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# Flow cytometric detection of leukemic stem cells in Acute Myeloid Leukemia: current status and future directions

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The assessment of measurable residual disease (MRD) plays a critical role in acute myeloid leukemia (AML) treatment response evaluation and prognosis. However, current AML MRD detection by flow cytometry (FC) is limited in sensitivity due to immunophenotypic variability, similarities to normal hematopoietic stem/progenitor cells, and the lack of stable leukemia-associated immunophenotypes. A significant proportion of AML patients classified as MRD-negative by FC eventually relapse, likely due to the persistence of therapy-resistant leukemic stem cells (LSCs) that are not sensitively detected by routine clinical flow panels. Flow cytometry panels designed to detect LSC antigens, while promising, face challenges like immunophenotypic heterogeneity across AML subtypes, lack of standardized marker panels across laboratories, and limited validation. Here, we summarize the current state of FC-based LSC detection in AML, discussing commonly used markers, immunophenotypic variability, assay setup challenges, and we review recent clinical studies on LSC assessment, outlining their main findings and implications for prognosis and MRD integration. We also consider advances in spectral flow cytometry for improved LSC detection.

## KEYWORDS

AML-acute myeloid leukemia, LSC-leukemic stem cells, flow cytometry, MRD-measurable residual disease, therapeutic targeting biomarkers

## Introduction

Acute myeloid leukemia (AML) is an aggressive cancer of hematopoietic stem and progenitor cells (HSPCs), affecting approximately 20,000 individuals annually in the United States (Dohner et al., 2015). Despite therapeutic advances, prognosis remains poor with a 60%–70% mortality rate (National Cancer Institute, 2024), primarily due to post-remission relapse.

Measurable residual disease (MRD) detection is essential in guiding treatment for several hematologic malignancies (Short et al., 2020). In B-lymphoblastic leukemia (B-ALL), MRD detection by flow cytometry (FC) achieves high sensitivity, often below 0.01% (Theunissen et al., 2017), and holds prognostic significance (Verbeek and van der Velden, 2024). Similarly, in multiple myeloma FC-based MRD detection is highly sensitive to 0.001%, offering strong prognostic value and thus routinely incorporated into response assessments (Medina-Herrera et al., 2023; Flores-Montero et al., 2017). However, in AML, MRD detection remains challenging, primarily due to patient-to-patient immunophenotypic variability and the lack of stable, aberrant leukemia-associated immunophenotypes (van der Linde et al., 2023). The sensitivity of FC for MRD

detection in AML is typically limited to around 0.1%, as estimated in clinical studies (Blachly et al., 2022; Hanekamp et al., 2020). A significant proportion of AML patients deemed MRD-negative by FC ultimately relapse (Ivey et al., 2016; Terwijn et al., 2013), indicating that current MRD assays fail to detect low-levels of clinically relevant AML blasts (Thakral et al., 2022), supporting the notion that therapy-resistant clones that include functional leukemic stem cells (LSCs) enriched for self-renewal, dormancy, and drug resistance (Ivey et al., 2016; Terwijn et al., 2013), persist after treatment and drive disease recurrence (Joshi et al., 2019). Detecting LSCs by FC remains a significant challenge due to their low frequency, antigen heterogeneity, and overlap with normal HSPCs (Terwijn et al., 2010; Srinivasan Rajsri et al., 2023). Moreover, the great majority of reported LSC antigens have not been incorporated into routine MRD panels in clinical flow cytometry laboratories.

In this review, we summarize current knowledge on the application of FC to identify LSCs in AML. We discuss the phenotypic characteristics of LSC-enriched fractions, commonly used surface markers, and how these profiles vary across AML molecular subtypes. We also highlight supporting evidence from functional assays such as xenotransplantation, address key technical considerations (e.g., required cell numbers, optimal sample processing), and explore the clinical implications and limitations of FC-based LSC detection in the context of MRD monitoring. Finally, we consider how advances in spectral (full-spectrum) FC are expanding the possibilities for deeper, more precise immunophenotyping of rare and heterogeneous LSC populations.

## Immunophenotypic identification of leukemic stem cells

The search for methods to identify LSC antigens has been based on the longstanding paradigm that AML blasts are transformed HSPCs expressing novel antigens or antigenic patterns that reflect the normal sequence of antigen expression in normal HSPCs. Efforts to identify normal human hematopoietic stem cells (HSCs) led to reports in 1991 that CD34<sup>+</sup>CD38<sup>-</sup> cells contain primitive progenitors (Terstappen et al., 1991), and in 1992, the addition of CD90 enriched for cells capable of multilineage engraftment in immunodeficient mice (Baum et al., 1992). This foundational work helped establish the commonly accepted phenotype of human HSCs as CD34<sup>+</sup>CD38<sup>-</sup>CD90<sup>+</sup>Lineage<sup>-</sup>, although subsequent studies further refined this phenotype and showed that HSCs are CD45RA<sup>-</sup> (Majeti et al., 2007) and can be further enriched by identifying CD49f<sup>+</sup> cells in the CD34<sup>+</sup>CD38<sup>-</sup>CD90<sup>+</sup>CD45RA<sup>-</sup>Lineage<sup>-</sup> fraction (Notta et al., 2011). In 1994, Lapidot et al. demonstrated that rare AML cells with a CD34<sup>+</sup>CD38<sup>-</sup> phenotype could initiate leukemia in immunodeficient mice (Lapidot et al., 1994), establishing this population as enriched for LSC activity. The CD34<sup>+</sup>CD38<sup>-</sup> compartment has since served as the primary focus for LSC identification in AML, although subsequent studies demonstrate that other blast fractions harbor LSC activity, albeit at reduced frequencies (Eppert et al., 2011; Ng et al., 2016). This raises important questions: Are LSCs defined by a uniform phenotype? Do distinct LSC populations arise in different AML subtypes? And do LSCs represent cell states without normal counterparts in normal hematopoietic development?

In a pivotal study, Goardon et al. demonstrated that LSCs are not confined to a single immunophenotype but can be found in two hierarchically related compartments (Goardon et al., 2011). In the majority of AML cases, they identified both a CD34<sup>+</sup>CD38<sup>-</sup>CD90<sup>-</sup>CD45RA<sup>+</sup> population resembling lymphoid-primed multipotent progenitors (LMPP-like LSCs) and a more differentiated CD34<sup>+</sup>CD38<sup>+</sup>CD123<sup>+</sup>/lowCD110<sup>-</sup>CD45RA<sup>+</sup> population resembling granulocyte-monocyte progenitors (GMP-like LSCs). Crucially, only LMPP-like LSCs were able to generate GMP-like cells *in vitro* and *in vivo*, but not the reverse, indicating a developmental hierarchy and suggesting greater stemness potential of the LMPP-like subset (Goardon et al., 2011).

Although CD45RA positivity has been proposed to distinguish LSCs from HSCs (Cloos et al., 2018; Boyer et al., 2018), CD45RA expression alone may not be sufficient to effectively distinguish LSCs from normal progenitors since AML blasts frequently lose CD90 expression (Holden et al., 1995; Inaba et al., 1997; Kozii et al., 1997). A study observed that LMPPs express CD38 at an intermediate level between CD38<sup>-</sup> stem cells and CD38<sup>+</sup> progenitors, recommending a narrower CD38 negative gate to reduce misclassification (Kersten et al., 2016). Therefore, using CD45RA in conjunction with other aberrant LSC markers may enhance the specificity and accuracy of LSC identification.

Although the CD34<sup>+</sup>CD38<sup>-</sup> compartment is frequently enriched for LSC activity in CD34<sup>+</sup> AMLs, LSCs have also been described in AMLs lacking CD34 expression, which show <1% CD34 expression and comprise approximately 20% of all AMLs (van der Pol et al., 2003; Zeijlemaker et al., 2015; van Rhenen et al., 2005). Therefore, alternative markers such as CD117 have been used to enrich for stem-like cells (Quek et al., 2016). In NPM1-mutated AML, which is often associated with low CD34 expression, studies have shown that some cases harbor LSCs exclusively within the CD34<sup>-</sup> fraction, while others contain LSCs in both the CD34<sup>+</sup> and CD34<sup>-</sup> fractions (Taussig et al., 2010; Quek et al., 2016). More recently, venetoclax resistance in monocytic AML has been attributed to a distinct monocytic LSC population (CD34<sup>-</sup>CD14<sup>+</sup>CD11b<sup>-</sup>CD36<sup>-</sup>), which is immunophenotypically distinct from previously described CD34<sup>-</sup> LSC subsets and characterized by reduced BCL2 dependence (Pei et al., 2023). Together, these observations underscore the immunophenotypic heterogeneity of AML LSCs and highlight the limitations of relying on single-marker gating. To address this, additional markers such as CD99 (Chung et al., 2017), CD133 (Reuvekamp et al., 2025a), CD32 (Ho et al., 2016), CLL-1 (Larsen et al., 2012), CD244 (Quek et al., 2014) have been explored in CD34<sup>-</sup> AML, either individually or in combination with CD38 (Ho et al., 2016). The most commonly reported markers that enrich for LSCs in AML are listed in Table 1.

## Functional validation and limitations of stemness assays

Functional validation serves as the gold standard for confirming that immunophenotypically defined cell populations truly represent LSCs. This process relies heavily on xenotransplantation into immunodeficient mice to initiate disease as well as serial

TABLE 1 Review of surface markers that enrich for leukemic stem cells in acute myeloid leukemia.

Marker	Definition/Biology	Findings	References
CD123 (IL-3Ra)	$\alpha$ -subunit of IL-3 receptor	Highly expressed on CD34 <sup>+</sup> CD38 <sup>-</sup> AML LSCs; absent/low on normal HSCs but present on committed progenitors. Expression is associated with adverse prognosis. Reportedly upregulated after therapy in regenerative marrow. Ongoing clinical trials of CAR-T cells and antibody-drug conjugate targeting CD123	Goardon et al. (2011), Sarry et al. (2011), Jin et al. (2009), Vergez et al. (2011), Jordan et al. (2000)
CD45RA	Isoform of leukocyte phosphatase CD45	Highly expressed on AML LSCs, particularly those with a LMPP-like phenotype. Absent on normal HSCs but present on some normal committed progenitors. Improves discrimination between LSCs and normal HSCs	Goardon et al. (2011), Boyer et al. (2018), Kersten et al. (2016)
CLL-1 (CLEC12A/CD371)	C-type lectin-like inhibitory receptor	Broadly expressed on AML LSCs in both CD34 <sup>+</sup> and CD34 <sup>-</sup> disease; xenograft studies confirm leukemia-initiating capacity. Absent on HSCs, but present on normal monocytes and granulocytes. Ongoing development of CAR-T cells and antibody-drug conjugates to target CLL-1	Larsen et al. (2012), van Rhenen et al. (2007b), Joudinaud and Boyer (2021), Noordhuis et al. (2010)
TIM-3 (CD366)	Immune checkpoint receptor	Upregulated on AML LSCs and implicated in maintaining self-renewal. Expression is associated with relapse risk and poor survival. Absent on HSCs, but present on activated immune cells (e.g., T cells, monocytes), which may limit therapeutic targeting	Jan et al. (2011), Kikushige et al. (2015), Darwish et al. (2016), Akashi (2015)
CD44	Hyaluronan receptor; adhesion molecule	Expressed on both AML LSCs and normal HSCs, but more highly and functionally upregulated on LSCs. Expression is associated with chemoresistant leukemic cells. Blockade eradicates LSCs in xenograft models, though expression on HSCs limits specificity	Jin et al. (2006), Das et al. (2024), Gutjahr et al. (2021), Charrad et al. (2002)
CD96	Ig superfamily receptor; binds CD155/PVR	Expressed on some AML LSCs, with expression associated with poor prognosis. Absent on HSCs but present on activated immune cells, reducing specificity	Hosen et al. (2007), Du et al. (2015)
CD47	Integrin-associated protein; "don't-eat-me" checkpoint	Overexpressed on AML LSCs, associated with inferior survival. Also broadly expressed on normal HSCs and blasts, which limits specificity	Majeti et al. (2009), Majeti et al. (2008), Sallman et al. (2019)
CD99	Cell adhesion/signal protein	Highly expressed on AML LSCs and implicated in leukemogenesis; enriched at relapse. Absent on HSCs. Therapeutically targetable <i>in vitro</i> , but expression on other normal tissues may limit specificity	Chung et al. (2017)
IL1RAP	Co-receptor for IL-1 $\alpha/\beta$ and IL-33 signaling	Highly expressed on AML LSCs, with higher levels in FLT3-mutated cases. Minimal on HSCs. Attractive target in preclinical models, but expression on some normal progenitors and non-hematopoietic tissues may limit therapeutic specificity	Zhang et al. (2024), Metois et al. (2025), Askmyr et al. (2013)
GPR56 (ADGRG1)	Adhesion GPCR.	Highly expressed on AML LSCs and is associated with poor prognosis. Also expressed on normal HSCs, which limits its specificity as a therapeutic target.	Ng et al. (2016), Pabst et al. (2016), Daria et al. (2016), Saito and Morishita (2015)
CD33	Sialic acid-binding Ig-like lectin (Siglec)	CD33 is strongly expressed on AML LSCs. Also expressed at low levels on normal progenitors, and its expression can change after therapy, which limits its reliability as an MRD marker	Zeijlemaker et al. (2016), Terwijn et al. (2014)
CXCR4 (CD184)	Chemokine receptor for CXCL12	Overexpressed on AML LSCs, mediating bone marrow niche retention and promoting survival. Associated with poor prognosis. Blockade of CXCR4 mobilizes LSCs into circulation, which may increase their susceptibility to chemotherapy. Also expressed on normal HSCs, which limits specificity	Biondi et al. (2021), Roboz et al. (2018), Maganti et al. (2020)

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TABLE 1 (Continued) Review of surface markers that enrich for leukemic stem cells in acute myeloid leukemia.

Marker	Definition/Biology	Findings	References
CD70	TNF ligand binding CD27	Aberrantly expressed on AML LSCs and often upregulated at relapse. Blocking CD70 reduces leukemic engraftment in preclinical models. Absent on HSCs, but inducibly expressed on activated immune cells, which limits specificity. Under investigation in antibody- and CAR-T-based therapeutic trials	Ochsenbein et al. (2019), Riether et al. (2017), Marques-Piubelli et al. (2024), Hinterbrandner et al. (2017)
CD36	Fatty acid transporter	Marks metabolically distinct, quiescent AML LSCs; expression is enriched after therapy and associated with poor survival. Reported as absent on HSCs, but its role as a discriminator remains under investigation. Further studies are needed to validate its utility as an AML LSC marker	Landberg et al. (2018), Farge et al. (2023), Abacka et al. (2024), Zhang et al. (2020), Guerrero-Rodriguez et al. (2022)
CD97	Adhesion GPCR of EGF-TM7 family	Highly expressed on AML LSCs, where it supports self-renewal and is associated with poor prognosis. Minimal on HSCs. Also expressed on normal leukocytes and not yet used in clinical MRD setting or therapeutic studies	Martin et al. (2016), Martin et al. (2019)
CD93	C-type lectin receptor	Expressed on AML LSCs, particularly in MLL-rearranged AML, where it promotes proliferation. Preclinical antibody blockade reduces AML progression. Also expressed on endothelial cells and some myeloid progenitors, which may restrict specificity and therapeutic applicability	Kinstrie et al. (2020), Iwasaki et al. (2015), Jia et al. (2022), Richards et al. (2021)
CD69	Early activation antigen (C-type lectin)	Marks chemoresistant, quiescent AML LSCs, with high expression linked to poor survival. Not yet incorporated into flow MRD assays. Widely induced on activated immune cells, which limits specificity	Antony et al. (2023), Zhou et al. (2025), Qiu et al. (2022), Zhang et al. (2023)
CD200	Immunoglobulin superfamily glycoprotein	Enriches for LSCs in both CD34 <sup>+</sup> and CD34 <sup>-</sup> AML and contributes to immune suppression within the marrow microenvironment. Also expressed on normal HSCs, limiting specificity for LSC detection or therapeutic targeting	Ho et al. (2020), Coles et al. (2012), Coles et al. (2011)
CD25 (IL-2Ra)	$\alpha$ -chain of IL-2 receptor	Enriched on quiescent AML LSCs and associated with early treatment failure. CD25 <sup>+</sup> LSCs can give rise to CD25 <sup>-</sup> leukemic progeny, underscoring intrapopulation heterogeneity. Absent on normal HSCs at the transcriptomic level, but FC testing in healthy donors remains limited	Saito et al. (2010), Kageyama et al. (2018), Cerny et al. (2013)
CD32 (Fc $\gamma$ RII)	Low-affinity Fc receptor	Present on LSCs in ~30% of AML. In some cases, CD32 <sup>+</sup> CD34 <sup>+</sup> CD38 <sup>-</sup> cells initiate AML in xenografts, but in others only the CD32 <sup>-</sup> fraction has leukemic potential and is enriched for quiescent LSCs. Absent on normal HSCs. Enriches LSCs in certain CD34 <sup>-</sup> AMLs, but heterogeneous expression patterns limit its reliability as a universal marker	Ho et al. (2016), Saito et al. (2010)
CD166 (ALCAM)	Ig superfamily adhesion molecule	Upregulated in subsets of AML and enriched in LSC-like transcriptomic clusters. However, also expressed on normal HSCs, stromal cells, and endothelial cells, limiting its specificity as an AML LSC marker	Shimura-Nukina et al. (2016)
EMR2 (CD312)	Adhesion GPCR in same family as CD97	Expressed on AML blasts and LSCs, with preclinical studies suggesting it may be targetable with CAR-T approaches. Absent on HSCs but expressed on normal mature myeloid cells, which limits specificity	Unglaub et al. (2023), Xie et al. (2025)
CD133 (Prominin-1)	Stem/progenitor marker	CD133 <sup>+</sup> cells display LSC-like activity and can help identify LSCs in CD34 <sup>-</sup> AML. However, CD133 is also expressed on normal HSCs and progenitors, limiting its specificity	Reuvekamp et al. (2025a), Heo et al. (2020)

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TABLE 1 (Continued) Review of surface markers that enrich for leukemic stem cells in acute myeloid leukemia.

Marker	Definition/Biology	Findings	References
CD90 (Thy-1)	Glycoprotein stem cell marker	AML blasts are frequently CD90 <sup>+</sup> , and the CD90 <sup>+</sup> fraction is enriched for leukemogenic activity. Normal HSCs are typically CD90 <sup>+</sup> , so loss of CD90 expression may help distinguish LSCs from normal HSCs	Holden et al. (1995), Inaba et al. (1997), Kozii et al. (1997), Richards et al. (2021), Brendel et al. (1999), Buccisano et al. (2004)
CD109	GPI-anchored glycoprotein; TGF- $\beta$ modulator	Identified in LSC transcriptomic signatures, with high expression correlated with poor prognosis. However, CD109 has not been widely used as a FC marker for LSCs	Wagner et al. (2019)
CD74	Invariant chain of MHC II; receptor for MIF	Upregulated in slow-cycling AML subsets and reported in pediatric AML. Explored as a potential therapeutic target, but evidence as an AML LSC marker remains limited	Menssen et al. (2024), Li et al. (2024a)
CD98	Amino acid transporter; integrin enhancer	Overexpressed in AML and implicated in LSC self-renewal and engraftment. Functional studies support its role as a therapeutic target, but it is not widely applied as a FC marker for LSC detection or MRD monitoring	Hayes et al. (2015), Bajaj et al. (2016), Reinisch and Majeti (2016)
CD26 (DPP4)	Dipeptidyl peptidase-IV enzyme	Selectively expressed on FLT3-ITD <sup>+</sup> AML LSCs. Largely absent in FLT3- wildtype AML.	Herrmann et al. (2020)
CD49d ( $\alpha 4$ integrin)	Integrin $\alpha 4$ subunit; forms VLA-4	Highly expressed on AML blasts, including LSC-enriched fractions, and linked to increased MRD burden and relapse risk. Therapeutically targetable but broad expression on normal immune cells and stroma limits its specificity as an LSC marker	Grenier et al. (2021), Ogana et al. (2024)
CD9	Tetraspanin family protein	Expressed on AML LSCs and enriched in the CD34 <sup>+</sup> CD38 <sup>-</sup> fraction. Absent on HSCs at the transcriptomic level but detected on normal multipotent and lymphoid-primed progenitors at levels comparable to LSCs, which limits its specificity	Liu et al. (2021), Touzet et al. (2019)
CD244 (2B4)	SLAM family receptor	Frequently expressed on AML LSCs, where it supports leukemia maintenance and is associated with poor prognosis. Also expressed on normal HSPCs, monocytes, and various non-hematopoietic tissues, which limits its specificity	Haubner et al. (2019)
Lineage markers (CD2, CD7, CD11b, CD14, CD15, CD19, CD22, CD56)	Lymphoid/myeloid differentiation antigens	Frequently aberrantly expressed on AML blasts, including within the CD34 <sup>+</sup> CD38 <sup>-</sup> compartment, but not specific for stemness. Absent on HSCs. Primarily useful for LAIP determination and distinguishing leukemic from normal progenitors in MRD assessment	Goardon et al. (2011), Kersten et al. (2016), Zeijlemaker et al. (2016)

engraftment in order to demonstrate self-renewal capacity. However, these assays have inherent limitations. Successful engraftment depends on multiple factors including homing efficiency, immune evasion, and responsiveness to murine cytokines. Thus, it is possible that some LSCs with true leukemogenic potential may fail to engraft due to species-specific barriers. Indeed, using xenograft models, LSC frequencies have been reported to range from as high as  $\sim 1$  in  $10^4$  cells to as low as  $\sim 1$  in  $10^7$  cells, underscoring both their rarity and the marked variability between patients (Sarry et al., 2011; Bonnet and Dick, 1997). This necessitates complementary approaches, such as fluorescence *in situ* hybridization (FISH), next-generation sequencing (NGS), and targeted mutational profiling, to confirm the presence of leukemia-specific cytogenetic or molecular abnormalities. Moreover, linking these features with clinical outcomes like disease-free and overall survival is crucial to establish their clinical relevance.

Exploration of alternative LSC identification strategies that do not rely solely on surface antigens has been pursued. Some studies highlight metabolic distinctions, with LSCs in the CD34<sup>+</sup>CD38<sup>-</sup> fraction usually exhibiting lower aldehyde dehydrogenase (ALDH) activity than residual normal HSCs in AML bone marrows (Schoorhuis et al., 2013). However, AML LSCs with high ALDH activity have also been described (Hoang et al., 2015; Ran et al., 2009; Blume et al., 2018), indicating that ALDH-based discrimination is variable across subtypes and lacks the specificity to serve as a standalone marker. Other strategies attempt to detect LSCs using similar methods to HSC detection, like Hoechst-low side population (SP) cells, which are enriched for stem cell activity based on dye efflux ability (Feuring-Buske and Hogge, 2001; Moshaver et al., 2008). Given that both malignant and normal stem cells exhibit these features (Moshaver et al., 2019), distinguishing between them remains a challenge requiring additional markers to distinguish between them.

## Challenges in quantifying LSCs in clinical settings

Accurate identification of LSCs by FC is challenging due to the immunophenotypic heterogeneity of AML. Although employing extended antibody panels can improve detection, the variation in surface marker expression across different cases may require customized panel designs guided by initial diagnostic screening. Current FC-based strategies for LSC detection are limited by antibody panel variability among labs, the substantial phenotypic overlap between leukemic and normal progenitor compartments, subjective gating, and the lack of incorporation of antibodies that can reliably identify LSCs.

But what markers would constitute optimal markers for LSC identification? This involves assessing the specificity of candidate markers by comparing their expression in blasts and putative LSCs versus normal HSCs. Normal HSCs can be identified using negative control bone marrow samples or in the AML specimen, as we and others have shown the presence of residual HSCs in AML diagnostic samples (Jan et al., 2012; Chung et al., 2017). Ideally, a useful marker should show clear differences in marker expression within the CD34<sup>+</sup>CD38<sup>-</sup> compartment, either as a distinct population (marker<sup>+</sup> vs. marker<sup>-</sup>) or by overexpression in LSCs relative to normal HSCs. Zeijlemaker and colleagues proposed a scoring system ranking markers by their ability to provide such distinction (Zeijlemaker et al., 2016), emphasizing the importance of minimizing contamination of LSCs in the marker-negative fraction. However, marker-negative fractions cannot always be assumed to be free of LSCs. For example, analysis of GPR56 in combination with CD34 expression revealed engraftment potential in both CD34<sup>-</sup> and CD34<sup>+</sup> fractions, demonstrating that not all LSCs express the classical CD34<sup>+</sup>CD38<sup>-</sup> phenotype (Pabst et al., 2016). Similarly, Saito et al. showed that CD32 is highly expressed in LSCs, yet the CD32<sup>-</sup> fraction in a subset of cases also retains leukemic potential and is enriched for quiescent LSCs (Saito et al., 2010). These findings underscore the need for cautious interpretation as well as validation of markers across diverse AML subtypes.

Markers with continuous rather than bimodal expression also requires carefully defined thresholds for positivity to differentiate true aberrancy from background noise. Common approaches include using mean fluorescence intensity (MFI) ratio or fold change relative to isotype controls (Chung et al., 2017; Haubner et al., 2019), setting cutoffs at least two standard deviations from appropriate negative reference (Loghavi et al., 2021), or applying receiver operating characteristic (ROC) curves to optimize discrimination (Guy et al., 2013).

Homogeneous CD38 expression in some AML cases poses challenges for gating as a clearly defined CD38<sup>-</sup> compartment may be absent. In such cases, internal controls such as residual erythroid cells or calibration beads for CD38-negative thresholds (Cloos et al., 2018; Quek et al., 2014) and monocytes (Terwijn et al., 2014) or hematogones (Ngai et al., 2025) as CD38-positive references, provide a biologically anchored framework for interpretation.

Another consideration lies in the limitation in cell number when using conventional FC. When large panels are split across multiple tubes, sufficient events may not be acquired to confidently detect rare LSCs. To overcome this, prior studies have attempted to

incorporate several LSC markers into a single fluorescence channel within a one-tube assay (Zeijlemaker et al., 2016; Li et al., 2022). However, this approach does not allow the flexibility to interpret complex expression patterns of markers and nonuniform expression of lineage markers. This issue is particularly significant for rare cells, as collective assessment of populations with different expression levels and/or autofluorescence may lead to decreased sensitivity and specificity of LSC detection (Boesch et al., 2018). Furthermore, post-therapy immunophenotypic shifts within the shared channel can affect accurate LSC measurement. However, when markers are assessed separately, it requires significant effort to determine which markers are co-expressed or absent to identify a residual LSC population as subsets may be negative for certain markers yet positive for others.

Different approaches have been used to define LSC burden. Some studies calculate the proportion of LSCs relative to total WBCs (Ngai et al., 2023; Zeijlemaker et al., 2019a; Terwijn et al., 2014), and others calculating LSCs as a percentage of CD34<sup>+</sup> cells or primitive compartments (Ngai et al., 2025). Regardless of the method used, LSC frequency cutoffs have been calculated based on clinical endpoints. Several studies have addressed this by correlating LSC frequency with remission and relapse outcomes. For example, a cutoff of 0.03% CD34<sup>+</sup>CD38<sup>-</sup> LSCs linked to higher relapse risk, with higher frequencies associated with inferior remission rates and survival (Zeijlemaker et al., 2019b). In the post-therapy setting, thresholds are typically pushed closer to the limit of detection of FC, since residual LSCs are expected to be rare (Zeijlemaker et al., 2019a).

As the disease progresses, shifts in immunophenotype may occur due to clonal evolution, relapse, or treatment effect. Ideally, LSC markers should maintain stable expression after chemotherapy (Saito et al., 2010). Yet some, such as CD123 are also reported to be expressed in regenerating marrow, limiting specificity (van Rhenen et al., 2007a). Further work is also needed to clarify how these markers behave in the context of clonal hematopoiesis or myelodysplasia, and how to interpret LSC-like phenotypes in these settings. One study reported that post-remission clonal hematopoiesis can produce immunophenotypic alterations in myeloid progenitors exceeding typical regenerative patterns yet remaining distinct from the original AML, underscoring the need to avoid misclassifying such cells as MRD (Loghavi et al., 2021).

## Flow cytometry studies of LSCs in AML

Several studies have applied multiparameter FC panels combining more than one marker to distinguish LSCs from normal HSCs and assess their prognostic relevance and ability to assess MRD. Table 2 summarizes many published studies in this area, ranging from single-center explorations to cooperative trial-based investigations. Please note that not all published studies could be included due to space limitations.

## Development of guidelines for LSC quantification in the clinical setting

In 2018, a consensus document from the European LeukemiaNet (ELN) MRD Working Party addressed key

TABLE 2 Multiparameter flow cytometry studies of leukemic stem cells in acute myeloid leukemia.

Study	Markers/panel used	Summary of findings
Hwang et al. (2012)	Panel includes: CD34, CD38, CD123, CD44, CD184	In 63 AML patients, the median proportion of CD34 <sup>+</sup> CD38 <sup>-</sup> leukemic blasts was 1.3%. Among CD34 <sup>+</sup> CD38 <sup>-</sup> cells, 74.6% expressed CD123, 100% expressed CD44, and 85.7% expressed CD184. Patients who achieved complete remission had significantly lower LSC proportions compared to those who did not achieve remission
Terwijn et al. (2014)	Multi-tube panel including: CD45, CD34, CD38; lineage markers (CD2, CD7, CD11b, CD19, CD22, CD56), CLL-1, CD13, CD33, HLA-DR, CD45RA. Marker choice for MRD was based on screening for >10% positivity at diagnosis	Approximately 75% of AML cases exhibited aberrant marker expression within the CD34 <sup>+</sup> CD38 <sup>-</sup> compartment (e.g., CLL-1, lineage markers). Using this combined immunophenotypic and light-scatter-based approach, putative leukemic and normal stem cell populations could be distinguished in ~47% of samples, with a median of 82% of the CD34 <sup>+</sup> CD38 <sup>-</sup> fraction identified as neoplastic, and higher LSC frequencies associated with inferior clinical outcomes
Angelini et al. (2015)	Multi-tube panel including: CD34, CD117, CD38, CD123, CD25, CD99, CD7	Identified a distinct CD34 <sup>+</sup> CD123 <sup>+</sup> CD99 <sup>+</sup> CD25 <sup>+</sup> immunophenotype that was present in ~30–40% of FLT3-ITD AML. This population was detectable at diagnosis in some patients who later acquired FLT3-ITD relapse, suggesting it may mark high-risk subclones. The study primarily focused on genetic correlation rather than LSC phenotyping
Cheng et al. (2016)	Multi-tube panel including: CD45, CD34, CD38, lineage markers, CD33, CD123, CD133, CD44, HLA-DR	In pediatric AML, CD33 and CD123 were highly expressed on AML LSCs, and demonstrated significant differences compared with their expression on normal HSCs
Kersten et al. (2016)	Multi-tube panel including: CD45, CD34, CD38, CD45RA, CLL-1, CD33, CD123	CD45RA was the most reliable marker for identifying LSCs within the CD34 <sup>+</sup> CD38 <sup>-</sup> compartment. Cases with >90% CD45RA <sup>+</sup> LSCs showed higher scatter values ( $P < 0.001$ ) of LSC compared to HSCs
Zeijlemaker et al. (2016) Zeijlemaker et al. (2019b)	8-color one-tube assay including: CD45, CD34, CD38, CD45RA, CD123, CD33, CD44, and a composite channel (CLL-1/TIM-3/CD7/CD11b/CD22/CD56)	Developed an 8-color one-tube LSC panel defining LSCs within the CD34 <sup>+</sup> CD38 <sup>-</sup> compartment as cells positive for aberrant markers (CD123, CD33, CD45RA, or a composite channel). CD123 and CD33 were aberrant in ~82% of cases; CD123 was reliable at diagnosis but upregulated post-therapy. CD44 was uniformly expressed on HSCs but often overexpressed in LSCs. A single channel combining multiple markers correlated well with individual markers for CD34 <sup>+</sup> CD38 <sup>-</sup> LSC quantification
Freeman et al. (2018)	One-tube assay including: HLA-DR, CD45, CD123, CD56/CD2, CD34, CD117, CD7 and CD38	In younger adults, a high frequency of LSCs at diagnosis was predictive of relapse risk. Patients with an LSC load >17.2% of CD34 <sup>+</sup> blasts were at especially high risk
Yabushita et al. (2018)	Panel includes CD25, CD96, CD123	In a cohort of <i>de novo</i> AML patients, expression of two or more of CD25, CD96, and CD123 was associated with poorer 3-year overall survival
Haubner et al. (2019)	Multi-tube panel including: CD34, CD38 + CD33, CD123, CLL-1, TIM-3, CD244 and CD7	CD33, CD123, CLL-1, TIM-3, and CD244 were broadly expressed on AML blasts at both diagnosis and relapse, independent of genotype. Dual-marker analysis showed that CD33/TIM-3 and CLL-1/TIM-3 combinations were most specific for AML compared with normal or non-hematopoietic tissues
Zeijlemaker et al. (2019a)	7 customized 8-color tubes with backbone markers (CD34, CD38, CD45, CD19, CD13, CD14, HLA-DR) supplemented with patient-specific markers (CD33, CLL-1, TIM-3, CD133, CD123, CD96, CD22, CD44, CD7, CD2, CD36, CD11b, CD15)	Median LSC percentage of CD34-positive cases was 0.0079%. Demonstrated that LSC frequency was an independent predictor of overall survival and combining LSC burden with MRD status after remission improved risk stratification. Notably, some patients who were initially LSC-negative became LSC-positive after therapy. However, the method required multiple patient-specific tubes, limiting its practicality for routine monitoring
Petersen et al. (2021)	15-color one-tube pediatric panel: CD45, CD3, CD117, CD34, CD38, CD123, CD45RA, CLL-1, TIM-3, IL1RAP, CD25, CD93, CD99, BCL-2, viability dye	Aberrant LSC fractions within the CD34 <sup>+</sup> CD38 <sup>-</sup> compartment were quantified and t-SNE was used to support analysis. The study proposed a framework for standardized pediatric LSC assessment, but data were limited
Li et al. (2022)	8-color Zeijlemaker et al. (2016) one-tube LSC assay	Compared the traditional FC-based MRD assay with the LSC-based approach by Zeijlemaker et al. in AML patients post-allogeneic transplantation. LSC marker expression within the CD34 <sup>+</sup> CD38 <sup>-</sup> compartment remained stable up to 1 year after transplant. Detection of CD34 <sup>+</sup> CD38 <sup>-</sup> LSCs after allografting was useful for predicting relapse, but not for leukemia-free survival or overall survival

(Continued on following page)

TABLE 2 (Continued) Multiparameter flow cytometry studies of leukemic stem cells in acute myeloid leukemia.

Study	Markers/panel used	Summary of findings
Ngai et al. (2023)	8-color Zeijlemaker et al. (2016) one-tube LSC assay	Prospectively validated the prognostic value of LSC burden at diagnosis and after the second chemotherapy cycle in intermediate- and adverse-risk AML. Findings demonstrated that incorporating LSC quantification into clinical trial frameworks provides additional value for identifying patient subgroups with poorer outcomes beyond standard risk stratification
Das et al. (2024)	10-color single-tube: CD45, CD34, CD38, CD117, sCD3, CD45RA, CD123, CD44, CD33; composite channel with CLL-1, TIM-3, CD25, CD11b, CD22, CD7, CD56	LSCs and HSCs were identified in 88.2% and 71.6% samples, respectively. LSCs showed significantly higher expression of composite, CD45RA, CD123, CD33, and CD44 compared with HSCs. No significant differences in LSC antigen expression were observed between CD34 <sup>+</sup> and CD34 <sup>-</sup> subsets. Multiple LSC subpopulations were detected in 4.4% of cases
Reuvekamp et al. (2025b)	8-color Zeijlemaker et al. (2016) one-tube LSC assay	In older AML patients treated with hypomethylating agents, LSC positivity was associated with higher relapse rates and shorter event-free survival. All patients who were both MRD <sup>+</sup> and LSC <sup>+</sup> relapsed within 6 months
Zhang et al. (2025)	29-color single-tube full-spectrum FC assay. Panel includes CD45RA, CD123, CD33, CD44, CD47, TIM-3, CD133 and CLEC12A	Developed a comprehensive spectral assay enabling simultaneous MRD and LSC detection in AML. When compared with a conventional 5-tube assay analyzing 20 AML BM samples, the panel showed high concordance and reduced sample and tube requirements

challenges in MRD assessment in AML (Schuurhuis et al., 2018). As part of their recommendations, the group proposed exploration of a separate LSC panel to assess total LSC burden at any point from diagnosis to relapse. They cited the approach described by Zeijlemaker et al. (2016) (Zeijlemaker et al., 2016), which included a panel combining CLL-1, TIM-3, CD7, CD11b, CD22, and CD56 in one channel, along with CD123, CD45RA, CD44, and CD33 in the same tube. Additional markers such as CD25, CD32, and CD99 were also noted to be potentially useful.

In the 2021 ELN MRD Working Party report (Heuser et al., 2021), evaluation of LSCs in FC-based MRD assessment was identified as a priority area for the future improvement of MRD recommendations. LSCs were defined immunophenotypically as CD34<sup>+</sup>/CD38<sup>-</sup> cells combined with aberrant markers not expressed on normal HSCs such as CD45RA, CLL-1, or CD123. The report highlighted that LSC measurements may have prognostic relevance and should be further validated in prospective clinical trials. For optimal detection, acquisition of approximately 4 million events was recommended, ideally using a single-tube assay. Building on this, the 2022 ELN international expert panel on AML diagnosis and management also acknowledged the potential prognostic relevance of residual LSCs and stressed the need for continued investigation and validation in this area (Dohner et al., 2022).

## Prognostic significance and multimodal integration of LSC and MRD assessment in AML

Recent studies report that LSC burden in AML at diagnosis and post-treatment can carry prognostic value in adult and pediatric cohorts (Zeijlemaker et al., 2019a; Hanekamp et al., 2018). High LSC frequency is associated with earlier relapse and shorter overall survival (Ngai et al., 2023; Zeijlemaker et al., 2019a; Terwijn et al., 2014). Incorporating LSC assessment into MRD monitoring modestly improves risk stratification. For example,

patients with both LAIP-MRD<sup>high</sup> and LSC<sup>high</sup> showed significantly reduced survival than patients with only one or neither of these features (e.g., MRD<sup>low</sup>/LSC<sup>high</sup>, MRD<sup>high</sup>/LSC<sup>low</sup>, or MRD<sup>low</sup>/LSC<sup>low</sup>) (Zeijlemaker et al., 2019a). In particular subsets such as LAIP-MRD-negative intermediate-risk patients, LSC positivity may guide considerations for transplant decisions or more intensive monitoring strategies (Li et al., 2022; Ngai et al., 2023).

Beyond its prognostic value, MRD assessment is emerging as a key component of AML management by offering enabling more risk-adapted and individualized treatment strategies. Persistently positive MRD after induction or consolidation identifies patients at elevated risk of relapse who may benefit from treatment intensification, such as allogeneic stem cell transplantation or enrollment in investigational protocols, whereas MRD negativity supports continuation of standard consolidation or therapy de-escalation (Freeman et al., 2018; Heuser et al., 2021).

Together, MRD and LSC analyses provide a complementary framework for predicting relapse and refining treatment decisions. Dual assessment enhances sensitivity and specificity over either approach alone (Li et al., 2022). Moreover, persistence or re-emergence of LSCs in the post-transplant setting has been associated with relapse (Li et al., 2025), suggesting that incorporating LSC monitoring into post-transplant surveillance could be beneficial. Collectively, these advances position LSC quantification as a powerful complement to MRD, bridging biological insight with clinical practice and refining therapeutic decision-making across the AML disease course.

Molecular MRD assessment adds an important layer of prognostic refinement in AML. Leukemia-specific assays targeting stable genetic lesions, such as NPM1 mutations, PML-RARA and core-binding factor (CBF) rearrangements, or other recurrent fusions, enable sensitive detection of residual disease in certain genetically defined AML subtypes (Heuser et al., 2021). NGS-based MRD monitoring can further enhance prognostic assessment when applied alongside multiparameter FC, detecting

persistent or emerging variants that may indicate molecular persistence or disease evolution (Walter et al., 2021).

## Advancing LSC detection with spectral flow cytometry

Spectral FC enables the simultaneous analysis of 20+ markers in a single tube (Bonilla et al., 2020), offering a powerful tool to interrogate rare leukemic subpopulations, including LSCs, without requiring multiple tubes or complex panel splitting. A recently developed 29-color single-tube spectral assay further exemplifies the potential of this technology (Zhang et al., 2025). Compared with conventional multicolor cytometry, spectral FC minimizes sample consumption and acquisition variability while enabling a more refined “different-from-normal” analysis strategy (Li K. et al., 2024). Dimensionality reduction algorithms further can enhance the resolution of immunophenotypic heterogeneity (Ferrer-Font et al., 2020), with LSCs occupying distinct and reproducible positions separate from normal HSPCs. Building on these advances, recent efforts have shifted from reliance on single surface markers to profiling co-expression patterns of multiple antigens (Haubner et al., 2019). Flow cytometric assessment of co-expression signatures improves diagnostic specificity, overcoming the phenotypic overlap between LSCs and normal HSCs, while also carrying therapeutic implications by informing the design of combinatorial targeted therapies that increase selectivity and minimize off-target toxicity. Collectively, these advances highlight how next-generation cytometry can refine LSC detection and accelerate translation into precision MRD monitoring and personalized therapeutic approaches in AML.

Future optimization of FC-based strategies is expected to further improve AML MRD detection sensitivity and specificity. For example, standardization of antibody panels across institutions, harmonized instrument settings and analysis thresholds, implementation of automated high-dimensional analysis pipelines, and integration with machine learning-based clustering tools will enhance reproducibility and objectivity in rare LSC detection. Increasing total event acquisition is likely to increase sensitivity of MRD detection as well. Finally, aligning FC-based and molecular MRD assessments may provide complementary information on residual disease and treatment response. Further studies are needed to determine whether single or combined MRD approaches offer the most accurate prognostic value and how they should be applied to guide personalized treatment decisions.

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MY: Writing – review and editing, Conceptualization, Writing – original draft. CP: Supervision, Writing – original draft, Writing – review and editing, Conceptualization.

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