

Leukemia Stem Cells in Blood Cells; Focused on Acute Myeloid Leukemia

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It is known that acute myeloid leukemia (AML) is a heterogeneous blood cancer, which is enormously propagated by self-renewing leukemia stem cells (LSCs). The persistence of LSCs after chemotherapy can contribute to minimal residual disease and relapse by LSCs can be evoked promptly. Elucidating special molecules and cellular activity of LSCs is an extremely important to eliminate AML. Despite an increasing understanding of the origin of LSCs by incessant study, AML still remains a notorious disease with high mortality. An exact identification of the LSCs that sustain the proliferation of neoplastic clone is a fundamental issue in AML treatment. CD34+CD38- conventional phenotype is overall regarded as LSCs, but it has a limitation that is still hard to demarcate exactly due to similarity with normal hematopoietic stem cells (HSCs). Not all primary blasts and progenitors have equal function, thus a bona fide marker for identifying LSCs from HSCs is needed in hematologic malignancy, especially in AML. These findings have direct important implications in both in mechanistic study of LSCs as well as in the strategies of more effective therapies. In this review, I briefly summarized current advances in LSCs biology, focusing on membrane markers and a functional behavior of LSCs in AML treatment with monoclonal antibodies. Ultimately, it may be helpful in overviewing the status of LSC research, while expecting the clinic benefits of target therapy by specific inhibition.

Key Words: Leukemia stem cells, Surface marker, Acute myeloid leukemia

INTRODUCTION

Acute myeloid leukemia (AML) is heterogeneous blood cancer, which is accumulated by malignant blasts and closely involved in proliferation of leukemia stem cells (LSCs) (Hope et al., 2003, Ferrara et al., 2013). A rare population of stem cells, having self-renewal and differentiation, allows for the maintenance and proliferation of cancer and has been studied in recent years (Hamburger et al., 1977; Jordan et al., 2006). Controversies for existence of cancer stem cells (CSCs) including LSCs have been still

ongoing, but evidence has continuously shown for restricted cells with leukemic clonogenic activity by xenograft mouse model in AML (Lapidot et al., 1994). In addition, a role of LSCs with mechanism of marker proteins has been specifically demonstrated (Dieter et al., 2011; Bonnet et al., 1997; Jin et al., 2006; Claudia et al., 2014). Most studies of LSCs showed suppression of their homing and engraftment without leukemic properties, when specific inhibition for LSC marker applied. Because LSCs can drive cancer growth and relapse, the deepening of understanding of leukemogenesis is crucial to treat AML. Since John Dick et al addressed the CD34+CD38- cells as a LSCs by immunophenotype (Lapidot

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et al., 1944), CD34+CD38- cells by NOD/LtSz-*scid* IL-2R γ^{null} mice (termed NSG) model have been referred to as LSCs (Bonnet et al., 1997; Hope et al., 2004). These immunodeficient mice can allow superior engraftment of human stem cells, unlike in immunocompetent mice. Because hematologic reconstitution does not completely achieve *in vitro*, repopulating should be confirmed via *in vivo* system. Thus NOD/LtSz-*scid* IL-2R γ^{null} mice, which can strongly provide a results for repopulation, has been regarded as available tool to identify LSCs. CD34+CD38- cells by FACS sorting are easily infiltrated into bone marrow (BM) of NOD/LtSz-*scid* IL-2R γ^{null} mice and generated all lineage hematopoietic cells including T, B and NK cells, indicating CD34+CD38- cells as a representative stem cells. However, fatal drawback of CD34+CD38- population is that it suggested as a representative phenotype in both LSCs and normal hematopoietic stem cells (HSCs) (Ailles et al., 1997; Bhatia et al., 1997). Besides, because AML-LSCs display functional heterogeneity against chemotherapy, not all LSCs could be detected in the only CD34+CD38- population. Some LSCs, CD34+CD38+ cells or other compartments were found to sustain LSCs, when more severe models induced. (Taussig et al., 2008; Taussig et al., 2010). Surface marker is important to confirm the biologic activity with LSC marker from clones of phenotypic cell fractions in LSC study. NOD/LtSz-*scid* IL-2R γ^{null} mice, which are deleted function of T, B, and NK cells by ablation of IL-2R receptor, are used to address the capacity of LSCs by repopulating of the cancer cells, it allows researcher to further investigate mechanism of development and mutation in leukemogenesis under human like condition (Lapidot et al., 1944; Saito et al., 2010; Ishikawa et al., 2005). Finding out these rare cell populations generating the leukemia in NOD/LtSz-*scid* IL-2R γ^{null} mice is the starting point of LSC study. The first experiment for AML-LSC in NOD/LtSz-*scid* IL-2R γ^{null} mice showed that LSCs are exclusively contained in CD34+CD38- cells (Ishikawa et al., 2007). Furthermore, both CD34- and CD34+CD38+ AML cells failed to stabilize engraftment, in spite of the transplantation with large number of cells, compare to those of CD34+CD38- cells. These data is not consistent with previously mentioned data from Taussig et al., 2010. Although these contradictions containing variation

exist, the reason LSCs study should continue is that because it could be provided therapeutic index separating LSCs from HSCs in definitive differences with functional properties. Although it may be difficult to exact identify LSCs due to several conundrums, suppression of LSCs by advanced protein inhibitors such as monoclonal antibody and peptides has also revealed effective treatment in AML. These outstanding discoveries of surface markers in LSCs let us isolate high enriched LSCs, resulting in advanced opportunities to investigate LSCs. Here, I briefly summarized the markers for LSC, which are ongoing studies and are trying in clinic application. Identifying marker protein also raises the possibility of therapeutic benefits through specific inhibition in cancer biology including solid cancer as well as hematologic malignancy.

Typical phenotypes of LSCs in AML I - from the beginning of LSC seeking

There are several types including M0, M1, M2, M3, M4, M5, M6, M7 subtypes in AML. Among them, M3 is known as acute promyelocytic leukemia (APL) accumulating immature promyeloblasts, but not stem cells. M6 and M7 are categorized form of acute erythroid and megakaryoblastic leukemia. Other groups possess LSC cells except M3, M6 and M7. The CD34+CD38- compartment in particular, was shown to contain both LSCs as well as HSCs. By reported data from Eppert K et al (Eppert et al., 2011), frequency of major CD34+CD38- phenotype in AML patients is diversely shown. Besides CD34+CD38- compartment, CD45^{dim/low} is expressed on leukemia blasts as well as LSCs, but not HSCs (Lacombe et al., 1997; Feller et al., 2004). In contrast, CD45^{bright}, hematopoietic marker, is expressed in normal hematopoietic stem cells but not LSCs. Thus, CD34+CD38-CD45^{dim} phenotype can distinctly point out the LSCs. Interestingly, unarguable difference between isolated CD34+CD38-CD45^{dim} cells and CD34+CD38-CD45^{bright} cells exists, when CFU-colony occurred. Typical CFU-colonies including granulocyte, erythrocyte, macrophage, megakaryocyte (GEMM), granulocyte, macrophage (GM), erythroid (E) were evenly found in HSCs. However, AML-LSCs derived colonies mainly formed GEMM and GM and aberrant morphologies were observed such as very tiny colonies and

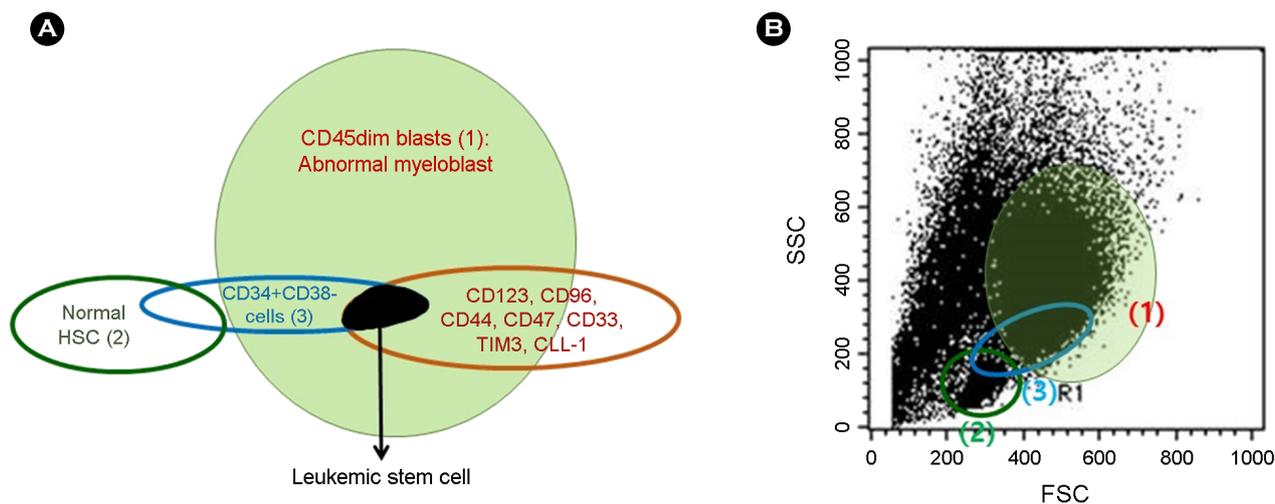


Fig. 1. (A) Schematic diagram showed LSCs in CD45^{dim} population (1) and normal HSCs (2). Typical marker for LSCs and HSCs, CD34+CD38⁻ cells (3) is depicted by blue circle. Emerging markers should be highly expressed on CD34+CD38⁻ cells. (B) Forward vs. scatter plot of whole cells in AML clearly divided into mononuclear cells (2) from abnormal myeloid blasts (1).

condensed clump (<0.4 mm) (Lee et al., 2016; Matsushita et al., 2008) unlike in HSCs. Prospective isolation of LSCs via surface markers is possible in mononuclear cells and immature myeloblast in AML and it reveals whether isolated positive cells can function as LSCs or not *in vitro*. To achieve complete remission and to develop diagnosis and therapy, one of the strategies is to identify cell surface markers that can distinct LSCs from HSCs. So far, confirmation for several marker proteins is addressed by using specific antagonists and monoclonal antibodies in both *in vitro* and *in vivo* experiments. By using of specific markers, isolation of pure LSCs could provide basic mechanism and therapeutic clues by targeting LSCs. However, because highly enriched populations of LSCs have still displayed heterogeneous properties, combinational treatment with conventional anti-cancer drugs should be recommended under continuous surveillance with specific inhibition.

Phenotypes of LSCs in AML II - differential expression between LSC and HSC

A phenotypic studies have shown that LSCs are typically: CD34⁺, CD38⁻, CD90⁻, CD117⁻, CD123⁺ (IL3Ralpha), CD96⁺, CD44⁺, CD47⁺, CD33⁺, TIM-3⁺ (CD366), C-type lectin like molecule-1 (CLL-1) (Blair et al., 1997; Blair et

al., 2000; Jin et al., 2009; Jin et al., 2006; Majeti et al., 2009; Walter et al., 2012; Kikushige et al., 2010; van Rhenen et al., 2007). These marker proteins are mainly distributed in LSCs, compared to that of HSCs, showing induction of leukemia. Based on these, many studies have presented rapid advance in phenotypic characterization against ambiguity of LSCs. Features of the LSC marker protein were as follows: First, LSCs with marker proteins revealed a frequent expression in CD34+CD38⁻ primitive leukemic cells with rare or no expression of HSCs and lineage markers. Second, isolated positive cells have cancer biologic activities *in vivo* and *in vitro*, compared to that of negative cells. Third is high ability for long-term repopulating in engrafting NOD/LtSz-*scid* IL-2R γ^{null} mice regardless of low number of injected cells. Furthermore, overexpressed proteins are directly associated with shorter overall survival in AML cohorts depending on specific subtypes and are correlated to AML linked mutation genes such as FLT-3, NPM1. Collectively, it indicated that specific inhibition of markers could exactly target LSCs in terms of FAB subtype and might pave the way in treatment with specific monoclonal antibody, when patients received autologous transplantation. Approximate distribution by markers and general scattering of cells is presented by Fig. 1. More detailed LSC phenotype, which

might coexist in Lin⁻CD34⁺CD38⁻CD90⁻CD45RA⁺ and Lin⁻CD34⁺CD38⁺CD123⁺CD45RA⁺ compartments, is announced after discovery of CD34⁺CD38⁻ cells. In common, these cell populations are mostly similar to normal granulocyte-macrophage progenitor (GMP) and lymphoid-primed multipotent progenitor (LMPP) (Goardon et al., 2011). Indeed, because not all AML-LSCs restricted in CD34⁺CD38⁻ phenotype, we cannot exclude phenotypic diversity with molecular complication of AML-LSCs. If dynamic status of LSCs including cytogenetic mutation, AML subtype, therapeutic status, response of chemo-resistance, and gene mutations is simultaneously and scrupulously considered, it may be helpful to target LSCs effectively.

Potential drugs, monoclonal antibodies that correspond to each target

Despite of heterogeneity of LSCs, target molecules and drugs have been continuously developed and are undergoing clinical trials. Perpetual challenge for developing drugs could ultimately provide LSC elimination to be completed. Among these, monoclonal antibodies were well positioned and their therapeutic potentials owing to their target antigen specificity and minimal toxicity were highlighted. In recent years, a number of surface antigens have been identified as mentioned above. Representative monoclonal antibodies applying clinical trials are CD44, CD47, CD33, CLL-1 and CD123. And these marker proteins have strongly demonstrated anti-tumoral effects in NOD/LtSz-*scid* IL-2R γ ^{null} mice and their application in clinic phase is in progress. CD123 is known

as interleukin-3 receptor alpha and is expressed on normal HSC as well as LSCs (Testa et al., 2002; Taussig et al., 2005). CD123 is over-expressed in AML patients, who have *fms*-like tyrosine kinase 3- internal tandem duplication (FLT3-ITD) and nucleophosmin 1 (NPM1) gene mutation with over 72% (Rollins-Raval et al., 2013). FLT3-ITD expressed patient have been regarded as high risk group with recurrence and Rollins showed that CD123 expression in CD34⁺CD38⁻ compartment may predict relapse in FLT3-ITD groups, implicating specificity of monoclonal antibody depending on patient's status. CD123 targeting monoclonal antibody can suppress LSC activity with effective inhibition, which is achieved by Fc dependent mechanism, resulting in blocking homing and engraftment of LSCs (Jin et al., 2009). When Fc receptor, but not Fab region was blocked either by immunoglobulin or immunosuppressive antibodies, AML-LSC repopulating activity was remarkably decreased in the CD34⁺CD123⁺ cells. It suggested the importance of receptor phosphorylation via Fc receptor. High enriched CD123 cells in CD34⁺CD38⁻ compartment is attested as a LSCs. CD44 is a family of transmembrane glycoprotein, mainly linked to hyaluronan acid, and promotes homing of LSCs and HSCs into endosteal niche (Lapidot et al., 2005). It is known that LSC homing is also dramatically suppressed by inhibition of CD44, leading to BM niche control (Jin et al., 2006). Monoclonal antibody for CD44 already entered into preclinical phase 1 in solid tumor, but not AML yet (Vugts et al., 2014; Willemien et al., 2016). In case of CD47, it is widely expressed in the human tissues and blood lineage

Table 1. Monoclonal antibodies in AML. - Clinicaltrials.gov

Target marker	Active and recruiting trials	Status	Expression on HSCs	Expression on LSCs
CD123	NCT00397579	Active, not recruiting	Positive	Strongly positive
	NCT01632852	Completed		
CD44	Clinical phase I in solid tumor, but not AML	Active, recruiting	Positive	Strongly positive
CD47	NCT02641002	Recruiting	Positive	Strongly positive
CD96	Preclinical phase	N/A	Positive	Strongly positive
CLL-1	NCT03038230	Currently recruiting	Negative	Positive
CD33	NCT02326584 Clinical phase III	Active, not recruiting	Strongly positive	Strongly positive

cells including myeloid cells and is over-expressed in myelodysplastic syndromes as well as severe AML. In xenografting, blocking of CD47 via monoclonal antibody or neutralization induces active status of macrophages and leads to anti-leukemic effect by activation of immune cell including macrophage (Majeti et al., 2009; Theocharides et al., 2012). CD96 is a type I membrane protein and immunoglobulin family, which is very similar to CD226. CD96 is overall enriched in 90% of LSCs, very low coexistence of HSCs is known with 5% (Hosen et al., 2007). Interestingly, CD96 is preferentially expressed on CD34+CD38-CD123+ cell population and can be explored diagnosis indicator for poor outcome (Chávez-González et al., 2014). Regardless of the ambiguous mechanism of CD33, it is considered as a key marker protein in AML due to strong expression on LSCs with CD34+CD38- fractions. CD33 is expressed both in LSCs and HSCs. but, CD33 is discriminated by CD34+CD38-CD123+ cells in AML-LSCs, but not HSCs.

Based on previous results, Clinical phase III for CD33 is completed in AML patients (Petersdorf et al., 2013). Based on above data, CD123+ phenotype is strongly highlighted in CD34+CD38- cells and showed results of the most upgraded clinic application with monoclonal antibody. In clinic, therapeutic targeted monoclonal antibodies were provided by Table 1. Ultimately, finding of decent marker for LSCs will need to investigate biology of LSCs and to advance clinic application.

CONCLUSION

I simply summarized a surface markers of LSCs and targeting monoclonal antibodies in clinical trials (Fig. 2). Monoclonal antibodies in clinical trials are undergoing to investigate their anti-leukemic effects in AML. Specific inhibition of LSCs by monoclonal antibody will present a novel leukemic therapeutic avenue, in spite of individual

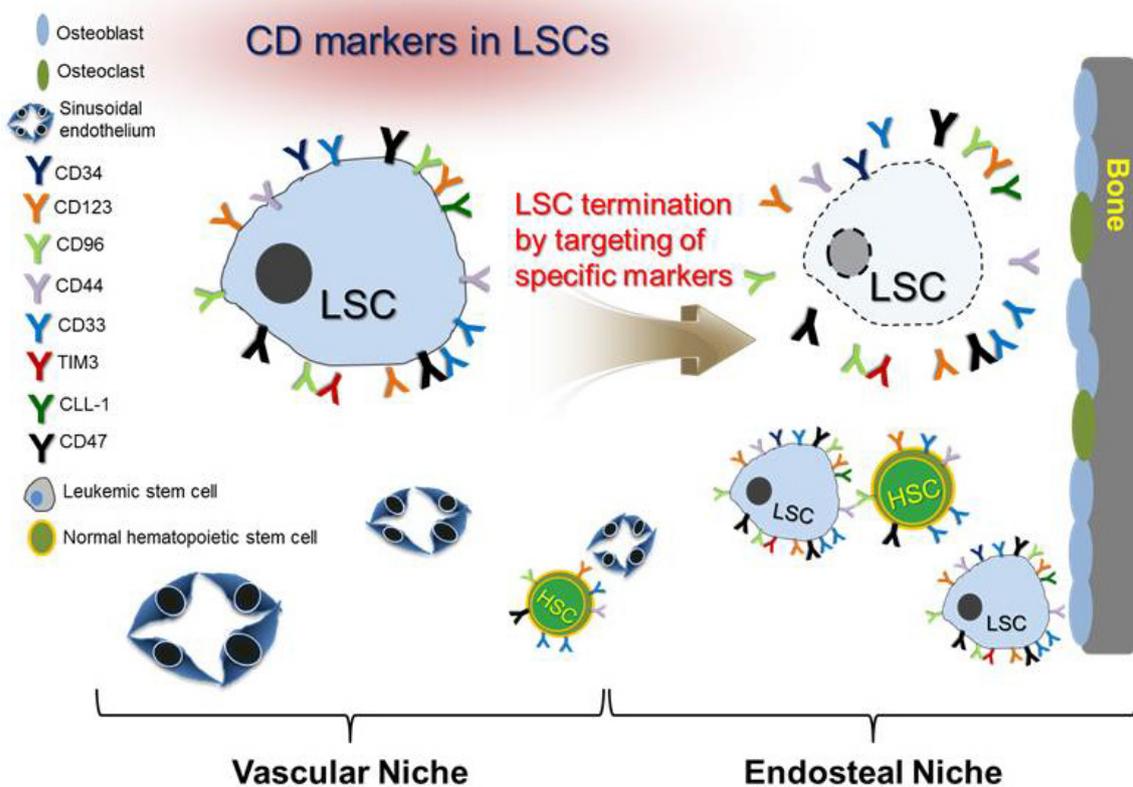


Fig. 2. Schematic diagram of markers for LSCs and HSCs in bone marrow.

variation of AML. Since LSCs are regarded as being resistant to chemotherapeutic agents, marker study in LSCs is crucial for clinical application. To completely eradicate AML, which is recurred by LSCs, a better understanding of LSCs properties and mechanism should be accompanied. Expected outcomes in clinic phase will be yielded by targeting LSCs using bona fide marker in the next strategies.

Conflict of interest

The author declares that I have no conflict of interests.

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