Original Article

Reduction in the numbers of CD33+ myeloid population in Egyptian children with B-linage acute lymphoblastic leukemia and its recovery after induction of chemotherapy

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ABSTRACT

Background: Acute lymphoblastic leukemia (ALL) is biologically and clinically considered as a heterogeneous neoplasm of lymphoid progenitor cells. About 85% of children with ALL are diagnosed as B-ALL, expressing CD19; the typical marker of normal B cells. Problem: Given that the chemotherapy associated with leucopenia, in particular myeloid cells (CD33+ cells), **Aim:** the main aim of this study was to analyze the numbers of these cells in children with B-ALL before and after induction of chemotherapy. **Materials and Methods:** The frequencies of CD33+ myeloid cells and CD19+ B-cells were analyzed in the peripheral blood patients before ($n = 10$) and after ($n = 10$) induction of chemotherapy as well as in healthy volunteers ($n = 10$) using multiparametric flow cytometry. **Results:** As expected, B-ALL patients showed high numbers of CD19+ cells before induction of chemotherapy; where the numbers of these cells were reduced upon the induction of chemotherapy. CD33+ myeloid cells showed decrease in numbers in B-ALL patients before chemotherapy as compared to healthy control volunteers. Interestingly, treatment of B-ALL patients with chemotherapy-induced almost recovery of the numbers of these cells. **Conclusion:** CD33+ myeloid cells are increased in numbers after induction of chemotherapy, indicating to a dynamic mobilization or differentiation of their precursors into circulation. This study opens a new avenue to characterize the phenotype and function of these cells in different hematological malignancies; in particular, they may harbor regulatory cells.

Key words: Acute lymphoblastic leukemia, B-acute lymphoblastic leukemia, cancer, CD19, CD33, chemotherapy, leukemia, myeloid derived suppressor cells, myeloid cells

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a malignant clonal proliferation of lymphoid progenitor cells. ALL is representing about 25% of cancer diagnosed among children younger than 15 years.[1] About 85% of cases are diagnosed as B-cell ALL.[2,3] The most immature cells (blast cells) in B-ALL mainly express B-cell markers such as CD19; the typical marker of normal B-cells.[4] It is particularly involved in B-cell development, activation, and differentiation. Alterations in the intensity of CD19 which are seen in some B-cell lymphoid neoplasms including B-ALL enable to distinguish between reactive and neoplastic cells.[5]

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There are various treatments of B-ALL, including chemotherapy, targeted therapy, and bone marrow (BM) transplant.[6] The aim of these treatments, particularly chemotherapy is to inhibit the production of leukemic blasts in BM resulting in clinical and hematological remission,[7,8] which can be detected by a moderately cellular BM with <5% blast cells, a moderate number of erythroid precursors and a moderate number of granulocytes including CD33+ myeloid cells.[9] CD33 is commonly expressed on myeloid populations including monocytes, granulocytes, dendritic cells, and mast cells, and it is absent on normal platelets, lymphocytes, erythrocytes, and hematopoietic stem cells.[10,11] The myeloid cells play a critical antimicrobial role in particular during chemotherapy of cancer patients.[12]

Given that CD33+ myeloid cells in cancer patients can harbor both normal cells and immunosuppressive cells, particularly myeloid derived suppressor cells (MDSCs),[13] this study was designed to investigate whether the numbers of CD33+ myeloid cells are affected by anti-B-ALL chemotherapy. To this end, we studied the frequencies of these cells in the peripheral blood of B-ALL pediatric patients before and after induction of chemotherapy. We also investigated whether there is any correlation between these cells and CD19+ cells in B-ALL pediatric patients.

To the best of our knowledge, this study is the first to investigate the numbers of CD33+ myeloid cells in Egyptian children with B-ALL. Therefore, these results open a new avenue for further studies to characterize the subsets of these cells in particular to test whether they contain immunoregulatory subsets.

MATERIALS AND METHODS

Patients

This study was conducted among B-ALL patients who were recruited before ($n = 10$) and after ($n = 10$) induction of chemotherapy with a mean age of 6.5 ± 3.4. We also recruited healthy volunteers (n = 10) with a mean age of 7 ± 2.2. Patients were recruited from Hematology and Oncology Unit, Pediatric Department, Tanta University Hospital; Tanta Cancer Center, Tanta, Egypt. The research study was approved by the Ethics Committee, Faculty of Medicine, Tanta University, and informed consent was obtained from all patients before participation.

Patients' diagnosis

Acute leukemia was routinely diagnosed by detection of blast cells in the BM film from the BM aspirate. BM or peripheral blood samples were then sent to the laboratory for immunophenotyping. Accordingly, acute leukemia was subdivided into B-ALL, T-ALL, and AML based on the surface and intracellular antigens of the blast cells.

B-Acute lymphoblastic leukemia treatment protocol

Patients included in this study were risk stratified according to risk classification system and 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 BM on day 21 after induction chemotherapy which included: Vincristine 1.5 mg/kg/m^2/week intravenous (IV) (days 0, 7, 14, 21, 28, 35), doxorubicin 25 mg/m^2/week IV infusion (days 0, 7, 14, 21, 28, 35), L-asparaginase 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, BM aspiration was done. In nonresponding 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

All monoclonal antibodies (mAbs.), including CD10, CD19, CD2, CD7, CD13, CD117, CD33, CD14, CD64, CD34, HLA-DR, and CD45 (BD biosciences) were used for immunophenotyping analysis. BD fluorescence-activated cell sorting (FACS) lysing solution was used for ribosomal binding sites lysis and phosphate buffer saline (PBS) was used in samples washing and suspension.

Flow cytometric analysis

For identification of CD19+ B-cells and CD33+ myeloid cells, fresh venous peripheral blood samples were collected in sodium heparin tubes. Briefly, 100 µL of blood was stained with human mAbs using concentrations recommended by the manufacturers of each antibody in staining tubes, the tubes were incubated in cold dark conditions for 20 min then BD FACS lysing solution (×1) was added for 15 min for red blood cells (RBCs) lysis. Samples were then centrifuged at 1250 rpm for 5 min, the supernatant was discarded to remove the lysed RBCs. The cells then were washed twice using PBS to remove any remained debris or RBCs, the pellets then re-suspended in PBS. Negative stained samples were used as internal controls all over the experiments. FACSCalibur or FACSCanto II (BD Biosciences, SanJose, CA, USA) were used for acquisition. FACSDiva, CellQuest (BD Biosciences) and Flowjo software were used for data analysis. The total white blood cells (WBCs) count in the peripheral blood was enumerated using an automated instrument for complete blood count (ABX Micros 60 hematology analyzer, Horiba medical, USA). Accordingly, the absolute numbers of CD19+, CD33+ cells were calculated, respectively as: (Total WBCs count [cells/µL] × CD19+, CD33+ %)/100.

Statistical analysis

The pediatric B-ALL patients were divided into two groups; before and after induction of chemotherapy. The clinical data were collected along the study and analyzed for each

patient, each value was calculated as the mean ± standard deviation results were analyzed using a paired Student's *t*-test. *$P \leq 0.05$, **$P \leq 0.01$; significant difference between two test groups. Linear regression test was used to detect whether there is a correlation between the two quantitative variables.

RESULTS

Patient demographics before and after induction chemotherapy

The mean age, sex, WBSs count, and relative size of spleen and liver are illustrated in Table 1.

Reduction in the numbers of CD19+ B-cells expression in the peripheral blood of B-acute lymphoblastic leukemia pediatric patients

Analysis of percentages and absolute numbers of CD19+ B-cells showed significant increases in the numbers of CD19+ B-cells in B-ALL patients before chemotherapy as compared to healthy control volunteers; (79.7 ± 9.8 vs. 16.4 ± 5.3, $P < 0.001$), respectively. The numbers of CD19+ cells decreased upon induction of chemotherapy as compared to before chemotherapy (8.9 ± 6.8 vs. 79.7 ± 9.8, $P < 0.001$), respectively, as well as compared to healthy volunteers; (8.9 ± 6.8 vs. 16.4 ± 5.3, $P < 0.001$), respectively, as shown in Figures 1 and 2.

Increases in the numbers of CD33+ myeloid cells in peripheral blood of B-acute lymphoblastic leukemia pediatric patients

We analyzed the total numbers of CD33+ myeloid cells in B-ALL patients before and after induction of chemotherapy as compared to healthy control volunteers. We found that the percentages and absolute numbers of CD33+ myeloid cells were significantly decreased in patients before induction of chemotherapy when compared to healthy control volunteers; (23.4 ± 15.09 vs. 43.6 ± 5.1, $P < 0.001$), respectively. Interestingly, these numbers were almost recovered upon induction of chemotherapy. Patients after induction of chemotherapy showed a slightly higher numbers of CD33+ myeloid cells when compared to healthy volunteers; (56.6 ± 13.9 vs. 43.6 ± 5.1, $P > 0.05$), respectively, as shown in Figures 3 and 4.

Correlation between CD33+ myeloid cells and CD19+ B-cells in B-Acute lymphoblastic leukemia Pediatric patients:

Linear regression test was used to determine the correlation between CD33+ and CD19+ subsets as quantitative variables in one group. We found that there is no direct correlation between the two subsets of cells either before or after induction of chemotherapy. However, the increases in CD19+ B-cells before induction of chemotherapy were associated with significant decreases in the numbers of CD33+ myeloid cells and vice versa, $P < 0.001$, as shown in Tables 2 and 3.

DISCUSSION

In this pilot study, the immunophenotypic analysis was performed on the peripheral blood of B-ALL pediatric patients in order to assess the numbers of CD33+ myeloid cells in parallel with CD19+ B-cells. We also investigated the impact of chemotherapy on these cells and determined the correlation between these two cell populations before and after the induction phase of chemotherapy. The analysis showed that there is no direct correlation between the two subsets of cells either before or after induction of chemotherapy. However, the increases in CD19+ B-cells were associated with significant decreases in the numbers of CD33+ myeloid cells and vice versa. Whereas leukemic state before chemotherapy and induction phase of chemotherapy both have a direct effect on the two subsets of cells. Therefore, as expected, B-ALL patients before chemotherapy showed high numbers of CD19+ B-cells since they harbor leukemic blasts which decreased upon the induction of chemotherapy. In contrast, significant decreases in the numbers of CD33+ myeloid cells were observed in B-ALL patients before chemotherapy. These

Table 1: Demographics data of B-ALL patients and the total WBCs count before and after induction of chemotherapy

Variant	Total	Before induction of chemotherapy	After induction of chemotherapy
Number of patients	*n*=20	*n*=10	*n*=10
Age (mean)	6.6	7.5	6
Sex			
Male	8	6	2
Female	12	4	8
WBCs count (mean)	2952	3995	1910
Hepatosplenomegaly			
Yes	14	8	6
No	6	2	4

ALL: Acute lymphoblastic leukemia, WBCs: White blood cells

Table 2: Differences between percentages of CD33+ myeloid cells and CD19+ B-cells in recruited B-ALL pediatric patients before and after induction of chemotherapy

Variant	Difference	*q*	*P*
CD33% before versus CD19% before	−73.853	37.523	<0.001
CD33% after versus CD19% after	4.233	2.151	<0.001

P≤0.05, *P*≤0.01, significant difference between the two subsets of CD33+ and CD19+ cells. ALL: Acute lymphoblastic leukemia

Table 3: Differences between absolute numbers of CD33+ myeloid cells and CD19+ B-cells in recruited B-ALL pediatric patients before and after induction of chemotherapy

Variant	Difference	*q*	*P*
CD33 absolute before versus CD19 absolute before	−2901.7	11.477	<0.001
CD33 absolute after versus CD19 absolute after	−2561.9	10.133	<0.001

P≤0.05, *P*≤0.01, significant difference between the two subsets of CD33+ and CD19+ cells. ALL: Acute lymphoblastic leukemia

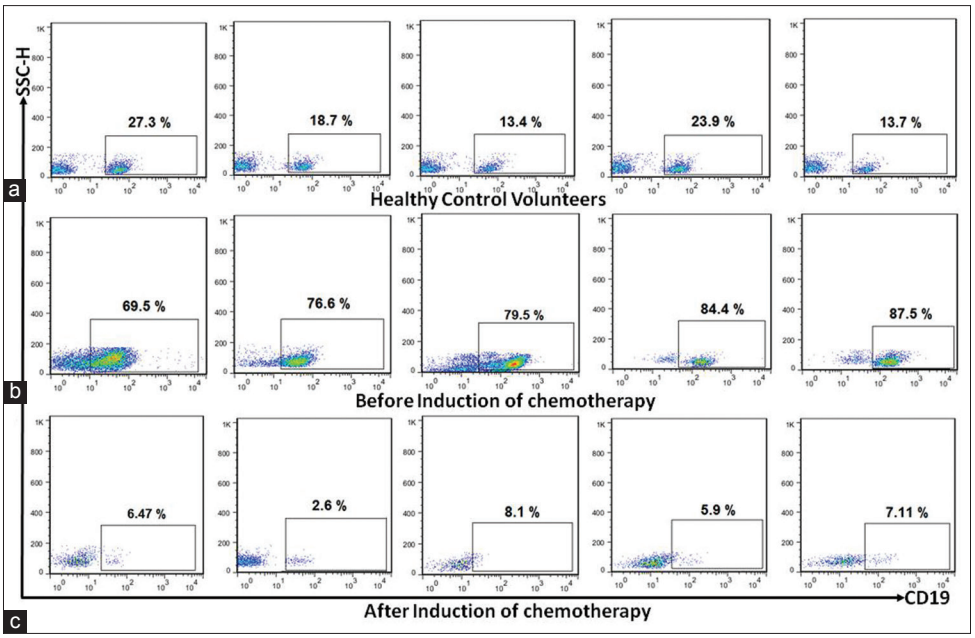

Figure 1: A representative flow cytometric analysis showing the frequencies of CD19+ B-cells in B-acute lymphoblastic leukemia pediatric patients. CD19+ B-cells numbers in five healthy control volunteers (a), 10 patients before (b), and 10 patients after (c) the induction of chemotherapy

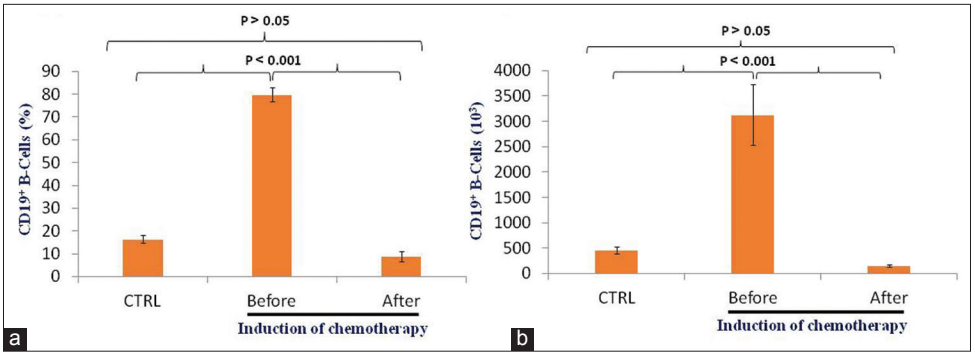

Figure 2: A representative statistical analysis showing the percentages and absolute numbers of CD19+ B-cells in B-acute lymphoblastic leukemia pediatric patients. CD19+ B-cells percentages (a) and absolute numbers (b) before and after induction of chemotherapy as compared to healthy volunteers "CTRL." The absolute numbers of CD19 cells were calculated as: (Total white blood cells [cells/μL] × CD19+ %)/100

numbers were almost recovered upon the induction of chemotherapy.

Our findings are consistent with previous studies[14,15] which showed that BM cells were replaced by more than 70% blasts, affecting the production of the normal blood cells including CD33+ myeloid cells.[16] The increases in the numbers of these cells after chemotherapy indicate mobilization of myeloid cell precursors from BM to circulation or their differentiation during anti-B-ALL chemotherapy. Given that CD33+ cells are expressed on both normal cells and regulatory myeloid cells in particular MDSCs,[13] these results warrant further studies to characterize the immunoregulatory phenotypes of these suppressive cells.

Of note, the blood samples were drawn from the recruited patients at different time points during induction of chemotherapy. This would explain why some of the recruited patients under treatment during the induction phase of chemotherapy showed decreased numbers of CD33+ cells than other patients. One possible explanation is that those patients were in "nadir" time point, whereas significantly decreased numbers of blood cells, particularly WBCs and platelets were reported at this point.[17] The nadir point usually extends to 10 days after the induction of chemotherapy, although this may vary depending on the drugs given.[14,17] The other possible explanation is that those patients might have not achieved a complete remission since 15–20% of children with B-ALL show relapse where progressive repopulation of blasts occur in excess of 5% to more than 25% in the BM of patients.[17]

Although the mechanism behind why B-ALL patients fail chemotherapy is not clear; one potential mechanism which has been suggested by our and other groups in solid tumors is

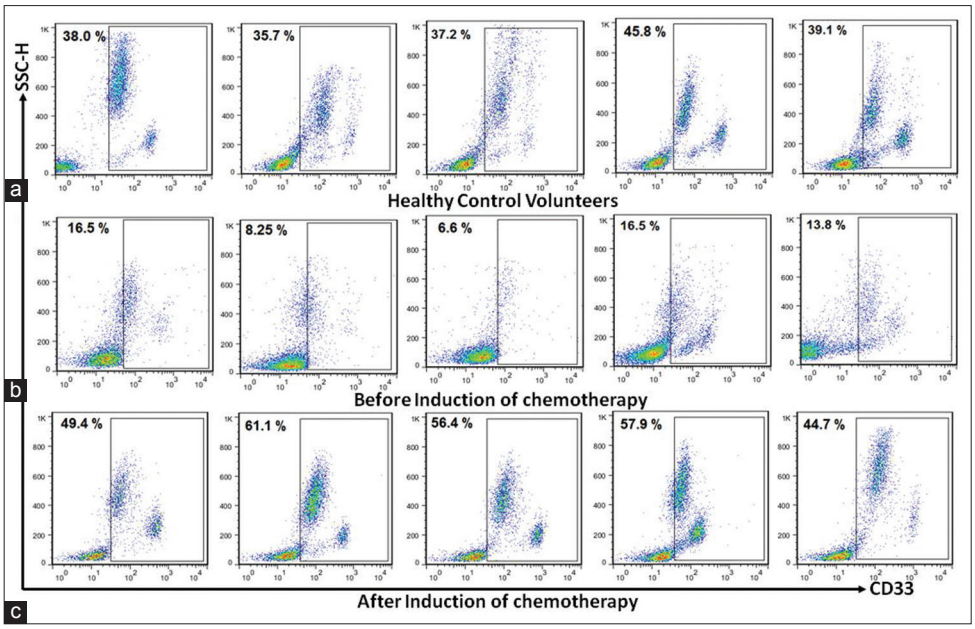

Figure 3: A representative flow cytometric analysis showing the frequencies of CD33+ myeloid cells. CD33+ myeloid cells numbers in five healthy control volunteers (a), 10 patients before (b), and 10 patients after (c) the induction of chemotherapy

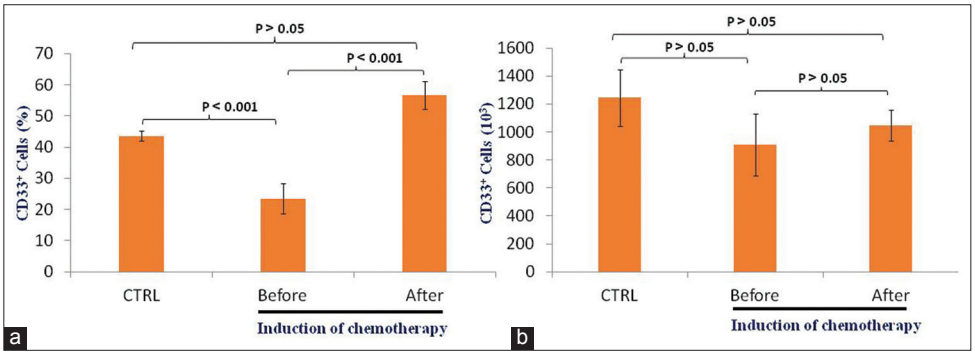

Figure 4: A representative statistical analysis showing the percentages and absolute numbers of CD33+ myeloid cells in B-acute lymphoblastic leukemia pediatric patients. CD33+ myeloid cells percentages (a) and absolute numbers (b) before and after induction of chemotherapy as compared to healthy volunteers "CTRL." The absolute numbers of CD33+ cells were calculated as: (Total white blood cells [cells/μL] × CD33+ %)/100

the emergence and accumulation of immune regulatory cells including MDSCs.[18] These MDSCs have the capability to shut down the functionality of anti-tumor T-cells both in a cell-cell contact and production of inhibitory mediators.[19] MDSCs are considered a heterogeneous population of myeloid cells with suppressive activity, containing precursors of CD33+ populations.[20] Previous studies including ours showed significant increases in MDSCs in cancer patients subsequent to cyclophosphamide-based chemotherapy,[21,22] resulting in suppression of immune responses. We and others also have recently reported increases in the numbers of MDSCs in hepatitis C virus patients as well as in cirrhotic and noncirrhotic liver cancer patients.[23] It might be possible that similar immunosuppressive CD33+ cells emerge in B-ALL in particular after induction of chemotherapy since it also includes cyclophosphamide. As such few previous studies focused on MDSCs suppressive role in hematologic malignancies including multiple myeloma.[24]

Given the crucial role of host microenvironment to shape the quality and quantity antitumor immunity in the setting of adoptive T cell therapy,[25-31] it is important to understand the immune cellularity, in particular MDSCs.

To the best of our knowledge, this study is the first to investigate the numbers of CD33+ myeloid cells in Egyptian children with B-ALL. Indeed, based on our preliminary results of the increases in the numbers of these cells, we are performing ongoing studies to characterize the numbers of MDSCs in B-ALL patients.

CONCLUSION

The data of this study shed a light on the effect of leukemia on CD33+ myeloid cells, and the impact of chemotherapy on these cells. Further studies are ongoing by our group to characterize the exact phenotypes of CD33+ cells in B-ALL patients.

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Conflicts of interest

There are no conflicts of interest.

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