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Translating Leukemia Stem Cells into the Clinic: Harmonizing the Heterogeneity

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Abstract

Considerable evidence suggests that rare leukemia cells with stem cell features, including self-renewal capacity and drug resistance, are primarily responsible for both disease maintenance and relapses. Traditionally, these so-called leukemia stem cells (LSCs) have been identified in the laboratory by their ability to engraft acute myeloid leukemia (AML) into immunocompromised mice. For many years, only those rare AML cells characterized by a hematopoietic stem cell (HSC) $CD34^+CD38^-$ phenotype were believed capable of generating leukemia in immunocompromised mice. However more recently, significant heterogeneity in the phenotypes of those AML cells that can engraft immunocompromised mice has been demonstrated. AML cells that engraft immunocompromised mice have also been shown to not necessarily represent either the founder clone or those cells responsible for relapse. A recent study found that the most immature phenotype present in an AML correlated with genetically-defined risk groups and outcomes, but was heterogeneous. The patients with AML cells expressing a primitive HSC phenotype ($CD34^+CD38^-$ with high aldehyde dehydrogenase activity) manifested significantly lower complete remission rates, as well as poorer event-free and overall survivals. Leukemias whose most primitive cells displayed more mature phenotypes demonstrated better outcomes. The strong clinical correlations suggest that the most immature phenotype detectable within a patient's AML might serve as a biomarker for "clinically-relevant" LSCs.

Keywords

leukemia stem cells; acute myeloid leukemia; hematopoietic stem cells

Introduction

Most acute myeloid leukemia (AML) patients achieve complete remissions (CRs) with standard induction chemotherapy, but the majority subsequently relapse and succumb to the disease. It is becoming clear that tumor heterogeneity is one of the important factors in the

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dissociation between response and survival in AML. Although some of this tumor heterogeneity can be explained by subclonal progression within the malignant cells, the presence of leukemia cells at various stages of differentiation is also a major contributor. [1] Most data suggest that AML retains some semblance of the normal hematopoietic hierarchical structure: i.e., rare leukemia cells with stem cell features, including self-renewal capacity, give rise to partially differentiated progeny that comprise the bulk of the leukemia but possess only limited proliferative potential. These rare leukemia-initiating cells, or so-called leukemia stem cells (LSCs), are postulated to be responsible for relapse by resisting traditional cytotoxic chemotherapies that are usually highly active against the leukemia bulk. [2–15] LSCs appear to exhibit inherent drug resistance at least in part by co-opting normal stem cells' intrinsic defense mechanisms such as quiescence, efflux pumps, and detoxifying enzymes. Moreover, LSCs, like normal hematopoietic stem cells (HSCs), upregulate the 'don't eat me signal' CD47, presumably as a mechanism to avoid immune-mediated killing. [16] Recent data also suggest a prominent role for the stem cell microenvironment or niche in protecting LSCs from both cytotoxic and immunologic therapies. Importantly, cross talk between LSCs and their niche has now been clearly shown to be critical to the growth and maintenance of the leukemic clone. [17, 18]

The LSC Concept: Historical Perspective

As initially postulated by Ashley, cancer-initiating cells must survive long enough to accumulate the 3–7 genetic mutations necessary to generate cancer. [19, 20] Nowell hypothesized that the inherent longevity and extensive proliferative capacity of stem cells made them ideal candidates for cancer initiating cells. [21] However, longevity and extensive proliferative capacity are not traits restricted to classical stem cells. To some degree, myeloid progenitors beyond the level of HSCs also retain these properties.

The first clear evidence supporting the LSC concept was published more than 40 years ago, when Fialkow *et al* demonstrated clonal hematopoiesis involving both the erythroid and myeloid lineages in patients with chronic myeloid leukemia (CML). [22] In 1994, Lapidot and colleagues [8] established the capability to recapitulate leukemia after transplantation into immunocompromised mice as the gold standard for identifying LSCs. In these early mouse experiments LSCs were located strictly within the $34^{+}38^{-}$ cell compartment, suggesting a homogenous HSC phenotype. [3, 8] Moreover, in most AML patients the leukemic $CD34^{+}CD38^{-}$ cells that engrafted immunocompromised mice could be separated from normal HSCs by their expression of the stem cell marker aldehyde dehydrogenase 1 (ALDH). Normal HSCs exhibited high ALDH expression ($CD34^{+}CD38^{-}ALDH^{high}$), while the putative LSCs expressed intermediate levels ($CD34^{+}CD38^{-}ALDH^{int}$). [5, 7, 12] However, in a significant fraction of AML patients no leukemia cell subset will engraft immunocompromised mice, even using the newer, more permissive mouse models. [2, 10, 14]

LSC Heterogeneity

Many studies have now suggested that the phenotype of putative LSCs is heterogeneous. AML cells of various differentiation phenotypes, including $CD34^{+}CD38^{+}$ and $CD34^{-}$, have

been shown capable of engrafting immunocompromised mice. [4, 10, 11, 13] Still other groups have suggested that putative LSCs can exhibit heterogeneous expression of ALDH. [5, 7, 23, 24] Sarry *et al* found that the engrafting AML cells can be heterogeneous even within the same patient. [11]

Our group found that the majority of core-binding factor (CBF) AML cells present in minimal residual disease (MRD) exhibited a $CD34^{+}CD38^{-}ALDH^{int}$ phenotype [5], even though such cells represented only about 1–10% of the total leukemia burden at diagnosis. [6] Moreover, their presence after therapy was highly associated with subsequent clinical relapse. [5] Thus, we hypothesized that the most primitive hematopoietic phenotype present in the AML may serve as a clinical biomarker for LSCs. [6] However, several patients had no detectable $CD34^{+}$ AML cells, as others have also described [4, 10, 11, 13], and others had leukemia cells that were $CD34^{+}CD38^{-}ALDH^{high}$. [5] To better understand the heterogeneity and clinical significance of the most immature phenotype present in a leukemia, patients with newly-diagnosed AML prospectively entered on a large multi-institutional clinical trial were studied. [6] As our earlier work predicted, the most immature hematopoietic cellular phenotype present within a specific leukemia was found to be heterogeneous, ranging from $CD34^{-}$ to that of primitive HSCs (i.e., $CD34^{+}CD38^{-}ALDH^{high}$). [6] In most patients, the most primitive AML phenotype found was $CD34^{+}CD38^{-}$. The $CD34^{+}CD38^{-}$ leukemia cells from about 60% of these patients displayed intermediate ALDH expression as previously described [5, 7, 12], while normal $CD34^{+}CD38^{-}$ HSCs expressed high levels of ALDH. In the other 40% of patients harboring $CD34^{+}CD38^{-}$ leukemia cells, the primitive AML cells exhibited high ALDH activity. No $CD34^{+}$ leukemia cells could be detected in about a quarter of patients. [6]

Clinical significance of LSCs

Despite abundant research around the LSC concept, there has been limited data that LSCs are indeed responsible for disease resistance or relapse. Several groups have reported that the frequency of $CD34^{+}CD38^{-}$ leukemia cells correlated with prognosis [14, 25], but as just described, some leukemias do not have a $CD34^{+}CD38^{-}$ population to assess. [4, 10, 11, 13] Engraftability of AML cells in immunocompromised mice has also been shown to be associated with a poor clinical outcome. [2, 9, 10] However, the mouse engraftment assay may more accurately reflect the proliferative potential of the leukemic cells [26] and/or their interactions with the mouse microenvironment [27], than it does their role in disease maintenance and relapse. Accordingly, a recent study showed that AML cells that engrafted into immunocompromised mice may not represent either the founder clone or those responsible for relapse. [1] Thus, these data together with the fact that no AML subset in many patients will engraft immunocompromised mice, suggest that other means for LSC identification are needed to allow their study clinically.

Regardless of their phenotype or tumorigenic potential in immunocompromised mice, leukemic cells that persist after therapy (i.e., MRD) are arguably the most clinically important. Our group studied the clinical significance of an AML's most primitive hematopoietic phenotype, since we found it to be enriched during MRD. [5] Despite demonstrating substantial heterogeneity overall within AML patients, the most immature

phenotypes were much more consistent within individual genetically-defined risk groups. [6] The majority of AML patients whose most immature phenotype was $CD34^{+}CD38^{-}ALDH^{high}$ harbored poor-risk cytogenetics or FLT3 internal tandem duplications. The most immature phenotype found in all CBF and most intermediate-risk AMLs was $CD34^{+}CD38^{-}ALDH^{int}$, and the most immature phenotype in the most favorable AMLs, NPM1 as a single mutation and APLs, was usually $CD34^{+}CD38^{+}$ or $CD34^{-}$. [6]

Not surprisingly given the strong association with poor-risk genetics, patients harboring AML cells with a primitive HSC phenotype ($CD34^{+}CD38^{-}ALDH^{high}$) display significantly lower event-free and overall survivals (Figure 1). [5–7, 23, 24] Patients whose most immature AML cells were $CD34^{-}$ displayed the best event-free and overall survivals [6], as others have also described. [28] Patients whose most immature AML cells had a $CD34^{+}CD38^{-}ALDH^{int}$ phenotype showed an intermediate prognosis (Figure 1). [6]

These data raise the possibility that the correlation of the AML's most immature phenotype with outcome may be a function of the stage of hematopoietic differentiation at which the leukemogenic mutation develops. As normal $CD34^{+}CD38^{-}ALDH^{high}$ HSCs differentiate into more committed progenitors, both CD34 and ALDH expression decrease while CD38 expression increases (Figure 2A). [29–33] In addition, expression of resistance mechanisms (e.g., quiescence, efflux pumps, and detoxifying enzymes) also decreases with differentiation. [34] The most favorable AMLs appear to arise from more differentiated progenitors ($CD34^{-}$) and the least favorable from primitive HSCs ($CD34^{+}CD38^{-}ALDH^{high}$) (Figure 2B – 2D). The differentiation state of the AML's cell of origin also appears prognostic within genetically-defined risk groups. Some NPM1-mutated AMLs and APLs appear to arise from $CD34^{+}$ progenitors, and they appear to do worse than the more common $CD34^{-}$ varieties of these AMLs. [5, 35–38]

The LSC Microenvironment

It is now clear that the pathophysiology of AML involves a complex interplay between the leukemic cells and their surrounding bone marrow microenvironment. [17] In fact, creating a more humanized bone marrow microenvironment allowed for engraftment of traditionally "hard to engraft leukemias," such as APL and CBF AML, further highlighting the importance of crosstalk between the human bone marrow microenvironment and the leukemia cells in disease pathology. [39] Like it does for normal hematopoietic progenitors, the bone marrow niche provides critical signals for the growth and maintenance of AML cells. However, the bone marrow microenvironment is highly complex, and thus the critical interactions between the niche and leukemia cells can also be expected to be multifaceted.

Increasing evidence suggests an important role for LSC interactions with the bone marrow microenvironment through the chemokine receptor CXCR4, which is important in the homing and support of HSCs. Normally, HSCs use CXCR4 on their surface to interact with CXCL12 secreted from various cells of the bone marrow to create HSC niches. Like normal HSCs, LSCs home to $CXCL12^{+}$ areas of the bone marrow and increased expression of CXCR4 on leukemic cells predicts poor outcomes in leukemia patients. [40, 41] Intriguingly, *in vitro* and *in vivo* models have shown that homing of LSCs to the marrow can

be disrupted by treatments targeting the CXCR4/CXCL12 axis, and chemotherapy resistance can be overcome. [42–46]

Combination therapies using LSC/microenvironment-targeted treatments have shown promise. The CXCR4 inhibitor, plerixafor, in combination with anti-TGFbeta and cytarabine was capable of prolonging survival in a mouse model of AML. [47] Interestingly, studies have shown an increase in CXCR4 expression on AML cells in response to chemotherapy, which further highlights the important role of microenvironment and leukemia cell interactions in AML pathogenesis. [48] Anti-VLA-4 antibodies used to disrupt the binding of VLA-4 on the surface of LSCs to fibronectin in the bone marrow microenvironment in combination with cytarabine significantly prolonged survival in a mouse model of AML. [49] Overall, these data demonstrate the potential of combinations targeting both LSCs and their interactions with the bone marrow microenvironment.

The bone marrow microenvironment's expression of CYP3A4 and cytidine deaminase was recently shown to be at least partially responsible for the bone marrow stroma's ability to protect leukemia cells from etoposide and cytarabine, respectively, both *in vitro* and *in vivo*. [50] Importantly, inhibiting CYP3A4 was able to restore the activity of etoposide against AML cells in the presence of bone marrow stroma. [50] Bone marrow stromal expression of CYP26, the major means of retinoid inactivation, also appears to protect LSCs from retinoids, a possible reason why these drugs have demonstrated little clinical activity in non-APL AML despite substantial *in vitro* activity. [51] Thus, expression of drug-metabolizing enzymes appears to be a novel mechanism of microenvironment-mediated drug resistance, allowing the bone marrow niche to create a sanctuary site from drugs. [50]

Clinically targeting LSCs

Despite the increasing evidence that relatively treatment-resistant LSCs are in part responsible for relapses following successful induction of complete remissions [5, 6, 10, 16, 25], there remains no clinical proof of the LSC concept: i.e., targeting these cells will result in improved outcomes. In order for an LSC-based target to have clinical utility, it must not only be expressed on LSCs, but if co-expressed by any normal cells, it must also have an acceptable toxicity profile. Given the heterogeneity of LSCs across different AMLs, it is also likely that one target will not be effective for all AML patients.

Several cell surface markers have been proposed as potential LSC-associated targets (Table 1). [29, 52–56] These phenotypic LSC markers have largely been identified based on their presence on AML cells that will engraft immunocompromised mouse models or from RNA sequencing of sorted cell populations. In some cases, including CD25, CD47, CD123, and CLL-1, the markers have been shown to correlate with AML patient outcomes. [55–58] CLL-1 expression on residual $CD34^{+}CD38^{-}$ leukemic cells after induction was a better predictor of outcome than traditional MRD monitoring in one small series. [56] In another report, the high expression of a combination of CD123 with CLL-1 on $CD34^{+}$ cells was also a strong prognostic marker for relapse in AML patients who had achieved remission. [59]

Several of these targets have already been, or are actively being, studied clinically. Monoclonal antibody therapy targeting $CD33^+$ cells did show efficacy in both relapsed and elderly AML patients. [60–62] However, lack of overall survival despite higher remission rates suggested CD33 was probably expressed primarily by differentiated leukemia cells and not LSCs. [65] Moreover, given the ubiquitous expression of CD33 on many blood cell types, cytopenias were a common side effect. [63, 64] Interestingly, the one subgroup that appeared to have an overall survival improvement was the favorable cytogenetic AMLs [65, 66]; these data may represent additional evidence that favorable AMLs arise from more differentiated, $CD33^+$, hematopoietic progenitors, while the LSCs from other subtypes arise from $CD33^-$ progenitors.

Anti-CLL-1 antibodies were capable of inducing *in vitro* complement-dependent cytotoxicity (CDC) in CLL-1 expressing AML cell lines and primary blasts in both *in vitro* and *in vivo* mouse models. [65] It does not appear that clinical trials targeting CLL-1 in AML have been undertaken. Anti-CD123 as an LSC-targeted therapy in AML has also shown promise. Though early experiments suggested a nonconventional role for upregulated CD123 on LSCs, more recent *ex vivo* studies have shown that anti-CD123 is capable of reducing IL-3-mediated proliferation in primary AML cells. [54, 66, 67] In addition, cytokine-induced killer cells transduced with a chimeric antigen receptor (CAR) targeting CD123 have shown potent killing of CD123 positive AML cell lines *in vitro* as well as primary AML blasts while sparing normal HSCs *ex vivo*. [68] Trials targeting CD123 are in progress, but thus far have generally involved patients that were refractory to standard therapy so its role in preventing relapses remains unclear. [66] The ‘don’t eat me signal,’ CD47, was shown to be expressed on most primary AML specimens, bulk tumor as well as LSCs, compared with normal bone marrow HSCs which expressed lower levels. [26] Clinical trials targeting CD47 are just beginning.

In addition to targeting LSCs directly, clinical trials targeting the LSC microenvironment are also in progress. Inhibition of the CXCR4/CXCL12 axis is being studied to mobilize leukemia from its protective environment as a means to increase its sensitivity to chemotherapy. A phase I/II clinical trial of plerixafor to inhibit CXCR4 in relapsed, refractory AML showed it to be safe and capable of mobilizing AML blasts [69], but definitive clinical activity awaits the results of ongoing trials. Targeting other adhesion molecules such as CD44 [70] or V-CAM [71] could also overcome microenvironment-mediated drug resistance. However, it is possible that the microenvironment-mediated drug resistant phenotype is maintained even after malignant cells are displaced from their bone marrow niche. [72] Based on the detoxifying effects of CYPs in the microenvironment [50, 51], our group has developed several clinical trials aimed at overcoming this potential mechanism for LSC resistance.

Conclusion

The failure of complete remissions to reliably translate into cures in AML can be explained by the LSC paradigm. Unfortunately, the definitive proof for the clinical importance of LSCs – that targeting them improves outcome – is currently lacking. Thus, the true clinical relevance of LSCs has remained the focus of considerable debate. The long-standing

definition of LSCs focusing on immunocompromised mouse models of engraftment has led to potentially contradictory results that have proven difficult to translate into the clinic and across AML subtypes. Not only are such assays cumbersome and non-quantitative, but they also reveal that varying cell phenotypes are capable of engrafting leukemia in mice; further, these assays may have little correlation to ultimate disease outcomes. [1, 4, 10, 11, 13] Beyond that, a significant fraction of AML patients has no leukemia cell subset that will engraft. [2, 10, 14]

Most studies have found that both phenotypic and genetic heterogeneity is less evident in MRD present during first CR than at disease diagnosis (Figure 2). [5, 73, 74] As a “more homogeneous” leukemia cell population, first CR may present an optimal time to target these cells with novel approaches. Moving forward, focusing on the most primitive cell phenotype present within the patients’ AML cells may provide a broadly applicable means of studying clinically relevant LSCs as well as appropriate therapies to target these cells. Moreover, about 30–40% of AML patients lack any usual cytogenetic or genetic prognostic factors, and even when present such prognostic factors may not be available for days or weeks. The most immature phenotype present within a patient’s AML can be readily determined in essentially all patients by flow cytometry within hours of diagnosis. Rapid risk-stratification may be particularly useful for patients harboring $CD34^{+}CD38^{-}ALDH^{high}$ leukemia cells, which appear to identify high-risk patients often refractory to induction chemotherapy. A $CD34^{+}CD38^{-}ALDH^{high}$ leukemic phenotype could also be used to guide patients toward allogeneic transplantation when no prognostic cytogenetic or genetic abnormalities are present.

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Highlights

- Relapses occur in acute myeloid leukemia because current treatments do not clinically target and eliminate leukemia stem cells.
- Several lines of evidence now question the clinical relevance of leukemia cells that engraft in immunocompromised mice, the “gold standard” for identifying LSCs.
- The leukemia clone’s most immature phenotype is heterogeneous for CD34, CD38, and aldehyde dehydrogenase expression, but correlates with genetically-defined risk groups and outcomes.
- Identifying the most immature phenotype within a patient’s leukemia and studying this population for actionable targets may bypass the reliance on mouse models to identify LSCs.

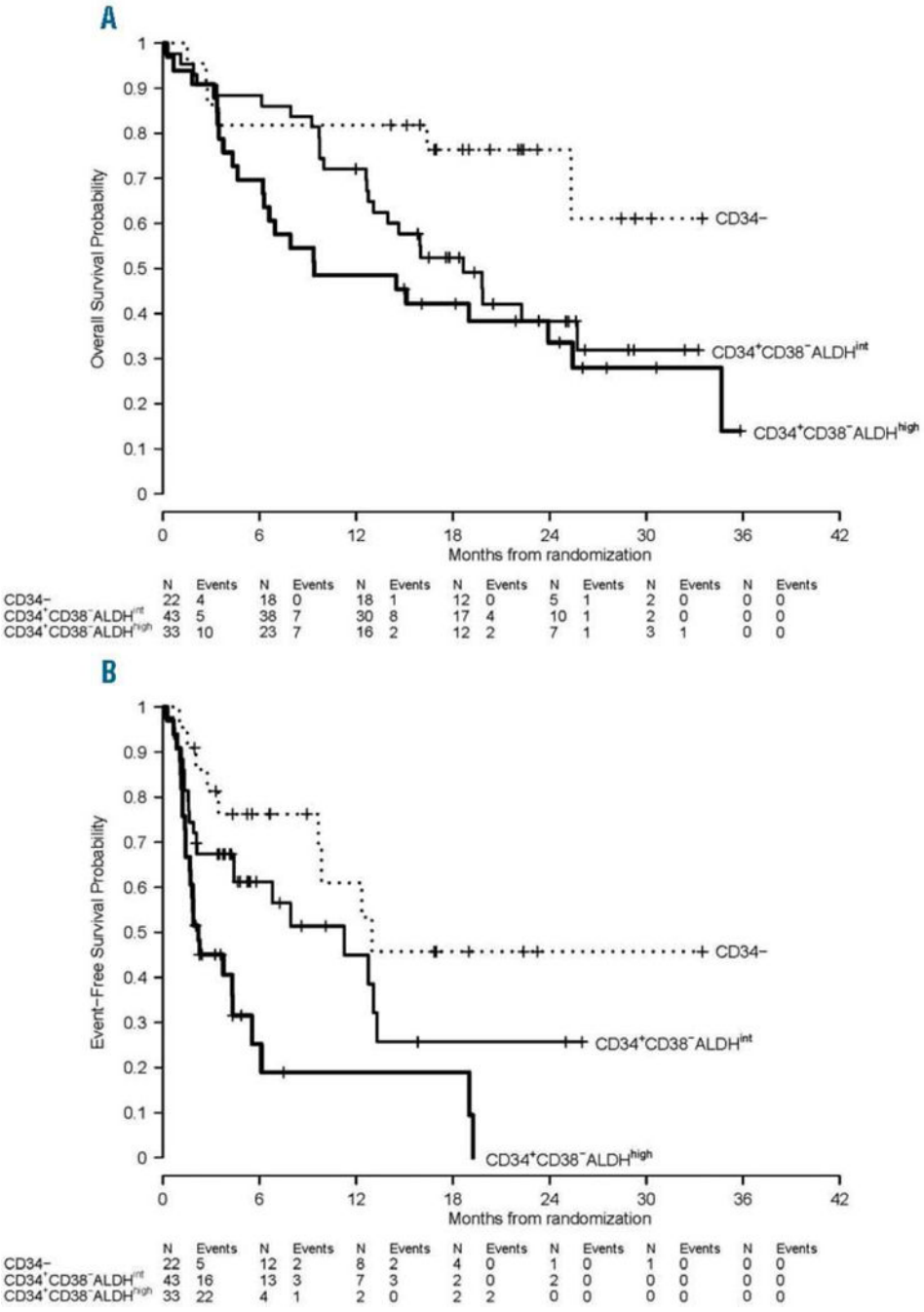

Figure 1. Clinical outcomes of AML patients based on the most immature leukemic cell phenotype

(A) Overall survival and (B) event-free survival by the most immature phenotype detectable in leukemia cells. (Adapted from Gerber *et al*, 2016 [6])

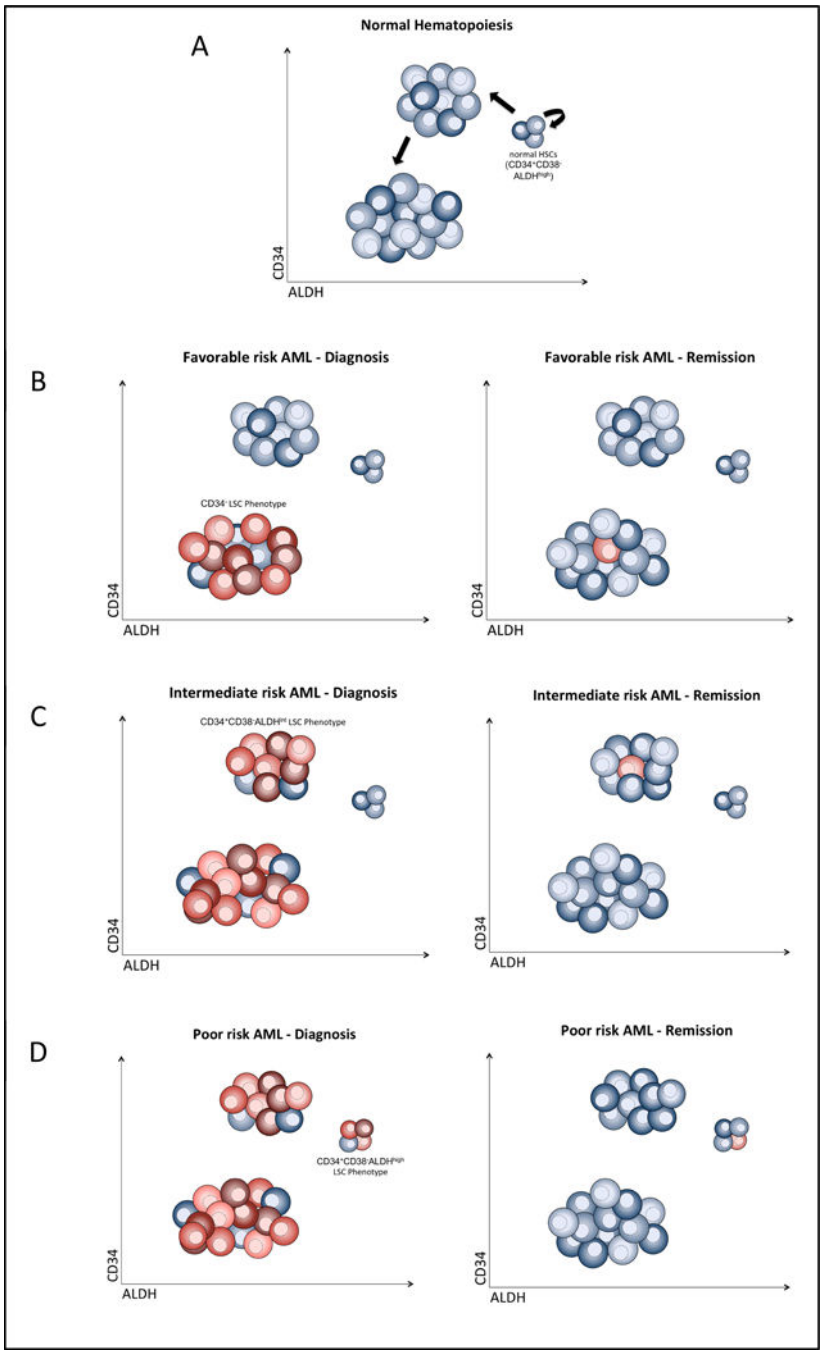

Figure 2. Leukemia stem cell heterogeneity as a function of the stage of hematopoietic differentiation at which the leukemogenic mutation develops

(A) As normal $CD34^{+}CD38^{-}ALDH^{high}$ HSCs (blue) differentiate into more committed progenitors, both CD34 and ALDH expression decreases. LSCs (red) are phenotypically heterogeneous with (B) the most favorable AMLs arising from more differentiated progenitors ($CD34^{-}$), (C) intermediate-risk AMLs from less differentiated $CD34^{+}CD38^{-}ALDH^{int}$, and (D) the least favorable AMLs from primitive HSCs

($CD34^{+}CD38^{-}ALDH^{high}$). At remission, MRD is enriched for the most immature phenotype present in the leukemia (i.e., LSCs).

Table 1

Proposed phenotypic LSC markers

A compilation of proposed phenotypic LSC-specific markers in AML, their alternate name(s), known biological function(s), and some of the cell type(s) they are known to be positive on.

	Alternate name(s)	Function(s)	Cell Type(s)
CD25	IL-2Rα	T cell proliferation, inflammatory responses	T cells, B cells
CD26	Dipeptidyl peptidase-4 (DPP4)	Immune regulation, T cell activation, apoptosis, and glucose metabolism	T cells, B cells, NK cells, macrophages
CD33	Sialic acid binding Ig-like lectin 3 (Siglec-3)	Sialic acid dependent binding, apoptosis in AML	Myeloid cells, few lymphoid cells
CD47	Integrin associated protein (IAP)	Apoptosis, proliferation, adhesion, migration, and immune recognition; phagocytosis block	T cells, B cells, NK cells, macrophages, progenitor cells, and many others
CD96	T cell activation, increased late expression (Tactile)	Immune adhesion, antigen presentation	T cells, NK cells, some B cells
CD123	IL-3R	Proliferation and differentiation during hematopoiesis	Hematopoietic progenitor cells
CLL-1	C-type lectin-like molecule 1, C-type lectin domain family 12 member A (CLEC12A)	Cell adhesion, cell-cell signaling, inflammation, and immune responses	Monocytes, macrophages, dendritic cells, granulocytes
TIM3	T cell immunoglobulin mucin-3	Autoimmunity and allergy	Th1 cells