

Re-evaluation of various molecular targets located on CD34⁺CD38⁻Lin⁻ leukemia stem cells and other cell subsets in pediatric acute myeloid leukemia

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Abstract. Leukemia stem cells (LSCs) are hypothesized to be capable of driving the development of leukemia, and are responsible for disease relapse. Antibody therapy targeting cell surface antigens has significantly improved the treatment outcomes of leukemia. Therefore, it is important to identify cell surface markers that are expressed on LSCs, and that are unexpressed or expressed at reduced levels on normal hematopoietic stem cells (HSCs), in order to establish novel therapeutic targets. In the present study, the immunophenotypic characteristics of cluster of differentiation (CD)34⁺CD38⁻lineage (Lin)⁻ stem cells were analyzed, and antigen expression levels were compared with the expression of other cell components, using multicolor flow cytometry, in 54 patients with newly diagnosed acute myeloid leukemia (AML) and 11 control patients with immune thrombocytopenia. The findings indicated that CD133 and human leukocyte antigen (HLA)-DR were expressed on normal HSCs and on AML LSCs, with no significant difference ($P>0.05$). By contrast, CD33, CD123 and CD44 were highly expressed on AML LSCs, and demonstrated significant differences compared with their expression on normal HSCs (CD33, 81.7 vs. 18.3%; CD123, 75.8 vs. 19.1%; CD44, 97.7 vs. 84.4%). Among the aforementioned antigens, CD33 and CD123 were promising candidates for targeted therapy for the treatment of AML. This was particularly evident for CD123 in immature AML subtype cells, which may require additional investigation within a clinical trial setting. CD44, CD133 and HLA-DR may not be suitable for leukemia targeting due to their broad and high expression levels on normal HSCs and other tissues.

Introduction

Acute myeloid leukemia (AML) is an aggressive and heterogeneous malignant disease, which accounts for 15-20% of observed pediatric leukemia cases (1). Utilizing current treatment strategies, which are similar to those proposed in adult AML treatment protocols, complete remission rates approach 95% and the overall survival rate is ~70% in developed countries; however, the survival rate is markedly lower in developing countries (2). In addition, the prognosis for children exhibiting therapy resistance or suffering from relapse is worse than that of patients without relapsed or refractory AML (3). Increasing evidence has indicated that small populations of leukemia cells consisting of early stem/progenitor cells, which are termed leukemia stem cells (LSCs) or leukemia initiating cells, are proposed to be resistant to currently used chemotherapeutic drugs, and to mediate disease relapse (4). Effective therapeutic strategies that specifically eliminate these LSCs may, therefore, be promising for the cure of this disease.

Previous studies have indicated that normal hematopoietic stem cells (HSCs) and LSCs are negative for the expression of lineage markers (Lin), positive for the expression of cluster of differentiation (CD)34 and negative for the expression of CD38 (5). Subsequent studies revealed that a number of markers [CD33 (6), CD44 (7), CD123 (8), CD133 (9), CD32 (10), CD47 (11) and C-type lectin-like molecule-1 (12)] are expressed aberrantly on LSCs, and may be considered to be specific markers indicating the presence of AML LSCs. However, CD33 (13), CD123 (13) and CD133 (14,15) are also reported to be expressed on normal HSCs. Gemtuzumab ozogamicin (GO), an antibody against CD33 that is conjugated with the cytotoxic agent calicheamicin, was withdrawn from the market in 2010 in the United States due to low improvement in survival outcomes and high levels of hepatic toxicity (16). Furthermore, the distribution of these markers on various cell fractions in AML, including CD34⁺, CD34⁺CD38⁺ and CD34⁺CD38⁻Lin⁻ cells, remains to be elucidated. In addition, antigen expression on AML LSCs in pediatric patients has not been extensively investigated.

Therefore, the high level of inconsistency regarding antigen expression and the observed failure of clinical treatment has suggested the need to re-evaluate the expression

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levels of various antigens on stem cells, and to investigate the potential cell differentiation stage selectivity of antigen expression. In the present study, multicolor flow cytometry was utilized in order to analyze the expression of the progenitor cell marker human leukocyte antigen (HLA)-DR, as well as additional well-known stem cell markers (CD33, CD123, CD133 and CD44) on a number of cell populations, including CD34⁻, CD34⁺CD38⁺, CD34⁺CD38⁻ and CD34⁺CD38⁻Lin⁻ cells, in order to explore the potential therapeutic benefits for childhood AML.

Materials and methods

Patient and control samples. A total of 54 patients newly diagnosed with AML [French-American-British (FAB) subtypes M0-M6] (17) were examined at the Department of Hematology-Oncology, Children's Hospital of Zhejiang University School of Medicine (Hangzhou, China), following receipt of informed consent from their parents or guardians. All of the patients were diagnosed based on morphologic, immunophenotypic, cytogenetic and molecular biology, in accordance with the World Health Organization diagnostic criteria (18). Patients exhibiting <1% CD34 expression were excluded from the present study to avoid the misgating of non-leukemia cells. Control bone marrow samples were obtained from 11 patients with immune thrombocytopenia following receipt of informed consent, in accordance with the Declaration of Helsinki (19). Patient characteristics are summarized in Table I.

Flow cytometry (FCM) analysis. Direct fluorescent immunostaining was performed on heparinized (Sangor Biotech Co., Ltd., Shanghai, China) whole bone marrow cells (1x10⁶ leukocytes/tube in 100 μ l volume) for 20 min at room temperature in the dark. Subsequently, erythrocytes were lysed using FACS Lysing Solution (BD Biosciences, San Jose, CA, USA) for 10 min and washed twice using phosphate-buffered saline (pH 7.4; Sangon Biotech Co., Ltd.), supplemented with 5% fetal calf serum (Sijiqing Biotech Ltd., Hangzhou, China) and 0.02% sodium azide (Sangon Biotech Co., Ltd.). Four colors of antibody conjugates [fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/peridinin chlorophyll (PerCP)/allophycocyanin (APC)] were systematically applied in various combinations, as follows: Lin1/CD33/CD34/CD38, Lin1/CD123/CD34/CD38, Lin1/CD133/CD34/CD38, Lin1/CD44/CD34/CD38 and Lin1/HLA-DR/CD34/CD38. The Lin 1 antibody cocktail included antibodies against CD3, CD56, CD19, CD20, CD14 and CD16, labeled with FITC (cat no. 340546; BD Biosciences). Additional individual fluorophore-conjugated mouse monoclonal antibodies, purchased from BD Biosciences include: CD34-PerCP (clone 8G12; cat no. 340430), CD38-APC (clone HB7; cat no. 345807), CD33-PE (clone P67.6; cat no. 347787), CD123-PE (clone 9F5; cat no. 340545), CD44-PE (clone G44-26; cat no. 555479) and HLA-DR-PE (clone L243; cat no. 347367). CD133-PE (clone AC133; cat no. 130-080-801) was purchased from Miltenyi Biotech (Bergisch Gladbach, Germany). Isotype-matched control antibodies were utilized for each antibody reaction. Antigen expression level expressed as the percentage of positive cells among the total gated cells. For each case,

Table I. Clinicopathological features of AML patients at diagnosis.

Clinicopathological feature	Value
No. of patients (male/female)	54 (34/20)
Median age, years (range)	5.5 (1.1-14.1)
Median WBC count, x10 ⁹ /l (range)	15.4 (0.9-460)
Median hemoglobin, g/l (range)	77.5 (42-124)
Median platelet, x10 ⁹ /l (range)	43.5 (2-295)
Median bone marrow blast, % (range)	72 (30-95)
French-American-British subtypes	
M0-M1	4
M2	14
M3	5
M4-M5	29
M6	2
Cytogenetics, % (n)	
Favorable ^a	27.7 (13/47)
Intermediate ^b	46.8 (22/47)
Unfavorable ^c	25.5 (12/47)
Molecular markers, % (n)	
Fms-related tyrosine kinase 3	14.3 (4/28)
CEBPA mutations	0.0 (0/28)
c-Kit mutation	3.6 (1/28)
Nucleophosmin 1 mutations	3.6 (1/28)

^aCases exhibiting t(8;21), t(15;17) or inv(16)/t(16;16); ^bcases demonstrating a normal karyotype and other non-complex changes; ^ccases exhibiting complex abnormalities (>3 distinct aberrations), -5, add(5q)/del(5q), 7/add(7q), t(6;11) or 3q abnormalities. AML, acute myeloid leukemia; WBC, white blood cells; CEBPA, CCAAT/enhancer-binding protein α .

positivity was defined as $\geq 20\%$ cell antigen expression in gated cells. Data acquisition was performed and analyzed with a FACSCalibur™ flow cytometer using CellQuest 2000 software (BD Biosciences).

Cell fraction definitions. CD34⁻ cells were considered to represent a more mature cell population compared with CD34⁺ cells. CD34⁺ cells were additionally gated as CD34⁺CD38⁺ progenitor cells, and more immature CD34⁺CD38⁻ cells. CD34⁺CD38⁻Lin⁻ cells demonstrated increased enrichment for stem cells compared with CD34⁺CD38⁻ cells. The gating strategy is shown in Fig. 1.

Statistical analysis. Data were analyzed using SPSS version 19.0 (IBM SPSS, Armonk, NY, USA). Variables were presented as the median and range, or the mean \pm standard error. For quantitative variables, the antigen expression differences between two groups were analyzed using Mann-Whitney U tests. Comparisons of antigen expression between different cell fractions were performed using the Wilcoxon signed-rank test. $P < 0.05$ (two-sided) was considered to indicate a statistically significant difference.

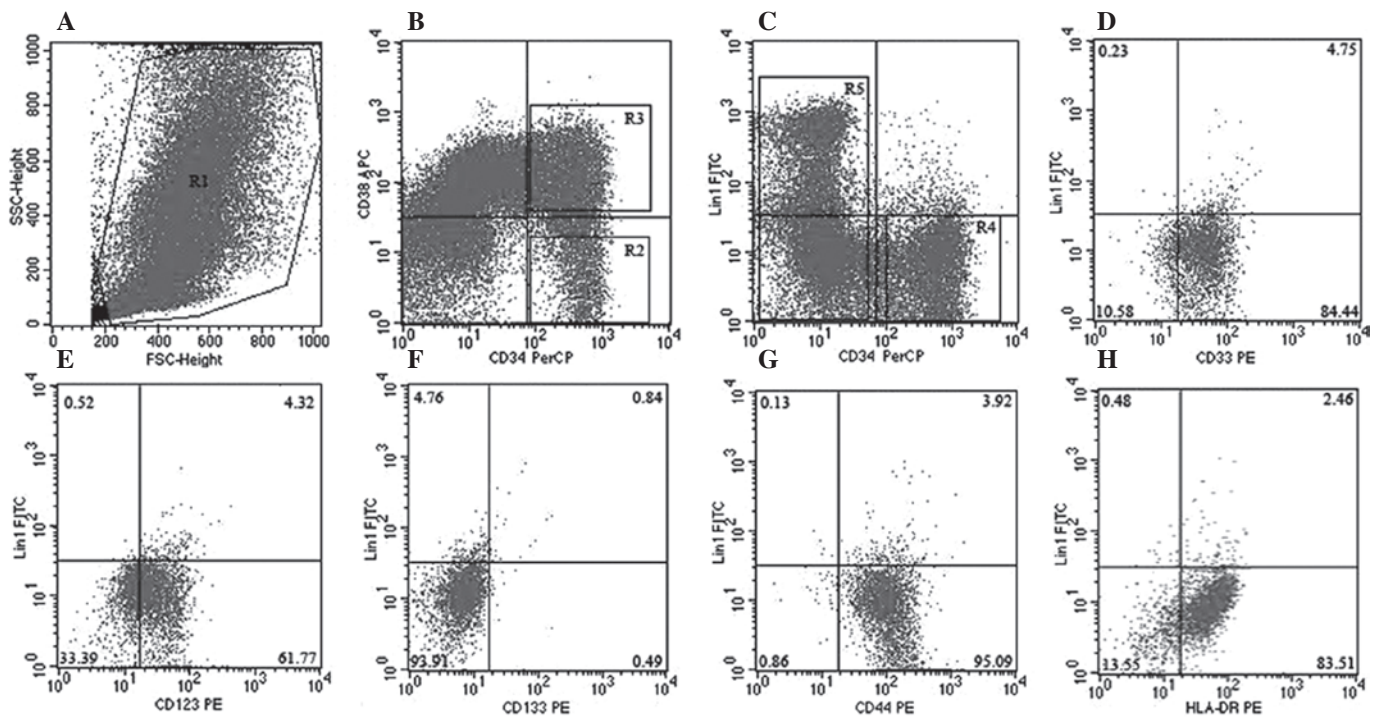


Figure 1. Gating strategy for antigen expression in acute myeloid leukemia at diagnosis. (A) Gating of cells was defined by FSC and SSC (R1). (B) Gating of the $CD34^+CD38^-$ cells (R2) and $CD34^+CD38^+$ cells (R3) within R1. (C) Gating of the $Lin1^-$ cells (R4) and $CD34^-$ cells (R5) within R1. (D) Expression of CD33 on $CD34^+CD38^-$ cells. (E) Expression of CD123 on $CD34^+CD38^-$ cells. (F) Expression of CD133 on $CD34^+CD38^-$ cells. (G) Expression of CD44 on $CD34^+CD38^-$ cells. (H) Expression of HLA-DR on $CD34^+CD38^-$ cells. SSC, side scatter; FSC, forward scatter; PerCP, peridinin chlorophyll; APC, allophycocyanin; PE, phycoerythrin; FITC, fluorescein isothiocyanate; CD, cluster of differentiation; Lin, lineage marker.

Results

Molecular targets are present on $CD34^+CD38^-$ and $CD34^+CD38^-Lin^-$ stem cells in AML samples. Patient clinicopathological features are summarized in Table I. The median age of AML patients at diagnosis was 5.5 years, with a range of 1.1-14.1 years. A total of 12/47 (25.5%) patients demonstrated unfavorable cytogenetics at diagnosis (Table I).

The median proportions of $CD34^+CD38^-$ and $CD34^+CD38^-Lin^-$ stem cells in AML at diagnosis were 1.28% (range, 0.01-20.32%) and 0.88% (range, 0.01-20.2%), compared with 0.45% (range, 0.01-3.03%) and 0.26% (range, 0.01-2.72%) in control samples ($P<0.05$), respectively. Antigen expression on $CD34^+CD38^-$ cells and $CD34^+CD38^-Lin^-$ stem cells is summarized in Table II. The expression levels of CD33 on $CD34^+CD38^-Lin^-$ cells (median, 81.7 vs. 18.3%; $P=0.002$) and $CD34^+CD38^-$ cells (median, 86.5 vs. 32.9%; $P=0.0016$) in AML samples were significantly increased compared with those of control samples. CD123 was found to be positively expressed on $CD34^+CD38^-$ cells in 49/54 (90.7%) AML cases, and on $CD34^+CD38^-Lin^-$ cells in 51/54 (94.4%) AML cases. Compared with control samples, CD123 exhibited increased expression levels on $CD34^+CD38^-$ cells (median, 75.9 vs. 21.6%; $P=0.003$) and $CD34^+CD38^-Lin^-$ cells (median, 75.8 vs. 19.1%; $P=0.003$) in AML samples. CD133 was additionally present on $CD34^+CD38^-Lin^-$ AML LSCs, however, it demonstrated reduced frequency (63% positive cases) and expression levels (median, 38.2%). In addition, no significant differences in the rates of expression of CD133 or HLA-DR on $CD34^+CD38^-Lin^-$ ($P=0.3241$ for CD133 and $P=0.4781$ for HLA-DR, respectively) and $CD34^+CD38^-$ cells ($P=0.1131$ for CD133 and $P=0.9423$ for

HLA-DR, respectively) between AML and control samples were observed. CD44 was observed to be positively expressed on $CD34^+CD38^-$ and $CD34^+CD38^-Lin^-$ cells in all AML and control samples; however, significant differences in the level of expression were identified between AML and control groups in $CD34^+CD38^-$ cells (median, 98.5 vs. 85.7%; $P=0.0003$) and $CD34^+CD38^-Lin^-$ stem cells (median, 97.7 vs. 83.1%; $P=0.0028$).

The expression of cell surface markers on stem cells differs in various subtypes of AML. When AML samples were grouped according to FAB classification, CD33 demonstrated higher expression levels on AML LSCs in M3 when compared with other AML subtypes. CD123 was preferentially expressed in M0-M1, M4-M5 and M6, but not in M2; similar observations were made for CD133. CD44 demonstrated similar patterns of expression in all AML subtypes. HLA-DR and CD133 demonstrated low expression levels on more mature M3 leukemia cells (Fig. 2). Due to the low number of samples in some subtype of AML, especially for M6 ($n=2$), we did not perform statistical analysis of the differences between different AML subtypes.

Antigen expression varies depending on the cell subset in AML. In order to demonstrate the selective expression of antigens on cells at certain stages of differentiation, cell subpopulations, characterized by the presence of specific immunophenotypes, including $CD34^-$, $CD34^+CD38^+$, $CD34^+CD38^-$ and $CD34^+CD38^-Lin^-$, were analyzed for the expression of various antigens in AML and control samples (Fig. 3).

Table II. Antigen expression analysis of leukemia stem cells exhibiting AML at diagnosis.

A, Positive/CD34 ⁺ CD38 ⁻ gated		
Antigen	Control ^a , % (range)	AML ^b , % (range)
CD33	32.9 (5.3-92.9)	86.5 (3.4-100) ^c
CD123	21.6 (1.2-84.1)	76.9 (13.9-100) ^c
CD133	15.3 (0.6-86.1)	47.3 (0-97.7)
CD44	85.7 (33.7-98.4)	98.5 (50.4-100) ^c
HLA-DR	90.5 (67-100)	93.5 (0.6-100)
B, Positive/CD34 ⁺ CD38 ⁻ Lin ⁻ gated		
Antigen	Control ^a , % (range)	AML ^b , % (range)
CD33	18.3 (8.1-88)	81.7 (3.3-100) ^c
CD123	19.1 (0-78.3)	75.8 (12.9-100) ^c
CD133	20.9 (0.2-91.6)	38.2 (0-97.9)
CD44	84.4 (60.8-99.4)	97.7 (48.7-100) ^c
HLA-DR	90.8 (56.8-94.7)	80.3 (0.7-100)

All values are presented as median percentage of cell surface marker expression on stem cells (range). ^an=11. ^bn=54. ^cP<0.05 vs. control group. AML, acute myeloid leukemia; CD, cluster of differentiation; HLA, human leukocyte antigen; Lin, lineage marker.

In general, the median expression levels of the antigens on CD34⁺ mature cells were decreased compared with that of more immature CD34⁺CD38⁺, CD34⁺CD38⁻ and CD34⁺CD38⁻Lin⁻ cell fractions, in AML and control samples. CD33 and HLA-DR were preferentially expressed on CD34⁺CD38⁺ leukemia cells in AML samples. CD133 was preferentially expressed on the most primitive CD34⁺CD38⁻Lin⁻ HSCs, whilst it was more likely to be highly expressed on CD34⁺CD38⁻ leukemia cells than CD34⁺CD38⁻Lin⁻ LSCs in AML. CD123 was preferentially expressed on CD34⁺CD38⁺ cells in AML and control samples. As an adhesion molecule, CD44 demonstrated similar expression level on the four cell populations (CD34⁻, CD34⁺CD38⁺, CD34⁺CD38⁻ and CD34⁺CD38⁻Lin⁻) in control samples, while it demonstrated increased expression levels on CD34⁺CD38⁻ leukemia cells in AML samples.

Discussion

In the present study, the expression of a variety of membrane markers was investigated in the CD34⁺CD38⁻Lin⁻ cell populations and additional cell subsets in AML samples, in order to quantify the antigen expression levels on LSCs and identify the cell subsets with preferential expression of certain antigens.

CD33 was observed to be highly expressed on AML LSCs, particularly in cases of the M3 subtype, which is similar to the results of a previous study that reported high expression of CD33 on AML blasts in acute promyelocytic leukemia cases (APL) (20). The relatively more selective expression of CD33 on CD34⁺CD38⁺ and not CD34⁺CD38⁻ leukemia

cells may lead to the ineffective elimination of LSCs by CD33-targeted therapy, thus resulting in disease relapse, and may partly explain why treatment with GO has proved to be ineffective in the majority of AML patients (21). In addition, the low antibody internalization rate and low cell surface density of CD33 presented challenges for the use of CD33 antibodies (22). Furthermore, consistent with Taussig *et al* (13), the present study identified the presence of CD33 on CD34⁺CD38⁻ and CD34⁺CD38⁻Lin⁻ cells in control samples. CD33-directed therapy has demonstrated efficacy *in vivo* against normal hematopoietic progenitor cells (23,24). The results of the present study have provided further evidence to support the hypothesis of Taussig *et al* (13), that utilizing CD33 antigen-targeted therapies may lead to potential HSC killing. Recently, a number of novel anti-CD33 antibody-based agents, including Seattle Genetics (SGN)-CD33A and bispecific monoclonal antibodies, have demonstrated high effectiveness and reduced side effects in preclinical studies; therefore, they may be useful new therapeutic tools for the treatment of AML (25,26). Subsequent clinical trials have demonstrated high efficacy of GO against APL, and that the use of low-fractionated doses of GO in combination with chemotherapy may improve survival with less toxicity and reduce the risk of relapse in AML patients (27,28). However, the value of CD33-targeted therapy in AML remains to be elucidated.

CD123, alternatively known as the interleukin-3 receptor- α subunit, has been reported to demonstrate high levels of expression on leukemia progenitors and LSCs in AML (29). However, it was also observed to be partially expressed on normal CD34⁺CD38⁻ cells in control samples, with a median expression level of 21.6% in the present study. The current findings are consistent with those of Taussig *et al* (13), who reported that CD123 was expressed on the majority of cord blood CD34⁺CD38⁻ cells and regenerating bone marrow (BM) CD34⁺CD38⁻ cells, but only on a minority of normal BM CD34⁺CD38⁻ cells (13). It was additionally demonstrated that CD123 expression was expressed on the majority of CD34⁺ hematopoietic progenitors during hematopoietic differentiation (30). In the present study, CD123 was observed to be expressed predominantly on CD34⁺CD38⁺ cells in AML samples, particularly in immature AML FAB subtypes (M0-M1 and M4-M5). Pizzitola *et al* (23) reported that anti-CD123 chimeric antigen receptor (CAR) cells demonstrate a reduced toxicity profile against normal hematopoiesis compared with anti-CD33 CAR cells, while sharing a similar effect against leukemia cells. CSL362, an anti-CD123 antibody engineered with increased affinity for human CD16, is able to potentially induce antibody-dependent cell-mediated cytotoxicity of CD34⁺CD38⁻CD123⁺ LSCs by natural killer cells, and inhibits leukemic cell growth in mouse models (31). However, in clinical trials, CD123-targeted therapies (<https://clinicaltrials.gov/>; NCT00401739 and NCT00397579) in AML have failed to generate favorable clinical responses within the safety profile (32). Therefore, as a potentially valuable therapeutic target for AML, it may be more beneficial to focus on the application of CD123 in immature AML subtypes, including M0-M1 and M4-M5, in future studies.

CD133 has been reported to be expressed on normal HSCs (14,15), endothelial (33) and neural epithelial cells (34). According to previous studies, CD133 is preferentially expressed

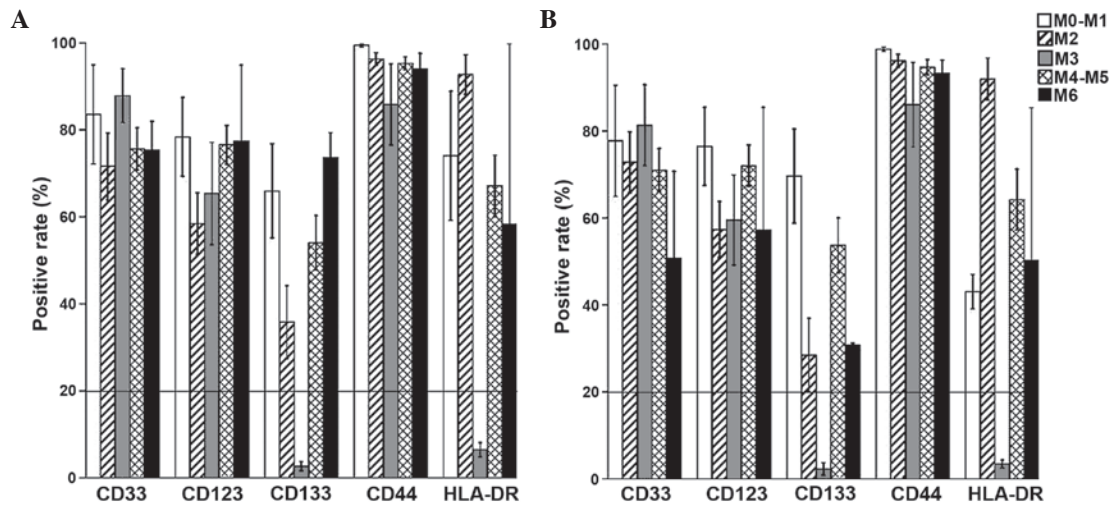


Figure 2. Antigen expression in various acute myeloid leukemia subtypes. (A) Antigen expression on CD34⁺CD38⁻ cells. (B) Antigen expression on CD34⁺CD38⁻Lin⁻ cells. Values are presented as the percentage of cell surface marker expression on gated cells (the mean \pm standard error). The dotted line represents the positive level (20%). CD, cluster of differentiation; Lin, lineage marker. M0-M1, n=4; M2, n=14; M3, n=5; M4-M5, n=29; M6, n=2. Statistical analysis was not performed due to the low sample numbers.

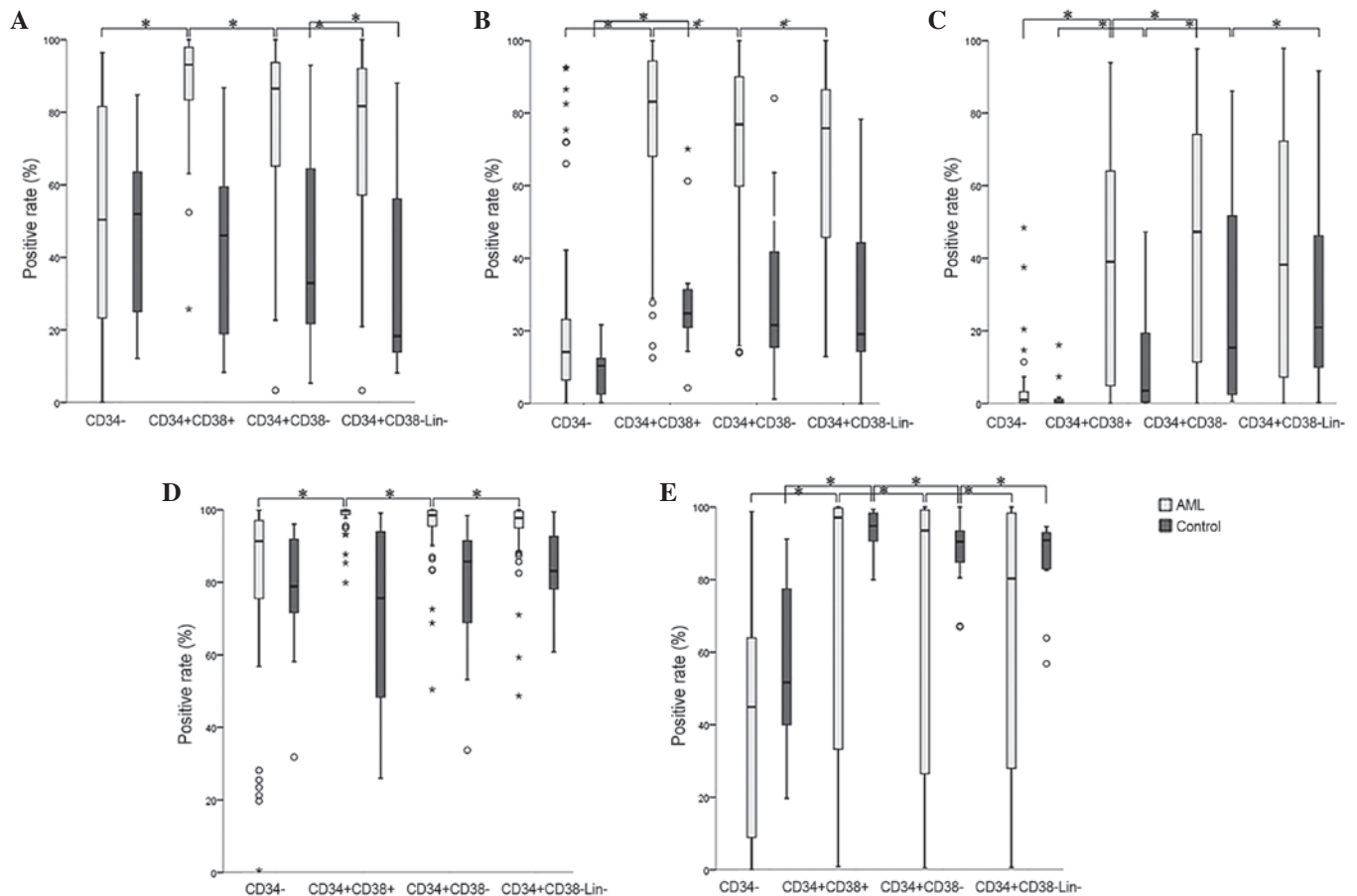


Figure 3. Antigen expression of various cell populations in AML and control samples. (A-E) Profiles of CD33, CD123, CD133, CD44 and HLA-DR expression on CD34⁻/CD34⁺CD38⁺, CD34⁺CD38⁺/CD34⁺CD38⁻ and CD34⁺CD38⁻/CD34⁺CD38⁻Lin⁻ cell populations (*P<0.05). (A) CD33 was preferentially expressed on CD34⁺CD38⁺ cells in AML; (B) CD123 was preferentially expressed on CD34⁺CD38⁺ cells in control and AML; (C) CD133 was preferentially expressed on the more primitive cell population in control; (D) CD44 demonstrated similar expression in different cell populations in control; (E) HLA-DR were highly expressed on CD34⁺CD38⁺ cell compartment in control and AML. AML, acute myeloid leukemia; CD, cluster of differentiation; Lin, lineage marker.

on CD34⁺CD38⁻Lin⁻ HSCs when compared with alternative more mature cell populations, and is frequently identified to be more

highly expressed in M0-M1 and M4-M5 AML subtypes (35). However, in the present study, CD133 was partially expressed

on CD34⁺CD38⁺LIN⁻ LSCs in 34/54 AML cases, with a median expression level of 38.2%. In addition, no significant difference was identified for CD133 expression between AML and control samples on CD34⁺CD38⁺LIN⁻ stem cells, which would make it difficult to use as a target for hematological malignancies. Therefore, regarding CD133, it is suggested that further studies should focus on prognostic association analyses in patients, and that a clinical targeted therapy for leukemia utilizing this antibody may not provide any benefit.

CD44 has been reported to be broadly expressed on normal CD34⁺CD38⁺ cells, more differentiated hematopoietic cells and cells from a number of other tissues (7). In line with the results of previous studies, CD44 was identified to be expressed on CD34⁺CD38⁺LIN⁻ stem cells, CD34⁺CD38⁺ cells, more mature CD34⁺CD38⁺ and CD34⁺ cells, in a similar expression pattern to control sample cells. Although CD44 demonstrated significant differential expression on stem cells between AML and control samples, it exhibited high expression levels on CD34⁺CD38⁺ cells (median 85.7%) and CD34⁺CD38⁺LIN⁻ HSCs (median 83.1%). An anti-human CD44 monoclonal antibody has been reported to be capable of eliminating human LSCs in mouse models, via disruption of cell homing capacity to the microenvironment or by induction of blast differentiation (7). However, to the best of our knowledge, a follow-up study investigating this antibody has not been performed. We hypothesize that potential toxicity induced by increased CD44 expression on HSCs and broad expression on other tissues may limit the use of this antigen as a target for AML or alternative anticancer therapies in clinical practice.

It has previously been demonstrated that CD34⁺CD38⁺ AML LSCs and normal HSCs do not express HLA-DR (36). In the present study, although it was observed that HLA-DR is expressed on CD34⁺CD38⁺ and CD34⁺CD38⁺LIN⁻ cell populations, HLA-DR was preferentially expressed on CD34⁺CD38⁺ cells of increased maturity.

In conclusion, CD33 and CD123 may be suitable for further investigation for use as targeted therapies, particularly CD123 in the immature AML subtype. It may also be necessary to identify and compare cell surface antigen expression on AML LSCs and normal HSCs, which may be of significant importance for the design of directed therapies and for predicting the clinical response. Furthermore, it may be important to identify novel potential cell surface markers in order to complement the current selection of antigen targets. As a result, personalized strategies may improve outcomes due to the heterogeneous expression of antigens in AML.

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