Identifying Leukemia-associated Immunophenotypes in Acute Myeloid Leukemia Patients using Multiparameter Flow Cytometry

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ABSTRACT

Objectives: To identify leukemia-associated immunophenotypes in fifty acute myeloid leukemia patients at diagnosis using an eight color multiparameter flow cytometry panel and to detect if they showed any alteration in relapse/refractory cases.

Methods: Eight color multiparameter flow cytometry panel with CD45/SSC log gating strategy was used for analysis of leukemia-associated immunophenotypes in fifty acute myeloid leukemia patients presenting to Alexandria University Hospitals at diagnosis, relapse and refractory cases. Twenty bone marrow samples from patients performing bone marrow aspirate for non-malignant hematological indications of matched age and sex were included as controls.

Results: Leukemia-associated immunophenotypes were observed in 43 cases (86%). Only one aberrant immunophenotype was identified in four cases (8%), while two to twelve aberrant immunophenotypes were found in the other 39 cases (78%). *Strong leukemia-associated immunophenotypes* were obtained by the combination of CD2, CD4, CD56 with either CD34 or CD117, in contrast to CD19 which has to be combined with CD117. Refractory cases showed the presence of same LAIPs at both initial diagnosis and persistent

disease, while one of the relapsed cases showed acquisition of new leukemia-associated immunophenotypes after relapse.

Conclusion: The good choice of leukemia-associated immunophenotypes depends on their specificity rather than their frequency. The results of this study can help in increasing the sensitivity of leukemia-associated immunophenotypes strategy in minimal residual disease using multiparameter flowcytometry in acute myeloid leukemia patients which is considered an important post-diagnosis parameter associated with prognosis and clinical outcome.

Keywords: Acute myeloid leukemia (AML); multiparameter flow cytometry (MFC); leukemia-associated immunophenotypes (LAIPs); minimal residual disease (MRD); complete remission; relapsed AML; refractory AML.

INTRODUCTION

Being a heterogeneous disease, acute myeloid leukemia (AML) is highly variable in cytogenetic and molecular characteristics, which are considered important prognostic factors during diagnosis, and play an essential role in risk stratification and clinical decision making.[1,2] Adult AML patients can achieve hematological complete remission CR with bone marrow blasts <5%,[3] through intensive chemotherapy regimens. Nevertheless, most of these patients, about 60% to 70%,[4] will eventually relapse.[5] The persistence of leukemic cells after chemotherapy unidentified by routine morphologic evaluation is known as minimal residual disease (MRD),[6] and is the main cause of relapse.[4]

The abnormal expression of immunophenotypic markers that distinguishes the leukemic cells is known as leukemia-associated phenotypes (LAIPs),[7] which are not present or are only very infrequently present on normal bone marrow cells.[6,8,9] LAIPs in AML include; asynchronous antigen expression, cross-lineage antigen expression, antigen under/over-expression and absence of lineage-specific antigens.[6,7] LAIPs can be used to determine MRD

by multiparameter flow cytometry (MFC).[5,6] It has been shown that detection of LAIPs varied from 50% to almost 100%, depending on different laboratories and protocols.[5,6] No standard method has been established using MFC. Recently some recommendations were published in a consensus document from the European LeukemiaNet (ELN) MRD Working Party.[10]

The change in aberrant immunophenotype during disease progression imposes a technical difficulty and limitation for MRD detection using LAIPs by MFC which limits its diagnostic performance and explains the high rate of relapse associated with MRD negative patients.[11] Increasing the number of fluorochromes (at least eight) in one tube allows identification of higher number of LAIPs thus improving the performance of MRD analysis using this strategy.[12,13]

Alteration of LAIPs can occur during disease relapse,[5,14-16] this suggests that relapse might be due to either clonal evolution or occurrence of secondary AML due to chemotherapy treatment effect.[5] However, in most of these studies the detection of immunophenotype changes was focused on the subgroup of patients who displayed highly aberrant phenotypes, which comprise 60% to 80% of all AML cases.[15] In the light of this data, we used an eight color MFC panel to identify LAIPs in fifty AML patients at diagnosis and to detect if they showed any alteration in relapse/refractory cases.

METHODS

Samples

The present study was conducted on fresh bone marrow samples obtained from fifty consecutive, unselected AML patients (de novo and secondary AML) at diagnosis. Twenty bone marrow samples from patients of matched age and sex performing bone marrow aspirate for non-malignant hematological indications were included as controls to determine the specificity of LAIPs. The study was performed in Alexandria University Hospitals (AUHs),

Egypt during the period of September 2019 to December 2020. Patients and controls gave written informed consent after the purpose and investigational nature of the study and its potential risks were explained. The study was carried out according to AUHs protocols and was approved by the Medical Ethics Committee of Alexandria Faculty of Medicine before it started. AML cases were diagnosed based on morphologic findings, immunophenotyping, and cytogenetics.[1,17] Patients characteristics are shown in **(Table 1)**. The cytogenetic/ molecular risk categories were defined according to ELN risk stratification by genetics.[3] Refractory AML was defined as inability to achieve complete remission following one or two cycles of standard combination chemotherapy. While, relapsed AML was defined as any proof of disease recurrence after complete remission is achieved. Complete remission was defined as <5% blasts present in the BM, absence of circulating blasts and blasts with Auer rods in addition to >1 x 10^9/L neutrophils and >100 x 10^9/L platelets in the peripheral blood (PB).[3]

Table 1 : Patient characteristics.

Clinical characteristics	**All patients (n=50)**
Age, median (range) (y)	28.5 (3-80)
Gender, No (%)	
Male	21 (42%)
Female	29 (58%)
WBC, median (range) (x10^3/μL)	49.375 (0.7- 362.26)
Hemoglobin, median (range) (g/dl)	8 (4.4-12.5)
Platelets, median (range) (x10^3/μL)	31.5 (10-418)
BM blasts, median (range) (%)	79.5 (24-97)
According to WHO Classification,[17] No (%)	
AML with recurrent genetic abnormalities	
AML with t(8;21)(q22;q22)	2(6)
APL with *PML-RARA*	2(4)
AML-MRC	1(2)
AML,NOS	
AML with minimal differentiation	1(2)
AML without maturation	21(42)
AML with maturation	2(4)
Acute myelomonocytic leukemia	7(12)
Acute monoblastic and monocytic leukemia	14(32)
FAB subtype, No (%)	
M0	1(2)

M1	21(42)
M2	4(8)
M3	2(4)
M4	8(16)
M5	14(28)
Cytogenetics/molecular risk stratification, No (%)	
Low risk	5(10)
Intermediate risk	36(72)
High risk	9(18)

WBC: white blood cells; No: number; y, years; APL: acute promyelocytic leukemia; AML-MRC: acute myeloid leukemia with myelodysplasia related changes; AML,NOS: acute myeloid leukemia, not otherwise specified; FAB: French-American-British classification.

MFC

Fresh bone marrow samples were lysed according to the EuroFlow standard operating protocol (SOP) for bulk lysis for MRD panels.[18] *(see www.EuroFlow.org for Version 1.3, updated on 25 june 2018)* The final volume of cells was counted using cell counter ADVIA 2021i *(Siemens, Germany)*, and the concentration was adjusted to 20,000 cells/μL in 1 ml PBS and well mixed by vortex. 100μL of the prepared sample solution was pipetted in each tube to be stained with all the fluorochrome-labeled monoclonal antibodies (MoAb) [19] shown in **(Table 2)** in the order of designed/screening panel shown in **(Table 3)**.

Table 2: Monoclonal antibodies used in immunophenotyping.

Antibody	Clone	Conjugation	Source
HLA-DR	TU36	FITC	BD biosciences, California, USA
CD15	HI98	FITC	BD biosciences, California, USA
CD22	HIB22	FITC	BD biosciences, California, USA
CD11c	B-ly6	FITC	BD biosciences, California, USA
CD13	WM15	PE	BD biosciences, California, USA
CD133	W6B3C1	PE	BD biosciences, California, USA
CD56	B159	PE	BD biosciences, California, USA
CD11b	ICRF44	PE	BD biosciences, California, USA
CD34	8G12	PerCP	BD biosciences, California, USA
CD117	104D2	PE-Cy7	BD biosciences, California, USA
CD14	M5E2	APC	BD biosciences, California, USA
CD19	HIB19	APC	BD biosciences, California, USA
CD4	SK3	APC	BD biosciences, California, USA

CD7	M-T701	APC-H7	BD biosciences, California, USA
CD2	RPA-2.10	APC-H7	BD biosciences, California, USA
CD33	WM53	BV421	BD biosciences, California, USA
CD45	HI30	V500	BD biosciences, California, USA

FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, Peridinin-Chlorophyll-Protein; PE-Cy7, phycoerythrin-Cy7 tandem conjugate; APC : Allophycocyanin ; APC-H7: Allophycocyanin-H7 tandem conjugate; BV421: Brilliant Violet 421; V500: Violet 500.

Table 3: Designed/screening panel uesd for identifying LAIPs at diagnosis.

Tube	FITC	PE	PerCP	PE-CY7	APC	APC-H7	BV421	V500
1	HLA-DR	CD13	CD34	CD117	CD14	CD7	CD33	CD45
2	CD15	CD133	CD34	CD117		CD2	CD33	CD45
3	CD22	CD56	CD34	CD117	CD19		CD33	CD45
4	CD11c	CD11b	CD34	CD117	CD4	CD64	CD33	CD45

FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, Peridinin-Chlorophyll-Protein; PE-Cy7, phycoerythrin-Cy7 tandem conjugate; APC : Allophycocyanin ; APC-H7: Allophycocyanin-H7 tandem conjugate; BV421: Brilliant Violet 421; V500: Violet 500.

Data Acquisition and analysis

A minimum of $1x10^5$ cells was acquired using BD FACS Canto II *(BD biosciences, California, USA).* Samples were analyzed using FACS Diva software v 8.0.2 *(BD biosciences, California, USA).*

Statistical analysis

Median percentages of LAIPs positive cells in AML and normal bone marrow were calculated and compared.

RESULTS

Using the CD45/SSC gating strategy

Identification of blasts was done using CD45/SSC log gating strategy excluding low FSC (non-viable cells, erythroid cells) within the FSC/SSC plot. Sequential-gating strategies using immature/primitive markers as CD34 and CD117 were used to better define the blast population especially in monocytic leukemia cases where there is no clear- cut between blasts and monocytic population. CD14 back-gating was performed to exclude any monocytes, and consequently the LAIPs were defined on blast populations. CD45/CD34/CD117 were used in addition to different myeloid and lymphoid markers in an eight color combination for LAIPs detection. The gating strategy used to identify LAIPs is shown in **(Figure 1, also see**

supplementary figures S1 for gating strategy in $CD34^{Negative}/CD117^{Negative}$AML, S2 for more examples of LAIPs).

Identification of LAIPs

Clinical and laboratory criteria of 50 AML patients are shown in **(Table 1)**. LAIPs were observed in 43 cases (86%). Only one aberrant immunophenotype was identified in four cases (8%), but in the other 39 cases (78%), two to twelve aberrant immunophenotypes were identified (2 LAIPs, 8 cases; 3 LAIPs, 8 cases; 4 LAIPs, 4 cases ; 5 LAIPs, 8 cases ; 6 LAIPs, 5 cases; 7 LAIPs,3 cases ; 8 LAIPs,1 case ; 9 LAIPs, 1 case ; and ;12 LAIPs, 1 case). The most frequent LAIPs identified was in order of frequency; lack of antigen expression (73 times), followed by asynchronous antigen expression of progenitor cell markers and differentiation markers (58 times) and cross-lineage antigen expression (41 times). The percentage of AML cells carrying specific LAIPs was assessed in each case and ranged from 7% to 99.6 % (median, 64.9%). (**Table 4)**

Table 4: Frequency of LAIPs in 50 AML patients and healthy bone marrow.

LAIPs	Number of cases*	% of positive cells from AML BM [a]	% of LAIPs from control BM [b]	LAIPs classification in categories of specificity[13]
Cross-lineage antigen expression (n=41)				
$CD34^+/CD7^+$	12	7- 96.6	0.01(0.00-0.03)	Good
$CD117^+/CD7^+$	9	7- 96.6	0.01(0.00-0.03)	Good
$CD117^+/CD19^+$	8	8- 82	<0.01(0.00-0.01)	Strong
$CD34^+/CD2^+$	5	8- 92	<0.01(0.00-<0.01)	Strong
$CD117^+/CD2^+$	4	8- 92	<0.01(0.00-<0.01)	Strong
$CD34^+/CD4^+$	1	89.3	<0.01(0.00-0.01)	Strong
$CD117^+/CD4^+$	1	82.8	<0.01(0.00-0.01)	Strong
$CD33^+/CD4^+$	1	94.8	<0.01(0.00-0.01)	Strong
Asynchronous Expression (n=58)				
$CD34^+/CD56^+$	8	20- 79	<0.01(0.00-0.01)	Strong
$CD117^+/CD56^+$	8	28- 87.1	<0.01(0.00-0.01)	Strong
$CD33^+/CD56^+$	9	17- 97	<0.01(0.00-0.01)	Strong

$CD34^+/CD11b^+$	5	23- 86.7	<0.01(0.00-0.09)	Good
$CD117^+/CD11b^+$	8	17.2- 84.9	<0.01(0.00-0.09)	Good
$CD34^+/CD64^+$	6	13- 91	<0.01(0.00-0.01)	Strong
$CD117^+/CD64^+$	11	14- 95	<0.01(0.00-0.01)	Strong
$CD34^+/CD15^+$	2	30.1-52	<0.01(0.00-0.02)	Good
$CD117^+/CD15^+$	1	57.2	<0.01(0.00-0.02)	Good
Lack of antigens (n=73)				
$CD34^+/CD13^-$	2	14.6- 98.2	0.04 (0.02-0.16)	Weak
$CD34^+/CD33^-$	5	46- 93%	0.04 (0.01-0.16)	Weak
$CD34^+/HLA\text{-}DR^-$	4	14- 95	0.04 (0.01-0.28)	Weak
$CD34^+/CD117^-$	4	9- 96.6	0.34 (0.02-1)	Weak
$CD117^+/CD13^-$	3	28- 92	0.06 (0.02-0.16)	Weak
$CD117^+/CD33^-$	5	41-91	0.05 (0.01-0.12)	Weak
$CD117^+/HLA\text{-}DR^-$	11	37- 99	0.04 (0.01-0.28)	Weak
$CD117^+/CD34^-$	21	13.3 - 99	0.31 (0.08-1.48)	Weak
$CD33^+/CD13^-$	6	39.8-98	0.03 (0.01-0.08)	Good
$CD33^+/HLA\text{-}DR^-$	12	75- 99.6	0.04 (0.01-0.16)	Weak

AML, acute myeloid leukemia; BM, bone marrow; LAIPs, leukemia-associated immunophenotypes; n, number of cases found for a defined aberrant phenotype.
*Some cases have more than 1 LAIP
[a] % of positive cells in AML BM is given as a range when more than one case was identified.
[b] Median % of LAIPs (minimum- maximum) in control bone marrow.

The most common LAIPs identified were $CD117^+/CD34^-$ in 21/50 (42%), $CD34^+/CD7^+$ in 12/50 (24%) and $CD33^+/HLA\text{-}DR^-$ in 12/50 (24%). Cross-lineage antigen expression was detected 41 times. The most frequent lymphoid antigen detected was CD7 in 12 (24%) of 50 cases, followed by CD19 in 8 (16%) cases, CD2 in 5 (10%) cases, and CD4 in 1 (2%) case. At least one myeloid marker was absent 21 times, with CD13 the most frequent marker absent 6/50 (12%) of cases.

Percentage of LAIPs positive cells within control BM samples were determined. **(Table 4)** and the median percentage for each LAIP was calculated ranging from 0.00% to 0.02%. LAIPs in control BM samples showing median percentage more than 0.1% as; $CD34^+/CD117^-$ (0.34%) and $CD117^+/CD34^-$ (0.31%) are considered to have poor specificity.

Seven Refractory cases were examined (14%), 2/7 showed no LAIPs neither at initial diagnosis nor at persistent disease, the remaining cases 5/7 showed the presence of same LAIPs

at both initial diagnosis and persistent disease. Two Relapsed cases were examined (4%), 1/2 showed no LAIPs neither at initial diagnosis nor at relapse while the other showed acquisition of new LAIPs after relapse. **(Table 5)**

Table 5: LAIPs at initial diagnosis and persistent/relapsed disease

Patient Number	LAIPs at initial diagnosis	LAIPs at persistent/relapsed disease
11 (Refractory)	CD45(dim)/CD34$^+$/CD117$^+$/ CD13$^+$ /CD33$^-$	CD45(dim)/CD34$^+$/CD117$^+$/ CD13$^+$ /CD33$^-$ **(No change)**
17 (Refractory)	CD45(dim)/CD34$^+$/CD117$^+$/ CD13$^+$ /CD33$^-$ /CD19$^+$	CD45(dim)/CD34$^+$/CD117$^+$/ CD13$^+$ /CD33$^-$ /CD19$^+$ **(No change)**
22 (Refractory)	CD45(dim)/CD34$^+$/CD117$^+$/ CD13$^+$/ CD33$^+$ /CD19$^+$	CD45(dim)/CD34$^+$/CD117$^+$/ CD13$^+$/ CD33$^+$ /CD19$^+$ **(No change)**
33 (Refractory)	CD45(dim)/CD34$^-$/CD117$^+$/ CD13$^+$/ CD33$^+$ /HLA-DR$^-$	CD45(dim)/CD34$^-$/CD117$^+$/ CD13$^+$/ CD33$^+$ /HLA-DR$^-$ **(No change)**
34 (Refractory)	CD45(dim)/CD34$^+$/CD117$^+$/ CD13$^+$/ CD33$^+$ /CD56$^+$	CD45(dim)/CD34$^+$/CD117$^+$/ CD13$^+$/ CD33$^+$ /CD56$^+$ **(No change)**
6 (Relapsed)	CD45(dim)/CD34$^+$/CD117$^+$/ CD13$^+$/ CD33$^+$ / CD4$^+$/ CD56$^+$/CD64$^+$ / CD11b$^+$	CD45(dim)/CD34$^+$/CD117$^+$/ CD13$^+$/ CD33$^+$ / CD4$^+$/ CD56$^+$/CD64$^+$ / CD11b$^+$/ **CD2$^+$**

DISCUSSION

The monitoring of MRD has become increasingly important to guide therapy in patients with AML. It plays an important role in the identification of patients with high risk of relapse. MRD detection is now used in some of recent protocols to guide patient-tailored therapy in AML.[20] However, the ability of MRD by MFC to predict relapse is still unsatisfactory, the main challenge depends on the correct choice of the number and types of LAIPs.[13] In this study we used an eight color MFC and an extensive panel of MoAb, blasts were identified with the help of CD45/SSC gating strategy depending on dim expression of the CD45 antigen. This method was validated by *AL-Mwali et al*,[6] LAIPs were detected 86% of cases. More than one LAIP was detected in 78% of cases ;one case showed up to twelve LAIPs. The

detection of several LAIPs in same case can help in cases of immunophenotypic switch during treatment, [21,22] and further improves the use of LAIPs strategy in MRD by MFC. Previously published articles,[13] used eight color MFC on normal BM with the aim of subdividing LAIPs in categories of specificity into; *strong LAIPs* (≤0.01%), *good* (>0.01% but <0.1%) and *weak* (>0.1%). Our results were similar to *Cui et al*,[5]in the order of LAIPs frequency and confirmed the findings of *Rossi et al,*[13] *and Al-Mwali et el*,[6] that *strong LAIPs* were obtained by combination of CD2, CD4, CD56 with either CD34 or CD117, in contrast to CD19 which has to be combined with CD117 to be classified as *'strong LAIPs'*. *Lack of antigen expression*, although was the most frequent group in our study (73 times), yet most of them included $CD117^{+}/CD34^{-}$ (42% of cases) and $CD33^{+}/HLA\text{-}DR^{-}$ (24% of cases) which are considered to be *'weak LAIPs'* due to their low specificity. CD33-based aberrancies unassociated with immature markers such as CD34 or CD117 were excluded from our study as they did not have a clear role as LAIPs.[13] Further investigation on a larger number of cases and correlation with cytogenetic/ molecular data should be done to conclude whether there is alteration in LAIPs in refractory/relapsed cases than those detected at diagnosis. To sum up, eight color MFC helped in better population identification and was found to be extremely useful in small specimens with fewer cells. Also, fewer tubes were used therefore, reagents and instrument time were saved. The good choice of LAIPs depends on their specificity rather than their frequency. Thus, it is important that the patients selected for MRD monitoring should have more than 2 LAIPs; at least one *'strong'*, two *'good'* or three *'weak'* LAIPs as stated by *Rossi et al*,[13] to avoid false negative results.

CONCLUSION

The good choice of leukemia-associated immunophenotypes depends on their specificity rather than their frequency. The results of this study can help in increasing the

sensitivity of leukemia-associated immunophenotypes strategy in minimal residual disease using multiparameter flowcytometry in acute myeloid leukemia patients which is considered an important post-diagnosis parameter associated with prognosis and clinical outcome.

DISCLOSURE

The authors declared no conflicts of interest whether personal or financial, no funding was received for this study.

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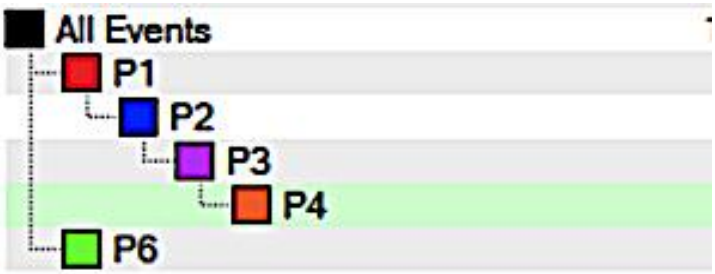
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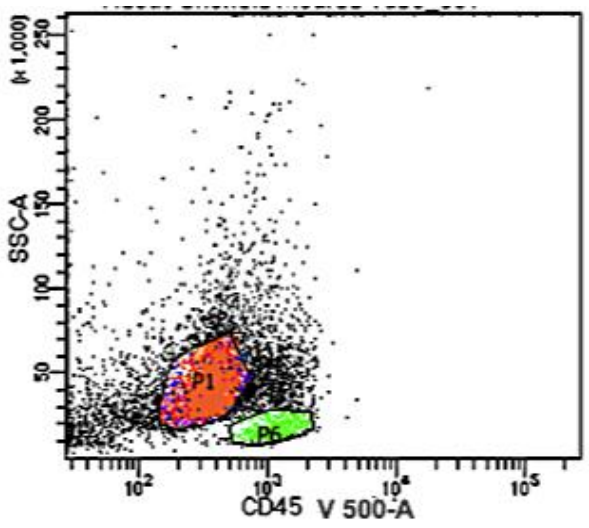
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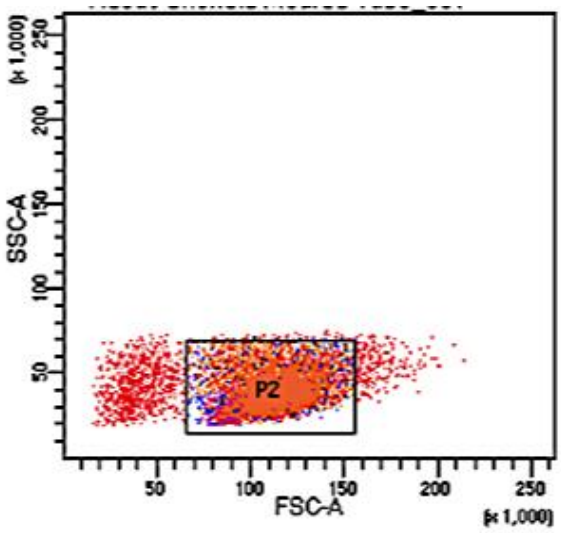
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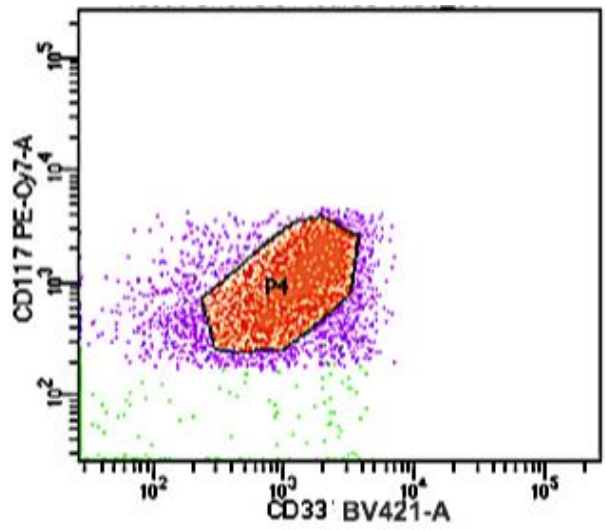
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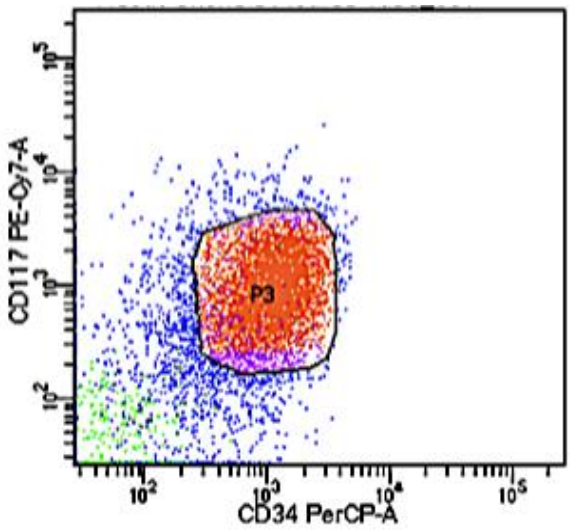
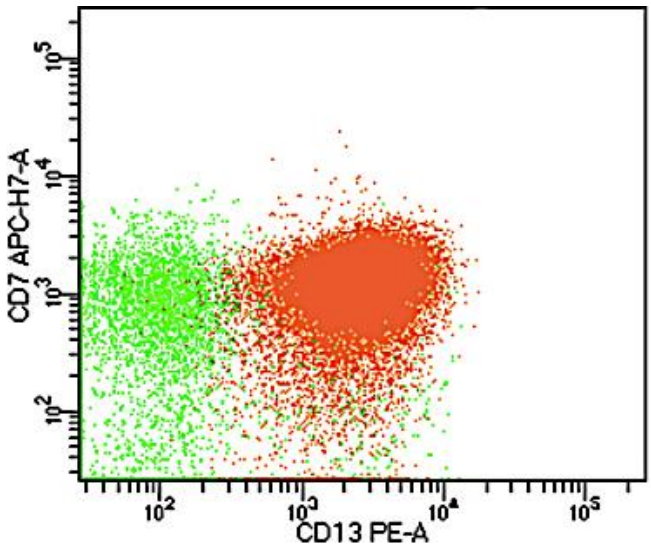
CD33 BV421-A

D

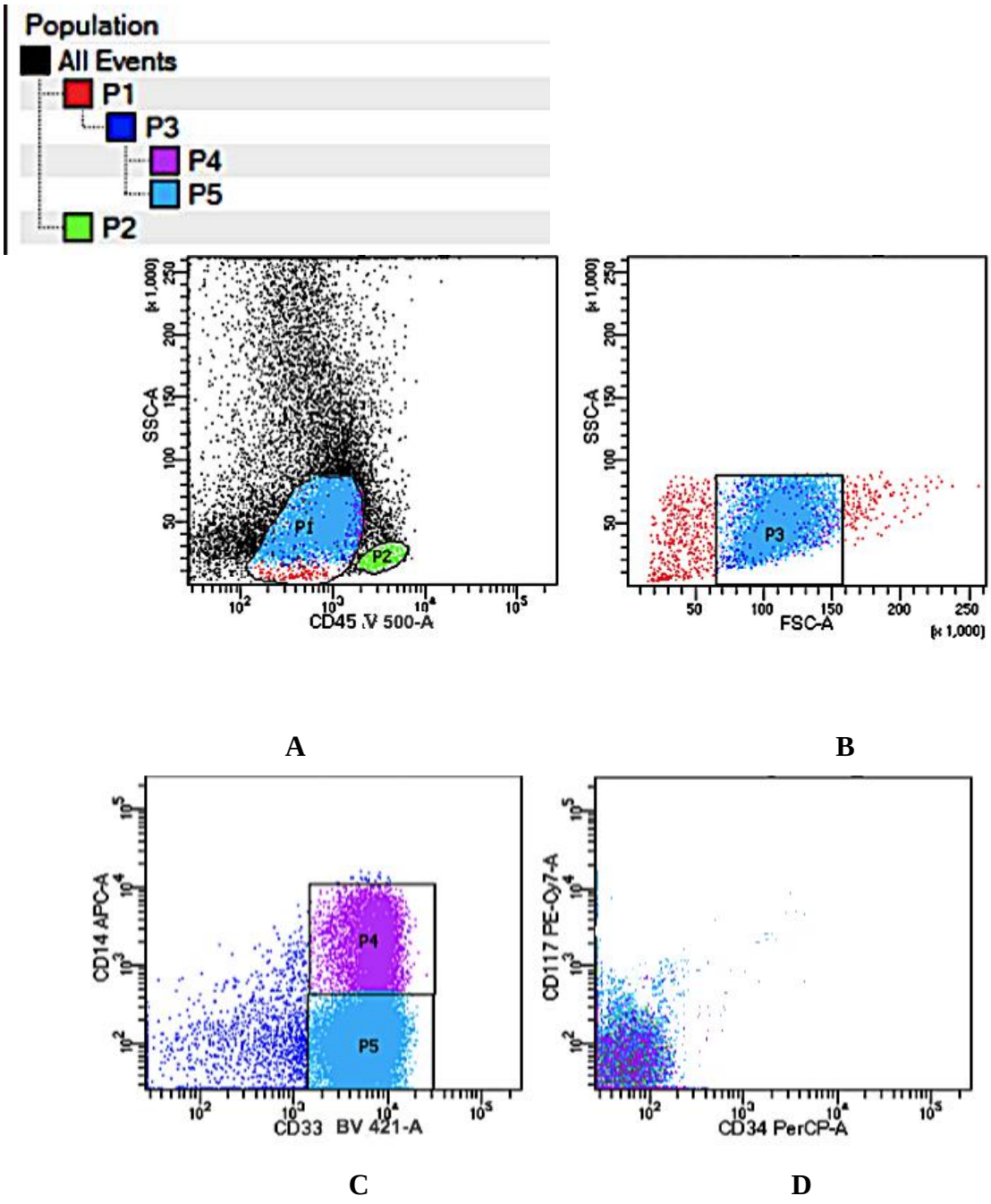

CD117 PE-Cy7-A
P3
CD34 PerCP-A

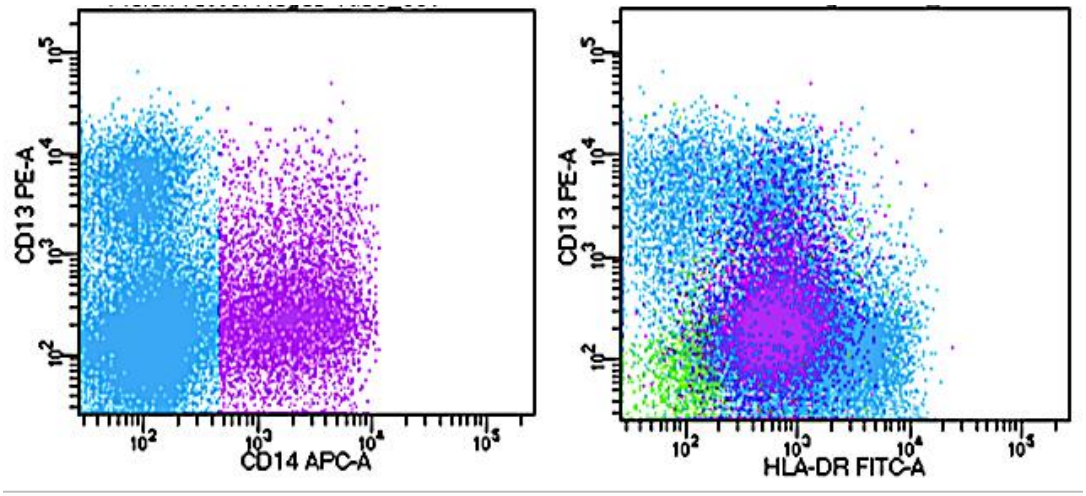
C



E

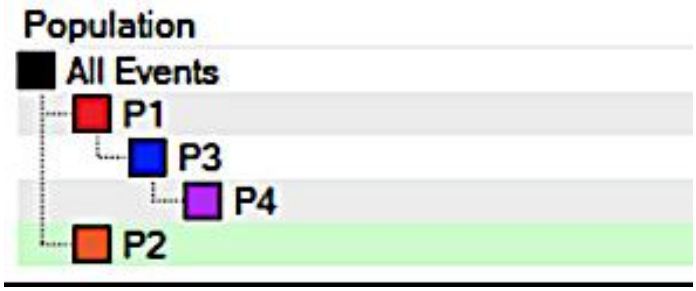
Figure 1: The gating strategy used to identify LAIPs; **A**: Identification of blasts with the help of CD45/SSC gating strategy depending on dim expression of the CD45 antigen and being low SSC (P1). Gating on lymphocytes was done as an internal control (P6/green in color) **B**: Low FSC (non-viable cells, erythroid cells) within the FSC/SSC plot were then excluded (P2). **C:** Sequential-gating strategy using immature/primitive markers CD34 and CD117 (P3). **D**: Plotting of CD117 against myeloid marker CD33 (P4). **E:** showing that our target population is CD13/CD7 double positive (Orange in color), while lymphocytes are CD7 positive only (green color).

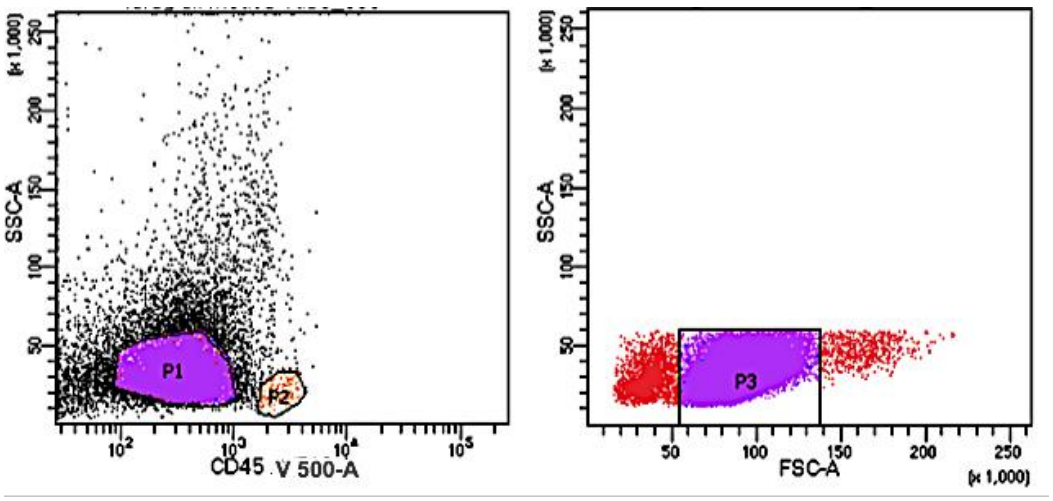

Population
All Events
P1
P3
P4
P5
P2
SSC-A
CD45 V 500-A
P1
P2
SSC-A
FSC-A
P3
A
B
CD14 APC-A
CD33 BV 421-A
P4
P5
CD117 PE-Cy7-A
CD34 PerCP-A
C
D



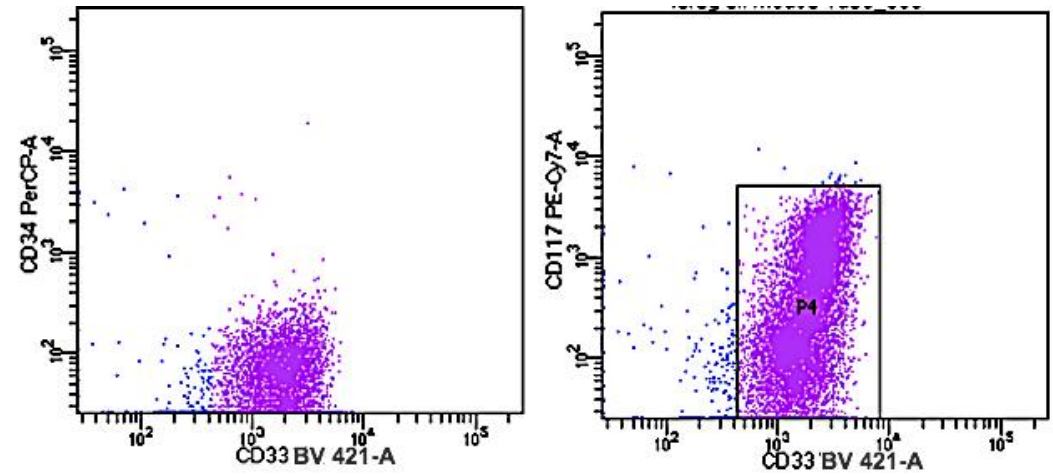
E **F**

Supplementary Figure 1 (S1): Gating strategy in CD34^{Negative}/CD117^{Negative} AML; A: Identification of blasts with the help of CD45/SSC gating strategy depending on dim expression of the CD45 antigen and being low SSC (P1). Gating on lymphocytes was done as an internal control (P2/green in color) **B**: Low FSC (non-viable cells, erythroid cells) within the FSC/SSC plot were then excluded (P3). **C:** An exclusion marker that has to be absent on the leukemic cells is used in order to exclude monocytes and/or granulocytes [23], in this case CD14 is used to exclude monocytes (P4) from blasts (P5). **D:** Shows that blasts are both CD34 and CD117 negative. **E and F**: Shows how CD14 differentiates monocytes (purple in color) from blasts (blue in color).

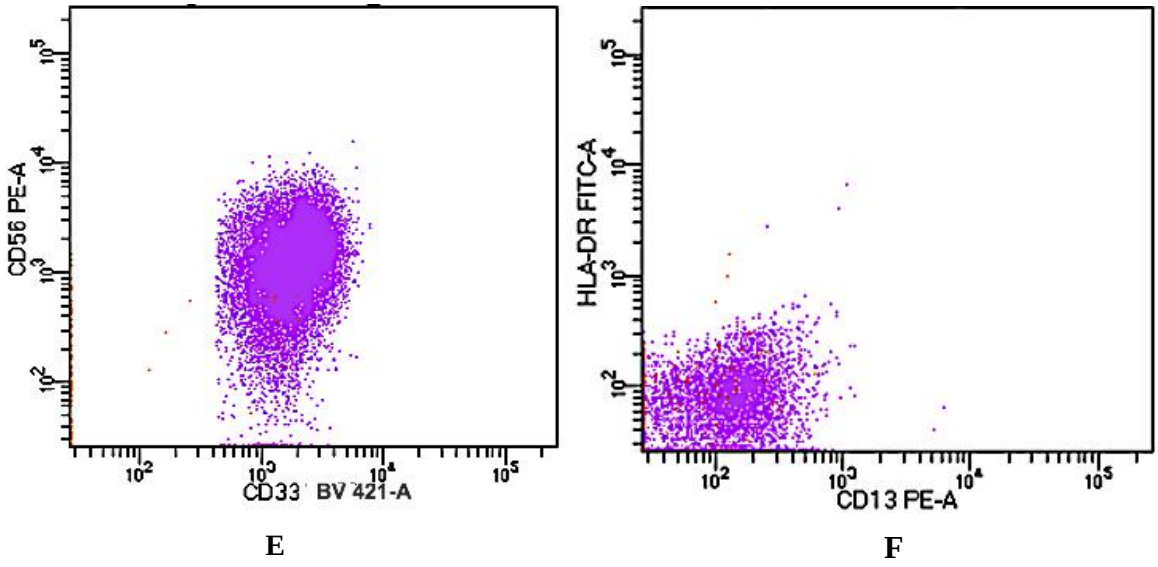

Population
All Events
P1
P3
P4
P2


SSC-A
(x 1,000)
P1
P2
CD45 V 500-A
SSC-A
(x 1,000)
P3
FSC-A
(x 1,000)

A **B**


CD34 PerCP-A
CD33 BV 421-A
CD117 PE-Cy7-A
P4
CD33 BV 421-A

C **D**



Supplementary Figure 2 (S2): A: Identification of blasts with the help of CD45/SSC gating strategy depending on dim expression of the CD45 antigen and being low SSC (P1). Gating on lymphocytes was done as an internal control (P2/orange in color/ very minimal in amount) **B**: Low FSC (non-viable cells, erythroid cells) within the FSC/SSC plot were then excluded (P3). **C:** Sequential-gating strategy using immature/primitive marker CD117 against CD33 as the case is CD34 negative. (P4). **D**: Plotting of CD33 against CD34 to demonstrate that blasts are CD34 negative. **E:** showing that our target population is CD33/CD56 double positive (purple in color), and is also **F:** both CD13 and HLA-DR negative (purple in color) , while lymphocytes are very minimal (orange in color in both **E** and **F**).