ORIGINAL ARTICLE

Regulatory T Cells in Egyptian Acute Myeloid Leukemia Patients

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ABSTRACT

Introduction: Current treatments for AML have not changed for several decades and have not resulted in satisfactory outcomes. Modulating the immune system may improve survival in AML patient. Increasing evidence shows that Treg cell may play an important role in immune evasion mechanisms employed by cancer This work was designed to measure the frequency of regulatory cells T cells (Treg(in newly diagnosed adult acute myeloid leukemia patients (AML) and to correlate its percent with cytogenetic study and clinical outcome. **Material and methods:** This study was conducted on 50 subjects divided into two groups: 25 AML cases(group 1) admitted to Hematology Unit, Alexandria Main University Hospital, Egypt and 25 healthy subjects(group 2) of matched age and sex. Detection of Treg was done to both groups by Multi-Color Flow Cytometry Kit. **Results:** Treg value was higher in AML patients at diagnosis compared to healthy controls. No significant difference was present in the percent of Treg between the patients with normal and abnormal karyotype. Patients who achieved complete response after induction chemotherapy had lower Treg percent compared to those with persistent leukemia. **Conclusion:** From this study we can conclude that higher pretreatment Treg percentage may be a poor predictor to response to induction therapy.

Key words: acute myeloid leukemia, flow cytometry, prognosis, T regulatory cells

INTRODUCTION

Acute myelogenous leukemia (AML) represents a group of clone hematopoietic stem cell disorders that result from genetic alterations in normal hematopoietic stem cells.[1,2] The AML microenvironment is immunosuppressive and anti-apoptotic favoring the survival of malignant hematopoietic cells.[3] In vitro studies have shown that AML cells secrete factors, which inhibit T cell activation and proliferation and limit pro-inflammatory T helper-1 cytokine production. This suppressive effect is reversed, when Tregs and other T lymphocytes are removed from the microenvironment in vitro, leading to augmented immune responses to AML.[4]

Current treatments for AML have not changed for several decades and have not resulted in satisfactory outcomes. Modulating the immune system may improve survival in AML patients.[5]

Access this article online	
Website: www.actamedicainternational.com	Quick Response code
DOI: 10.5530/ami.2015.1.23	

Treg cells consist of an anergic lymphocyte population representing 1–10% of total CD4 T cells in thymus, peripheral blood and lymphoid tissues.[6] CD4 Treg cells express the transcription factor FOXP3[7] andexist in two categories: Naturally occurring (nTreg) and induced (iTreg). nTreg cells develop during the normal process of T cell maturation in the thymus and survive in the periphery.[8] Treg cells are genetically controlled and are influenced by antigen recognition, and various signals, in particular, cytokines such as interleukin-2 and transforming growth factor-Beta 1, which control their activation, expansion, and suppressive effectors activity.[9] In contrast, i Treg CD4 cells acquire their suppressive activity as a consequence of in vivo or ex vivo activation under unique stimulatory conditions.[10]

Treg cells contribute to the maintenance of self-tolerance. They can actively suppress the function of auto-reactive cells that escape thymic negative selection.[11] Increasing evidence shows that Treg cell may also play an important role in immune evasion mechanisms employed by cancer.[12]

The aim of the present work was to measure the percent of Treg cells in newly diagnosed adult AML and to correlate it with cytogenetic study and clinical outcome.

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MATERIALS AND METHODS

This study consisted of 50 adult subjects divided into two groups. Group 1 consisted of 25 newly diagnosed AML cases admitted to Hematology Unit, Alexandria Main University Hospital, Egypt. Sixteen (64%) were males and 9 (36) were females. Their age ranged from 20-52 years with a mean (SD) of 30.72 (9.46) years. Group 2 consisted of 25 healthy subjects of matched age and sex.AML patients were classified according to FAB classification.[13] Patients were treated by 7+3 protocol. Patients were considered in complete remission (CR) if bone marrow blasts is less than 5%.[13]

All patients were subjected to full history taking and clinical examination, complete blood count[14] using an automated cell counter (ADVIA2120), bone marrow aspiration[15], cytogenetic study[16] using G-banding and immune-phenotyping[17] using MACS Quant Flow-cytometer analyzer equipped with MACS Quantify software version. A panel of M0Abs specific to myeloid, lymphoid and cytoplasmic clusters of differentiation antigens was used. Detection of Human Regulatory T Cell was done to both groups by Multi-Color Flow Cytometry Kit.[18, 19]

Measurement of Human Regulatory T Cell (CD4+CD25+ Foxp3+)

This kit contains three conjugated antibodies.that can be used for single-step staining of human regulatory T cells: CD25-PE (Clone 24212; mouse IgG2A), CD4-PerCP (Clone 11830; mouse IgG2A), and FoxP3-APC (goat IgG). The kit also contains Flow Cytometry FoxP3 Staining Buffer.

Procedure

1. Human peripheral blood mononuclear cells (PBMCs) (about 1 x 10^6 cells per sample) were washed with 2 mL of phosphate buffer saline (PBS), by spinning at 300 x g for 5 minutes, using 5 mL flow cytometry tubes.
2. All remaining PBS was removed and the samples tube re-suspended in 100 µL of Flow Cytometry Staining Buffer.
3. 10 µL of CD4-PerCP and 10 µL of CD25-PE antibodies were added (R&D Systems, Catalog IC003P).
4. The mixture was incubated for 30 - 45 minutes at 2 - 8° C in the dark.
5. Following the incubation, excess antibody was removed by washing the cells with 1 mL of Flow Cytometry FoxP3 Staining Buffer.
6. After decanting the Flow Cytometry FoxP3 Staining Buffer, 10 µL of FoxP3-APC antibody was added to the samples in the remaining small volume of buffer (about 100 µL).
7. The mixture was incubated for 1 hour at 2 - 8° C in the dark.
8. Following the incubation, excess antibody was removed by washing the cells with 1 mL of Flow Cytometry FoxP3 Staining Buffer.
9. The final cell pellet was re-suspended in 200-400 µL of Flow Cytometry Staining Buffer for flow cytometric analysis
10. During analysis, a gate was set around the required cell population and then percent of T reg was detected.

Data Analysis

Data were analyzed using the Predictive Analytics Software (PASW Statistics 18). Qualitative data were described using Chi-square test. Quantitative data were described as mean and standard deviation. For normally distributed data, comparison between two independent populations was done using independent t-test. For abnormally distributed data, Mann-Whitney Test was used to analyze two independent populations. Correlations between two quantitative variables were assessed using Spearman coefficient. Significance of the obtained results was judged at the 5% level.

RESULTS

Demographic data and laboratory findings of the studied groups are shown in Tables 1 and 2 respectively. Extra-medullary manifestations such as spleenomegaly, lymphadenopathy and gum hypertrophy were found in 13 (52%) of studied patients. Of all AML patients, 8 cases were M1 (32%), 7 cases were M2 (28%), 6 cases were M4 (24%) and 4 was M5 (16%) according to FAB classification. Of the AML patients, 16 had normal karyotypes (64%) and 9 had abnormal karyotypes (36%). Correlation between Treg percent and bone marrow blasts percent is shown in Table 3 while comparison between Treg

Table 1: Demographic data of the two studied groups

Parameter	Cases	Controls	Statistics
Age (years), mean (SD)	30.72 (9.46)	31.92 (7.82)	t=2.57, P=0.627
Male	16	15	χ^2=0.085, P=0.771
Female	9	10	

P is significant at≤0.05, χ2: Chi-square test, t: Student t-test

Table 2: Comparison of laboratory data and percent of Treg in AML patients and controls

Parameter	Cases	Controls	MW	P value
	Mean (SD)	Mean (SD)		
WBCs× 10^9/l	11.27 (32.12)	6.26 (1.96)	116	p<0.001*
Platelets× 10^9/l	70.64 (46.98)	237.55 (48.29)	0	p<0.001*
Hb g/dl	7.56 (2.19)	13.02 (0.92)	22	p<0.001*
BM blasts %	53.48 (18.11)			
CD4 of total lymphocytes cell	9.39 (11.17)	15.64 (8.10)	155.5	p=0.002*
CD4/CD25/FOXP3%	0.32 (0.43)	0.02 (0.04)	66.5	p<0.001*

MW: Mann Whitney test; *: Statistically significant at P≤0.05, BM: Bone marrow

percent and response to chemotherapy and cytogenetic study is shown in table 4

Patients who achieved complete remission after induction chemotherapy had significantly lower percent of Treg at presentation compared with patients who had persistent leukemia (P = 0.016). There was no significant difference in the percentage of circulating Treg between patients with normal and abnormal karyotypes. But there was a statistically significant difference between the two groups regarding CD4+% (p=0.036)

DISCUSSION

In this study, we evaluated the percentage of Treg obtained from the peripheral circulation of AML patients at diagnosis by human regulatory cell multi-color flow cytometry kit. Treg have been classically defined based on high FoxP3 expression [20].The flow cytometry-based method accurately gate for CD4.[21,22] From a technical point of view, peripheral blood sampling for Treg measurement may be better than bone marrow in routine clinical settings since bone marrow processing is more difficult due to its contents of fat tissues and its lower lymphocyte proportions.[23]

As evident from the results, the mean value of the frequency (SD) of circulating CD4+ T cells was higher in the healthy controls 15.64 (8.10) than in AML patients 9.39 (11.17) while the frequency (SD) of circulating CD4+CD25+ Foxp3+ Treg was higher in AML patients 0.32 (0.43) compared with normal persons 0.02 (0.04). To gain further insights into the role played by Treg in acute leukemia, we also evaluated the percentage of Treg in relation to response to induction chemotherapy. We found that patients who achieved complete remission after induction chemotherapy had a significantly lower pretreatment Treg percentage compared with patients who had persistent leukemia. In comparison with solid malignancies, relatively little information is available about functional characteristics of Treg or their clinical significance in patients with acute leukemia.[24]

AML is clinically, cytogenetically and molecularly heterogeneous.[25] Age at the time of diagnosis has the largest impact on the probability of remission and on duration of survival. Our AML patients consisted of 9 females and 15 males with mean (SD) age of 30.72 (9.46) years. No significant difference was present between AML cases and healthy controls as regards age and sex.

Karyotype diagnosis provides the most important prognostic information.[26, 27] We have not detected any significant difference in the percentage of circulating Treg between the patients who have a normal karyotype and those with abnormal karyotypes (p= 0.202).

AML cases are divided into 8 subtypes (M0-M7) according to FAB classification.[13] In our study, the patients fell into four of eight subtypes of the FAB classification: 8 M1 (32%), 7 M2 (28%), 6 M4 (24%) and 4 M5 (16%). There was no significant difference in the percentage of circulating Treg between different subtypes of FAB classification. Furthermore, we did not detect any correlation between the percentage of circulating Treg cells and blast cells percent in the bone marrow. The percentage of Treg at diagnosis was lower in patients who had achieved complete remission compared to those with persistent leukemia.

The frequency of Treg cells in the peripheral blood may thus be used as a biomarker for predicting sensitivity to chemotherapy, monitor disease status and evaluate disease progression.[28] The elimination of Tregs appears to increase the rate of complete response. The increased CR rate may allow more patients to become eligible for potentially curative therapies such as stem cell transplantation.[29]

Table 3: Correlation between percent of T reg cells and percent of blast cells in bone marrow

Parameter	Blast cells % in bone marrow	
	r_s	P
CD4 of total lymphocytes	0.181	0.386
CD4/CD25 and FOXP3	−0.233	0.263

r_s: Spearman coefficient; *: Statistically significant at P≤0.05

Table 4: Comparison between Treg cells and response to therapy and Cytogenetic study results

Parameter	CD4 of total PMNC	CD4 CD25 FOXP3 of total PMNC
	Mean (SD)	Mean (SD)
Response to therapy		
Complete remission (n=9)	5.73 (4.54)	0.10 (0.12)
Persistent leukemia (n=16)	11.46 (13.26)	0.44 (0.49)
MW	52	29.5
P value	0.258	0.016*
Cytogenetic		
Normal (n=16)	12.25 (13.02)	0.24 (0.34)
Abnormal (n=9)	4.31 (3.32)	0.46 (0.55)
MW	35	69.5
P value	0.036*	0.202

MW: Mann Whitney test., *: Statistically significant at p≤0.05

CONCLUSION

Higher pretreatment Treg percentage may be a poor predictor to response to induction therapy. However, further studies on a large number of patients are still needed.

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How to cite this article: Hamed NA, El-Halawani N, Nafie D, Swelem R, Araby AA. Regulatory T Cells in Egyptian Acute Myeloid Leukemia Patients. Acta Medica International. 2015;2(1):134-137.

Source of Support: Nil, **Conflict of Interest:** None declared.

Handling Editor: Nidhi Sharma