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$CD34^+CD38^-$ subpopulation without CD123 and CD44 is responsible for LSC and correlated with imbalance of immune cell subsets in AML

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Abstract: Acute myeloid leukemia (AML) is regarded as a stem cell disease. However, no one unique marker is expressed on leukemia stem cells (LSC) but not on leukemic blasts nor normal hematopoietic stem cells (HSC). $CD34^+CD38^-$ with or without CD123 or CD44 subpopulations are immunophenotypically defined as putative LSC fractions in AML. Nevertheless, markers that can be effectively and simply held responsible for the intrinsical heterogeneity of LSC is still unclear. In the present study, we examined the frequency of three different LSC subtypes ($CD34^+CD38^-$, $CD34^+CD38^-CD123^+$, $CD34^+CD38^-CD44^+$) in AML at diagnosis. We then validated their prognostic significance on the relevance of spectral features for diagnostic stratification, immune status, induction therapy response, treatment effect maintenance, and long-term survival. In our findings, high proportions of the above three different LSC subtypes were all significantly characterized with low complete remission (CR) rate, high relapse/refractory rate, poor overall survival (OS), frequent FLT3-ITD mutation, the high level of regulatory T cells (Treg) and monocytic myeloid-derived suppressor cells (M-MDSC). However, there was no significant statistical difference in all kinds of other clinical performance among the three different LSC groups. It was demonstrated that $CD34^+CD38^-$ subpopulation without CD123 and CD44 might be held responsible for LSC and correlated with an imbalance of immune cell subsets in AML.

Introduction

Acute myeloid leukemia (AML) is generally regarded as a stem cell disease characterized by an accumulation of undifferentiated and functionally heterogeneous populations of leukemic blasts (Lapidot *et al.*, 1994; Bonnet and Dick, 1997; Dick, 2005). The study of antigen expression patterns in leukemic stem cells (LSC) has a great significance for understanding the mechanisms of leukemogenesis and developing novel therapeutic strategies in AML (Thomas and Majeti, 2017; Pollyea and Jordan, 2017). A number of cell surface markers are expressed on LSC for AML, but thus far, no one unique marker has been discovered that is universally expressed on LSC across the patients with AML, but not on bulk leukemic blasts nor on normal hematopoietic stem cells (HSC) (Thomas and Majeti, 2017).

CD34 is recognized as one of the most important immunophenotypic and functional markers of HSC either in bone marrow or in peripheral blood (Furness and McNagny, 2006; AbuSamra *et al.*, 2017). Compared with $CD38^+$ subpopulation in $CD34^+$ HSC, $CD38^-$ subpopulation seems to possess a higher leukemogenic ability, more therapy-resistant, and less immunogenic characteristics *in vitro* and *in vivo*. These contribute the $CD34^+CD38^-$ section not combined with other surface markers is immunophenotypically defined as the putative LSC fraction in AML at an early date (Sarry *et al.*, 2011; Eppert *et al.*, 2011; Ng *et al.*, 2016; Zeijlemaker *et al.*, 2019). Another reported specific immuno-phenotypic property is the expression of CD123 or CD44 antigen within the $CD34^+CD38^-$ compartment (Jordan *et al.*, 2000; Jin *et al.*, 2009; Jin *et al.*, 2006). Thus, the panels of $CD34^+CD38^-CD123^+$ and $CD34^+CD38^-CD44^+$ are also widely used for LSC characterization and even separating

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LSC from HSC in AML (Thomas and Majeti, 2017; Pollyea and Jordan, 2017; Mastelaro de Rezende *et al.*, 2020). Nevertheless, which of these subpopulations can be effectively and simply be held responsible for the intrinsical LSC leading to the pathogenesis, relapse/ refractory disease progression, and poor prognosis of AML are delusive.

In this study, we tested the frequency of three different LSC subtypes ($CD34^+CD38^-$, $CD34^+CD38^-CD123^+$, $CD34^+CD38^-CD44^+$) in the patients with AML at diagnosis, and then validated their prognostic significance on the relevance of spectral features for diagnostic stratification, immune status, induction therapy response, treatment effect maintenance and long-term survival. Finally, we found that $CD34^+$ $CD38^-$ subpopulation without CD123 and CD44 was held responsible for LSC and correlated with an imbalance of immune cell subsets in AML.

Materials and Methods

Patients

In this study, 60 patients newly diagnosed with AML with CD34 positive leukemic blasts from June 2017 to August 2019 at the Second Hospital of Anhui Medical University, with no congenital/acquired immuno-deficiency, were enrolled. According to Chinese guidelines for diagnosis and treatment of adult AML (not acute promyelocytic leukemia, APL) (2017) (Leukemia Lymphoma Group CSoHCMA, 2017) and Chinese guidelines for diagnosis and treatment of APL (2014) (Jun, 2014), patients were characterized by their own diagnostic stratification and received standard treatment consisting of one to two cycles of induction chemotherapy followed by either consolidation therapy or allogeneic hematopoietic stem cell transplantation (allo-HSCT), based on risk stratification and pretreatment risk assessment. The detailed clinical characteristics of the 60 patients with AML are shown in Tab. 1. This study was approved by the Institutional Review Board (IRB) Institutional of the Second Hospital of Anhui Medical University (No. LLSC20140009). All patients enrolled in the study have signed informed consent.

LSC analysis

Heparinized bone marrow (BM) cells and peripheral blood (PB) cells were analyzed for three kinds of LSC subtypes by CytExpert 2.0 flow cytometer (Beckman Coulter, Miami, FL, USA). In Fig. 1, $CD34^+CD38^-$ LSC were defined by the phenotype of $CD34^+CD38^-CD45^{dim}$ using CD45 and CD34 gating strategy, which included all cells with or without CD123 or CD44. $CD34^+CD38^-CD123^+$ LSC were defined by the phenotype of $CD34^+CD38^-CD123^+CD45^{dim}$ using CD45 and CD34 gating strategy and CD38 backgating strategy. $CD34^+CD38^-CD44^+$LSC were defined by the phenotype of $CD34^+CD38^-CD44^+CD45^{dim}$ using CD45 and CD34 gating strategy and CD38 backgating strategy. Control tubes stained with an isotype-matched control were included in all experiments and were used to define the cutoff point of 98%

TABLE 1

Characteristics of the patients with AML

Group	Sub-group	Number	Group	Sub-group	Number
Gender	Male	32	Cross lineage	Yes	24
	Female	28		No	36
Age	<60 y (16–59)	37	Fusion gene	RUNX1-RUNX1T1	7
	≥60 y (60–85)	23		PML/RARα	6
FAB classification	M0	1		Others	47
	M1	2	Gene mutation	FLT3-ITD	9
	M2	31		CEBPA	8
	M3	6		NMP1	6
	M4	13		ASXL1	7
	M5	4		RUNX1	6
	M7	1		Others	24
	Mu	2	Extramedullar	Yes	9
WBC	Low	16		No	51
	Middle	17	First induction	CR	26
	High	27		Non-CR	23
			Other therapy	Supportive care	7
Karyotype	Low risk	13		Early death	4
	Medium risk	37	Curative effect	CCR	26
	High risk	7		R	8
	No data	3		NR	15

Note: Mu: unclassifiable AML in morphology; WBC: white blood cell; CR: complete remission; Non-CR: not complete remission; CCR: continued complete remission; R: relapse; NR: non-response after two induction therapy, refers to refractory.

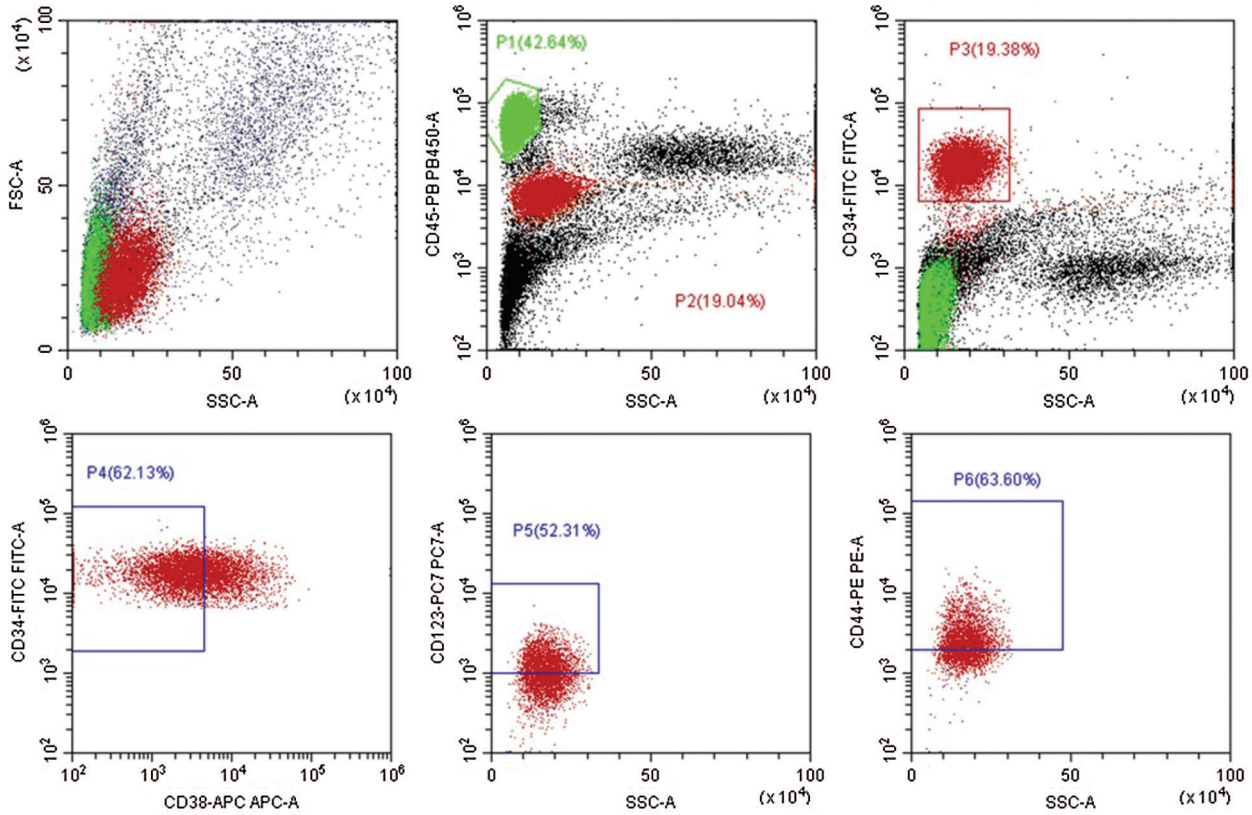

FIGURE 1. Gating strategy in AML to identify different LSC subtypes.
P4 gate was defined as CD34$^+$CD38$^-$LSC. P5 gate was defined as CD34$^+$CD38$^-$CD123$^+$LSC using P4 gate. P6 gate was defined CD34$^+$CD38$^-$CD44$^+$LSC using P4 gate. For the gating of CD34$^+$CD38$^-$ compartment, the proportion of CD44$^-$CD123$^-$ was 21.22%, CD44$^+$CD123$^-$ was 26.47%, CD44$^-$CD123$^+$ was 15.18%, CD44$^+$CD123$^+$ was 37.13%.

for positive/negative staining. All commercial monoclonal antibodies used in the present experiments are shown in Tab. 2.

Immune cell subsets analysis

Peripheral circulated immune cell subsets including Th, CTL, Treg, Teff, B, NK, and M-MDSC were detected by Cytomics® FC500 flow cytometer (Beckman Coulter, Miami, FL, USA). Th subsets were identified by the phenotype of CD3$^+$CD4$^+$CD8$^-$CD45^{++}. CTL subsets were identified by the phenotype of CD3$^+$CD4$^-$CD8$^+$CD45^{++}. Treg subsets were identified by the phenotype of CD4$^+$CD25$^+$ CD127$^{low/-}$CD45^{++}. Teff subsets were identified by the phenotype of CD4$^+$CD25$^+$CD127^{high}CD45^{++}. B subsets were identified by the phenotype of CD19$^+$CD20$^+$CD45^{++}. NK subsets were identified by the phenotype of CD3$^-$CD16$^+$CD56$^+$CD45^{++}. M-MDSC subsets were identified by the phenotype of CD14$^+$HLA-DR$^{low/-}$CD45^{++}. The isotype-matched control was used to define the cutoff point of 98% for positive/negative staining. All commercial monoclonal antibodies used in the present experiments are also shown in Tab. 2.

Statistical analysis

Statistical power was calculated using PASS 11.0, and the significant level (alpha) was 0.05. Statistical analysis was performed with SPSS 16.0. The chi-squared test, Fisher's exact probability tests, and the Student's *t*-test were used when appropriate to evaluate the significance of differences in data between groups. If variances within groups were not homogeneous, a non-parametric Mann-Whitney test was used. The prognostic value was evaluated by Kaplan-Meier survival curves. Overall survival (OS) was used and defined as the time from date of diagnosis until the date of death, and the median follow-up time was 8 months. We used continued complete remission (CCR) as an indicator to generate the ROC curve and determined the optimal cut-off value based on the Yoden index. The cut-off values of CD34$^+$CD38$^-$LSC, CD34$^+$CD38$^-$CD123$^+$ LSC and CD34$^+$CD38$^-$CD44$^+$ LSC were 3.83%, 1.68% and 3.19%, respectively. More than or equal to the respective cut-off value was defined as the high-frequency group, and less than the respective cut-off value was defined as the low-frequency group. The *p*-value less than 0.05 was considered statistically significant.

Results

LSC status at diagnosis in the patients with AML

Baseline characteristics of the 60 patients with AML at diagnosis in the present study were shown in Tab. 1. Of these 60 cases, BM samples of all cases and PB samples of 25 cases with significant peripheral circulation blasts (more than 10% leukemic blasts, median 33%, range 11–95%) were used for three kinds of LSC subtype analysis. In Fig. 2A, CD34$^+$CD38$^-$LSC, CD34$^+$CD38$^-$CD123$^+$LSC and CD34$^+$CD38$^-$CD44$^+$LSC were respectively 4.04 ± 6.80%, 2.62 ± 5.68% and 3.34 ± 6.29% posed of all mononuclear cell percentage, and there was no significant statistical difference in the three different groups of LSC subtype

TABLE 2

Specification of monoclonal antibodies

Cell subsets	Antibody	Clone	Conjugate	Source
LSC	CD34	581	FITC	Beckman Coulter
	CD38	HB-7	APC	BD Biosciences
	CD123	7G3	PE-CY7	BD PharmingenTM
	CD44	515	PE	BD PharmingenTM
	CD45	J.33	PB	Beckman Coulter
Th/CTL	CD4/CD8/CD3	13B8.2/B9.11/VCHT1	FITC/PE/PC5	Beckman Coulter
	CD45	J.33	PC7	Beckman Coulter
Treg/Teff	CD4	13B8.2	PC5	Beckman Coulter
	CD25	B1.49.9	FITC	Beckman Coulter
	CD127	R34.34	PE	Beckman Coulter
B	CD19	J3.119	FITC	Beckman Coulter
	CD20	B9E9	PE	Beckman Coulter
	CD45	J.33	PC5	Beckman Coulter
NK	CD3/CD(16+56)	VCHT1/3G8+N901	FITC/PE	Beckman Coulter
	CD45	J.33	PC5	Beckman Coulter
M-MDSC	CD14	RMO52	FITC	Beckman Coulter
	HLA-DR	Immu-357	PE	Beckman Coulter
	CD45	J.33	PC5	Beckman Coulter

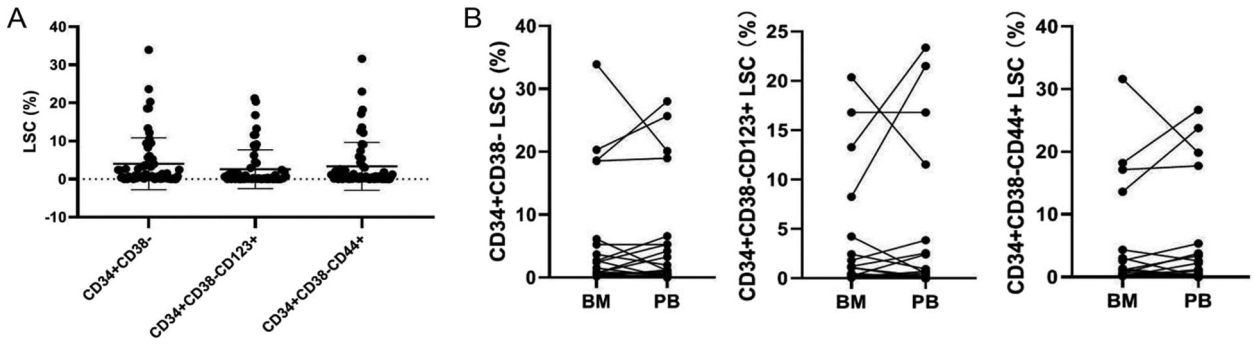

FIGURE 2. LSC status at diagnosis in patients with AML.
(A) The frequency of $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$LSC and $CD34^+CD38^-CD44^+$LSC at diagnosis in BM samples of 60 patients with AML. (B) The frequency of $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$LSC and $CD34^+CD38^-CD44^+$LSC at diagnosis in BM samples and PB samples of the same 25 patients with AML. The *p*-value of different groups were all >0.05.

($p > 0.05$). In Fig. 2B, the data of BM and corresponding PB samples in the same 25 cases showed that some cases had a higher LSC frequency, but some cases had a lower LSC frequency in PB compared with that in BM. There was also no significant statistical difference in the three kinds of LSC subtype between BM and PB samples ($p > 0.05$), hence, we alternatively used the proportion of LSC in BM samples for subsequent analysis.

Validation of the prognostic relevance of LSC

Of the above 60 cases in the present study, 49 cases (82%) were received standard treatment consisting of one to two cycles of induction chemotherapy followed by either consolidation therapy or allo-HSCT, 7 cases (12%) were received only supportive care because of their old age or poor physical condition, the other 4 cases (6%) died of fatal bleeding or infection in a short time (within 2 weeks) due to their disease progression. In Fig. 3A, after the first induction chemotherapy of 49 cases, 26 cases (53%) obtained complete remission (CR) and 23 cases (47%) did not attain CR (Non-CR). The frequency of $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$LSC and $CD34^+CD38^-CD44^+$LSC in the CR group (1.93 ± 3.00%, 1.16 ± 2.73%, 1.28 ± 2.79%, respectively) were all significantly lower than the Non-CR group (6.83 ± 9.04%, 4.31 ± 6.20%, 5.84 ± 8.23%, and $p = 0.0115$, $p = 0.0209$, $p = 0.0118$, respectively). In Fig. 3B, after the re-induction therapy or consolidation therapy or allo-HSCT of the same 49 cases, 26 cases (53%) preserved continued complete remission (CCR) and 23 cases (47%) were eventually progressed to non-respond/refractory or relapsed disease (NR/R). The frequency

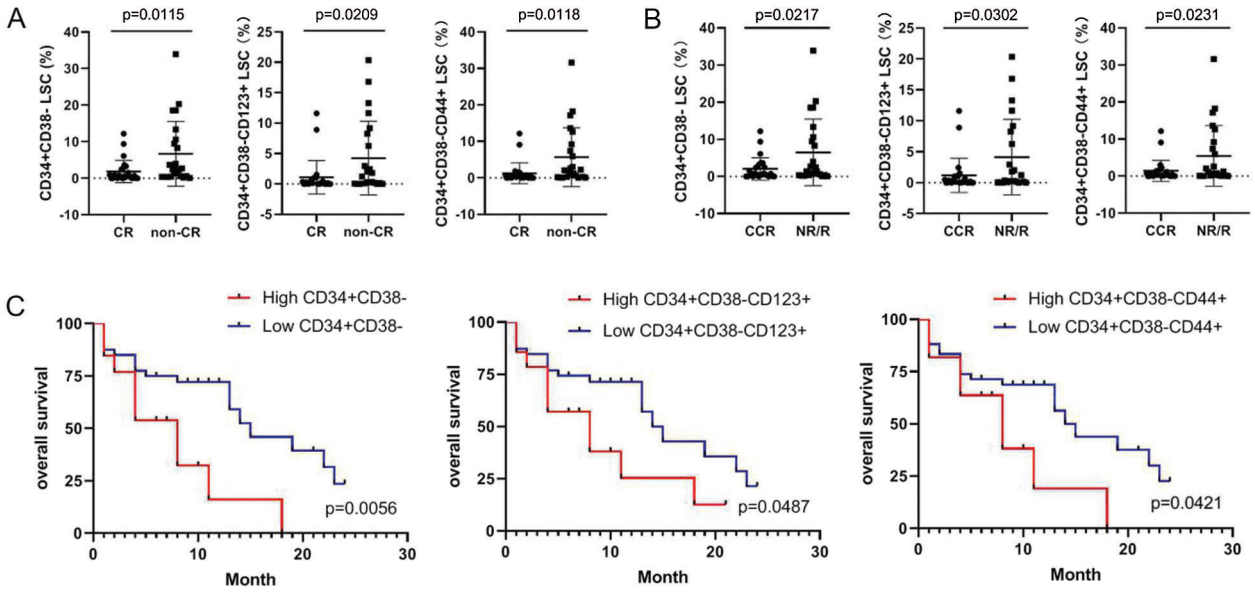

FIGURE 3. The significance of LSC on the features of prognostic relevance.
(A) The significance of $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$LSC and $CD34^+CD38^-CD44^+$LSC in the first induction chemotherapy. (B) The significance of $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$LSC and $CD34^+CD38^-CD44^+$LSC in the treatment effect maintenance. (C) The significance of $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$LSC and $CD34^+CD38^-CD44^+$ LSC in the long-term survival. The *p*-value of different groups were all shown in the figure.

of $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$LSC and $CD34^+CD38^-CD44^+$LSC in the CCR group (2.05 ± 3.02%, 1.18 ± 2.76%, 1.44 ± 2.84%, respectively) were all significantly lower than the NR/R group (6.84 ± 8.98%, 4.15 ± 6.11%, 5.46 ± 8.19%, and $p = 0.0217$, $p = 0.0302$, $p = 0.0231$, respectively).

Follow-up analyses were performed for all 60 patients, including those who received standard treatment, supportive care, and early death. In Fig. 3C, analysis for the above three kinds of LSC subtype showed that the low LSC frequency groups all had significant survival advantages compared with the high LSC frequency groups ($p = 0.0056$, $p = 0.0487$, $p = 0.0421$, respectively). Together, these findings suggested that these three different LSC subtypes, including $CD34^+CD38^-$ subpopulation with or without CD123 or CD44, were all specific and could individually serve as prognostic indicators to predict outcome in AML.

LSC frequency and clinic risk categories

In this study, we analyzed the correlation between the proportion of LSC and common clinical risk factors in all 60 AML cases. In Figs. 4A–4C, our data demonstrated that there were no significant statistical differences in $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$LSC and $CD34^+CD38^-CD44^+$ LSC regarding age, WBC count and extramedullary infiltration (all $p > 0.05$). We also analyzed the correlation between the proportion of LSC and the cross-linage expression, morphological typing, and leukemic blasts percentage. The above indicators are very important factors reflecting the malignancy and tumor burden of leukemic blasts. In Figs. 4D–4F, we found that there was no significant statistical difference in the three kinds of LSC subtypes among cross linage expression, morphological typing, and leukemic blasts percentage (all $p > 0.05$). Together, it was suggested that the prognostic values of these three different LSC subtypes, including $CD34^+CD38^-$ subpopulation with or without CD123 or CD44, all had no statistical correlation with common clinic risk categories.

LSC frequency and cytogenetic/molecular risk categories

Total 59 of the 60 cases with AML were detected by G-banded chromosome recognition (2 cases without chromosome division), gene panels of 44 kinds of fusion genes, gene panels of FLT3-ITD, CEBPA, NPM1, C-kit mutation. We further conducted next-generation sequencing (NGS) testing on 21 of the 60 cases. Then, we analyzed the correlation between the proportion of LSC and karyotype stratification, fusion gene, and gene mutation. In Fig. 5, we found there was no significant statistical difference in $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$LSC and $CD34^+CD38^-CD44^+$LSC subtypes among the groups of karyotype stratification, fusion gene and gene mutation (Figs. 5A–5G, all $p > 0.05$), except for the FLT3-ITD mutation group (Fig. 5H, $p < 0.05$). Together, it was suggested that the prognostic values of $CD34^+CD38^-$ LSC with or without CD123 or CD44 all had no statistical correlation with common cytogenetic/molecular risk categories. But LSC might interact with the FLT3-ITD mutation contributing to the pathogenesis in AML.

LSC frequency and immune cell subsets

Firstly, 27 cases were detected of peripheral circulated immune cell subsets. Then, we analyzed the correlation between the proportion of LSC and the above immune cell subsets. We found there was no significant statistical difference in $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$ LSC and $CD34^+CD38^-CD44^+$LSC subtypes among the groups of B, NK, Th, CTL, Th/CTL ratio, Teff (Figs. 6A, 6B, 6D–6F, and 6H, all $p > 0.05$). However, Treg percentage in the high-frequency groups of $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$LSC and $CD34^+CD38^-CD44^+$LSC (6.68 ± 3.01%, 6.51 ± 2.79%, 6.68 ± 3.37%, respectively) were all significantly higher than the low-frequency groups of $CD34^+CD38^-$LSC,

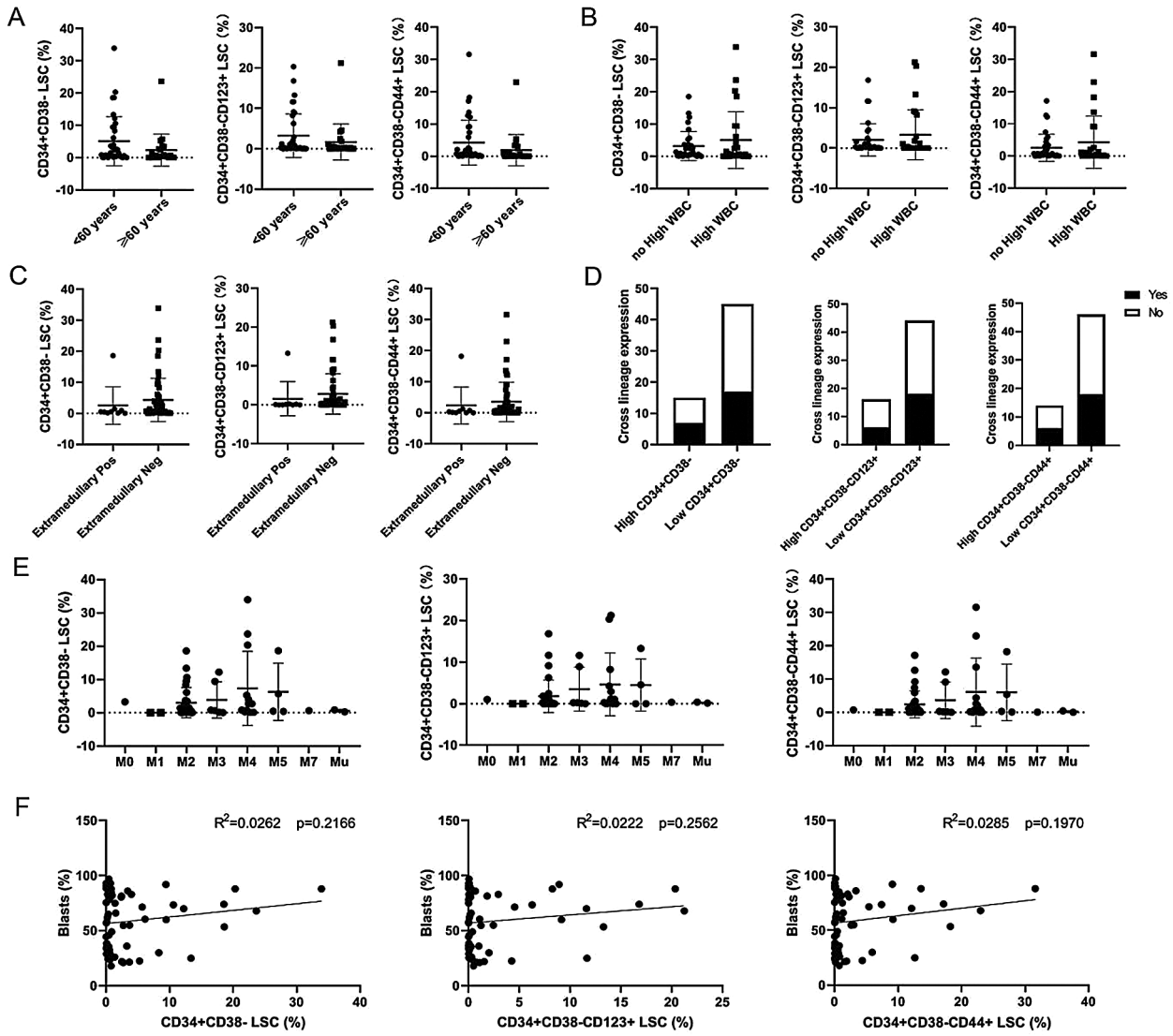

FIGURE 4. The correlation between LSC frequency and clinic risk categories.
(A–F) The frequency of $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$LSC and $CD34^+CD38^-CD44^+$LSC in different groups of age, WBC count, extramedullary infiltration, cross linage expression, morphological typing, and leukemic blasts percentage, respectively. The *p*-value of different groups were all >0.05.

$CD34^+CD38^-CD123^+$LSC and $CD34^+CD38^-CD44^+$LSC (5.38 ± 1.30%, 5.38 ± 1.33%, 5.44 ± 1.30%, and $p = 0.0053$, $p = 0.0120$, $p = 0.0023$, respectively, Fig. 6G). Moreover, Treg/Teff ratio were also increased with the increase of Treg percentage (Fig. 6I). M-MDSC percentage in the high-frequency groups of $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$LSC and $CD34^+CD38^-CD44^+$LSC (9.33 ± 2.88%, 8.56 ± 2.64%, 9.37 ± 2.86%, respectively) were all significantly higher than the low-frequency groups of $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$ LSC and $CD34^+CD38^-CD44^+$LSC (2.16 ± 0.90%, 2.14 ± 0.96%, 2.15 ± 0.90%, and $p = 0.0045$, $p = 0.0091$, $p = 0.0041$, respectively, Fig. 6C). Together, it was suggested that LSC subtypes were correlated with the imbalance of peripheral circulated immune cell subsets, especially for significantly increased Treg and M-MDSC.

Discussion

LSC is defined as cells that are capable of initiating the disease when transplanted into immunodeficient animals and can self-renew by giving rise to leukemia in serial transplantations and also partially differentiate into non-LSC bulk blasts that resemble the original disease (Lapidot *et al.*, 1994; Bonnet and Dick, 1997; Dick, 2005). In AML, the concept of LSC has been widely accepted and developed with the idea to explain the clonal hierarchies and architectures (Thomas and Majeti, 2017; Valent *et al.*, 2019). Interestingly, LSC are theoretically distinct from the bulk of leukemic blast populations and normal HSC but are actually responsible for managing all the hierarchies (Thomas and Majeti, 2017; Valent *et al.*, 2019). Therefore, the study of the different phenotypes of LSC, leukemic blasts, and normal HSC in AML with the aid of up-to-date flow cytometric techniques is important for the deep insight into the mechanisms of leukemogenesis and disease progression (Ivanivska *et al.*, 2019).

It is currently known that the $CD34^+CD38^-$ subpopulation exhibits higher LSC characteristics compared with $CD34^+CD38^+$ subpopulation and $CD34^-$ subpopulation (Sarry *et al.*, 2011; Eppert *et al.*, 2011), and is usually

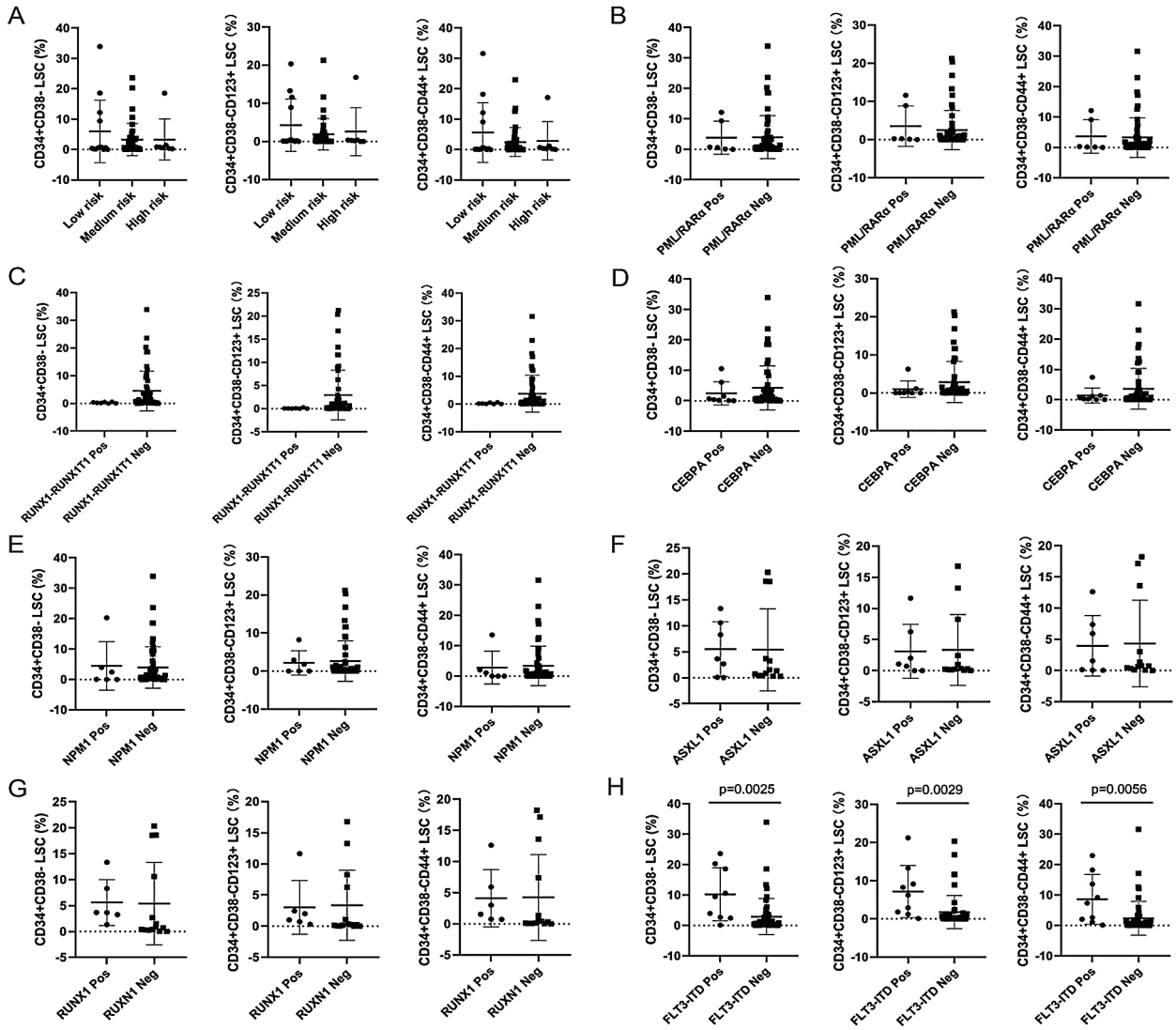

FIGURE 5. The correlation between LSC frequency and cytogenetic/molecular risk categories.
(A–H) The frequency of $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$LSC and $CD34^+CD38^-CD44^+$LSC in different groups of karyotype, PML/RARa fusion gene, RUNX1-RUNX1T1 fusion gene, CEBPA gene mutation, NPM1 gene mutation, ASXL1 gene mutation, RUNX1 gene mutation and FLT3-ITD gene mutation, respectively. The *p*-value of different groups were all >0.05, except for the FLT3-ITD group. The *p*-values of the FLT3-ITD group were all shown in the figure.

immunophenotypically defined as LSC fraction in AML (Thomas and Majeti, 2017; Zeijlemaker *et al.*, 2019; Karantanos and Jones, 2019). Gene expression profiling from $CD34^+CD38^-$LSC is also used to define an LSC score and is an independent predictive value of poor prognosis in patient cohorts (Eppert *et al.*, 2011; Jung *et al.*, 2015; Lechman *et al.*, 2016). Moreover, many other surface markers have also been identified that are up-regulated on $CD34^+CD38^-$LSC (Thomas and Majeti, 2017; Gentles *et al.*, 2010). As an anti-IL-3 receptor alpha chain, CD123 is considered as a novel specific marker expressed on traditional LSC and even an important therapeutic target for the treatment of AML (Jin *et al.*, 2009; van Rhenen *et al.*, 2007; Abdollahpour-Alitappeh *et al.*, 2018). As a cell adhesion molecule, CD44 plays an important role in the interaction with a variety of extracellular matrix components, cytokines, and growth factors secreted by cells present in the tumor environment. It is therefore not surprising that many studies have reported CD44 is also been identified as a putative marker for LSC in AML (Jin *et al.*, 2006; Morath *et al.*, 2016).

However, few studies have evaluated the prognostic effect of traditional $CD34^+CD38^-$LSC with or without CD123 or CD44 in patients with AML. It is also not clear that which of these different subpopulations can be effectively and simply held responsible for the intrinsical LSC in AML. Herein, we examined the above LSC subtypes ($CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$ LSC and $CD34^+CD38^-CD44^+$LSC) and the combined effect of their expression on clinical outcome. Our data elucidated that $CD34^+CD38^-$LSC, with or without CD123 or CD44, were all present in a significant proportion of the patients with AML at diagnosis and they all could be responsible for resistance to traditional treatments, and the high percentage of minimal residual disease (MRD) that was translated into the significantly high number of Non-CR, relapse/refractory and poor OS. Together, it was suggested that $CD34^+CD38^-$ subpopulation without CD123 or CD44 might be held

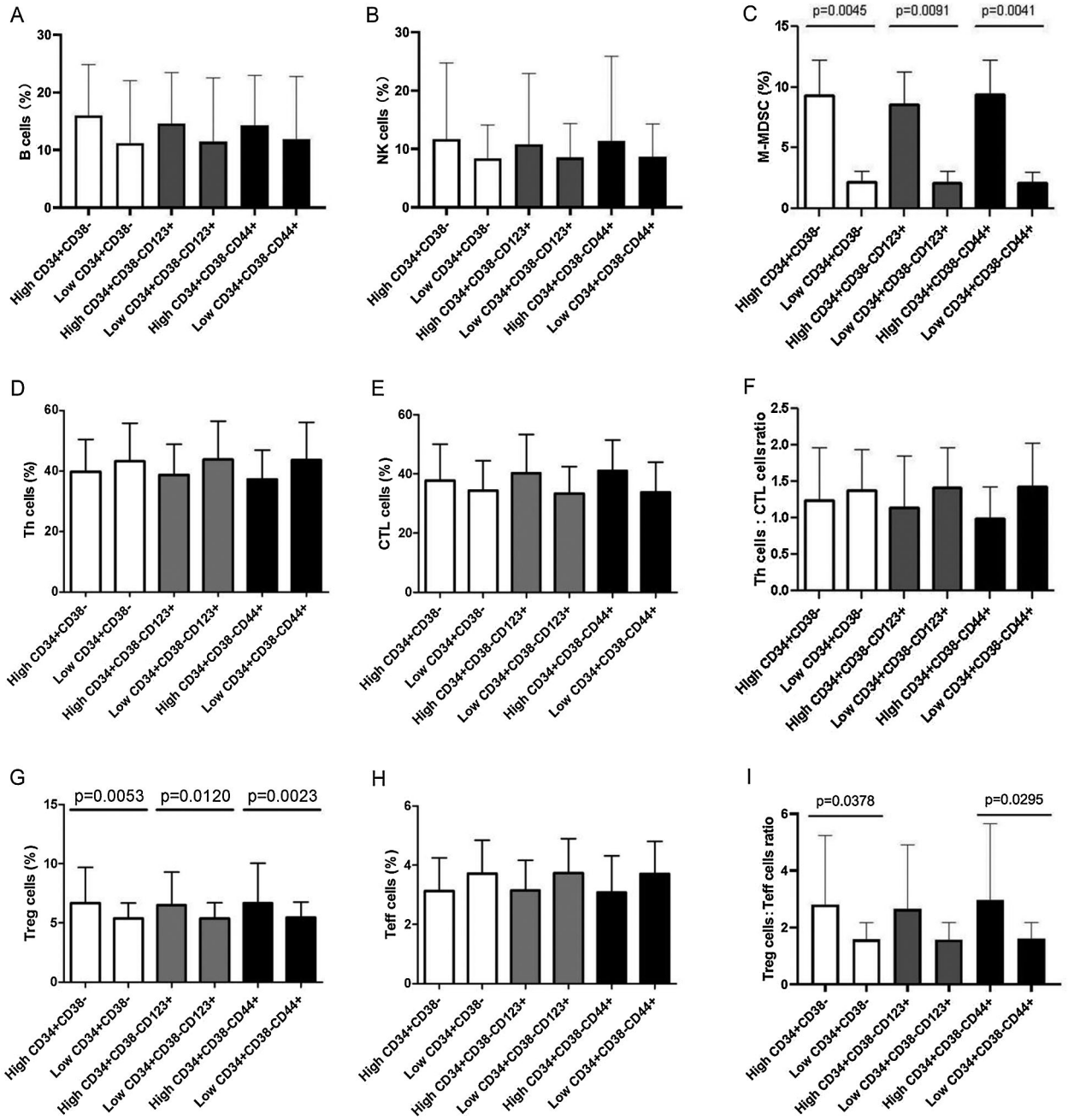

FIGURE 6. The correlation of LSC frequency and immune cell subsets.
(A–I) The frequency of B, NK, M-MDSC, Th, CTL, Th/CTL ratio, Treg, Teff and Treg/Teff ratio in different groups of $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$LSC and $CD34^+$ $CD38^-CD44^+$LSC, respectively. The *p* value of Treg, Treg/Teff ratio and M-MDSC groups were all shown in the figure, the *p*-values of other groups were all >0.05.

responsible for the most feasible LSC in AML considering from a clinical prognostic significance perspective.

As we all know that AML is a heterogeneous disease with different clinical manifestations and different disease prognosis. It is generally believed that patients with a poor prognosis are usually characterized by some high clinical risk factors at the time of diagnosis. These high clinical risk factors include old age, high WBC count, AML secondary to myelodysplastic syndromes (MDS) or myeloproliferative neoplasms (MPN), therapy-related AML, and AML with extramedullary infiltration (Leukemia Lymphoma Group CSoHCMA, 2017). Additionally, except for cytomorphology, cytochemistry and immunophenotyping, state-of-the-art AML diagnostics also relies on cytogenetics and molecular genetics. Especially, most prognostic models like NCCN and ELN have stratified AML patients using cytogenetic and molecular features into different prognostic groups based upon predicted response to standard therapy and survival. However, it is unknown that which of these clinical factors, cytogenetic and molecular factors are related to the prognostic significance of $CD34^+CD38^-$ subpopulation with or without CD123 or CD44.

In the present study, our data showed that $CD34^+CD38^-$LSC, $CD34^+CD38^-CD123^+$LSC and $CD34^+$ $CD38^-CD44^+$LSC frequencies were all significantly increased in the FLT3-ITD mutation-positive group compared with

the FLT3-ITD mutation-negative group. This phenomenon fitted the previous study perspectives of FLT3-ITD mutations represent and express within LSC (Levis *et al.*, 2005; Levis, 2017). Moreover, the signaling of LSC represents a combined signaling node of the FLT3-ITD driven leukemogenesis, and the FLT3-ITD signaling network also promotes the survival of LSC (Yoshimoto *et al.*, 2009; Tam *et al.*, 2013; Al-Mawali *et al.*, 2016; Zhang *et al.*, 2019). So, LSC is potentially an important parameter to be evaluated in the FLT3-ITD mutation-positive AML patients undergoing treatment with FLT3 inhibitors. In our previous study, we have discovered a highly potent FLT3 kinase inhibitor, CHMFL-FLT3-165, which exhibits strong biochemical inhibition against FLT3-ITD mutation-positive leukemic blasts and significant *in vivo* tumor suppression (Wu *et al.*, 2016). So, it provides a rationale for the treatment of our novel FLT3-ITD inhibitor and might directly against LSC in our future experiments on LSC. However, there was no significant statistical difference for the above three LSC frequencies among the groups of morphological typing, leukemic blasts percentage, cross linage expression, karyotype stratification, fusion gene, and other gene mutation. Together, it was suggested that the prognostic value of $CD34^+CD38^-$ subpopulation with or without CD123 or CD44 all had no statistical correlation with common clinic risk categories and cytogenetic/molecular risk categories (except for FLT3-ITD mutation), and $CD34^+CD38^-$ subpopulation without CD123 and CD44 might be held responsible for the most feasible LSC in AML. From an objective point of view, CD123 was not essential for the diagnostic evaluation of LSC but was still important because offered the opportunity to target these cells therapeutically.

Population dynamics of Treg are crucial for the underlying interplay between leukemic and immune cells in the progression of AML, as shown by a model incorporating promotion of Treg expansion by leukemic blast cells, LSC, and progenitor cells (Nishiyama *et al.*, 2018). Meanwhile, M-MDSC has attracted a lot of attention in the field of tumor immunology in recent years (Lv *et al.*, 2019), but little is known about its roles in AML especially for LSC as opposed to its multiple roles in solid tumors. In our previous study, we have reported that tumor immune escape mechanism mediated by Treg and Treg-related cytokines is an important pro-tumorigenic factor for in AML (Tao *et al.*, 2015; Wang *et al.*, 2015). We herein further found that the $CD34^+CD38^-$LSC with or without CD123 or CD44 were significantly correlated with peripheral circulated Treg and M-MDSC proportion, although our data demonstrated there were no statistical differences in all kinds of LSC subtype among the groups of peripheral circulated B, NK, Th, and CTL cells. It was suggested that the immune tolerance status of peripheral circulation might interact with LSC for the pathogenesis and progression of AML.

Conclusion

In the present study, we have illustrated that $CD34^+CD38^-$ subpopulation without CD123 and CD44 is held responsible for the most feasible LSC in AML considering from a clinic prognostic perspective and is also correlated with the imbalance of peripheral circulated immune cell subsets, especially for Treg and M-MDSC. We believed that our study has an important application value in the clinical prognosis evaluation of patients with AML.

Ethics Approval: This study was approved by the Institutional Review Board (IRB) Institutional of the Second Hospital of Anhui Medical University (No. LLSC20140009).

Availability of Data and Materials: The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Author's Contributions: Study conception and design: Zhai Z, Dong Y; data collection: Tao Q, Zhang Q, Wang H, Xiao H, Zhou M, Liu L, Wang J, Qin H, An F; analysis and interpretation of results: Tao Q, Zhang Q; draft manuscript preparation: Tao Q. All authors reviewed the results and approved the final version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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