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Leukemic stem cells: identification and clinical application

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Abstract Leukemic stem cells (LSCs) in acute myeloid leukemia (AML) represent a low-frequency subpopulation of leukemia cells that possess stem cell properties distinct from the bulk leukemia cells, including self-renewal capacity and drug resistance. Due to these properties, LSCs are supposed to facilitate the development of relapse. The existence of LSCs is demonstrated by the ability to engraft and initiate human AML in immune-compromised mouse models. Although several lines of evidence suggest the complex heterogeneity of phenotypes displayed by LSC, many studies consider the CD34+/CD38− compartment as the most relevant. To increase the understanding of the true LSC, techniques such as multicolor flow cytometry, side-population assay and ALDH assay are utilized in many laboratories and could aid in this. A better understanding of different LSC phenotypes is necessary to enhance risk group classification, guide clinical decision-making and to identify new therapeutic targets. These efforts to eliminate LSC should ultimately improve the dismal AML outcome by preventing relapse development.

Keywords Leukemic stem cells · Acute myeloid leukemia · Flow cytometry · Immunophenotypic

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by a multitude of molecular abnormalities. Despite increasing understanding of the pathogenesis no significant changes in treatment have been achieved as yet. With current treatment strategies, the percentage of adult patients achieving complete remission has increased, mainly due to better risk classifications and improvements in supportive care. However, with still roughly half of these patients relapsing, survival rates remain low. A rare population of therapy resistant cells is believed to be at the origin of the relapse. Since these cells have the self-renewal capacity to repopulate a leukemia despite their low frequency, they are considered leukemic stem cells (LSC), also referred to as the leukemia-initiating cells (LIC). Whether these cells originate from normal hematopoietic stem cells (HSC) or from more mature progenitors that gained stemness features remains elusive and may differ among patients. Currently, many studies reveal the importance of estimating LSC burden for prognostic purposes and strategies to eradicate these cells in order to completely eliminate the leukemia. In this review, we will focus mainly on the identification of these LSC using flow cytometry and summarize novel opportunities for elimination of these LSC.

Identification of leukemic stem cells

In order to identify LSC, knowledge of their specific characteristics is essential. The recognition of stemness features (e.g., drug resistance, self-renewal and undifferentiated state) [1] alone is not sufficient since those features are also characteristics for HSC coexisting in the bone marrow (BM)

[2]. The existence of leukemic cells that meet these criteria was first demonstrated over 20 years ago. In the 1990s, Dick and colleagues demonstrated that a rare fraction of AML cells (i.e., cells with the CD34+/CD38− immunophenotype, similar to HSC) was capable of generating leukemia in immunodeficient mice [3, 4]. Since this discovery, these putative LSC have been the focus of extensive research. Golden standard property of LSC populations is the ability to engraft and initiate leukemia in a recipient mouse (initiation), to grow out after re-transplantation into secondary recipients (self-renewal) and preferable in tertiary recipients. Since normal CD34+/CD38− cells possess similar features as LSC and the design of new therapies require the specific eradication and monitoring of CD34+/CD38− LSC it is crucial to specifically discriminate LSC-containing fractions from HSC using cell-surface markers. Table 1 presents a summary of markers that are commonly used to distinguish LSC from HSC, thereby allowing to define the contribution of both the LSC and the HSC to the total CD34+/CD38− compartment. However, each marker allowed identification of LSC only in part of the AML patient population and often identified only part of the total LSC population in a particular patient [5, 6]. Combining all markers and other properties that distinguish LSC from HSC [5] allows to robustly identify the LSC and to estimate its frequency. Since the use of all markers to identify CD34+/CD38− LSC fraction of the total CD34+/CD38− compartment in each patient would require a dramatic amount of work and money, we developed a simplified comprehensive panel of markers that included only non-redundant LSC markers [6].

The existence of LSC outside the CD34+/CD38− fraction has been proposed as early as 1996, when CD34− cells were shown initiating leukemia in immune-deficient mice [6, 7]. Indeed, in less immunodeficient mice strains, both CD34+ and CD34− populations engraft [8–10]. Besides the influence of distinct properties of the mouse models used on engraftment, important studies of Bonnet et al. showed that there is another phenomenon that determines engraftment: they showed that the anti-CD38 antibody (used to purify CD34+/CD38−, CD34+/CD38+, CD34−/CD38− and CD34−/CD38+ cells prior to transplantation) inhibited subsequent engraftment [11, 12]. This is suggesting that earlier results could be based on technical interference rather than true biological differences of the CD38− and CD38+ populations.

Taken together, these studies advocate that LSC might co-exist in all CD34/CD38 defined subpopulations. In a recent study of Ng et al., the four CD34/CD38 defined cell populations of AML patients were sorted and were subsequently injected into mice and screened for their leukemia-initiating ability [13]. This exquisite approach confirmed that LSC activity was detected in all fractions; however, with a majority of CD34+ fractions, especially CD34+/CD38−, and minority of CD34− fractions containing LSC. The fact that there were hardly cases in which leukemia-initiating cells originated from CD34− and/or CD34+/CD38+ without concomitant activity in CD34+/CD38− suggests that the CD34+/CD38− fraction contains the most important leukemia-initiating cells when the other fractions are concomitantly present.

Table 1 Distinct leukemic stem cell markers

Marker	Identified as	Expression				References
		Normal	In AML (%)	HSC	CD34+ CD38− LSC	
IL1RAP	IL1R3	T cells	79	−	+	[70–72]
CLL-1	CLEC12A, MICL, DCAL-2	Myeloid cells	70	−	+	[6]
TIM-3	T-cell Ig Mucin 3	Activated T cells, NK cells	91	−	+	[73]
CD2	SRBC, LFA2, T11	T cells, NK cells	87	−	+	[14]
CD7	GP40, TP41, LEU-9	T cells	43	−	+	[6]
CD11b	Integrin alpha M, Mac-1	Myeloid cells	55	−	+	[6]
CD22	BL-CAM, Siglec-2	B cells	51	−	+	[6]
CD25	IL2RA, TAC	Activated B and T cells	25	−	+	[74]
CD33	P67, Siglec-3	Myeloid cells, NK cells	82	+	++	[6] [75]
CD44	Adhesion molecule	Ubiquitously	100	+	++	[6]
CD45RA	Tyrosine phosphatase receptor type C	T cells, myeloid cells	65	−	+	[76]
CD47	Integrin-associated protein (IAP)	Ubiquitously	100	+	++	[77]
CD56	N-CAM, MSK39	NK cells, activated T cells	32	−	+	[6]
CD96	TACTILE	Activated T cells	33	−	+	[6]
CD99	MIC2, single-chain type-1 glycoprotein	Myeloid cells	83	−	+	[78]
CD123	IL3R	Myeloid cells	82	+	++	[6] [48] [79]

This hypothesis is confirmed by other observations: in transplantation experiments using NOD/SCID mice of unfractionated AML, engraftment correlated only with the CD34+/CD38− frequency in the original sample, but not with the CD34+/CD38+ or CD34 frequency [14]. In addition, in line with the finding of in vitro and in vivo therapy resistance [15, 16], it was found that it is only the CD34+/CD38− LSC frequency that correlates with therapy outcome and minimal residual disease (MRD) levels, i.e., number of leukemic blasts detected after therapy [5]. This suggests that it is this fraction, and not the CD34+/CD38+ and CD34− fractions, that preferentially survives therapy and recapitulates leukemia.

The frequency of LSC within all mononuclear cells is shown to vary widely between patients (1 in 1.6×10^3 in 1.1×10^6) [10]. Since the CD34+/CD38− population frequency is 1 in 5×10^3 [17], we need, at least in part of the patients, assays to identify the smaller subpopulation of LSC within this population. Exploiting other (non-immunophenotypical) features of the LSC allows this.

About 20% [18] of AML cases are characterized by absence of neoplastic CD34+ cells [18, 19]. In these cases the commonly small CD34+ (<1%) blast population does not contain leukemic cells [18, 20, 21]. By definition, these CD34− patients lack CD34+/CD38− or CD34+/CD38+ leukemic populations; however, a potential LSC population should be found within the remaining CD34− fraction. Apparently, there are small and yet unidentified subpopulations to consider as most leukemogenic and therapy resistant in these AML cells.

Since LSC are supposed to be relatively chemotherapy resistant, the finding of a very small cellular compartment that is defined by high ABC drug transporter activity is of particular interest. Indeed a specific cell population [i.e., side population (SP)] could be identified using flow cytometry analysis in which the specific Hoechst dye 33342 [22, 23] is extruded efficiently by these drug pumps. These SP cells are resistant to AML therapies that include drugs that are used for treatment of AML patients like anthracyclines [24]. Purified SP cells were shown to have leukemic initiating capacity in NOD/SCID mouse models [24, 25] and contained both CD34+ cells and CD34− cells [26, 27] which are indeed in part neoplastic [28]. Although this suggest that a small part of the CD34− cells are therapy resistant, it remains to be established whether the SP cells are candidates for the leukemia-initiating cells in so-called CD34− leukemia.

Since the SP population can contain both HSC and LSC, inclusion of LSC-specific surface markers should aid in distinction between the LSC and HSC within the SP.

Next, stem cells are known to protect themselves by high expression of aldehyde dehydrogenase (ALDH), which is a cytosolic enzyme involved in retinoic acid metabolism maintaining cellular homeostasis. ALDH is shown to protect against DNA damage induced by reactive oxygen species and reactive aldehydes. In normal BM CD34+/CD38− HSC display high levels ALDH activity ($ALDH^{high}$) [29]. In both normal BM and in the majority of AML BM cells, the CD34+/CD38−/$ALDH^{high}$ population is considered to contain only HSC [21, 29, 30]. In contrast to normal BM, in AML a second population can be discriminated with cells having intermediate ALDH expression [29]. When purified, this population was most potent in AML engraftment in immunodeficient mice and was generally found positive for leukemic cytogenetic markers [29]. Furthermore, presence of this population after therapy was highly predictive for relapse [29]. In conclusion, ALDH activity can be used as a functional stem cell marker, identifying HSC population and LSC population in AML. Validity to therapeutically target ALDH in AML treatment is controversial; a recent paper showed that in vitro and in vivo inhibition of ALDH selectively eradicates CD34+/CD38−ALDH+ cells [31]. In this study, the authors used the CD34+/CD38−/ALDH+ phenotype to describe LSC, which is distinct from most other studies that define CD34+/CD38−/ALDH+ to reflect HSC. To reveal whether these CD34+/CD38−/ALDH+ cells that are targeted, are indeed neoplastic cells, additional genetic characterization might be insightful.

LSC heterogeneity of LSC within a patient

Recently it was shown that the constitution of AML at relapse may differ from diagnosis due to clonal changes including clonal evolution, clonal regression and clonal selection, with possible changes on immunophenotypic [32, 33], cytogenetic [34], genetic [34, 35] and epigenetic [35] level. Detailed whole genome sequencing studies, analyzing paired diagnosis–relapse samples, showed that at time of diagnosis, patients could present with a wide array of small subclones of which some remained in relapse [36]: indicative for clonal selection under therapy pressure. In 2012, we showed that immunophenotypically defined subpopulations of cells prominent at relapse could be traced back as very minor immature (CD34+/CD38−/dim) subpopulations of cells at diagnosis [32], again suggesting the importance of the CD34+/CD38− leukemic stem cell fraction. Since LSC are currently followed during therapy as biomarker of treatment efficacy and as prognostic factor for relapse, it is of great relevance to identify all (possibly minor) LSC populations that are potentially capable of causing relapse [37].

Clinical relevance of LSC load for prognosis

The CD34+/CD38− burden of AML patients is of strong prognostic value. In adult AML, patients with CD34+/CD38− frequencies higher than 3.5% at diagnosis had a median relapse-free survival of 5.6 months, compared to 16 months in those with lower CD34+/CD38− frequencies [14]. These results were later confirmed in other studies in adult AML [14] and in pediatric AML [38]. As knowledge on the makeup of the CD34+/CD38− fraction increased, other markers and properties were included anticipating better selectivity in defining LSC as previously summarized [5, 29]. In studies on the prognostic impact of CD34+/CD38− LSC on disease outcome, the prognostic influence of complete absence of this fraction was also discovered: CD34− status, characterized by the complete absence of neoplastic CD34+ cells [18], turned out to be an independent prognostic factor identifying patients with better prognosis in adult [17] and pediatric AML [39] compared to patient with high or low CD34+/CD38− LSC frequencies.

Despite the accumulating evidence of the prognostic relevance of LSC load at diagnosis, this feature is currently not included in risk group stratification. It is our assumption that implementation of flow cytometric quantification of LSC could be implemented without great effort using our one-tube assay [6]. Since prediction of outcome also greatly depends on many different factors during therapy, including LSC measurements during therapy (for instance at MRD time points) is warranted [40].

Impact of LSC frequency during therapy

Assessment of the frequency of remaining leukemic cells present during and after therapy (measurable/minimal residual disease, MRD) is increasingly used as an early read-out of therapy efficacy [6, 41]. MRD frequency measurement has been shown to have independent prognostic impact across different cytogenetic and molecular subgroups [42–45], and is currently used to refine risk group classifications after induction therapy. In particular, MRD is implemented in the HOVON/SAKK H132 study to guide decisions for transplantation type in intermediate risk patients. In this study, immunophenotypic MRD measurements are complemented with mutation analysis in NPM1-mutated patients, in which NPM1 status at MRD is leading for the clinical decision. In fact, many MRD studies are currently being performed, which use (or include) molecular assays [57].

It is remarkable that in all immunophenotype and/or molecular MRD studies still a proportion of MRD-negative patients develop a relapse. There are multiple possible reasons for this, e.g., low assay sensitivity, occurrence of mutational/immunophenotypic shifts or different kinetics of MRD disappearance. There may, however, also be a biological explanation: it may not only be the number of leukemic blast cells, reflecting MRD, that defines the risk of relapse, but also the number of LSC present within this blast cell population. As we argued earlier, stem cells have been demonstrated to be more therapy resistant than leukemic blast cells. The MRD population is thus likely enriched with LSC, but these are too low in frequency to contribute significantly to the total frequency of MRD cells. Indeed, when the number of CD34+/CD38− LSC after therapy was determined, LSC load was an independent predictive factor for patient survival [5]. Such was found by others too, be it with different assays and different immunophenotypical and functional definitions of stem cells [29, 46]. Remarkably, assessment of both LSC and MRD led to better separation of patients risk group classification than either MRD or LSC alone [5, 17].

For newly tested therapies, survival end point is the most important determinant of the therapeutic effectiveness. However, large clinical trials are needed with high numbers of included patients. At best, it than takes approximately 2–3 years to predict survival [47]. With increasing numbers of tested therapies, specifically targeting LSC, usage of LSC frequencies as surrogate intermediate endpoint for survival would be highly beneficial [48].

Therapeutic opportunities eliminating LSC

General principles and challenges faced by targeting LSC

With the poor prognosis of AML and only little improvements in therapeutic options, there is a pressing need for novel therapies. Therapies targeting LSC offer hope for such improvement. Fundamental to LSC therapy is the selection of the target and the timing of the therapy. Ideally, the target is highly expressed by LSC, highly selective, i.e., absence of expression on other cells in particular HSC and no circulating antigens, and preferentially expressed by high numbers of patients. Acknowledging the many similarities that LSC and HSC share, it is not surprising that current treatment approaches are limited. As the specific identification and, with that, the characterization of LSC has become more detailed, therapies directed to LSC, while sparing normal HSC, are becoming reality and are currently investigated as delineated below.

Distinct cell-surface markers have been proposed as potential LSC-specific targets (Table 1) and several approaches targeting some of these LSC surface markers are currently in clinical trials (see Table 2). Of these markers, therapies targeting CD33 are possibly the most studied

Table 2 Evaluation of novel leukemic stem cell directed drugs

Target	Antibody/small molecule	Efficacy	Trials	References
Therapy targeting stem cell-specific surface markers				
CD33	AMG330 (CD33-CD3 BiTE)	Reduced in vitro CFU	I	[80]
	Gemtuzumab ozogamicin	Selective kill of CD34+ CD38−CD123+ LSC, sparing HSC	I–III	[51]
	SGN-CD33A	Activity requires CD33 expression, activity does not correlate with expression levels	I–III	[81]
IL1RAP	IgG mAb 81.2	Selective kill of IL1RAP-positive leukemic blasts and LSC-enriched populations	N/A[a]	[70]
TIM3	ATIK2a	Selective block of LSC engraftment/development, sparing HSC	N/A	[56]
CLL-1	CLL1-CD3 BiTE	Internalization leads to stem cell death, induction of CDC and ADCC activity	I	[82]
CD123	SL-101	Selective suppression of leukemic progenitors (in CFU)	N/A	[48]
	SGN-CD123A	Anti-leukemic activity in preclinical AML models	I	[83]
CD44	IgG1 H90	Specificity towards leukemic cells over normal CD34+ cells, inhibits mTOR	I	[84]
Therapy targeting LSC-related molecular pathways				
AKT	MK-2206	Impaired leukomogenesis and reduced LIC frequency in vivo	I–II	[85]
	Perifosine	Reduced clonogenic activity, sparing normal CD34+ cells	I	[86]
mTOR	Torkinib, PP242	Reduced proportion of CD34+ cells in vivo	N/A	[87]
	MLN0128	CFU inhibition in LSC isolated from primary and secondary xenograft	N/A	[88]
BCL-2	ABT-263	Selectively targets LSC mitochondrial energy generation, induced cell death	N/A[b]	[89]
XPO1	KPT-8602	Selective kill of blasts and LSC in AML patient-derived xenograft models	N/A[c]	[90]
	Selinexor, KPT-330	Selective decrease of LIC frequency in AML cells isolated from xenografts	I–II	[91]
NF-kB	Parthenolide	Preferentially targets AML progenitors (in vitro CFU) and stem cell in SCID xenografts	N/A	[92]
Smoothened	PF-913	Reduced fraction of CD34+ CD38− cells, sensitized AML cells to cytosine arabinoside	N/A	[93]
Proteasome	Carfilzomib	Reduced long-term survival of AML CD34+ cells	I	[94]
	Bortezomib	Bortezomib-treated mice showed significant decrease in LIC-enriched populations	I–III	[95]
Histone deacetylase	Chidamide	Induced apoptosis in LSC-like cells and primary AML CD34+ cells	I–II	[96]
DOT1L	EPZ004777	CFU inhibition in primary samples with DNMT3A mutation, not affecting cells without this mutation	N/A	[97]
Therapy targeting the LSC microenvironment				
CXCR4	Plerixafor, AMD3100	Decreasing bone marrow homing	I–II	[66]
	AMD3465		N/A	[66]
	BMS-936564		I	[98]
VLA4	Natalizumab		II	[99]

CDC cell-dependent cytotoxicity, *ADCC* antibody-dependent cell-mediated cytotoxicity, *CFU* colony forming unit

[a] In chronic myeloid leukemia

[b] In chronic lymphoid leukemia

[c] In multiple myeloma

in AML patients. Although targeting CD33 was originally not meant as an anti-LSC therapy, it turned out that CD33 was overexpressed in LSC compared to HSC [6]. Treatment with Gemtuzumab ozogamicin (GO) treatment was associated with reduced relapse risk and improved overall survival in patient subgroups [49, 50]. Whether GO targets

CD33+ LSC, causing the reduction in relapse risk, remains unclear [50] as higher numbers of CD34+/CD38−/CD33+ cells and high CD33 expression levels decreased GO sensitivity in vitro [51]. One lesson that can be learned from GO treatment is clear: high specificity of the therapy is important. CD33 is, next to leukemic cells, also present on most HSC, on mature and immature myeloid cell and on various progenitors [52], possibly underlying toxicities as found in earlier studies [53]. Anti-CD123 therapy may have similar disadvantages [6], while results of clinical trials targeting newer discovered surface markers more specific for LSC (including CCL-1 [54, 55], TIM3 [55–57], CD96 [58]), will provide important insights in validity of therapies targeting immunophenotypic markers.

Next to specificity, the design of the antibody in terms of conjugates is of importance for effectiveness. Novel engineering of antibodies has potential to improve efficacy and reducing immunogenicity (mechanisms and constructs are reviewed by Scott et al. [59]. and Tiller and Tessier [60]).

One alternative way of direct LSC targeting is with the use of small-molecule inhibitors interfering in key signaling pathways altered in LSC (see Table 2). Using this strategy the leukemic progenitor cells are also targeted since mutations found in signaling pathways in AML are not limited to the LSC, but are inherited by their progeny. Recent studies have also indicated the relevance of splicing on signaling pathways [61]; therefore, small molecules that affect the spliceosome are also investigated as novel therapeutics to eradicate LSC [62].

Future perspectives

LSC maintenance and functioning is related, at least in part, to signals from the BM microenvironment [63–65]. Therapeutic targeting is therefore not only directed to LSC. Initial studies inhibiting factors necessary for LSC homing (e.g., CXCR4, CXCL12) have shown to abrogate chemoresistance [66], suggesting combination therapies with LSC-specific targets. Clinical trials targeting the LSC niche are in progress [67] (see Table 2).

In this review, we have conveyed the important role of LSC in AML with emphasis on the identification of LSC using flow cytometry. As the identification of CD34+/CD38− LSC allows for the identification of patients with a poor prognosis, we consider LSC measurements as valuable asset for clinical decision-making. This concerns both risk group classification at diagnosis or definition of risk groups after therapy (in an MRD situation). In view of the large heterogeneity of LSC within and among patients, the identification of all specific LSC would be too costly in terms of AML cells, time and money. For that reason, a broadly applicable simple one-tube approach has been developed, which can easily be implemented in routine diagnostics [6]. Additionally, screening for CD34+/CD38− LSC also enables identification of CD34− patients, who generally have a better prognosis [18]. Furthermore, as LSC-specific therapies—targeting LSC-specific surface markers—become available, individualized therapy may come in view. To select the most effective marker-directed therapy, the LSC phenotype of the individual patients needs to be determined. With increasing numbers of markers becoming available, innovations in flow cytometers will continue to support a growing number of channels/colors available in simultaneous measurements. The currently available multicolor flow cytometry approach used in AML does not exceed ten colors [68]. While this allows a universal screening, for precise characterization of the most pure (very minor) LSC population, multiple markers are needed. Current technological advances will come from high-number-multicolor flow cytometry or CyTOF approaches [69] in which an extensive panel of LSC markers will be available.

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