Bone marrow lymphocyte subsets in newly diagnosed acute myeloid leukemia patients and their relation to standard prognostic factors and response to induction therapy

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ABSTRACT

Aims: Immune responses act as a surveillance and protective system against malignant cells. Thus, the aim of this work was to study different lymphocyte subsets in bone marrow (BM)of newly diagnosed acute myeloid leukemia (AML) patients and identify their prognostic significance.

Study design: Cohort study.
Place and Duration of Study: Hematology department of Ain-Shams University Hospital (ASUH) from July 2017 till March 2018.
Methodology: This study was conducted on 33 newly diagnosed AML patients, all were subjected to peripheral blood count and flow cytometric immunophenotyping on bone marrow (BM) blasts (using acute leukemia panel in addition to monoclonal antibodies to detect different BM lymphocyte subsets); whereas cytogenetic studies using fluorescence in situ hybridization (FISH) technique were performed to determine risk groups. The patients' remission status following induction therapy (day28) was determined.

Results: Natural killer (NK) cells were relatively elevated (median 15.9%) in t (15:17), while the median percentage of T- cytotoxic (Tc) [43.5%], T-helper (Th) [39.5%] and NK-T cells [39.9%] were higher in t (8:21). Percentage of BM total lymphocytes showed a significant negative correlation with both total leukocyte count (r=-0.645, p<0.001) and percentage of BM blasts (r=-0.348, p=0.047), with positive correlation with platelet count (r=0.42, p=0.015). A numeric cutoff of 5% and 48% for both total BM lymphocytes and T-cytotoxic cells, respectively were associated with good response to induction.
Conclusion: Total BM lymphocytes and their subsets in BM of newly diagnosed AML patients were different from normal values. High total BM lymphocytes, T-helper, cytotoxic and B-cells were associated with complete remission to induction therapy.

Keywords: Acute myeloid leukemia (AML), Fluorescence in situ hybridization (FISH), Natural killer (NK), lymphocyte subsets

1. INTRODUCTION

Acute myeloid leukemia (AML) represents a group of clonal hematopoietic stem cell disorders with uncontrolled proliferation and accumulation of myeloblasts [1]. The discovery of new prognostic and predictive markers is mandatory to improve prognostication and help inventing novel therapeutic strategies. Immune responses act as a surveillance and protective system against malignant cells for their eradication [2]. Concerning the role of different immune cells in many neoplasms; both natural killer (NK) cells and CD8+ T-cytotoxic (Tc) act by their cytolytic activities in elimination of neoplastic cells, while B-cells act by releasing anti-tumor antibodies and other inhibitory effects [3].
The proportions of various immune cells in the bone marrow (BM) vary in different types of myeloid neoplasms and their relative numbers at diagnosis may correlate with prognosis [4]. In AML, many researchers have focused on the immunophenotypic and genetic aberrations

of neoplastic cells, lacking behind the surrounding non-neoplastic immune system cells, therefore a particular focus has been placed on NK cells, identifying functional links between NK cell activity and AML prognosis; likewise T-cells have been shown to be critical players in AML progression [5, 6]. Thus understanding the different lymphocyte subsets at beginning of AML is critical for development of new immunotherapeutic strategies. Therefore in this study, we aimed to study different lymphocyte subsets in BM of newly diagnosed AML patients and their relation to standard prognostic factors and response to induction therapy.

2. MATERIAL AND METHODS

2.1 Patient cohort

The present study was conducted on thirty-three newly diagnosed AML patients presented to hematology department of Ain-Shams University Hospital (ASUH) from July 2017 till March 2018. This study was approved by the ethical committee of faculty of medicine Ain-Shams University, a verbal informed consent was taken from all subjects participating in this study. Their ages ranged from 19-81 years with a mean of 41 years, eighteen were males and fifteen were females. All patients were subjected to full medical history and thorough clinical examination, the diagnosis of AML was established following the WHO classification [7]. In all cases, a retrospective review of their hemogram data, peripheral blood smears, bone marrow aspirates, results of flow-cytometric immunophenotyping and cytogenetic analysis (FISH) in selected cases for risk group stratification. Clinical follow up was done for all studied patients to detect response to induction therapy at day 28. Patients were treated with cytarabine and daunorubicin or idarubicin, with the exception of AML with t (15; 17) (q24; q21); PML/RARA who all received all-Trans retinoic acid [8]

2.2 Multiparametric flow cytometry

Flow cytometry (FCM) was performed on (1 ml EDTA) BM samples by NAVIOS 2 laser 6 color FCM [Beckman coulter, USA]. The acute leukemia panel of fluorescein isothiocyanate (FITC)/ Phycoerythrin (PE) - conjugated monoclonal antibodies (Beckman coulter, life science, Hielach, USA) were used for diagnosis and sub-classification of AML. Sample was considered positive for any marker if ≥20% of cells were expressing it, except for CD34 and MPO if only ≥10%. Gating was done on the residual normal BM lymphocyte population based on forward and side scatters and their bright expression of CD45. Those gated lymphocytes were analyzed for the percentages of CD3 + (PC5) CD4+ (FITC) [T-helper], CD3+(PC5)CD8+ (PE) [T-cytotoxic], CD3+(FITC)CD16+CD56+(PE) [NK-T], CD3-CD16+CD56+(PE) [NK] and CD19+(PE) CD20+(FITC) [B-cells] (Figure1).

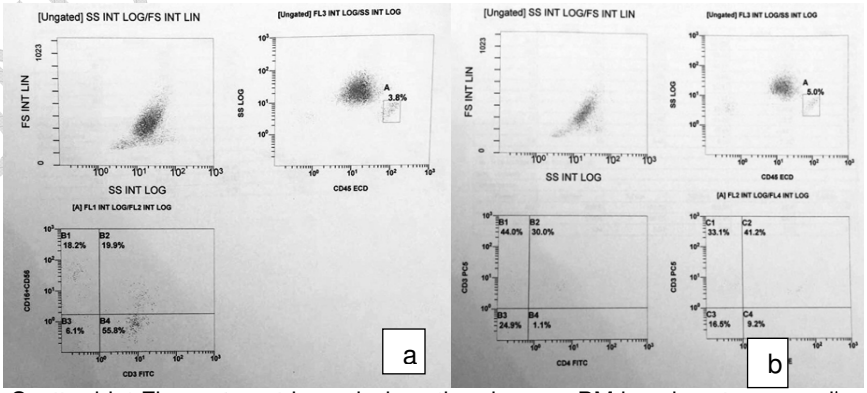

Figure (1): Scatter blot Flow cytometric analysis gating done on BM lymphocytes according to forward (FS) versus side scatter (SS) and CD45 versus SS (a) CD3/CD16CD56 (b) CD3CD4 / CD3CD8 expression.

2.3 Cytogenetic studies

FISH analysis was performed on BM samples collected on Li-heparin tubes; at least 100 interphase nuclei were scanned for the detection of the signals by cytovision automated cytogenetis platform [Leica Biosystems Richmond, USA]. The used probes were Vysis RUNX1/RUNX1T1 double fusion probe, PML/RARA single fusion and BCR/ABL single fusion probe. A cut off value for diagnosis of positive results was > 10% for single fusion probe and >3% for double fusion probe. This research did not receive any specific grant from funding agencies in the public, commercial, or not for profit sectors.

2.4 Statistical analysis

In addition to descriptive analysis, data was analyzed using SPSS version 20 (international business machines corporation, New York, 2010) statistical package, for analytical statistics; Mann Whitney test, Kruskal Wallis test, Fisher's exact test, correlation analysis (using spearman's method) were used in addition to logistic multi-regression analysis and a receiver operating characteristic (ROC).

3. RESULTS AND DISCUSSION

Clinical cohort

Patients were classified by WHO 2016 classification as AML with recurrent genetic (RGA) abnormalities (11 of 33; 33.3%), AML not otherwise specified (NOS) (19 of 33; 57.6%) and AML with myelodysplastic related changes (MRC) (3 of 33; 9.1%). Patients were further grouped according to response to induction therapy into responders (11 of 33; 33.3%) and non-responders (22 of 33; 66.7%). Other clinical and laboratory data are summarized in (Table1&2).

Table (1): Clinical data of the thirty-three newly diagnosed AML patients

Clinical Parameter			**Range (Mean ± SD)/ Number(percentage)**
Age (years)			19-81 (41.45 ± 17)
Gender	Male		18 (54.5%)
	Female		15 (45.5%)
AML subtypes	NOS N=19 (57.6%)	M1	5 (15.2%)
		M2	10 (30.3%)
		M4	3 (9.1%)
		M5	1 (3.0%)
	RGA N=11 (33.3%)	t (15; 17)	6 (18.2%)
		t (8; 21)	2 (6.1%)
		11q23 rearrangement	2 (6.1%)
		inv(16)	1 (3.0%)
	MRC		3 (9.1%)
Cytogenetic risk group	Favorable		9 (81.8%)
	Unfavorable		2 (18.2%)
Response to induction therapy	Complete remission		11 (33.3%)
	Partial remission		6 (18.2%)
	Death		16 (48.5%)

RGA: recurrent genetic abnormalities, NOS: not otherwise specified, MRC: myelodysplasia related changes.

Table (2): Laboratory data of the thirty-three newly diagnosed AML patients

Laboratory Parameter		Range [(Mean ± SD) or (Median IQR)*]
Hematological Parameters	TLC (x 10^9/L)	0.8-327 [24 (4-44)]*
	Hb (g/dL)	4.8 - 12.1 (8.03 ± 1.9)
	PLT (x 10^9/L)	6 - 119 (36.55 ± 32)
	Peripheral Blast (%)	0-96 (67.21 ± 25.28)
	BM blast (%)	40-99 (76.42 ± 17.8)
Total lymphocytes in BM by FCM (%) [a]		1-46 [4.95 (3-7.4)]*
Lymphocyte subsets	NK cells (%)	1-35 (12.43 ± 8.85)
	NK-T cells (%)	3 - 57 (20.01 ± 13.55)
	T-helper cells (%)	18-77 (34.61 ± 14.63)
	T-cytotoxic cells (%)	19-73 (39.84 ± 12.93) [41 (30.6- 48.1)]*
	B cells (%)	1-35 [2 (1-7)]*

[a] Total BM lymphocyte percentage is out of the total BM cells and lymphocyte subsets percentages are out of the BM lymphocytes.TLC: total leukocytic count, Hb: hemoglobin, PLT: platelets, BM: bone marrow, NK: natural killer cells, NK-T cells: natural killer T cells, SD: standard deviation, *IQR: interquartile range.

Lymphocyte subsets in AML patients

Total BM lymphocytes percentage (by FCM) in the studied AML patients ranged from 1-46% with a median of 4.9%; of which the mean of NK cells' percentage was 12.43% and that for NK-T, Th and Tc were 20%, 34.6% and 39.8%,respectively while the median of B cells was 2%. Although there was no statistically significant difference between different AML subtypes and both total BM lymphocytes percentage and their different subsets, but it seemed that B cells percentage was higher in AML-RGA especially in t (8; 21) with a median of 12%.

NK cells in t (15;17) showed their highest percentages (median 15.9%), NK-T cells' percentage was increased in t(8;21), FAB M4 and M5 with a median of 40% and 25% respectively. T-helper cells' percentage was increased in t(8;21), 11q23 rearrangement and AML-MRC with median value of 39.5%, 35% and 40%, respectively. T-cytotoxic cells showed higher percentages among all AML patients in comparison to other lymphocyte subsets in contrast to B-cells that showed the lowest percentage (Figure 2).

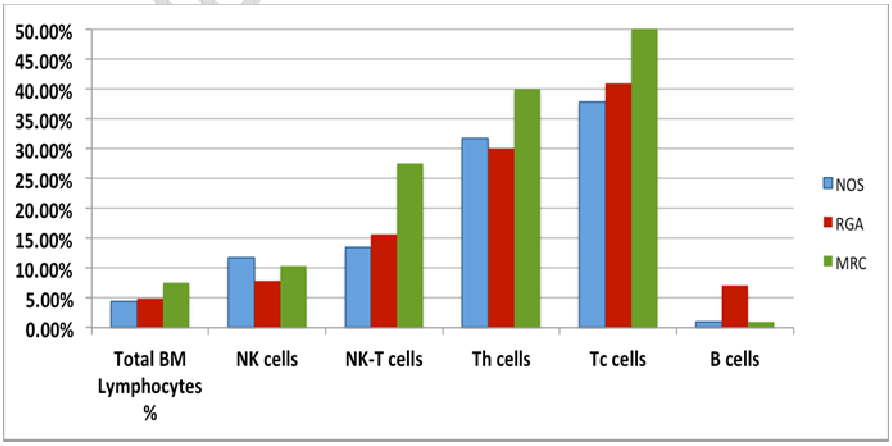

Figure (2): Lymphocyte subsets in different AML main subtypes (y-axis indicates percentages of all lymphocytes. NK-T, T-helper and T-cytotoxic cells' percentages were highest in AMLwith myelodysplastic related changes).

Lymphocyte subsets and relationship to different parameters and prognosis

There was a significant negative correlation between percentage of BM total lymphocytes and both total leukocytic count (TLC) (r = -0.645, p<0.001) and BM blasts percentage (r= -0.34, p=0.047), while a significant positive correlation was found with the platelet count (r= 0.42, p<0.001). B cells showed weak negative correlation with patients' age (r= 0.34, p=0.025) (table 3). Comparing responders and non-responders to induction therapy, the TLC and BM blasts were significantly higher in non-responders group (p<0.001 & p= 0.047), although no statistically significant difference was found in different lymphocyte subsets in both groups, the percentages of total BM lymphocytes, NK-T, Tc and B-cells were higher in responders than non-responders (table 4). On performing multiple logistic regression analysis, it revealed that Th, Tc and B cells together were significantly higher in responders (p=0.026), also both low BM blast cells percentage and high total BM lymphocytes percentage associated significantly with responders group (P= 0.001). Using receiver operating characteristic curve (ROC), it was found that a cut off of 5% for total BM lymphocytes can discriminate between responder and non-responder groups being higher than 5% in responder group. As for Tc, the best cut off value for discrimination was 48% (Table 5; Fig.3).

Table (3): Correlation between bone marrow lymphocyte subsets and clinical and laboratory parameters in 33 acute myeloid leukemia patients

parameter		Total lymphocytes %	NK cells %	NK-T cells %	T-helper cells %	T-cytotoxic cells %	B cells %
Age (years)	rs	0.15	0.24	0.02	-0.032	-0.21	-0.34
	p value	0.41	0.18	0.92	0.859	0.24	0.025*
TLC (x 10^9/L)	rs	-0.645	0.241	-0.233	-0.005	-0.005	-0.158
	p value	<0.001*	0.176	0.192	0.978	0.978	0.189
Hb (g/dL)	rs	0.21	-0.23	0.13	0.212	0.04	0.17
	p value	0.25	0.20	0.47	0.236	0.82	0.169
PLT (x 10^9/L)	rs	0.42	-0.27	-0.15	0.271	-0.25	0.10
	p value	0.015*	0.12	0.42	0.127	0.16	0.289
Peripheral blast %	rs	-0.199	0.292	-0.256	-0.132	-0.239	0.013
	p value	0.351	0.166	0.227	0.464	0.262	0.471
BM blast %	rs	-0.348	0.200	-0.142	-0.071	-0.262	-0.141
	p value	0.047*	0.265	0.431	0.695	0.141	0.217

rs: Spearman rank correlation coefficient, Asterisk indicates P-value <0.05.

Table (4): Comparison between responders and non-responders in 33 acute myeloid leukemia patients regarding clinical, laboratory parameters and lymphocyte subsets

Parameters	Responders (complete remission) N=11 Median (IQR)	Non-responders (partial remission and death) N=22 Median (IQR)	p value[a]	Sig
Age (years)	37 (24-50)	39.5 (28-57)	0.276	NS
TLC (x 10^9/L)	9 (4-24)	39 (11.5-93.5)	0.028*	S
Hb (g/dL)	9 (7-10)	8 (6-9)	0.072	NS
PLT (x 10^9/L)	29 (19-35)	19.5 (13-53.5)	0.528	NS
Peripheral blast %	6 (0-77)	67 (29.5- 87.75)	0.067	NS
BM blast %	70 (52-76)	87.5 (75- 94.25)	0.003*	S

Total Lymphocytes % in BM	6.75 (5 - 7.5)	4.08 (2.35 - 6)	0.054	NS
NK cells %	9.4 (5.2 - 12.6)	12.4 (5.2 - 18.2)	0.390	NS
NK-T cells %	19.2 (10.7 - 32.2)	13.75 (7.2 - 27.9)	0.222	NS
T-helper cells %	32 (23 - 41)	31.3 (25 - 40)	0.674	NS
T-cytotoxic cells %	43.8 (36.6 - 55.2)	39.3 (27.5 - 43.3)	0.113	NS
B cells %	4 (1 - 14)	1 (1 - 5)	0.068	NS

[a] Mann whitney test, Asterisk indicates P-value <0.05, N= number of patients, Sig.: significance, NS: non-significant, S: significant, IQR: interquartile range, TLC: total leukocytic count, Hb: hemoglobin, PLT: platelets, BM: bone marrow, NK: natural killer cells, NK-T cells: natural killer T cells.

Table (5): Best cut-off value of lymphocyte subsets for the prediction of non-responders in 33 acute myeloid leukemia patients.

Parameters	AUC	95% CI	p value	Sig.	Cutoff point	Sensitivity	Specificity
Total lymphocytes % in BM	0.709	0.525-0.853	0.028*	S	4.95	68.18	81.82
T-cytotoxic cells %	0.671	0.487-0.824	0.116	NS	48.3	90.91	45.45

Asterisk indicates P-value <0.05, AUC: area under the curve, Sig.: significance, NS: non-significant, S: significant, BM: bone marrow.

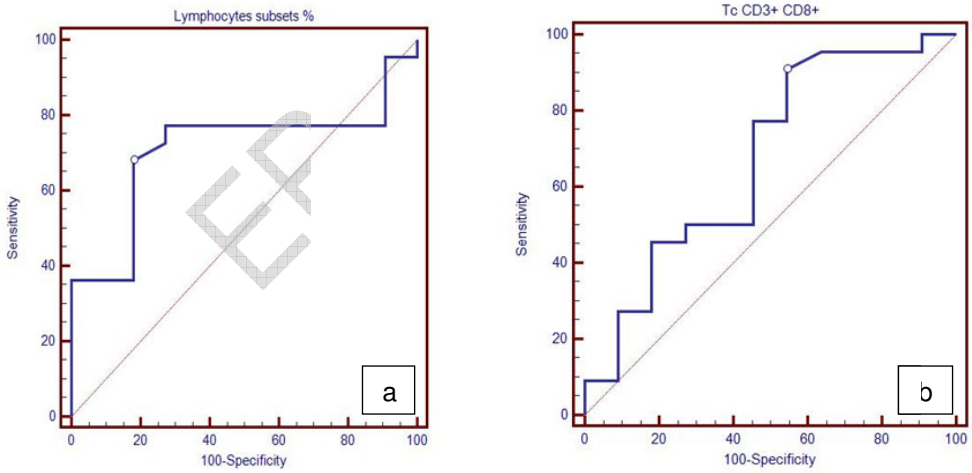

Figure (3): ROC curve analysis for discriminating responders from non-responders to induction therapy in 33 acute myeloid leukemia patients.
a) BM total lymphocytes percentage.
(b) T-cytotoxic cells percentage.

Discussion:

Many researches in AML has focused on understanding the immunophenotypic and genetic aberrations of neoplastic cells, resulting in better risk- stratification but still the treatment modalities does not change [9]. In this context, the role of the surrounding non-neoplastic immune system cells has gained increasing interest in AML. Our study of different

lymphocyte subsets in 33 newly diagnosed AML patients revealed a median total BM lymphocytes percentage of about 5% which were lower than previously reported normal percentages of 15.6%, 10 and 15.1% [10, 11, 4]. This could be explained by BM infiltration with the hostile microenvironment created by AML. The mean value of NK-T cells was 20%, higher than normal values (11%) reported by **Aggarwal et al** [4], that was suggested to be a compensation for the deficient CD1d molecule [12], however, this increase was not found to prevent disease progression due to lack of their cytotoxic function. In our study the B-cells showed the lowest percentages (2%) among all other lymphocyte subsets compared to the mean value of 6.5% and 12.5% reported in AML [11,13].

In this study, although there was no statistically significant difference between lymphocyte subsets in all AML subtypes. However NK cells were relatively higher in those with t(15;17) versus other AML-RGA, the same as reported by **Alcasid et al [14] and Ismail and Abdulateef [13];** this could be related to special compensatory mechanisms from the immune system to overcome immune escape from T-cell. The percentages of Tc, Th and NK-T cells were relatively higher in t(8;21) cases versus other AML-RGA, Previous researches focused on detailed relation of *RUNX1* gene on T-cell development [15, 16, 17]. NK-T and NK cells were found to be relatively higher in FAB M4/M5 than other AML-NOS subtypes, which could be attributed to the frequent association of CD56 with FAB M5, also in addition to uniform expression of CD1d antigen by the monocytes and myelomonocytic leukemic cells [18].

In our study, AML with MRC showed a lower percentage in B-cells with highest median percentages in Th and Tc, a finding not matching with the fact that AML-MRC is of poor prognosis [19]. However investigation for NPM and bi-allelic *CEBPA* is essential to exclude those specific groups that have a more favorable prognosis, unfortunately, no molecular studies were done to our patients.

On correlating different lymphocytes subsets with different hematological parameters, we found that the total BM lymphocytes percent showed a negative correlation with TLC and BM blast percentage and a positive correlation with platelet count. Those parameters were of prognostic value in AML patients as reported by **Greer et al [20].** This assumes that high total BM lymphocytes percentage is correlated to good prognosis that was shown in our results where total lymphocytes percentage was apparently higher in the group who achieved complete remission. We additionally identified that a numeric cut off of 5% for total BM lymphocytes and 48% for Tc were associated with good response to induction**; Ismail and Abdulateef [13]** reported an elevated T-cell percentage in responder group, they considered these lymphocytes an effective frontline in the host's immune response to leukemic blasts.

4. CONCLUSION

Total BM lymphocytes and their subsets in BM of newly diagnosed AML patients were different from normal values reported in the literatures. High total BM lymphocytes, T-helper, cytotoxic and B-cells were associated with complete remission to induction therapy.

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COMPETING INTERESTS

No conflict of interest.

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