prednisone 40 mg/m^2/day for 6 weeks orally. On day 21, bone marrow aspiration was done. In non responding cases, we added etopsoide 100 mg/m^2/dose IV (days 22, 25, 29), cyclophosphamide 750 mg/m^2/dose IV infusion (days 22, 25, 29), aracytin 100/m^2/dose IV (days 22, 25, 29), and high-dose methotrexate 5 g/m^2 over 4 h on day 28.

Reagents and antibodies

Monoclonal antibodies against surface markers, including CD10, CD19, CD2, CD7, CD13, CD117, CD33, CD14, CD64, CD34, HLA-DR and CD45 (BD Biosciences, CA, USA), were used in diagnosis of acute leukemia. For identification of T-Cell populations, anti CD4 (BD Biosciences), anti CD8 (BD Biosciences) were used in samples processing. BD FACS lysing buffer was used in RBSs lysis and phosphate buffer saline (PBS) was used in sample washing and suspension.

Flow cytometric analysis

Bone marrow biopsy and immunophenotyping were performed in order to identify the type of acute leukemia using the routinely diagnostic antibodies. For identification of T-cells subsets, fresh venous peripheral blood samples were collected in EDTA tubes. 100 μL of blood was stained with 5 μL of each antibody in the staining tubes, the tubes were incubated in dark conditions for 20 min then samples was mixed with BD FACS lysing solution (1X) then incubated for 15 min in the dark conditions. Samples then centrifuged at 1250 r/min for 5 min, the supernatant was discarded to remove the lysed RBCs. PBS was added then samples were centrifuged at 1250 r/min for 5 min then the supernatant was discarded to remove any remained debris or RBCs then the pellets were resuspended in 350 μL of PBS.

The absolute numbers of cells were calculated using the following formula: Percentage of cells × total number of white blood cells/100.

Statistical analysis

The clinical data were collected along the study and analyzed for each patient. The data were represented as mean ± standard deviation and analyzes of frequencies for statically significant differences were performed with a one-way analysis of variance (ANOVA). We assumed that frequencies of T-cell and B-cell in the peripheral blood of B-ALL pediatric patients did not follow a normal distribution; experimental differences over the healthy control volunteers were analyzed by the Student's *t*-test. Significant differences were defined as $P \leq 0.05$ (P values below 0.05 were considered significant).

RESULTS

Patient demographics before and after induction chemotherapy

The mean age, sex, total blast count and whether these patients diagnosed with splenohepatomegaly [Table 1].

Increases in the numbers of B cells expressing CD10+ B-cells in the peripheral blood of B-acute lymphoblastic leukemia patients

As shown in Figures 1 and 2a, analysis of the percentages showed significant increases in the numbers of CD10+ B-cells in B-ALL patients before chemotherapy as compared to healthy donors (75 ± 28.2 vs. 15.8 ± 3.23, $P < 0.001$). The percentages of CD10+ cells decreased upon induction of chemotherapy as compared to healthy donors; (10.5 ± 6.8 vs. 15.8 ± 3.23). The absolute numbers of CD10+ B-cells showed significant increases before chemotherapy as compared to healthy donors (3343.6 ± 2353 vs. 463.8 ± 223.4, $P < 0.001$) and these numbers turned to decrease after induction of chemotherapy when compared

Table 1: B-ALL pediatric patients' demographic data showing age, sex, diagnosis with hepato-splenomegaly and total blast count

Age	Gender	Hepato-splenomegaly	Total blast
Before induction of chemotherapy			
7	Male	No	91
7	Male	Yes	90
6	Male	Yes	72
7	Male	Yes	70
5	Female	Yes	70
12	Female	No	58
5	Female	Yes	55
12	Female	Yes	35
11	Female	Yes	25
0.5	Female	No	13
After induction of chemotherapy			
6	Male	No	4
12	Female	No	10
5	Female	Yes	2
12	Female	Yes	8
3	Female	No	4
6	Female	Yes	4.8
4	Male	Yes	4.64
2	Female	Yes	4.01
5	Male	No	3.8
10	Female	Yes	3.54

B-ALL: B-acute lymphoblastic leukemia

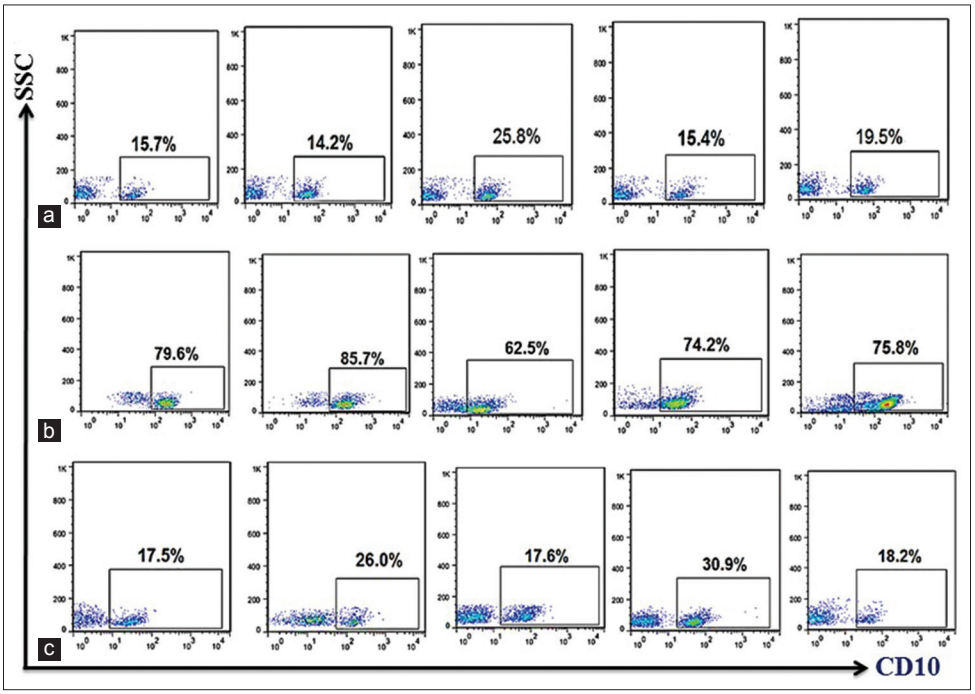

Figure 1: A representative flow cytometric analysis of CD10+ B-cells in pediatrics patient with B-acute lymphoblastic leukemia showing the expression of CD10+ B-cells in five healthy donors (a), five patients before induction of chemotherapy (b) and five patients after induction of chemotherapy (c)

to healthy donors (151.4 ± 77.4 vs. 463.8 ± 223.4) as shown in Figure 2b.

Increases in the numbers of CD4+ T-cells in the peripheral blood of B-acute lymphoblastic leukemia pediatric patients

As shown in Figures 3, 4a and b, the percentages of CD4+ T-cells increased in patients with B-ALL before induction phase of chemotherapy when compared to healthy donors (17.6 ± 5.58 vs. 14.6 ± 1.86) and these percentages decreased after induction of chemotherapy when compared to healthy donors (14.4 ± 1.23 vs. 14.6 ± 1.86). As shown in Figure 4c, patients with B-ALL showed significantly higher absolute numbers of CD4+ T-cell before chemotherapy as compared to healthy donors (858.6 ± 517.7 vs. 472.15 ± 295.12, $P \leq 0.05$) and decreases in absolute numbers after induction of chemotherapy as compared to healthy donors (293.8 ± 105.9 vs. 472.15 ± 295.12).

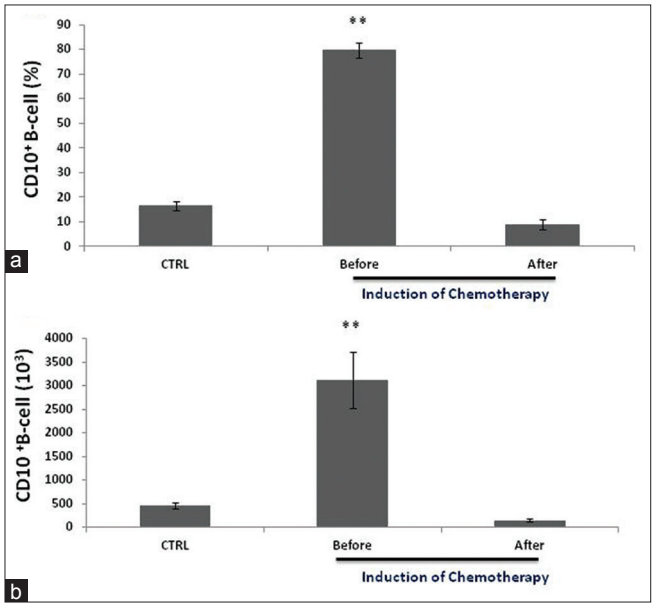

Figure 2: The numbers of CD10+ B cells in B-acute lymphoblastic leukemia patients. (a) The percentages of CD10+ B-cells in B-acute lymphoblastic leukemia pediatric patients before and after induction of chemotherapy as compared to healthy control donors "control." (b) The absolute (abs.) numbers of these cells in the same patients as compared to healthy control volunteers "control." The absolute numbers of CD10+ cells were calculated as: (Total white blood cells count [cells/μL] × percent of CD10+)/100

Increase in expression of CD8+ T-cells in the peripheral blood of B-acute lymphoblastic leukemia pediatric patients

As shown in Figures 3, 4a and b, the percentages of CD8+ T-cells increased in patients with B-ALL before induction phase of chemotherapy as compared to healthy donors and these percentages decreased after induction of chemotherapy as compared to healthy. As shown in Figure 4c, patients with B-ALL showed significantly higher absolute numbers of CD8+ T-cell before chemotherapy as compared to healthy donors (590.31 ± 317 vs. 472.15 ± 295.12, $P \leq 0.05$) and these numbers turned to decrease after induction of chemotherapy as compared to healthy donors (234.5 ± 90.16 vs. 472.15 ± 295.12).

The ratio of CD4/CD8 in the peripheral blood of B-acute lymphoblastic leukemia pediatric patients

We investigated the ratios of CD4/CD8 in pediatric patients before and after induction of chemotherapy. We found that the ratio of CD4:CD8 before chemotherapy was 1.3 as

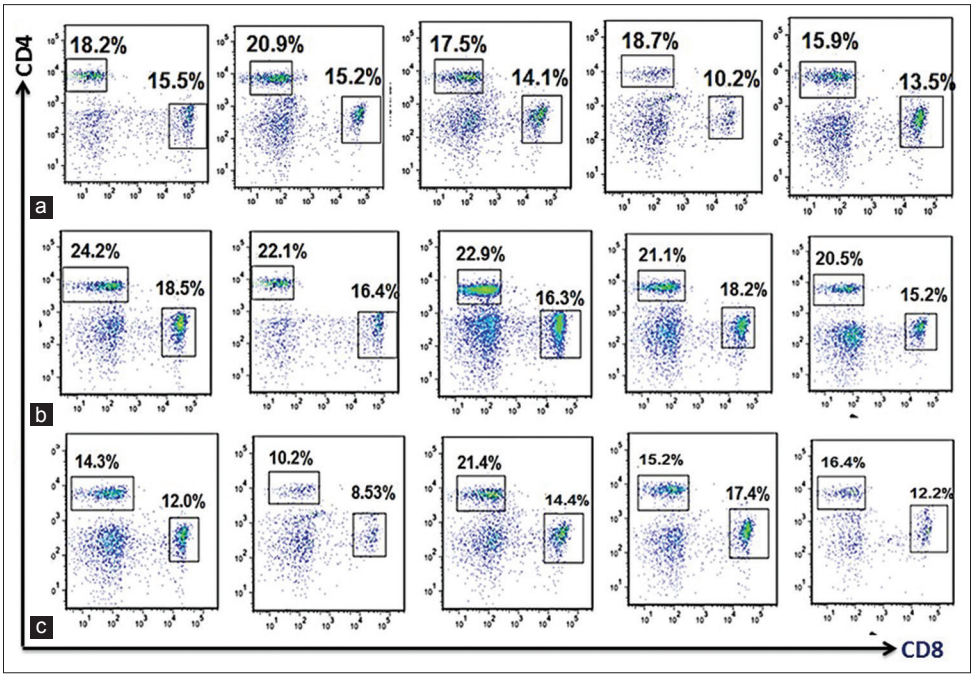

Figure 3: A representative flow cytometric analysis of CD4+ and CD8+ T-cells in pediatric patients with B-acute lymphoblastic leukemia showing the expression of CD4+ and CD8+ in five healthy donors (a), five patients before induction of chemotherapy (b) and five patients after induction of chemotherapy (c)

compared to 1.4 in healthy donors. The ratio of CD4:CD8 after induction of chemotherapy was 1.35 as compared to 1.4 in healthy donors as shown in Table 2.

Correlation between CD4+, CD8+ T-cells and CD10+ B-cells in the peripheral blood of B-acute lymphoblastic leukemia pediatric patients

ANOVA test was used to analyze the correlation between CD4+, CD8+ T-cells and CD10+ B-cells in the peripheral blood of B-ALL pediatric patients. We found that the increase in the numbers of CD4+ and CD8+ T-cells before induction of chemotherapy was associated with an increase in the numbers of CD10+ B-cells. The differences between the percentages and absolute numbers of CD4+, CD8+ T-cells versus CD10+ B-cells are shown in Table 3.

DISCUSSION

We performed immunophenotyping analysis on the peripheral blood of children patients with B-ALL based on surface markers detection. This analysis was carried out to determine the frequencies of CD4+, CD8+ T-cells and CD10+ B-cells and to show the correlation between these cells before and after induction of chemotherapy. The results showed that the increase in the numbers of CD10+ B-cells was associated with significant increases in the numbers of CD4+, CD8+ T-cells. As expected, we found that the numbers of CD10+ B-cells increased in B-ALL patients before chemotherapy coinciding with increase in the numbers of CD4+ and CD8+ T-cells. Induction of chemotherapy induced dramatic decreases in the numbers of CD10+ B-cells, coinciding with increases in the numbers of both CD4+ and CD8+ T-cells. Our results conclude that anti B-ALL chemotherapy induces alterations in the T-cells subsets, opening a new avenue to investigate the phenotypes of different subsets of these cells in particular regulatory T-cells.

Consistent with previous studies,[13-18] we found that the ratios of CD4+, CD8+ T-cells and CD10+ B-cells are higher in patients with B-ALL at diagnosis. These numbers were reduced upon treatment with chemotherapy. CD4+ effector T-cells have previously been suggested to play an important role in anti-tumor immunity in different malignancies including B-ALL[19,20] since they offer antigen-specific aid to tumor-reactive CD8+ T-cells, resulting in elimination of the leukemic lymphoblast during the induction of chemotherapy. Considerably, protective immune responses may actively hindered by local immunosuppression[21] in the peripheral blood of B-ALL patients. One of the

Table 2: The ratios of CD4/CD8 in the peripheral blood of healthy CTRL volunteers as well as B-ALL pediatric patients before and after induction of chemotherapy

	CD4/CD8		
Number	CTRL	Before chemotherapy	After chemotherapy
1	0.89	2.2	1.14
2	1.7	1.6	1.4
3	1.2	1.7	1.2
4	1.5	1.1	1.7
5	1.3	1.1	1.4
6	1.5	1.3	1.5
7	1.2	1.12	1.2
8	1.5	1.12	1.2
9	1.2	1.02	1.2
10	1.6	1.15	1.3
Mean	1.4	1.34	1.3
SD	0.24	0.37	0.17
P		>0.05	>0.05

CTRL: Control, SD: Standard deviation, B-ALL: B-acute lymphoblastic leukemia

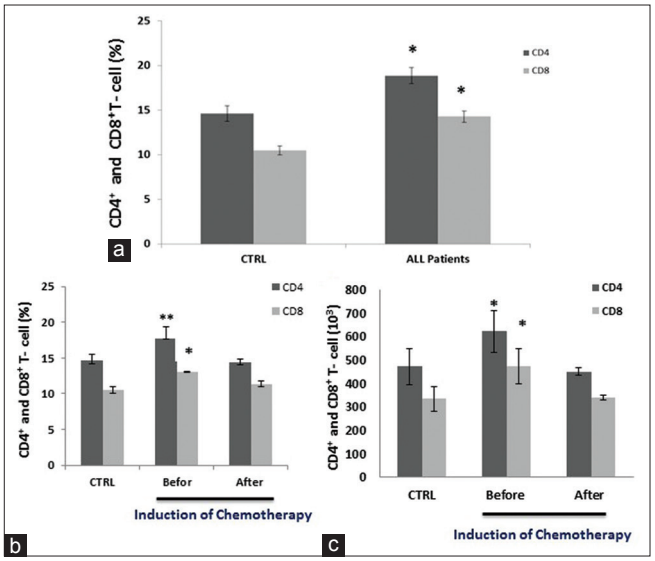

Figure 4: The numbers of CD4+ and CD8+ T-cells in B-acute lymphoblastic leukemia patients as compared to healthy control volunteers. (a) The percentages of CD4+ and CD8+ T-cells in all B-acute lymphoblastic leukemia pediatric patients as compared to healthy donors "control." (b) The percentages of CD4+ and CD8+ T-cells in patients before and after induction of chemotherapy as compared to healthy donors "control." (c) The absolute (abs.) numbers of CD4+ and CD8+ T-cells in patients before and after induction of chemotherapy as compared to healthy donors "control"

Table 3: Differences between CD4+, CD8+ T-cells versus CD10+ B-cells, percentages and absolute numbers in B-ALL pediatric patients before and after induction of chemotherapy

Correlation	Difference		*Q*		*P*	
	Percentages	Absolute numbers	Percentages	Absolute numbers	Percentages	Absolute numbers
CD4+ and CD8+ T-cell (before) versus CD10+ (before)	−43.009	−2252.5	21.521	8.552	<0.001	<0.001
CD4+ and CD8+ T-cell (after) versus CD10+ (after)	20.561	376.91	10.288	1.431	<0.001	>0.05

P≤0.05, *P*≤0.01; significant difference between the two subsets of CD4+, CD8+ T-cell and CD10+ B-cell. B-ALL: B-acute lymphoblastic leukemia

best-defined mechanisms of local immunosuppression is the accumulation of T_{regs} with suppressive functions[22] due to either increases in the numbers of natural T_{reg} cells or conversion of CD4+ T-cells to CD4+ T_{reg} cells.

The immunosuppressive function of T_{reg} cells are considered as part of CD4+ effector T-cells.[12] Although we have identified the phenotype of CD4+ T-cells in this study, it could be suggested that the increases in the numbers of CD4+ T-cells in our patients before chemotherapy might be a result of partial conversion of CD4+ T-cells into immunosuppressive T_{regs}.[23] Current studies by our group are ongoing to test this hypothesis. Furthermore the decreases in the numbers of CD4+ T-cells subsequent to induction of chemotherapy might be due to the cyclophosphamide that is included in this treatment protocol since this drug has been reported in previous studies to decrease the frequencies of CD4+ T-cells and T_{reg} cells.[24]

The affinity of CD4+ and CD8+ T-cells to functionally interact with leukemic cells needs to be determined in order to develop effective immunotherapeutic strategies in B-ALL patients.[25]

CD8+ co-receptor is predominantly expressed on the surface of cytotoxic T-cells. Previous studies have reported that T-cell numbers, particularly CD8+ in diagnosis correlate with prognosis in some hematological malignancies, including lymphoma and acute lymphoblastic leukemia.[26] Furthermore, previous publications by our and other groups established that cytotoxic CD8+ T-cells are the main killer cells that attack cancer cells specifically and cure advanced tumor.[27-33] Accordingly, adoptive cell therapy utilizing CD8+ T-cells has shown promising application in clinical setting.[34] This application has been reflected recently by the use of chimeric antigen receptor therapy for immunotherapy that shows great promise for ALL by engineering CD8+ T-cells to recognize CD19+ B cells resulting in specific killing of B cell leukemia and lymphoma.[25] As such, the alteration in both CD4+ and CD8+ T-cells in B-ALL patients before and after chemotherapy is of a great importance for the clinical application of immunotherapy.

CONCLUSION

Our study illustrates the alterations in both CD4+ and CD8+ T-cell subsets in B-ALL patients before and after induction of chemotherapy, where these cells increased and decreased before and after induction of chemotherapy, respectively. Further studies with larger numbers of patients are considered to specifically determine the subsets of T-cell subsets, in particular T_{reg} cells in B-ALL at different stages of chemotherapy.

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Conflicts of interest

There are no conflicts of interest.

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