Cell Communication and Signaling

REVIEW

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Decoding the archipelago: single-cell biomarkers rechart the molecular geography of acute myeloid leukemia

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Abstract

Acute myeloid leukemia (AML) is a molecularly archipelagic disease, characterized by profound cellular heterogeneity that fuels pathogenesis and therapy resistance. Traditional bulk sequencing approaches, by averaging signals across thousands of cells, have obscured the complex geography of leukemic stem cells (LSCs), dynamic clonal evolution, and critical ecosystem interactions within the bone marrow niche. This review explores how single-cell multi-omics technologies, including scRNA-seq, scDNA-seq, and high-dimensional cytometry, are fundamentally recharting the molecular landscape of AML. These approaches reveal that LSCs are not fixed entities but dynamic states shaped by genetic, epigenetic, and metabolic drivers. They decode the intricate cell-cell communication networks and immunosuppressive mechanisms that define the tumor microenvironment, uncovering novel vulnerabilities. Furthermore, single-cell profiling is revolutionizing the tracking of clonal dynamics in response to therapy, elucidating both genetic and non-genetic pathways to resistance and enabling a paradigm shift in measurable residual disease detection. By translating these high-resolution insights into novel biomarkers and therapeutic targets, single-cell technologies are paving the way for functional precision medicine in AML. In this review we explicitly (i) outline a practical roadmap to move single-cell candidate biomarkers from discovery through analytical and clinical validation to regulatory qualification, (ii) summarize evidence that single-cell assays are already used as correlative endpoints in clinical trials and indicate where they are being piloted as decision-enabling tools, and (iii) compare platform readiness (cost, standardization, turnaround and clinical trial feasibility) and the practical role of lower-dimensional assays for clinical validation. These translational perspectives aim to accelerate the responsible clinical adoption of single-cell biomarkers.

Keywords Single-cell sequencing, Acute myeloid leukemia, Leukemic stem cells, Biomarkers, Tumor microenvironment

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Introduction: the need for single-cell resolution in AML heterogeneity

Acute myeloid leukemia (AML) is characterized by extensive genetic and cellular heterogeneity that drives clinical variability and therapeutic failure, a pattern demonstrated by large genomic cohorts and high-resolution single-cell atlases. These primary datasets show that early epigenetic lesions (DNMT3A/TET2/ASXL1) establish founder clones that later diversify into branching, often convergent subclones with distinct transcriptional programs, explaining why identical driver mutations can yield divergent clinical phenotypes [1–3]. Despite advancements in genomic profiling and targeted therapies, AML continues to portend a poor prognosis, with five-year survival rates remaining below 30% for most adult patients and even lower for elderly populations [4, 5]. This sobering reality underscores the limitations of current diagnostic and risk stratification frameworks, which predominantly rely on bulk molecular analyses that obscure the cellular diversity and dynamic clonal evolution inherent to AML [6, 7]. While bulk sequencing has been invaluable for defining the core somatic landscape of AML [2, 8], its averaged profiles fail to capture the subclonal architectures and rare cell populations that drive progression and resistance, underscoring its limitations in resolving intratumoral heterogeneity [5, 7].

The "average cell" fallacy inherent to bulk analyses becomes particularly problematic in AML, where rare leukemic stem cells (LSCs), a self-renewing population residing at the apex of the cellular hierarchy, orchestrate disease initiation, propagation, and relapse [4, 5]. These LSCs often exhibit distinct molecular features, including quiescence, enhanced drug efflux capabilities, and resistance to conventional chemotherapy, enabling them to persist after treatment and drive eventual disease recurrence [4, 7]. Moreover, bulk profiling methods cannot resolve the intricate cellular ecosystems within the bone marrow (BM) microenvironment, where immune cell interactions, stromal signaling, and metabolic adaptations contribute to AML survival and immune evasion [9, 10]. Consequently, critical biomarkers and therapeutic targets embedded within these rare subpopulations remain undetected, limiting the efficacy of precision medicine approaches [7, 11].

The emergence of single-cell technologies has revolutionized our ability to deconvolute AML heterogeneity, offering unprecedented resolution to explore the molecular geography of this disease at a cellular level [5, 6]. Single-cell RNA sequencing (scRNA-seq) enables transcriptomic profiling of thousands of individual cells, revealing distinct cellular states, differentiation trajectories, and expression signatures associated with treatment resistance and poor prognosis [9, 10]. Complementarily, single-cell DNA sequencing (scDNA-seq) elucidates clonal phylogenies and mutational co-occurrence patterns, tracing the evolutionary dynamics of leukemic clones under therapeutic pressure [5]. Multi-modal approaches, such as cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) or RNA expression and protein sequencing (REAP-seq), concurrently measure gene expression and surface protein abundance, providing integrated views of cellular identity and function [7, 10]. Additionally, single-cell assay for transposase-accessible chromatin using sequencing (scATAC-seq) maps epigenetic landscapes and regulatory elements, uncovering mechanisms of transcriptional dysregulation in AML subclones. T-cell and B-cell receptor sequencing (TCR/BCR-seq) at single-cell resolution further illuminates adaptive immune responses and clonal immunodynamics within the tumor microenvironment [10].

Integrative multi-omic applications of these technologies have begun to redefine AML taxonomy, moving beyond static genetic classifications toward dynamic, cell-state-based frameworks that reflect functional biology and therapeutic vulnerabilities [7, 10]. For instance, recent single-cell studies have identified biomarkers and cellular states associated with venetoclax resistance: Pei et al. used CITE-seq and functional assays to describe a distinct monocytic-LSC (m-LSC) population that is developmentally and metabolically distinct from primitive LSCs and shows primary resistance to venetoclax-based regimens; and Wang et al. used integrated bulk and single-cell analysis to show that IFN-γ-driven inflammatory signaling is enriched in monocytic AML, correlates with ex-vivo venetoclax resistance, and yields a parsimonious IFN-γ/IFITM3 signature with prognostic value [12, 13]. These insights are paving the way for next-generation risk stratification systems that incorporate cellular diversity, clonal architecture, and microenvironmental interactions to predict clinical outcomes with greater accuracy [5, 11].

This review explores how single-cell technologies are transforming our understanding of AML. By mapping cellular heterogeneity, clonal evolution, and ecosystem interactions, these approaches uncover novel biomarkers and therapeutic vulnerabilities, for example, single-cell studies have identified monocytic leukemia stem cells and an IFN-γ/inflammatory microenvironment that are associated with resistance to the BCL-2 inhibitor venetoclax. They enable functional precision medicine through ex vivo drug testing, guiding the development of tailored therapies and refining prognostic models for improved patient outcomes. Figure 1 shows an overview of single-cell technologies that can explore the molecular geography of leukemia.

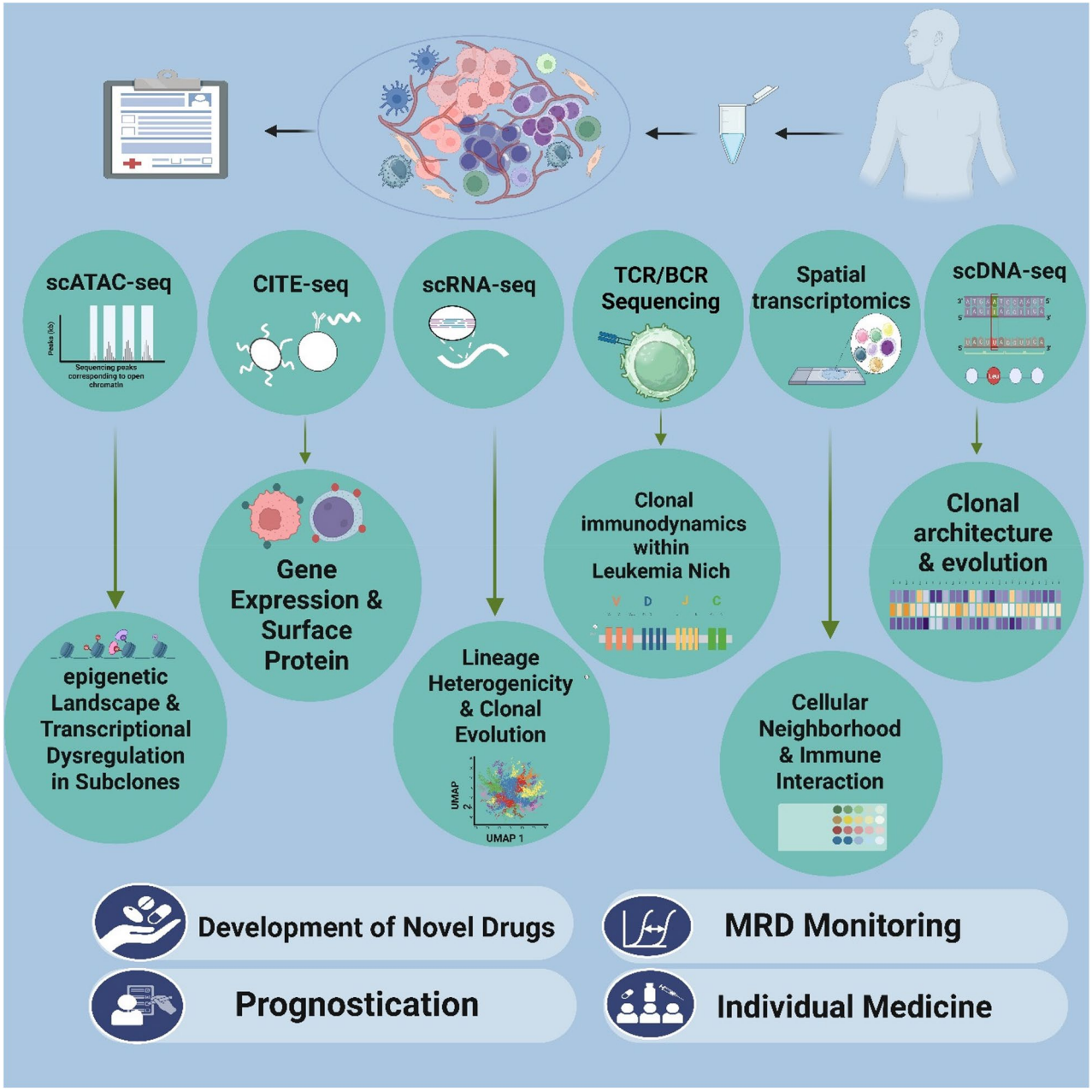

Fig. 1 Overview of single-cell molecular technologies in AML. Each "island" represents a distinct single-cell approach, including scATAC-seq, CITE-seq, scRNA-seq, TCR/BCR Sequencing, spatial transcriptomics scDNA-seq. These approaches contribute to understanding AML heterogeneity and have applications in prognostication, novel therapeutic development, measurable residual disease (MRD) evaluation, and ultimately, personalized medicine. AML (Acute Myeloid Leukemia), BCR (B-Cell Receptor), CITE-seq (Cellular Indexing of Transcriptomes and Epitopes by Sequencing), MRD (Measurable Residual Disease), scATAC-seq (Single-Cell Assay for Transposase-Accessible Chromatin Sequencing), scDNA-seq (Single-Cell DNA Sequencing), scRNA-seq (Single-Cell RNA Sequencing), TCR (T-Cell Receptor)

Beyond the bulk: charting AML's cellular cartography

The profound heterogeneity of AML has long constituted a fundamental barrier to understanding its pathogenesis and improving patient outcomes. Traditional bulk sequencing approaches, while invaluable, inevitably obscure the intricate cellular diversity and molecular relationships governing disease biology. The emergence of sophisticated single-cell technologies has initiated a paradigm shift, enabling researchers to deconvolute the genetic, transcriptomic, and proteomic complexity of AML with unprecedented resolution. Multi-modal dissection reveals a dynamic ecosystem of leukemic and immune cell states, continuous differentiation trajectories, and cellular interactions that bulk analyses miss. By moving beyond the bulk perspective, we can now

begin to construct a high-fidelity cellular cartography of AML, a detailed map that captures the true molecular geography of the disease and provides a transformative framework for refining diagnostics, prognostication, and therapeutic intervention [14, 15]. This conceptual shift has been driven by rapid technological progress. Over the past decade, single-cell platforms have achieved marked improvements in throughput, multimodal integration, and spatial contextualization, enabling the construction of comprehensive cellular atlases from clinically relevant patient cohorts. These advances have made high-resolution "cellular cartography" a practical reality, moving the field beyond bulk analyses.

Decoding the genetic mosaic: scDNA-seq uncovers clonal architecture and evolution

Modern scDNA approaches (targeted high-throughput platforms and plate-based deep genotyping) enable mutation co-occurrence mapping at single-cell resolution and can be combined with surface-protein panels to assign genotype to immunophenotype. In AML, these capabilities let investigators reconstruct patient-specific phylogenies, detect minor resistant clones and map mutational events to functional cell states described below.

scDNA-seq has fundamentally altered our understanding of AML's genetic architecture by enabling the direct observation of mutation co-occurrence and evolutionary trajectories within individual cells. Beyond direct scDNA-seq, endogenous mitochondrial-SNV (mtSNV) profiling offers a complementary method for clonal tracing from standard scRNA-seq data [16, 17]; its applications and caveats are discussed in "Clonal dynamics in response to therapy" section. Traditional bulk sequencing infers clonal composition indirectly through variant allele frequencies (VAFs), a approach that relies on mathematical deconvolution and often fails to accurately reconstruct complex subclonal hierarchies, especially when clones possess similar fitness or mutation burdens. In contrast, scDNA-seq provides a direct, unambiguous view of which mutations co-reside in the same genome, thereby revealing true clonal relationships and phylogenetic patterns without inference [14].

Studies applying scDNA-seq have demonstrated that AML evolution is frequently characterized by branching heterogeneity and convergent evolution. For instance, investigations in myelodysplastic syndrome (MDS) and AML have revealed that pre-malignant stem cells and MDS stem cells can contribute to AML transformation through nonlinear and parallel clonal evolutionary patterns [14]. This challenges the traditional linear model of clonal evolution and underscores the role of diverse, often minor, aberrant stem cell subpopulations in disease progression. Furthermore, scDNA-seq analyses have illuminated that driver mutations in AML often exhibit specific patterns of co-occurrence and mutual exclusivity, reflecting underlying biological constraints or synergies. Epigenetic modifiers, particularly mutations in DNMT3A, TET2, ASXL1, and IDH1/2 (collectively termed DTAI mutations), are frequently identified as early initiating events that establish a pre-leukemic foundation upon which additional mutations accumulate [14, 18, 19]. These foundational mutations often confer clonal dominance and shape the subsequent acquisition of mutations in signaling genes (e.g., FLT3, RAS), which tend to occur later in distinct subclones and can evolve independently multiple times within the same patient, indicating strong selective pressure [7, 14]. High-throughput single-cell genotyping studies directly reconstruct patient-specific phylogenies and show a reproducible pattern: epigenetic regulator mutations are often early, trunk events, whereas signaling mutations (FLT3, NRAS/KRAS) recur independently in parallel branches. This recurrent division between early epigenetic founders and later signaling diversification explains why therapeutic targeting of late-branch events often fails to eradicate MRD [1, 20].

The clinical implications of this refined genetic understanding are profound. Accurate reconstruction of clonal phylogenies enables the identification of persistent pre-leukemic or founder clones that may survive therapy and serve as reservoirs for relapse. Moreover, scDNA-seq can clarify the mechanisms of drug resistance, such as the outgrowth of minor subclones harboring specific resistance mutations that are undetectable by bulk sequencing prior to treatment. For example, integrative single-cell multi-omic profiling of relapsed/refractory AML has revealed that resistance to venetoclax can arise through both innate and acquired mechanisms, often linked to specific clonal evolutionary patterns [7]. By moving beyond VAFs, scDNA-seq provides a genuinely high-resolution view of AML's genetic mosaic, offering critical insights into disease origins, evolution, and resistance that are essential for advancing precision medicine approaches.

Mapping the transcriptomic landscape: scRNA-seq reveals novel cell states and plasticity

scRNA-seq has unveiled an exceptionally detailed and previously obscured view of the transcriptional landscape of AML, revealing novel cell states, continuous differentiation trajectories, and extensive phenotypic plasticity. Unlike bulk RNA-seq, which averages expression across thousands of cells, scRNA-seq captures the transcriptional individuality of each cell, enabling the identification of rare populations, transitional states, and the full spectrum of cellular heterogeneity within the leukemic ecosystem [15, 21].

A pivotal application of scRNA-seq has been the construction of comprehensive reference maps of normal

hematopoiesis and their use to project AML samples; van Galen et al. and subsequent single-cell atlases (Zeng et al.; Wegmann et al.) map AML transcriptional states onto hematopoietic reference trajectories and show that distinct stem-like and monocytic programs recur across patients. Together these primary studies demonstrate that (i) AML cells frequently converge on a limited set of aberrant differentiation programs, and (ii) transcriptional state rather than mutation alone better predicts immune microenvironment interactions, a finding with direct implications for MRD phenotyping and immunotherapy stratification [3, 15, 21]. When AML patient cells are projected onto these refined reference maps, they display profound deviations from normal differentiation, converging onto approximately twelve recurrent patterns of aberrant differentiation [21]. These patterns include unconventional lineage priming, such as erythroid priming linked to TP53 mutations and lymphoid-like states associated with RUNX1 mutations, blurring the traditional diagnostic boundaries between AML, mixed-phenotype acute leukemia (MPAL), and acute erythroid leukemia (AEL) [15, 21].

Furthermore, scRNA-seq enables the reconstruction of continuous differentiation trajectories and the identification of unique LSC states. Through pseudotime analysis, studies have revealed that AML cells often traverse distorted developmental pathways that are blocked or diverted relative to normal hematopoiesis [9, 15]. For instance, in AML with KMT2A rearrangements, scRNA-seq has identified two distinct subgroups that differ in the identity of their LSCs, likely reflecting divergent cellular origins and mechanisms of pathogenesis [15]. This phenotypic plasticity is further highlighted by the discovery that distinct LSC-driven hierarchies can coexist within individual patients, providing insights into clonal evolution and functional heterogeneity [15, 22].

The transcriptomic diversity captured by scRNA-seq also has direct prognostic and therapeutic implications. Studies have identified specific gene expression programs and novel cell states associated with poor outcomes, such as a stem-like program characterized by high expression of SPINK2 or a monocyte-like state expressing immunomodulatory genes that suppress T cell activity [9, 22]. Moreover, scRNA-seq of the AML BM microenvironment has revealed extensive remodeling of immune cell phenotypes and functions, including the presence of exhausted T cells, immunosuppressive macrophages, and unique dendritic cell subsets that contribute to immune evasion [22, 23]. These findings provide a roadmap for targeting specific cell states or microenvironmental interactions to overcome therapy resistance.

Importantly, computational tools for gene-regulatory network (GRN) inference now allow investigators to move beyond co-expression and infer the transcription factors and regulatory circuits that underpin the cell states and plasticity revealed by scRNA-seq. The application of these methods in AML studies has successfully identified key regulons and prognostic regulatory modules, linking transcriptional control to clinical outcomes [24–26].

Defining proteomic and immune contexture: high-dimensional cytometry profiles functional cell subsets

While genomic and transcriptomic analyses provide deep insights into cellular identity and potential, proteins are the primary functional executers in cells, defining signaling networks, immune responses, and therapeutic targets. High-dimensional cytometry technologies, particularly CyTOF and CITE-seq, have dramatically expanded our ability to profile protein expression simultaneously with nucleic acids at single-cell resolution, enabling a comprehensive functional definition of cellular subsets and the immune contexture in AML [27, 28].

CyTOF, which utilizes metal-tagged antibodies and mass spectrometry for detection, allows for the simultaneous measurement of over 40 protein parameters with minimal spectral overlap, overcoming a key limitation of conventional fluorescence-based flow cytometry [28, 29]. This high-dimensional capability is crucial for dissecting the immense heterogeneity of immune and leukemic cells. For example, CyTOF studies have identified previously unrecognized immune subsets in the AML microenvironment, such as exhausted CD8 + T cell phenotypes, altered NK cell subsets with impaired cytotoxicity, and expanded regulatory T cells (Tregs) with heightened immunosuppressive activity [7, 22]. Moreover, CyTOF has been instrumental in characterizing functional signaling states and phosphoprotein networks in response to therapies, revealing dynamic adaptations that contribute to drug resistance [7, 29, 30].

CITE-seq represents a powerful integration of protein and transcriptome measurement, whereby oligonucleotide-labeled antibodies enable the simultaneous detection of surface protein expression and whole transcriptome sequencing within the same single cell [28]. This technology provides a robust link between cellular identity (defined by surface markers) and transcriptional state, allowing for precise annotation of cell types and the discovery of novel subsets that would be missed by either modality alone. In AML, CITE-seq has been applied to dissect the surface antigen landscape of LSCs and blasts, revealing heterogeneity in marker expression that refines minimal residual disease (MRD) monitoring and identifies potential immunotherapeutic targets [7, 14].

The integration of these high-dimensional proteomic technologies has profoundly advanced our understanding of the AML immune microenvironment's composition and functional state. Detailed cellular censuses have

uncovered significant differences between normal and AML BM, including expansions of immunosuppressive myeloid populations such as myeloid-derived suppressor cells (MDSCs), altered dendritic cell (DC) subsets with impaired antigen presentation, and the presence of unique T cell states like TH17-like intermediates and cytotoxic CD4 + T cells [22]. Furthermore, functional analyses have revealed that AML cells actively remodel their microenvironment by secreting factors that drive immune dysfunction, such as upregulating checkpoint molecules like PD-L1 and CD276 (B7-H3), and promoting the expansion of immunosuppressive subsets [7, 22].

These insights are catalyzing new immunotherapeutic strategies. For instance, high CD36 expression has been identified on venetoclax-resistant blasts and associated with sensitivity to CD36-targeted antibody treatment ex vivo [7]. Similarly, the identification of specific immune evasion mechanisms suggests rational combinations, such as targeting checkpoint molecules alongside conventional therapies to restore immune surveillance. The ability to profile dozens of proteins simultaneously at single-cell resolution ensures that high-dimensional cytometry will remain an indispensable tool for defining predictive biomarkers, understanding therapy resistance, and designing novel immunotherapies for AML. Figure 2 displays the CyTOF (mass cytometry) workflow as an exemplar of high-dimensional single-cell proteomic profiling, chosen because CyTOF directly measures dozens of protein markers per cell and therefore best illustrates how protein-level single-cell data complement transcriptomic atlases. While many single-cell platforms are discussed in text (scRNA-seq, scDNA-seq, CITE-seq, spatial proteomics), CyTOF is shown here as a concrete, translationally-proximal example of how proteomic single-cell readouts are generated and analyzed. For a broader view of single-cell technologies (scRNA-seq, scDNA-seq, CITE-seq and spatial methods) see "Decoding the genetic mosaic: scDNA-seq uncovers clonal architecture and evolution"–"Defining proteomic and immune contexture: high-dimensional cytometry profiles functional cell subsets" sections and Table 5.

Redefining the quintessential biomarker: LSC

The enigmatic LSC represents both the cornerstone of AML pathogenesis and a principal barrier to cure; however, recent single-cell studies have moved the field from a concept of a single 'LSC population' to a mapped spectrum of LSC states with distinct transcriptional programs, niche interactions and drug sensitivities. Single-cell RNA-seq and multimodal single-cell atlases have (i) defined primitive/'stem-like' cellular compartments and their marker sets, (ii) mapped how bulk stemness scores (e.g., LSC17) project onto particular primitive cell states, and (iii) identified previously unrecognized therapy-resistant LSC states, findings that directly link single-cell resolved states to prognosis and therapy response. For example, van Galen et al. generated a single-cell AML atlas that mapped stem-like programs to primitive compartments and identified rare stem-like populations associated with immune suppression and poor outcome; subsequent single-cell analyses have extended and functionally validated these observations [3, 31].

This section examines how single-cell analyses are redefining LSC biology through the lens of cellular heterogeneity, novel biomarker identification, and non-genetic mechanisms of stemness plasticity.

Unmasking LSC heterogeneity

Single-cell transcriptomic and proteomic technologies have fundamentally dismantled the idea of a single LSC phenotype and instead revealed a spectrum of LSC states with distinct stemness programs, metabolic dependencies and chromatin landscapes. Importantly, single-cell studies have functionally linked these states to outcomes and drug sensitivity, e.g., van Galen et al. (single-cell atlas) identified primitive stem-like programs enriched in chemo-resistant compartments, and subsequent single-cell functional profiling (including CITE-seq and matched ex vivo drug assays) revealed monocytic-LSC states that confer venetoclax resistance [3, 12]. This heterogeneity manifests functionally through variable self-renewal capacities, differential chemotherapy resistance, and distinct microenvironmental interactions that collectively influence disease progression and therapeutic outcomes. Research demonstrates that even within individual patients, LSCs exist in multiple transcriptional states with divergent functional properties, suggesting that therapeutic strategies must account for this diversity to achieve durable remissions [32, 33].

The metabolic heterogeneity of LSCs represents a critical dimension of their functional diversity, with significant implications for therapeutic targeting. Quiescent LSCs demonstrate a distinct metabolic configuration characterized by enhanced oxidative phosphorylation and reduced glycolytic activity compared to their more differentiated progeny. This metabolic profile contributes to chemotherapy resistance, as most conventional anti-leukemic agents target rapidly dividing cells. Single-cell metabolic profiling has revealed that LSC populations contain subsets with differential dependence on mitochondrial function, fatty acid oxidation, and amino acid metabolism, suggesting that metabolic targeting strategies must address this heterogeneity to effectively eradicate all LSC subpopulations [33–35].

Epigenetic heterogeneity further compounds the complexity of LSC populations, with distinct chromatin accessibility patterns correlating with differential gene

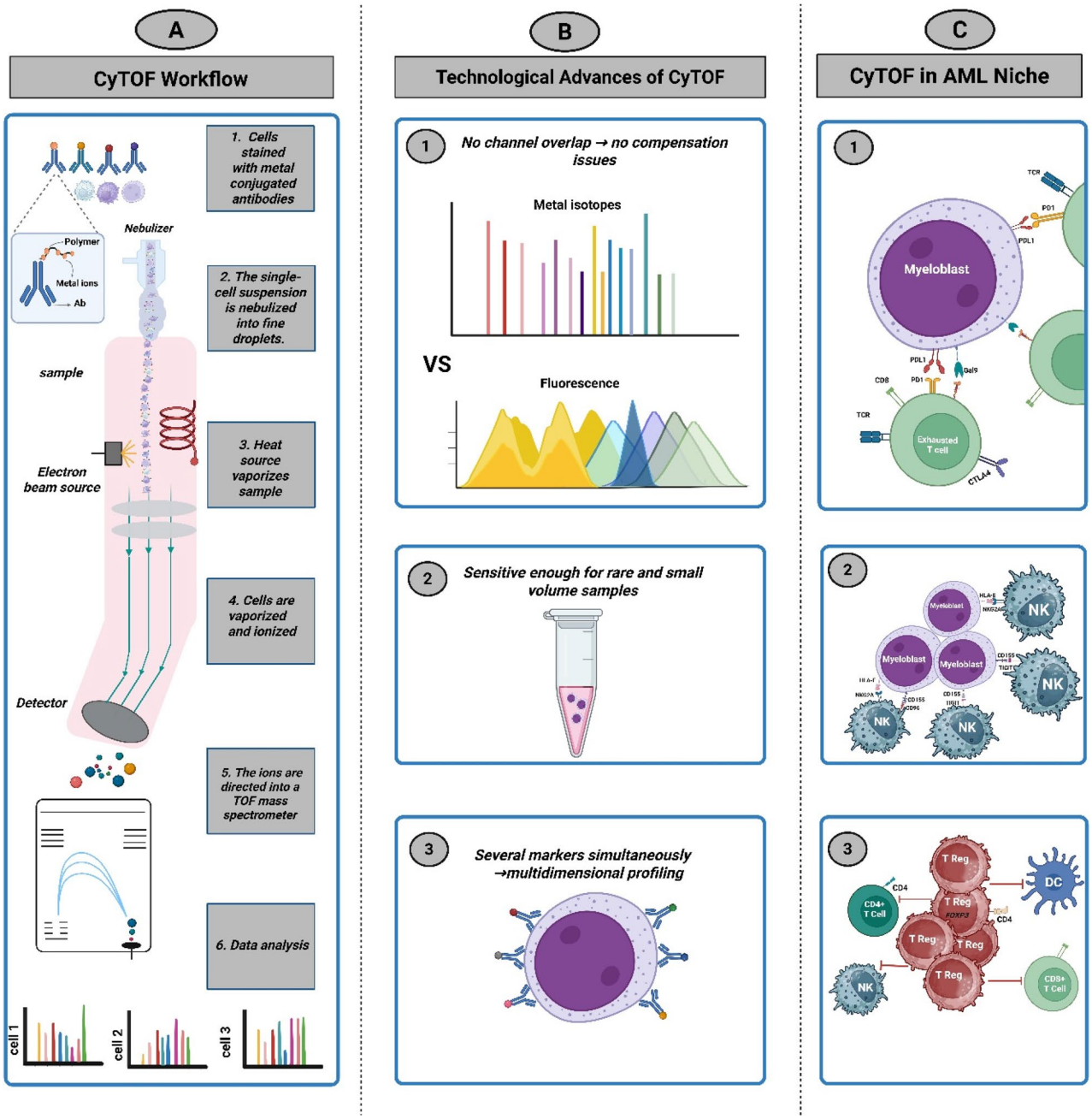

Fig. 2 CyTOF as an exemplar high-dimensional single-cell proteomic workflow. **A** Summary workflow: cells are stained with metal-conjugated antibodies, nebulized and ionized; metal isotopes are detected by time-of-flight mass spectrometry producing single-cell metal-tag counts that map to protein expression after normalization and clustering. **B** Key technical advantages: (1) minimal spectral overlap enabling > 40 simultaneous parameters, (2) capacity for small/rare cell populations, (3) direct protein (including phospho-protein) readouts that complement transcriptomic measurements. **C** Translational value: CyTOF has revealed immune phenotypes in AML (exhausted CD8 T cells, altered NK subsets, expanded regulatory myeloid populations) and serves as a bridge to clinical flow cytometry panels used in MRD pipelines. Note: CyTOF is shown as an exemplar proteomic platform; the manuscript also discusses other single-cell platforms (droplet scRNA-seq, scDNA-seq, CITE-seq and spatial methods) that provide complementary molecular information. (Figure created with BioRender)

expression and functional behavior. Single-cell epigenomic analyses have identified subpopulations of LSCs with varying accessibility at key transcription factor binding sites, suggesting differential regulatory circuit activity within seemingly homogeneous populations. This epigenetic plasticity enables rapid adaptation to therapeutic pressures and microenvironmental changes, representing a fundamental mechanism of treatment resistance. Research indicates that epigenetic heterogeneity often precedes genetic heterogeneity, creating

a permissive landscape for clonal evolution and disease progression [18, 36, 37].

The functional consequences of this multidimensional heterogeneity are profound, influencing therapeutic response, disease trajectory, and relapse risk. Studies tracking LSC subpopulations through treatment and relapse have demonstrated dynamic shifts in LSC composition, with selective pressure enriching for resistant subclones possessing specific metabolic and epigenetic configurations. This observation suggests that effective LSC-directed therapies must simultaneously target multiple LSC states or employ strategies that prevent adaptation through state switching. The clinical translation of these findings is evidenced by the development of prognostic tools that incorporate LSC heterogeneity metrics, such as the LSC score, which quantifies stemness expression signatures and correlates with clinical outcomes across multiple AML genotypes [32, 38].

Identifying LSC-specific biomarkers

The historical immunophenotypic definition of LSCs as CD34+/CD38- cells has proven insufficient for precise identification and targeting, as this population encompasses both LSCs and normal HSCs while missing LSCs that reside outside this phenotypic compartment. Single-cell comparative analyses of LSCs and HSCs have revealed numerous novel, functionally validated surface markers that enable more specific discrimination of leukemic from normal stem cells. These advancements have critical implications for MRD monitoring, therapeutic targeting, and patient stratification [39, 40].

CD123 (IL3RA) is among the best-validated LSC surface markers in primary datasets: flow and single-cell analyses show enrichment of CD123 on leukemic stem populations and correlation with inferior outcomes in intensive chemotherapy cohorts [34, 41].

Beyond surface markers, transcriptional signatures remain powerful for LSC identification and prognostic stratification. The LSC17 stemness signature remains the most extensively validated prognostic program; single-cell studies have since mapped LSC17 activity to specific primitive cell states, clarifying its cellular origin and limitations when used as a bulk proxy for LSC biology [31]. Although the LSC17 signature was derived from bulk analyses and validated as a clinical prognostic test [42, 43], single-cell studies have since mapped its activity to specific primitive cell states, clarifying its cellular origin and limitations as a bulk proxy [13, 20]. Applying such bulk signatures to single-cell data requires adapted scoring methods (e.g., AUCell, GSVA) to account for technical noise and dropouts. Notably, van Galen et al. used scRNA-seq to define conserved AML cellular hierarchies and mapped stem-like transcriptional programs to primitive cellular compartments and immune-suppressive niches, illustrating how single-cell resolution separates LSC-like states from bulk averages [3]. More recently, Zeng et al. proposed a cellular-hierarchy framework that identifies multiple, functionally distinct LSC states across patients and directly relates these states to differential drug sensitivities, emphasizing that bulk stemness scores such as LSC17 capture important prognostic signal but do not fully resolve the multiplicity of LSC states revealed by single-cell profiling [15]. When applying bulk-derived signatures (like LSC17) to single-cell data it is important to recognize technical and interpretational caveats: single-cell transcriptomes suffer from dropouts, variable detection sensitivity, and cell-level noise, so investigators often score bulk gene-sets on single cells using adapted methods (for example pooled/pseudobulk scoring, AUCell/GSVA variants or smoothed neighborhood scores) and validate results across orthogonal assays.

More recently, an eight-gene leukemia stem cell prognostic death score (LSCD) signature has been developed through integration of programmed cell death genes and LSC-related genes, offering enhanced prognostic capability and drug sensitivity prediction. This signature includes genes such as OAZ1, S100A4, MPG, IL2RA, MMRN1, CDK6, HOXA9, and XIRP2, which collectively capture the functional state of LSCs and their susceptibility to various cell death modalities [32, 33].

The clinical implementation of these refined biomarkers is already transforming patient management strategies. Flow cytometric detection of LSCs using expanded marker panels (e.g., CD34 + CD38-CD45RA+CD123 + TIM-3+) enables more sensitive MRD monitoring than conventional approaches, with studies demonstrating that persistence of these populations after treatment strongly predicts relapse. Similarly, digital PCR and RNA-based detection of LSC-specific signatures in peripheral blood offers a less invasive approach for disease monitoring and early relapse detection. The progressive refinement of these biomarkers through single-cell technologies continues to enhance their precision, moving the field closer to truly personalized LSC-directed therapies [38, 44]. The expanding arsenal of LSC-specific biomarkers, moving beyond the conventional CD34 + CD38- phenotype, is detailed in Table 1.

The plastic stem cell: a dynamic state

Perhaps the most paradigm-shifting revelation from single-cell studies is the recognition that stemness in AML represents a dynamic cellular state rather than a fixed cellular identity, with capacity for bidirectional conversion between stem and non-stem states through non-genetic mechanisms. This plasticity enables more differentiated blasts to reacquire stemness properties under appropriate environmental conditions, representing a potent mechanism of therapeutic resistance that operates

Table 1 Novel LSC-specific biomarkers identified via single-cell technologies

Biomarker	Type	Function / Significance	Prognostic/Therapeutic utility	Ref.
CD123 (IL3RA)	Surface Protein	Interleukin-3 receptor alpha chain; consistently overexpressed on LSCs vs. normal HSCs.	Target for antibody-drug conjugates (e.g., Tagraxofusp) and CAR-T cells. Predicts inferior outcomes.	[34, 41, 45, 46]
CLL-1 (CLEC12A)	Surface Protein	C-type lectin-like molecule-1; highly expressed on LSCs with minimal expression on normal HSCs.	A promising target for CAR-T and antibody-based therapies; improves MRD detection sensitivity.	[45, 47, 48]
TIM-3 (HAVCR2)	Surface Protein	Immune checkpoint molecule; expressed on LSCs and implicated in immune evasion.	Combination target with other immune checkpoints; associated with poor prognosis.	[49, 50]
LSC17 Score	Transcriptomic	17-gene expression signature derived from LSC-enriched populations.	Powerful independent prognosticator of survival across AML genetic subtypes.	[31]
LSCD Score	Transcriptomic	8-gene signature (OAZ1, S100A4, MPG, IL2RA, MMRN1, CDK6, HOXA9, XIRP2) integrating programmed cell death genes.	Predicts survival and drug sensitivity; refines risk stratification.	[33]

independently of genetic evolution. The quantification of this phenomenon at single-cell resolution has revealed its surprising frequency and clinical significance, fundamentally altering our understanding of AML pathogenesis [36, 37].

Epigenetic reprogramming represents a primary mechanism underlying stemness plasticity, with dynamic modifications to DNA methylation and histone acetylation patterns enabling state transitions without genetic alteration. Single-cell multi-omics approaches have demonstrated that epigenetic heterogeneity within leukemic populations creates a permissive landscape for cellular reprogramming, with certain epigenetic configurations predisposing cells to reacquire stemness properties under therapeutic pressure. Key epigenetic regulators such as DNMT1, TET2, EZH2, and IDH1/2 play crucial roles in this process, with their mutational status or aberrant expression influencing the plasticity potential of leukemic populations. These findings explain the limited efficacy of conventional therapies and support the development of epigenetic maintenance therapies that prevent or limit cellular reprogramming [36, 44].

Metabolic reprogramming represents another critical enabler of cellular plasticity, with shifting metabolic requirements facilitating transitions between stem and non-stem states. Quiescent LSCs maintain distinct metabolic dependencies compared to their proliferative counterparts, preferentially utilizing oxidative phosphorylation over glycolysis and demonstrating enhanced flexibility in fuel source utilization. Single-cell metabolic analyses have revealed that metabolic heterogeneity within leukemic populations creates reservoirs of cells poised to acquire stemness properties under appropriate conditions, with certain metabolic configurations conferring enhanced plasticity potential. Microenvironmental cues, including hypoxia, nutrient availability, and stromal interactions, further influence this metabolic plasticity, creating tissue-level patterns of cellular behavior that extend beyond cell-autonomous programming [33, 37].

The therapeutic implications of stemness plasticity are profound, suggesting that successful eradication of AML must address not only existing LSCs but also the potential for reconstructed stemness from more differentiated populations. Strategies targeting the epigenetic and metabolic regulators of cellular plasticity hold particular promise, with preclinical models demonstrating that inhibition of DNMT1, EZH2, or mitochondrial metabolism can limit cellular reprogramming and enhance chemotherapy efficacy. Similarly, microenvironment-directed therapies that disrupt the niche support for stemness acquisition represent another promising approach. The clinical translation of these concepts has been most clearly demonstrated by venetoclax-based regimens in selected patient populations. In the phase 3 VIALE-A trial, azacitidine plus venetoclax produced higher complete remission rates and improved overall survival compared with azacitidine alone in patients with newly diagnosed AML who were ineligible for intensive induction, establishing HMA+venetoclax as a standard option for older or unfit patients. Similarly, venetoclax plus low-dose cytarabine showed benefit in the VIALE-C setting. However, these benefits are context-dependent: trial populations were largely older and deemed unfit for intensive chemotherapy, and response rates and durability vary by molecular and cytogenetic features (for example, IDH1/2- and NPM1-mutant disease often shows favorable responses, whereas TP53-mutated or some RAS-pathway-mutant cases have inferior outcomes). Retrospective comparisons with intensive chemotherapy yield mixed results and suggest that treatment selection should be individualized based on age, fitness, comorbidities, cytogenetic risk, and actionable mutations. Therefore, while venetoclax combinations represent an important clinical advance in many settings, the statement that they are 'superior' to conventional therapies should be qualified by these clinical and molecular considerations [33, 44, 51–54].

Recent single-cell and multi-omic investigations have specifically characterized cellular and microenvironmental contributors to venetoclax resistance. Pei et al. define a monocytic-LSC (m-LSC) subtype, immunophenotypically and transcriptionally distinct from primitive LSCs, that preferentially drives monocytic disease progression and shows selective resistance to venetoclax, with unique metabolic dependencies (notably purine metabolism) that may offer alternative therapeutic targets [12]. Complementing this, Wang et al. integrate bulk and scRNA-seq data to demonstrate that an IFN-γ/inflammatory niche is enriched in monocytic AML and that an IFN-γ/IFITM3 signaling program correlates strongly with ex-vivo venetoclax resistance; experimentally, IFN-γ exposure increased venetoclax resistance in primary blasts, suggesting a causal microenvironmental contribution. Together these studies indicate that both lineage-program (monocytic differentiation/m-LSCs) and local inflammatory signaling (IFN-γ) are important mediators of venetoclax resistance and should be considered when developing predictive biomarkers and combination strategies to overcome resistance [13].

The ecosystem view: LSCs in dialogue

AML progression is not merely a cell-autonomous process but rather a dynamic ecosystem orchestrated through sophisticated dialogue between leukemic cells and their microenvironment. This complex interplay creates specialized niches that support LSCs, promote immune evasion, and foster therapeutic resistance. Single-cell technologies have revolutionized our ability to decode these interactions, revealing an intricate network of cellular communication, immunosuppressive mechanisms, and metabolic adaptations that collectively sustain the malignant niche. By mapping this molecular geography with unprecedented resolution, we can identify novel therapeutic vulnerabilities that disrupt these supportive networks while sparing normal hematopoiesis.

Deciphering cell-cell communication networks

The leukemic microenvironment is characterized by a complex signaling network mediated through ligand-receptor interactions between AML subclones and various stromal and immune components. scRNA-seq technologies have enabled the systematic mapping of these communication pathways using Computational tools for cell-cell communication inference, which infer potential cellular crosstalk based on ligand-receptor co-expression patterns [55, 56]. However, these computational inferences are limited: RNA expression does not guarantee functional protein interactions, and they lack spatial context. Thus, CCC maps should be treated as hypothesis-generating [55, 57, 58]. Orthogonal validation, through spatial confirmation, protein-level assays, and functional perturbation, is essential to establish causal interactions [59–61]. These analyses have revealed remarkable heterogeneity in signaling networks both within and between AML patients, with distinct subclones engaging different communicative programs with their microenvironment [62].

AML blasts actively manipulate their surroundings through multiple mechanisms. They secrete various immunomodulatory cytokines including IL-10, IL-35, TGF-β, and indoleamine 2,3-dioxygenase 1 (IDO1), which collectively promote Treg expansion and suppress effector T cell function. Additionally, AML cells dysregulate antigen presentation machinery through epigenetic silencing of HLA class II genes and genomic loss of HLA haplotypes, effectively evading T cell-mediated recognition [63]. The CXCL12-CXCR4 axis emerges as a particularly critical communication pathway, facilitating LSC retention within protective BM niches while excluding normal hematopoietic elements [64]. Recent studies applying hypergraph learning frameworks to scRNA-seq data have revealed extensive crosstalk between signaling pathways, with shared signaling components enabling efficient information transfer between different communication networks [62]. The key ligand-receptor interactions that mediate the dialogue between AML cells and their microenvironment, along with their functional significance and therapeutic targeting potential, are summarized in Table 2.

Advanced computational methods are now moving beyond simple ligand-receptor mapping to incorporate downstream intracellular signaling events. Tools like SigXTalk employ machine learning approaches to quantify signal fidelity and specificity within communication networks, revealing how pathway crosstalk influences transcriptional outputs in receiver cells [62]. These analyses demonstrate that AML cells create autocrine and paracrine signaling loops that activate pro-survival pathways such as NF-κB and β-catenin signaling while simultaneously suppressing anti-leukemic immune responses [63, 64, 72]. Spatial methods (spatial transcriptomics and spatial proteomics) provide the tissue context that is essential to move from transcript-level ligand–receptor inference to plausible in-situ signaling hypotheses. However, applying spatial omics to BM poses specific technical and analytic challenges that influence study design and interpretation: BM trephines and cores are calcified and heterogeneous (hematopoietic islands interspersed with adipocytes, vasculature and bone), sample processing (FFPE vs. fresh frozen vs. OCT) strongly affects RNA quality and probe penetration, and many spatial platforms trade single-cell resolution for breadth (e.g., Visium spot sizes > single cell) while high-resolution approaches (MERFISH/seqFISH, Slide-seq2, Slide-HD) require specialized workflows and are often lower throughput.

Table 2 Key cell-cell communication pathways in AML microenvironment

Ligand-receptor pair	Sender cell	Receiver cell	Functional significance	Therapeutic targeting	Validation level	Ref.
CXCL12-CXCR4	Stromal cells	LSC	Stem cell retention in protective niches	Plerixafor (AMD3100)	Clinical / translational evidence (multiple early-phase combination trials; functional in-vivo models). Clinical benefit mixed and context-dependent; combinations most promising.	[64, 65]
Galectin-9 - TIM-3	AML blast	T cell / NK cell	Induction of immune exhaustion and dysfunction	TIM-3 inhibitors	Preclinical + early clinical evidence; immune-modulating effects shown, single-agent efficacy variable; combination strategies in development.	[63, 66]
PD-L1 (CD274) - PD-1 (PDCD1)	AML blasts / myeloid cells / stromal cells	T cells (PD-1+)	Ligand-mediated inhibition of T-cell activation and cytotoxicity → T-cell exhaustion / immune evasion	PD-1 / PD-L1 inhibitors (e.g., nivolumab, pembrolizumab, atezolizumab); combinations with HMAs (azacitidine) tested in early-phase trials	Preclinical + early-phase clinical evidence (notable HMA + PD-1 signals); responses context-dependent; biomarkers under study	[67–69]
CD47 - SIRPα	LSC	Macrophage	"Don't eat me" signal; phagocytosis avoidance	CD47 blockers (e.g., Magrolimab)	Substantial translational evidence; promising phase 1/2 activity; phase-3 data maturing.	[63, 70]
IL-10 - IL-10R	Myeloid SSPC / MDSC	T cell	Promotion of immunosuppression and Treg expansion	IL-10 pathway inhibitors	Primarily preclinical and correlative human evidence; therapeutic use still exploratory.	[63, 71]
ATP - P2RX7	Damaged cells	Macrophage / T cell	Pro-inflammatory signaling; immune activation	P2RX7 modulators	Preclinical evidence in AML (mixed; isoform-dependent effects). Limited clinical validation; requires additional functional studies.	[63]

Computationally, spot deconvolution and cell-assignment rely on scRNA references and produce results that depend on reference quality and segmentation accuracy; orthogonal validation (smFISH, multiplexed IF, or imaging mass cytometry) is therefore commonly needed. Because of these caveats, investigators studying AML BM should (i) plan pre-analytical SOPs (decalcification, fixation) tailored to their chosen platform, (ii) combine spatial readouts with single-cell suspensions for robust deconvolution, and (iii) report limits of detection and spot deconvolution metrics to help readers assess sensitivity. Recent studies applying multimodal spatial profiling to human BM and AML illustrate both the power and the pitfalls of these approaches [73–75].

Spatial omics, state-of-the-art, technical difficulties, and examples in AML BM

Spatial transcriptomic and proteomic technologies extend single-cell atlases by mapping cellular states within their native tissue architecture. This is critical in AML, where cellular positioning dictates niche interactions and therapeutic access. While powerful, applying these methods to BM presents distinct challenges, including tissue processing, resolution trade-offs, and complex computational deconvolution [73, 74, 76, 77]. Despite these hurdles, recent multimodal spatial studies have successfully delineated the cellular biogeography of human BM, revealing specialized niches and immunotherapy-driven remodeling in AML [73, 78]. These approaches provide essential context for validating cell-cell communication hypotheses generated from single-cell suspensions.

State-of-the-art examples and what they show

Mapping of the cellular biogeography of human BM using combined single-cell and proteomic imaging has yielded high-resolution niche maps and defined micro-anatomical neighborhoods that control hematopoiesis, demonstrating the feasibility of multimodal spatial profiling in human BM [73]. Dedicated methodological reviews and practical workflows now exist for BM spatial transcriptomics that describe pre-analytic SOPs and pitfalls (e.g., trephine handling, decalcification choices), and these should be used as templates for future studies [74, 77]. Recent multimodal spatial studies in AML have demonstrated immunotherapy-driven remodeling of BM niches and identified TLS-like aggregates and CXCL12-rich stromal niches associated with leukemic persistence; these works show both the power of spatial approaches and the need for orthogonal validation (protein imaging, functional assays) [75, 78, 79].

In short, spatial omics are now practical and highly informative for BM niche, but they require deliberate sample handling, matched single-cell references, careful computational deconvolution, and orthogonal protein/

image validation to yield robust biological and translational inferences.

Characterizing the immunosuppressive niche

The AML BM microenvironment is more than a passive background, it is an actively immunosuppressive ecosystem that supports leukemic survival, therapy resistance and relapse. Single-cell studies have shown that this niche is composed of multiple cooperating cell types (expanded regulatory T cells, functionally exhausted CD8 + T cells, myeloid-derived suppressor cells (MDSCs), leukemia-associated macrophages and dysfunctional dendritic cells) as well as dysregulated B cell subsets, all of which contribute distinct but overlapping suppressive mechanisms [3].

Single-cell transcriptomics and high-dimensional cytometry have repeatedly demonstrated (i) coordinated upregulation of inhibitory receptors and their ligands (PD-1/PD-L1, TIM-3/galectin-9, TIGIT and others) on T cells and myeloid compartments, (ii) cytokine and metabolic programs (e.g., IL-10, TGF-β, IDO1, adenosinergic CD39/CD73) that blunt effector responses, and (iii) reprogramming of antigen presentation machinery (downregulated HLA class II, altered dendritic-cell states) that limits T-cell priming and recognition, mechanistic features that help explain clinical patterns of resistance to checkpoint monotherapy [12, 68].

Importantly, single-cell and spatial multimodal platforms provide the mechanistic bridge between descriptive immune atlases and clinical action: scRNA-seq and CITE-seq assign phenotype, activation/exhaustion status and surface antigen co-expression to individual immune and leukemic cells (enabling nomination of clone-restricted surface targets and scMRD readouts), while spatial transcriptomics and multiplexed proteomic imaging place these states in physical context (identifying CXCL12-rich stromal niches, TLS-like aggregates, or perivascular immune deserts associated with persistence and resistance) [13, 73].

Clinically this expanded view has three immediate implications: (1) predictive biomarkers should include niche features (for example, an IFN-γ/inflammatory signature, presence of m-LSC with adjoining exhausted T cells, or high adenosinergic activity) because these features influence response to targeted and immune therapies; (2) translation pathways must be multimodal, single-cell discovery → protein confirmation (CITE-seq/CyTOF) → reduced protein panels for clinical assays; and (3) therapeutic strategies should rationally combine agents that target leukemic vulnerabilities and niche-mediated suppression (e.g., metabolic/epigenetic priming + checkpoint or macrophage-directed therapies), with patient selection guided by combined tumor + niche biomarkers [3, 12, 13, 68, 73].

T cells within the AML microenvironment exhibit distinct dysfunctional states characterized by exhaustion and senescence markers. Exhausted T cells show progressive loss of effector functions, sustained expression of multiple inhibitory receptors (including PD-1, TIM-3, CTLA-4, LAG-3, and TIGIT), and impaired cytotoxic capacity [63, 80]. In particular, the PD-1 / PD-L1 axis, with PD-L1 frequently upregulated on AML blasts and tumor-associated myeloid/stromal elements, contributes to T-cell inhibition and clinical immune evasion; blockade of this axis in combination with hypomethylating agents (e.g., azacitidine + nivolumab) has produced encouraging early-phase signals in relapsed/refractory AML, although benefit appears context-dependent and predictive biomarkers remain under study [68, 69]. Senescent T cells demonstrate irreversible cell cycle arrest and secrete a characteristic repertoire of pro-inflammatory factors that further shape the immunosuppressive microenvironment. Single-cell analyses have revealed that these dysfunctional T cell states are not uniform but rather represent a spectrum of differentiation trajectories with distinct transcriptional programs [71]. AML blasts actively promote T cell dysfunction through multiple mechanisms, including impaired immune synapse formation, altered actin cytoskeletal dynamics, and secretion of immunomodulatory factors that drive Treg polarization [63].

Myeloid cells within the AML niche contribute significantly to immunosuppression through various mechanisms. MDSCs expand dramatically in AML and suppress T cell function through arginase-mediated L-arginine depletion, reactive oxygen species production, and nitrosative stress [63]. LAMs exhibit alternative activation markers and secrete anti-inflammatory cytokines that further dampen antitumor immunity. Recent scRNA-seq studies have revealed novel immunosuppressive populations such as CD39 + T cells with impaired effector functions that are expanded in response to leukemic extracellular vesicles [81–85]. These immunosuppressive populations create multiple layers of regulation that collectively establish an immune-privileged sanctuary for leukemic cells.

The immunosuppressive niche is not static but dynamically evolves during disease progression and in response to therapy. Pre-leukemic mutations in epigenetic regulators such as DNMT3A, IDH1/2, and TET2 in hematopoietic stem cells create a permissive microenvironment that facilitates subsequent leukemic transformation [64]. Following chemotherapy, residual AML cells remodel the niche through increased production of immunosuppressive factors that promote relapse. Understanding this dynamic evolution is critical for developing immunotherapeutic strategies that can reverse immunosuppression and restore effective anti-leukemic immunity.

Unraveling metabolic cross-talk

Metabolic reprogramming represents a fundamental adaptation that supports leukemic survival and creates nutritional competition within BM microenvironment. Single-cell metabolomic technologies such as SCENITH (Single Cell ENergetIc metabolism by profilIng Translation inHibition) have revealed remarkable heterogeneity in metabolic dependencies across AML subclones and between leukemic and stromal cells [81, 82, 86]. This method utilizes puromycin incorporation coupled with flow cytometric detection to measure global protein synthesis rates as a surrogate for cellular energy consumption under various metabolic inhibitions, allowing functional assessment of metabolic dependencies at single-cell resolution [81, 82].

AML cells exhibit diverse metabolic strategies that often mirror their cellular origins while adapting to microenvironmental constraints. LSCs particularly rely on oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) to meet their energetic demands, expressing high levels of the fatty acid transporter CD36 to uptake lipids derived from BM adipocytes [63, 87]. More differentiated blasts frequently demonstrate increased glycolytic flux, resembling the Warburg effect observed in many solid tumors. This metabolic heterogeneity creates opportunities for symbiotic relationships where glycolytic cells produce lactate that fuels oxidative metabolism in neighboring cells [87].

Metabolic competition represents a significant mechanism of immune evasion in AML. Leukemic cells consume essential nutrients including glucose, glutamine, and tryptophan, creating localized deficiencies that impair immune cell function [63, 87]. T cells within the leukemic microenvironment experience metabolic stress due to glucose restriction and accumulation of immunosuppressive metabolites such as kynurenines (from tryptophan catabolism) and lactate [87]. This metabolic rewiring directly suppresses cytotoxic function while promoting regulatory T cell expansion. AML blasts further exacerbate this competition through expression of ectonucleotidases such as CD39 and CD73 that convert extracellular ATP to immunosuppressive adenosine [63]. The distinct metabolic configurations of different cellular players in the AML niche, which underpin survival, proliferation, and immunosuppression, are consolidated in Table 3.

The metabolic landscape of AML is further complicated by the presence of specialized stromal cells that provide metabolic support to leukemic cells. BM adipocytes undergo lipolysis to release fatty acids that fuel β-oxidation in LSCs [63]. Mesenchymal stromal cells enhance mitochondrial transfer to leukemic cells through tunneling nanotubes, supporting oxidative metabolism and chemoresistance [87–89]. Endothelial cells maintain leukemic cell quiescence through Notch signaling activation, promoting a metabolic state that favors survival over proliferation [64]. This metabolic cross-talk creates a supportive niche that maintains LSCs while impairing immune effector function, representing a promising therapeutic target for disrupting the leukemic ecosystem.

Technological advances in single-cell metabolomics are rapidly improving our ability to characterize these metabolic interactions. Methods such as Met-Flow, SCENITH, and integration of scRNA-seq with mass spectrometry imaging (MSI) enable correlation of metabolic states with cellular phenotypes while preserving spatial context [81, 82, 87]. These approaches have revealed that metabolic features do not always align with conventional lineage markers, identifying metabolically defined subpopulations with distinct functional capabilities [81, 82]. Applying these technologies to clinical samples will be essential for understanding how metabolic heterogeneity contributes to treatment resistance and identifying metabolic vulnerabilities that can be therapeutically exploited.

The metabolic heterogeneity described above has direct translational consequences. First, metabolic phenotypes (for example, high OXPHOS / FAO dependency, elevated CD36 expression or purine-metabolism signatures) can serve as predictive biomarkers for response or resistance to existing therapies (notably BCL-2 inhibition with venetoclax combinations) and for candidate metabolic-targeting agents. Clinical proof-of-concept exists: venetoclax plus azacitidine demonstrates substantial efficacy in unfit/older AML but response durability varies by molecular and metabolic context (e.g., IDH and NPM1 cases often respond while some TP53/RAS contexts do not), arguing that metabolic state partially determines therapeutic benefit. Translationally, metabolic profiling at single-cell or functional single-cell

Table 3 Metabolic features of cellular components in AML microenvironment

Cell type	Primary metabolic pathway(s)	Key metabolites	Functional consequences	Ref.
LSCs	Oxidative Phosphorylation (OXPHOS), Fatty Acid Oxidation (FAO)	Lipids, Lactate	Quiescence, therapy resistance, self-renewal	[33, 63, 87]
Differentiated blasts	Glycolysis	Glucose, Lactate	Rapid proliferation, biomass production	[63, 87]
Effector T cells	Glycolysis, OXPHOS	Glucose, Glutamine	Cytokine production, cytotoxic function	[63, 87]
MDSCs	FAO, Amino acid metabolism	Arginine, ROS	T-cell suppression, immunosuppression	[63, 71]
BM adipocytes	Lipolysis	Free Fatty Acids (FFAs)	Provision of fuels for LSC OXPHOS and FAO	[63, 87]

levels (e.g., SCENITH/Met-Flow) can (i) identify patients whose AML is OXPHOS- or FAO-addicted and therefore plausible candidates for co-targeting strategies, (ii) reveal microenvironmental metabolic mechanisms (nutrient competition, adenosine production, lipid transfer) that can be targeted to restore immune function, and (iii) provide ex-vivo readouts to nominate rational drug combinations for a given patient. Finally, early clinical translation is underway for OXPHOS inhibitors and CD36/FAO pathway blockers, suggesting realistic near-term trial opportunities for metabolism-stratified therapy [52, 90].

The dynamic biomarker: tracking evolution and resistance

Clonal dynamics in response to therapy

Longitudinal single-cell DNA (scDNA-seq) studies have been particularly informative for clinically targeted therapies, directly tracing which genetic clones expand or contract during FLT3- or IDH-directed therapy. scDNA profiling of patients treated with FLT3 inhibitors has revealed selection for subclones with RAS-pathway activation and secondary FLT3-TKD or other kinase alterations at relapse, demonstrating that resistance often arises via clonal selection rather than a single uniform mechanism [91]. Similarly, integrated single-cell proteogenomic and scDNA analyses in IDH-mutant AML have shown that pre-existing stem-like programs and co-occurring mutations (for example RUNX1/CEBPA or RAS-RTK pathway variants) can mediate both primary and acquired resistance to IDH inhibitors, and scDNA approaches have enabled precise mapping of these events to specific immunophenotypic clones [92, 93]. Together, these scDNA-based studies provide direct evidence that (i) clinically actionable resistance can be clone-specific, (ii) resistance mechanisms frequently involve expansion of minor pre-existing subclones or selection for cooperating pathway mutations, and (iii) single-cell genotyping can therefore inform rational combination strategies to prevent or overcome resistance [91, 94].

In addition to scDNA-based genotyping, endogenous mitochondrial single-nucleotide variants (mtSNVs) have recently been developed as a powerful, complementary approach for clonal tracing in single-cell datasets. Methods such as those used by Velten et al. (MutaSeq) and targeted mitochondrial transcriptome enrichment approaches (e.g., MAESTER) enable recovery of mtDNA-derived clonal markers from standard single-cell RNA libraries or with modest targeted enrichment, allowing lineage assignment without exogenous barcoding. These approaches are attractive because they are compatible with widely used droplet- and plate-based scRNA-seq workflows, provide endogenous lineage barcodes that can be applied retrospectively to archived data, and can be combined with transcriptomic state information to link clone identity to cell phenotype. However, important limitations persist: mtDNA coverage is frequently uneven in standard scRNA-seq libraries, heteroplasmy and sequencing errors can confound variant calling, and mtSNVs alone do not always resolve recent nuclear mutation phasing; dedicated enrichment protocols and robust computational callers (e.g., maegatk/M A E S T E R and MQuad / CloneTracer pipelines) mitigate some issues but require careful validation in primary human samples. Importantly, applications in hematopoietic and leukemic samples have shown good concordance with nuclear genotypes and immune clonotypes while revealing lineage biases and clonal expansions, but sensitivity and scalability remain dependent on library chemistry and enrichment strategy [16, 17].

Single-cell multiomics approaches have further elucidated the complex patterns of clonal evolution in AML with complex karyotypes, revealing distinct evolutionary trajectories including monoclonal, linear, and branched polyclonal growth patterns [95]. These investigations demonstrate that approximately 75% of complex karyotype AML cases harbor multiple subclones that frequently display ongoing karyotype remodeling through mechanisms such as breakage, fusion–bridge cycles and chromothripsis. The functional significance of this clonal diversity is profound, as patient-derived xenograft models demonstrate varied clonal evolution of LSCs and subclone-specific drug-response profiles, enabling identification of targeted therapies including BCL-xL inhibition [95].

The technological advances in single-cell genotyping have enabled unprecedented resolution in tracking clonal dynamics. Integrated scDNA-seq and immunophenotyping approaches show high sensitivity of approximately 0.01%, enabling deconvolution of clonal architecture and providing clone-specific immunophenotypic data [96]. For example, single-cell mutational profiling of hundreds of samples (Miles et al., Nature 2020) showed that AML is frequently dominated by a small number of clones with recurrent co-occurrence of epigenetic regulator mutations and recurrence of signaling mutations in parallel subclones, highlighting how single-cell genotyping resolves clinically important clonal patterns [1]. This granular view of clonal evolution has revealed that rapid AML drug resistance is primarily driven by epigenomic regulation with minimal contribution from genetic mutations, challenging conventional mutation-centric models of resistance evolution [97]. A comprehensive overview of LSC heterogeneity, the evolutionary patterns of resistance, and the trajectories of clonal evolution, as revealed by single-cell multi-omics, is presented in Fig. 3.

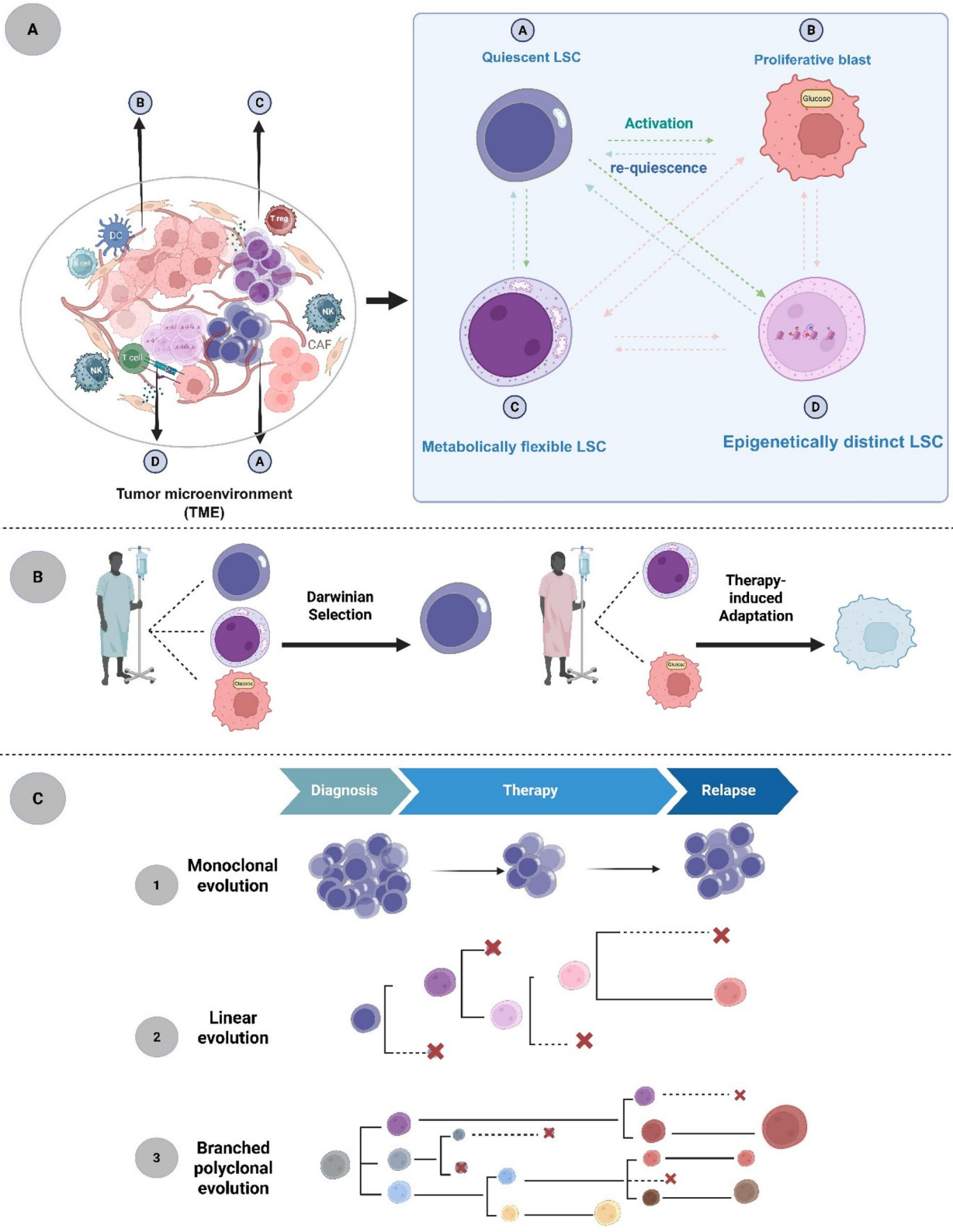

Fig. 3 (See legend on next page.)

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Fig. 3 **A** Single-cell analyses have revealed that LSCs exist in diverse metabolic, transcriptional, and epigenetic states, ranging from quiescent stem cells to proliferative glycolytic blasts, metabolically flexible subsets, and epigenetically distinct populations. These heterogeneous states are not fixed but interconnected through dynamic plasticity, enabling differentiated blasts to reacquire stemness properties. **B** treatment results two predominant evolutionary patterns: Darwinian selection, where pre-existing minor subclones harboring resistant traits expand under therapeutic pressure, and therapy-induced adaptive evolution, where leukemic cells undergo transcriptional and epigenetic reprogramming in response to treatment, generating new resistant states. **C** Single-cell multiomics analyses demonstrate that leukemic clones can evolve through three predominant trajectories, 1: monoclonal evolution, characterized by persistence of a dominant clone over time, 2: linear evolution, where sequential acquisition of alterations drives stepwise clonal replacement, and 3: branched polyclonal evolution, in which multiple subclones emerge and coexist through divergent evolutionary paths. A more refined classification has been incorporated in part C based on recent insights into LSC evolutionary patterns. Abbreviation: AMPK (AMP-activated protein kinase), CAF (Cancer-associated fibroblast), DC (Dendritic cell), DNMT3A (DNA methyltransferase 3 alpha), EZH2 (Enhancer of zeste homolog 2), HIF-1α (Hypoxia-inducible factor 1 alpha), Hedgehog (Hedgehog signaling pathway), IDH1/2 (Isocitrate dehydrogenase 1 and 2), LSC (Leukemic stem cell), NK cell (Natural killer cell), Notch (Notch signaling pathway), OXPHOS (Oxidative phosphorylation), PGC-1α (Peroxisome proliferator-activated receptor gamma coactivator 1 alpha), PDK1 (Pyruvate dehydrogenase kinase 1), SIRT1 (Sirtuin 1), TET2 (Ten-eleven translocation 2), Wnt/β-catenin (Wnt signaling pathway / β-catenin) (This figure is created in https://BioRender.com)

Mechanisms of non-genetic resistance

The emergence of non-genetic resistance mechanisms represents a formidable challenge in AML therapeutics, enabling leukemic cells to evade targeted therapies through transcriptional and epigenetic reprogramming rather than genetic mutation. Non-genetic resistance manifests in various forms, including drug persistence, unstable non-genetic resistance, and stable non-genetic resistance, each with distinct clinical implications [98]. This adaptive plasticity is rooted in two fundamental variables: epigenetic heterogeneity, which refers to variability in epigenetic states across cell populations, and epigenetic plasticity, which denotes the capacity of cells to alter their epigenetic state with a degree of heritability in response to internal or external stimuli [98].

scRNA-seq studies of AML cells subjected to chemotherapeutic agents have revealed substantial cellular heterogeneity and dynamic transcriptomic trajectories, uncovering a tendency for reprogramming toward a more stem-like state. This reprogramming is characterized by alterations in DNA methylation, chromatin architecture, and transcription factor activity rather than exonic mutations [97]. Research indicates that epigenetic alterations can drive resistance to targeted therapeutic agents through multiple mechanisms, including alterations of the transcriptome, transcription factors, DNA, and chromatin regulatory proteins. For instance, in epidermal growth factor receptor (EGFR)-mutant lung adenocarcinoma cells resistant to gefitinib, comprehensive transcriptomic analysis identified 865 mRNAs with altered expression, highlighting the profound transcriptional reprogramming associated with non-genetic resistance [99].

The role of transcription factors in mediating non-genetic resistance is particularly significant. Studies in acute lymphoblastic leukemia have identified DNA sequence recognition motifs for RUNX, ETS, and E-protein families in more accessible chromatin regions of drug-sensitive cells, with reduced binding of transcription factors RUNX2, ETV5, and TCF4 in resistant cells [99]. Similarly, in breast cancer, activating transcription factor 2 (ATF2) has been implicated in endocrine therapy resistance through the emergence of an alternative hormone-independent mechanism characterized by decreased estrogen receptor 1 (ESR1) levels and reduced ER-regulatory gene enrichment [99]. These findings underscore how transcriptional and epigenetic reprogramming enables cancer cells to adapt and resist various treatment modalities through lineage plasticity and cellular identity alteration [100].

The MRD paradigm shift: towards single-cell detection

MRD assessment is shifting from bulk assays to integrated scMRD that link genotype and phenotype in single residual cells, improving sensitivity and enabling clone-specific therapeutic decisions, although scMRD is currently resource-intensive and largely a research tool [96, 101–105]. These limitations are particularly problematic in AML, where approximately 50% of patients who achieve complete morphological remission eventually relapse due to persistent leukemic clones below conventional detection thresholds [101–104].

Next-generation sequencing-based MRD detection represents a significant advancement, offering unprecedented sensitivity (detecting VAFs ≤ 0.01%) and the ability to monitor multiple mutations simultaneously, including challenging targets like FLT3-ITDs [101–104]. Studies demonstrate that NGS-based MRD assessment provides critical prognostic information, with patients exhibiting a mean VAF of somatic mutations (excluding clonal hematopoiesis of indeterminate potential) ≤ 0.004 at consolidation therapy initiation and ≤ 0.020 during MRD monitoring having significantly better outcomes. The combination of multiparameter flow cytometry and NGS-based MRD assessment further refines prognostic stratification, with patients negative by both methods showing longer survival than those positive by either method alone [106].

The emergence of scMRD technologies represents a further evolution in residual disease monitoring, combining flow cytometric enrichment of targeted precursor/blast populations with integrated scDNA-seq and

immunophenotyping [96], notably, integrating single-cell metabolic/functional profiling (for example SCENITH or Met-Flow) with scMRD adds orthogonal information: residual clones can be characterized not only by genotype/phenotype but also by metabolic dependencies that may predict imminent outgrowth and inform targeted maintenance strategies. This approach provides not only high sensitivity (approximately 0.01%) but also deconvolutes clonal architecture and provides clone-specific immunophenotypic data, effectively offering a direct readout of the resistant clone [96]. The translational significance of this approach is profound, as it enables identification of minor, co-occurring, or emerging clones that would be missed by bulk methods, providing a fuller picture of clonal evolution that informs personalized disease management [101–104].

The clinical implications of this MRD paradigm shift are substantial, with regulatory agencies including the FDA and EMA increasingly recognizing MRD as an exploratory or supportive endpoint in AML trials [101–104]. The ability to track clonal dynamics in near real-time using scMRD approaches provides unprecedented opportunities for guiding therapeutic decisions, identifying patients who might benefit from maintenance regimens, allogeneic transplantation, or molecularly targeted agents. Importantly, because allogeneic HSCT remains the only curative option for many patients, single-cell studies that profile post-transplant immune reconstitution, clonal immune escape (including HLA loss), and early MRD have direct clinical relevance, they identify candidate biomarkers that predict relapse or GvHD and suggest avenues for pre-emptive, immunologically informed interventions [7, 107–109]. Furthermore, the integration of functional drug sensitivity testing with scMRD assessment offers a comprehensive framework for identifying actionable vulnerabilities and predicting treatment response, ultimately enabling more effective targeting of the resistant clones responsible for disease relapse [7]. The technical characteristics, advantages, and limitations of current and emerging MRD detection methodologies are compared in Table 4.

Translating single-cell insights to the clinic: challenges and opportunities

The integration of single-cell technologies into clinical practice holds substantial promise for refining AML diagnosis, monitoring and therapeutic selection by providing higher-resolution views of clonal architecture, cell-state heterogeneity and microenvironmental interactions. However, this promise is incremental and context-dependent: most single-cell methods remain primarily discovery or correlative tools, and their routine clinical utility requires demonstration of analytic robustness, reproducible clinical validity and, ultimately, clinical benefit [110]. However, translating these insights into routine care requires overcoming significant technical, analytical, and practical hurdles while seizing opportunities for personalized intervention.

Diagnostic and prognostic applications

scRNA-seq has enabled the construction of a high-resolution reference atlas of normal hematopoiesis, which serves as an essential benchmark for identifying aberrant differentiation trajectories in AML [4, 21, 111]. Large single-cell atlases (for example, recent studies that collectively profile on the order of a million cells across hundreds of samples) have identified a limited set of recurrent aberrant differentiation programs, including lymphoid-like and erythroid-like transcriptional patterns, that can add prognostic information beyond genotype in selected cohorts. The precise number of samples/cells and the clinical generalizability of these patterns vary by study and require independent validation in prospectively collected cohorts [111]. This has facilitated the development of novel biomarkers that quantify intra-tumoral heterogeneity, such as a Shannon Diversity Index for clonal complexity, which correlates with adverse outcomes and chemoresistance [4, 44]. Importantly, these biomarkers can refine existing risk models by incorporating MRD detection at single-cell resolution, revealing phenotypically distinct resistant subclones that are otherwise obscured in bulk assays [44, 112]. Early pilot programs and translational efforts have incorporated scRNA-seq into multidisciplinary tumor boards

Table 4 Evolution of MRD detection technologies in AML

Technology	Sensitivity	Key advantages	Key limitations	Ref.
Multiparameter flow cytometry (MFC)	$10^{-3} - 10^{-4}$	Rapid turnaround, provides phenotypic data on live cells.	Lack of standardization, subjective gating, cannot track specific genetic mutations.	[101, 104]
qPCR (Mutation-specific)	$10^{-4} - 10^{-6}$	High sensitivity and specificity for tracked mutations; standardized.	Only tracks 1–2 mutations per test; not applicable for ~ 40–50% of AML patients without suitable markers.	[103, 104]
Next-generation sequencing (NGS)	$\leq 10^{-4}$	Unbiased, can track multiple mutations simultaneously (including FLT3-ITD); provides clonal information.	May detect clonal hematopoiesis (CHIP) variants not derived from the leukemic clone; longer turnaround time.	[101, 103, 106]
Single-cell MRD (scMRD)	~ 10^{-4} (0.01%)	Gold standard future direction: Links genetic mutations to specific immunophenotypes at the single-cell level.	Currently expensive, low-throughput, and complex; primarily a research tool moving towards clinical application.	[96]

to provide additional correlative insights in challenging cases (e.g., ambiguous genotype or mixed phenotype disease). These are promising proof-of-concept efforts, but published evidence that scRNA-guided decisions improve patient outcomes remains limited and should be framed as preliminary [4, 113, 114].

Novel avenues for therapeutic targeting

Therapeutically, single-cell technologies are unlocking three key strategies for targeting AML diversity. First, neoantigen discovery is being advanced through integrated scRNA-seq and scTCR-seq analyses, which identify clonally restricted immunogenic peptides presented on major histocompatibility complex molecules in specific subclones [10, 115]. For instance, multi-omic profiling of t(8;21) AML uncovered cytotoxic T cell subsets with expanded clonality and high expression of cytotoxic molecules, suggesting exploitable immune vulnerabilities [10]. Second, surface markers enriched on LSCs or specific subclones provide targets for antibody-drug conjugates (ADCs) or cellular therapies [116]. CD33 and CD123 are among the most clinically developed surface targets (e.g. gemtuzumab ozogamicin for CD33), but single-cell data reveal marked heterogeneity in expression between patients and among subclones within individual patients. This heterogeneity argues for careful multimodal validation (RNA + protein + spatial data) of candidate targets and supports strategy options such as combinatorial targeting, patient selection, or focusing on more uniformly expressed antigens (e.g., CLEC12A/CLL-1) under clinical evaluation [117–119]. Third, scRNA-seq facilitates the design of rational combination therapies that co-target co-existing vulnerable pathways in different subclones [120]. For example, the simultaneous inhibition of MCL-1 and SRC kinases was shown to overcome paradoxical MCL-1 upregulation and induce synergistic apoptosis in preclinical models, highlighting the potential of "clone-combo" therapy [121–123]. Similarly, integrating BCL-2 inhibition with hypomethylating agents has proven effective in eradicating specific subclones, although scRNA-seq of relapsed patients has uncovered transcriptional adaptations driving resistance [4, 44, 124]. Importantly, single-cell metabolic readouts provide actionable stratification for these combination strategies. For example, identification of OXPHOS-dependent LSC compartments or CD36-high subpopulations can (a) nominate combinations that pair venetoclax with agents that blunt mitochondrial respiration or FAO, (b) prioritize enrollment into early-phase trials of OXPHOS inhibitors (e.g., complex-I inhibitors) or emerging CD36/FAO inhibitors, and (c) guide adaptive trial designs that use metabolic MRD (functional readouts or single-cell metabolic signatures) as enrichment or early-efficacy biomarkers. Embedding single-cell metabolic assays (SCENITH or Met-Flow) into correlative programs will accelerate identification of patients likely to benefit and streamline biomarker qualification for subsequent interventional testing [52, 90, 125, 126].

Expanded multimodal approaches and complementary technologies

While scRNA-seq remains a powerful discovery tool for transcriptional states and neoantigen discovery, a growing number of multimodal single-cell methods directly link transcriptomes to protein expression, genotype, and epigenetic state, information that is often essential when proposing therapeutic targets. Cellular Indexing of Transcriptomes and Epitopes by sequencing (CITE-seq) and its expanded variants (e.g., ECCITE-seq) enable simultaneous measurement of whole-transcriptome RNA and dozens to hundreds of surface protein markers by using oligonucleotide-conjugated antibodies, thereby improving the accuracy of cell-type annotation and enabling protein-level validation of candidate surface targets nominated by RNA alone [127, 128].

Integrating genotype and proteotype

Methods that combine single-cell genotyping with transcriptome or protein readouts (for example G&T-seq, TARGET-seq and targeted scDNA platforms) let investigators assign driver mutations to exact transcriptional and surface-protein states in the same cell. TARGET-seq and G&T-seq permit parallel mutation detection and RNA profiling from single cells and have been used to map how specific mutations map to cell states; targeted high-throughput scDNA platforms (e.g., Mission Bio Tapestri) further enable joint DNA + protein panels for large numbers of cells, permitting high-sensitivity tracking of clone-specific surface markers and drug-resistant genotypes. These genotype→phenotype linkages are particularly important for rational therapeutic design (for example to prioritize ADC or CAR-T targets that are truly expressed on the malignant clone) [129–131].

Proteogenomics, single-cell proteomics and spatial multimodality

Complementary single-cell proteomics (mass-spec-based single-cell proteomics and imaging mass cytometry/CyTOF) and spatial proteogenomic workflows provide orthogonal evidence of translated protein expression, post-translational modifications and in-situ cell neighbourhoods that are required when advancing a cell-surface target toward a therapeutic (e.g., ADC or CAR-T) or when validating ligand–receptor hypotheses derived from transcript data. Single-cell proteogenomics and modern spatial methods therefore convert scRNA-driven hypotheses into clinically actionable targets with higher confidence. Readers should view scRNA-based target

nomination as the beginning of a multimodal validation pipeline, scRNA → CITE-seq/CyTOF → scDNA/proteogenomics → spatial protein validation → functional perturbation (blockade, CRISPR), before proposing interventional clinical testing [129, 132].

The scRNA-seq workflow and its pivotal role in identifying novel therapeutic avenues are illustrated in Fig. 4.

Overcoming technical and analytical hurdles

Despite this promise, widespread clinical adoption faces formidable challenges. Pre-analytic variables (collection tube, time-to-processing, cryopreservation and decalcification) measurably alter cell recovery and RNA quality; therefore, studies should report these parameters and follow harmonized SOPs to enable valid cross-site

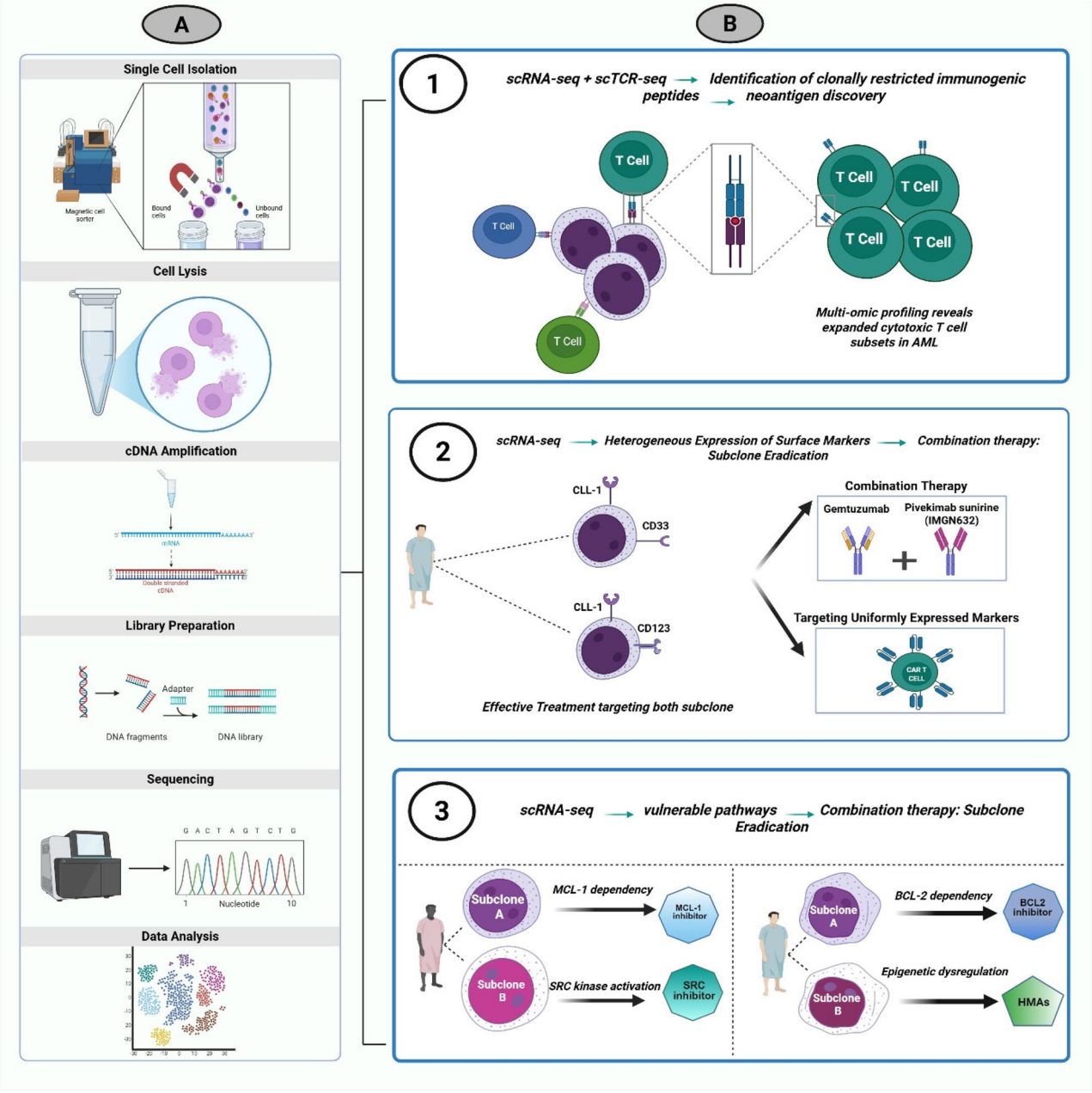

Fig. 4 **A** schematic of the procedures of scRNA-seq including: single cell isolation, cell lysis, cDNA amplification, Library preparation, sequencing, and data analysis. **B** scRNA-seq provides Novel avenues for therapeutic targeting. 1: scRNA-seq identifies neoantigen, providing novel treatment development. Accurate neoantigen identification requires paired scRNA-seq and scTCR-seq to link antigen presentation with T-cell receptor clonality. 2: scRNA-seq has revealed heterogeneous expression in different subclones, therefore combination targeting or more uniformly expressed markers (CLL-1-CAR-T therapies) could eradicate different subclones. 3: scRNA-seq provides information on vulnerable pathways in different clones in AML patients, therefore clever selection of combination therapy could target these subclones. (This figure is created in https://BioRender.com)

comparisons and pooled analyses [133]. Moreover, the computational analysis of single-cell data necessitates robust pipelines for batch effect correction, dimensional reduction, and clustering, which are often confounded by technical noise and the rarity of biologically relevant cell populations [4, 10, 134]. The integration of multi-omic data (e.g., scRNA-seq with scATAC-seq) further compounds analytical complexity, requiring advanced machine learning approaches to link transcriptional states to epigenetic drivers [10]. Data interpretation is another major hurdle, as distinguishing driver mutations from passenger events and differentiating malignant from non-malignant cells in a heterogeneous mixture demands sophisticated bioinformatic tools and validated reference databases [111]. Finally, cost and throughput limitations must be addressed to enable rapid turnaround times compatible with clinical decision-making. Current scRNA-seq workflows remain expensive and low-throughput compared to bulk sequencing or flow cytometry, though technological advances are steadily improving scalability [4, 135]. Finally, cost and throughput limitations must be addressed for clinical compatibility. A pragmatic translational path involves reducing high-dimensional discoveries to robust marker panels compatible with mature clinical platforms (e.g., flow cytometry, qPCR). This requires bridging studies to demonstrate concordance between the discovery signal and the simplified assay [136].

Envisioning a future clinical workflow

A plausible future clinical workflow would integrate rapid genetic and phenotypic readouts, for example targeted single-cell genotyping and protein panels with fast turnaround, rather than depend solely on current whole-transcriptome scRNA-seq. Such an integrated pipeline could help identify dominant clones and immune-escape features to inform treatment selection in some settings. However, practical implementation must address sample logistics (fresh vs. cryopreserved marrow), assay validation, regulatory pathways, cost/reimbursement and, crucially, demonstration that use of these data improves patient outcomes compared with current standards [110].

From discovery to clinic: practical steps to validate single-cell biomarkers

Translating a single-cell discovery into a clinically actionable biomarker requires a staged evidentiary pathway, progressing from unbiased discovery and rigorous analytical validation to demonstration of clinical utility and, ultimately, regulatory qualification [136, 137]. This structured approach is necessary to bridge the gap between high-dimensional discovery tools and robust, clinically deployable diagnostic assays.

Are single-cell omics being used in clinical trials, correlative vs. interventional?

Single-cell omics are increasingly incorporated into clinical trials, most commonly as correlative exploratory endpoints to characterize treatment effects, MRD dynamics or immune responses. A growing number of oncology trials use scRNA-seq, scDNA-seq or combined approaches to profile baseline heterogeneity and longitudinal response; some groups have also embedded single-cell functional assays (ex vivo drug sensitivity) to prioritize therapies for individual patients. Recent translational studies demonstrate that patient-tailored scRNA-based profiling can inform individualized drug selection and that scRNA-derived predictions can be experimentally validated ex vivo, illustrating a path toward decision-enabling clinical applications [138–140].

To date, the majority of single-cell applications in clinical trials remain correlative (i.e., used to explain mechanisms of response/resistance or to discover biomarkers), whereas interventional trials that use single-cell readouts as primary decision rules are still uncommon but emerging in pilot studies. When used as decision tools, single-cell assays must meet strict analytical validation and be integrated into the trial design (pre-specified endpoints, adaptive decision rules, and clear regulatory oversight). Active trialists should consider staged designs where single-cell assays are first used to stratify or enrich cohorts, and only after strong prospective evidence, to serve as triggers for treatment assignment [138, 141].

These distinctions (correlative vs. interventional) highlight why an explicit translational pathway (see "From discovery to clinic: practical steps to validate single-cell biomarkers" section) is essential prior to routine clinical use.

Single-cell insights relevant to allogeneic hematopoietic stem cell transplantation (allo-HSCT)

Allogeneic HSCT remains the principal curative option for eligible patients with AML, but relapse and immune complications (notably graft-versus-host disease, GvHD) limit its curative potential. Single-cell studies over the past several years have begun to illuminate both *why* transplant fails in some patients and how the procedure might be optimized. Key themes that emerge from these studies include: (i) clonal immune escape and HLA loss in relapsing leukemias, (ii) specific T- and NK-cell reconstitution signatures that associate with relapse versus durable graft-versus-leukemia (GvL), and (iii) cell states that presage acute GvHD or impaired immune reconstitution.

Several recent single-cell investigations demonstrate these points directly. Mathioudaki et al. performed scRNA-seq of BM T cells and $CD34^+$ cells from AML patients after allo-HCT and identified distinct T-cell transcriptional signatures that distinguish patients in

durable remission from those who relapsed after transplant; these signatures implicate defective GvL immune states in relapse [107]. Obermayer and colleagues applied single-cell TCR and transcriptome profiling to track persistent donor-derived T cell clones after allo-HSCT and showed that expanded, persistent T cell clones can be associated with both beneficial (GvL) and deleterious (GvHD) outcomes depending on phenotype and tissue localization [108].

Mechanistic work has also exposed genetic routes to immune escape after transplant: studies combining deep sequencing and single-cell approaches document selective loss or downregulation of HLA alleles and antigen-presentation machinery in relapsed leukemic clones, a direct tumor strategy to evade donor immunity. Pagliuca et al. and related immunogenetic analyses provide an integrated picture of how reduced HLA diversity or acquired HLA defects facilitate post-transplant relapse [109, 142].

Single-cell profiling has been applied to the study of immune reconstitution and GvHD as well: Mathews et al. used scRNA-seq to identify NK and other innate immune cell phenotypes after allograft that correlate with protection from acute GvHD, while other studies have reported atypical monocyte or MAIT-cell states that precede clinical onset of GvHD, offering candidate cellular biomarkers for early intervention [143, 144].

Clinically, these single-cell findings suggest several actionable directions to optimize allo-HSCT: (1) integrate single-cell immune profiling into early post-transplant monitoring to detect loss of GvL signatures or emergence of immune-escape clones; (2) use single-cell MRD and clonal-tracking (including mtSNV or scDNA genotyping) to detect early leukemic clones that survive conditioning and might be targeted pre-emptively; and (3) design immunomodulatory strategies (donor lymphocyte infusions, checkpoint blockade, cellular therapies) informed by the phenotype and clonality of the reconstituting donor T/NK repertoire. Early translational and preclinical results already support these approaches and argue for embedding single-cell correlative assays in transplant trials [107, 108, 142].

Technological readiness and platform comparison for clinical translation

Single-cell platforms differ in throughput, sensitivity, dimensionality and clinical maturity; platform choice should therefore be guided by the intended clinical use (exploratory discovery vs. diagnostic assay). High-throughput droplet 3′ end methods (commercially exemplified by Chromium-style workflows) provide large cell numbers at lower per-cell cost and are optimal for population-scale discovery, but they provide limited transcript coverage per cell and can show inter-laboratory variability that must be controlled by standardized SOPs and benchmarking. In contrast, plate-based full-length approaches (e.g., SMART-seq family) give higher gene detection per cell and superior isoform resolution, advantages for targeted mechanistic studies, but incur higher cost and lower throughput, which limit routine clinical scalability. These tradeoffs have been repeatedly documented in head-to-head benchmarking studies [133].

For single-cell DNA genotyping, targeted microfluidic platforms (e.g., Mission Bio Tapestri) offer sensitive, locus-focused detection and enable genotype→phenotype linkage when combined with protein panels, but they remain primarily research/precision-medicine tools because of cost, locus coverage limits and workflow complexity; independent analytical validation is required before routine diagnostic use [145]. Spatial transcriptomics adds crucial tissue context but faces practical constraints in mineralized tissues (including trephine BM biopsies): decalcification and other pre-analytic steps reduce RNA quality and gene detection per spot, which degrades single-cell resolution unless optimized protocols and orthogonal validation (smFISH, multiplexed protein imaging) are applied. Investigators should report pre-analytic methods and deconvolution metrics when presenting BM spatial data [146, 147]. A concise, side-by-side comparison of platform readiness, cost, typical turnaround and trial-feasibility is provided in Table 5, which readers may use to match discovery platforms with downstream clinical assay strategies.

Conclusion and future perspectives

Synthesis of key findings

Single-cell technologies have fundamentally transformed our understanding of AML, revealing an intricate molecular geography characterized by remarkable cellular heterogeneity and ecosystem dynamics [4, 5]. This review has synthesized how scRNA-seq and associated multi-omic platforms have decoded the complex architecture of AML, moving beyond bulk analyses to uncover the diverse cellular subpopulations, including LSCs, that drive disease pathogenesis, therapy resistance, and relapse [5]. The emergence of high-resolution single-cell biomarker profiling has enabled unprecedented mapping of clonal architecture and evolutionary trajectories, providing critical insights into the molecular mechanisms underlying AML progression and treatment failure [4, 5]. These technological advances have facilitated the identification of novel biomarkers with potential prognostic and therapeutic significance, ultimately refining risk stratification and guiding more precise therapeutic interventions [4]. The archipelagic model of AML emphasizes the interconnected yet distinct cellular communities that constitute the disease ecosystem, offering a novel

Table 5 Pragmatic comparison of single-cell platforms for clinical translation (qualitative categories)

Platform (typical)	Relative cost per sample	Standardization level (inter-lab)	Turnaround time (typical)	Feasibility in clinical trials (correlative / interventional)	Notes
Droplet-based scRNA-seq (high-throughput commercial)	Moderate	Moderate → improving	1–3 weeks (lab + analysis)	High for correlative; low for immediate interventional use	Best for discovery/screening; needs bridging for diagnostic use [148].
Plate-based / SMART-seq (full-length)	High	Low–Moderate	2–4 weeks	Correlative; rarely interventional	High sensitivity per cell, costly for large cohorts [148].
scDNA-seq	High	Low	2–4 weeks	Correlative; targeted uses in trials	Excellent for clonal architecture; variable coverage and complexity [149].
CITE-seq (RNA + protein tags)	High	Low–Moderate	2–3 weeks	Correlative; emerging interventional pilots	Enables direct RNA/protein links; bridging assays needed for clinics [150].
CyTOF (mass cytometry)	Moderate–High	Moderate	1–2 weeks	High for correlative; potential translational workflows	Protein readouts closer to clinical flow cytometry; infrastructure needed [150].
Targeted clinical assays (flow cytometry, qPCR, ddPCR, IHC), validation stage	Low–Moderate	High	< 48–72 h	High (routine clinical use)	These are the practical endpoints for clinical deployment after discovery [136].

framework for understanding its spatial and functional organization.

Clinical implications and translational potential

The clinical translation of single-cell biomarkers is rapidly progressing toward improving patient outcomes through enhanced diagnosis, monitoring, and therapeutic targeting [4, 5]. ScRNA-seq has demonstrated significant utility in MRD detection, enabling more sensitive monitoring of treatment response and early identification of relapse-prone patients beyond the limitations of conventional methods [5]. The technology's capacity to characterize immunosuppressive niches and cellular crosstalk mechanisms has unveiled promising immunotherapeutic targets, including those applicable to CAR T-cell therapy and immune checkpoint inhibition [4]. Furthermore, single-cell functional assays have revealed metabolic dependencies and drug resistance mechanisms, providing a rationale for novel targeted therapies and combination strategies. The integration of single-cell data into clinical decision-making promises to advance personalized medicine approaches in AML, potentially guiding patient-specific therapeutic selections based on individual disease ecology [4, 5].

Technological challenges and future directions

While single-cell approaches are transformational, several specific and currently limiting gaps must be addressed before routine clinical translation. First, pre-analytical heterogeneity (sample collection, anticoagulant, time-to-processing, cryopreservation, and decalcification of trephines) materially alters cell recovery and RNA quality and remains widely unharmonized across centers; standardized SOPs and reporting checklists are therefore required. Second, platform and batch variability (library chemistry, instrument, and bioinformatic pipelines) continues to produce inter-laboratory differences that confound clinical interpretation; community benchmarking and reference material programs are needed to quantify and reduce this variability. Third, data reporting and metadata standards for single-cell experiments are incomplete, adoption of minimum-information checklists (e.g., minSCe / MINSEQE-like standards) and FAIR data practices is essential for reproducibility. Fourth, sensitivity and limit-of-detection issues (critical for scMRD) need analytically validated workflows with CLIA-style performance metrics (LOD, linearity, precision, specificity) and bridging studies to clinically mature assays (flow, ddPCR). Fifth, regulatory and evidentiary gaps remain: biomarker qualification pathways (FDA/EMA) require pre-specified intended uses and prospective clinical evidence. Finally, cost, turnaround and workforce constraints limit real-world use; pragmatic solutions include centralized CLIA labs for clinical-grade testing and development of reduced, validated marker panels for rapid decision-making. Addressing these discrete gaps requires coordinated technical, clinical and regulatory efforts [151–153].

Outstanding questions and priority research directions

To accelerate clinical translation, the field should prioritize: (1) multicenter, prospective studies that embed standardized single-cell pipelines and include clearly pre-specified clinical endpoints; (2) robust bridging/assay-reduction studies that convert discovery signatures into clinically deployable assays; (3) harmonized pre-analytical SOPs and inter-laboratory proficiency testing; (4) development of regulatory and reimbursement frameworks that accommodate complex multi-omic signatures; and (5) studies that quantify cost-effectiveness and decision-impact compared to current standards. Addressing these gaps will be essential to move single-cell biomarkers from fascinating research tools to reliable clinical tests [136, 154, 155].

Concrete action plan: what needs to be done (priority items)

To translate single-cell discoveries into clinically reliable biomarkers, we recommend the following prioritized, concrete steps:

Harmonize pre-analytic SOPs and metadata reporting. Funders and consortia should establish and mandate minimum pre-analytical standards and structured metadata reporting (e.g., minSCe-style) to reduce technical variance and enable pooled analyses [152]. *Create reference materials and multicenter benchmarking.* Launch coordinated multicenter benchmarking using defined reference materials (cell-line mixes, "Quartet/MAQC"-style controls) to measure inter-lab variability and to drive standardized pipelines. Past multicenter benchmarking studies demonstrate this is feasible and informative [151, 156]. *Define analytical validation standards for scMRD and sc-assays.* For any clinical intended-use, require CLIA-style analytical validation (LOD, linearity, precision, specificity, reproducibility) and cross-platform bridging studies demonstrating equivalence (single-cell discovery → targeted clinical assay). *Mandate orthogonal and spatial validation before clinical claims.* Ligand–receptor inferences and surface-target nominations must be validated by protein-level assays (CITE-seq/CyTOF), spatial confirmation (smFISH/imaging mass cytometry) and functional perturbation (blocking or genetic perturbation) prior to therapeutic development [153]. *Engage regulators early and use formal qualification pathways.* For specimens intended as drug-development biomarkers, engage with the FDA Biomarker Qualification Program and EMA scientific advice early to define evidentiary expectations and trial designs. *Publish minimal reporting checklists and adopt FAIR data principles.* Require submission of raw data, processed matrices, and complete metadata in public repositories to enable reanalysis and independent validation [152]. *Design prospective multicenter clinical trials embedding standardized single-cell pipelines.* Trial consortia should prespecify single-cell endpoints, power for MRD/clonality outcomes, and explicit plans for assay bridging to clinically deployable tests (flow, ddPCR). This will generate the clinical utility evidence required for adoption and reimbursement [151].

Concluding remarks

In conclusion, single-cell technologies have recharted the molecular geography of AML, exposing complex clonal and microenvironmental architectures that were previously unresolvable. Realizing clinical impact requires moving beyond discovery: harmonized pre-analytic SOPs, reference materials and multicenter benchmarking, CLIA-style analytical validation for scMRD and nominated assays, prespecified trial endpoints, and early regulatory engagement are all essential. We provide a concrete, prioritized action plan ("Concrete action plan: what needs to be done (priority items)" section) to guide these efforts. If adopted, this pragmatic roadmap, discovery, analytical validation, prospective clinical validation, regulatory qualification, will accelerate responsible translation of single-cell biomarkers into clinical tests that improve decision making and patient outcomes [151, 153].

Acknowledgements

This study was supported by the "Tianshan Talents" Medical and Health High-Level Personnel Training Plan (project number: TSYC202301B022) AND The "Outstanding Talents" training plan of the First Affiliated Hospital of Xinjiang Medical University.

Authors' contributions

Hamed Soleimani Samarkhazan wrote the main manuscript text and Farzaneh Tavakoli prepared figures. Fengjie Kang, Hailong Yuan Revise the manuscript. All authors reviewed and approved the final submitted manuscript and agree to be accountable for all aspects of the work.

Funding

This research did not receive any financial support from public, commercial, or nonprofit organizations.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 6 October 2025 / Accepted: 7 February 2026
Published online: 17 February 2026

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