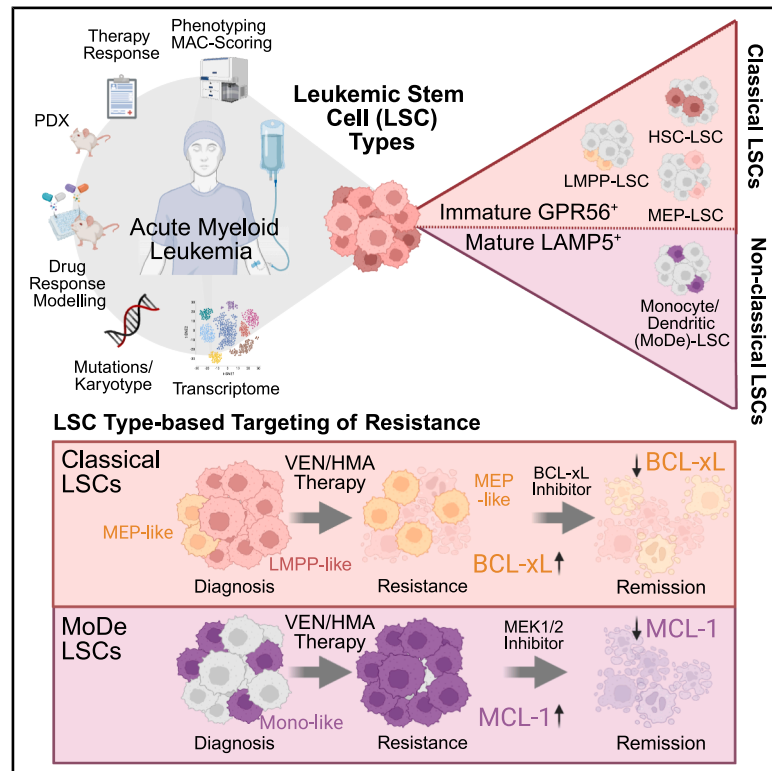


Leukemic stem cell subtypes determine venetoclax resistance and therapeutic vulnerabilities in AML

Graphical abstract



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In brief

Waclawiczek et al. describe four distinct leukemic stem cell (LSC) subtypes in acute myeloid leukemia that determine response or resistance to the BCL-2 inhibitor venetoclax. Targeting subtype-specific vulnerabilities—MEK1/2 inhibition in LAMP5⁺ monocytic/dendritic LSCs and BCL-xL inhibition in megakaryocytic/erythroid-biased LSCs—overcomes resistance and supports biomarker-guided therapeutic strategies.

Highlights

- Therapy resistance is heterogeneous and links to LSC differentiation state in AML
- Defining four LSC states provides a framework to target BCL-2 inhibitor resistance
- MEK1/2 inhibition targets MCL-1-driven resistance of LAMP5⁺ monocytic/dendritic LSCs
- BCL-xL inhibition targets plasticity into the megakaryocytic/erythroid state even in *TP53*-mut AML



Article

Leukemic stem cell subtypes determine venetoclax resistance and therapeutic vulnerabilities in AML

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SUMMARY

The BCL-2 inhibitor venetoclax has transformed the treatment of acute myeloid leukemia (AML), but relapse due to resistance of leukemic stem cells (LSCs) remains a major challenge. By molecular and functional profiling of LSCs from >150 patients, we identify four LSC subtypes. These mirror distinct hematopoietic lineage stages, which determine the expression ratio between the venetoclax target BCL-2 and resistance-inducing proteins MCL-1 and BCL-xL (MAC-score). Longitudinal analyses reveal that venetoclax resistance mostly arises in LSCs through plasticity toward a megakaryocytic/erythroid-progenitor (MEP)-LSC state that switches survival dependency from BCL-2 to BCL-xL. In rare cases, mature monocytic/dendritic (MoDe)-LSCs, found within LAMP5⁺ monocytic AMLs, drive venetoclax resistance. LSC subtyping improves genetic risk stratification and provides subtype-specific therapies: venetoclax-resistant MEP-LSCs respond to BCL-xL inhibitors, whereas MoDe-LSCs are sensitive to MEK1/2 inhibition. Our findings reveal four distinct LSC types with unique vulnerabilities and propose biomarker-guided treatment strategies that complement genetic profiling to overcome venetoclax resistance.

INTRODUCTION

Acute myeloid leukemia (AML) remains a cancer with a dismal prognosis, particularly in elderly or frail patients ineligible for allogeneic stem cell transplantation, as well as in patients with high-risk disease. Current upfront treatment regimens include

standard induction chemotherapy consisting of cytarabine and anthracyclines (e.g., daunorubicin; A/C) or the BCL-2 inhibitor venetoclax (VEN) in combination with hypomethylating agents (HMAs) (e.g., 5-azacitidine [5-AZA]). VEN/HMA also demonstrates remarkable response rates as salvage therapy in patients relapsing after A/C. Nevertheless, over 60% of unfit patients



receiving VEN/HMA succumb to AML within 24 months, with 35% of patients experiencing upfront resistance, demanding novel therapeutic approaches.^{1–5}

Over the past decades, the concept of self-renewing leukemic stem cells (LSCs) that drive a hierarchical disease has emerged. This concept argues that curative treatment of AML requires the eradication of LSCs and focused translational research on this most potent cell type.^{6–12} Multiple LSC markers differing in sensitivity, specificity, and generalizability have been proposed.^{13–15} To allow detection of LSCs across genetic subclasses, we previously reported CD64 and CD11b expression to distinguish two subpopulations in AML: “mature” CD64⁺CD11b⁺ and “immature” (*not* CD64⁺CD11b⁺) leukemic cells. Immature leukemic cells can be subdivided based on expression of LSC marker GPR56, which overlaps with CD34, but also labels LSCs in CD34⁺ AML (e.g., *NPM1c-mut.*).^{6,16} Classically, LSCs are enriched among immature GPR56⁺ leukemic cells, whereas those resembling mature myeloid cells are unable to initiate AML in immunocompromised mice.^{6,16}

Several VEN/HMA resistance mechanisms are already described. These include metabolic changes (oxidative phosphorylation,⁹ fatty acid,¹⁷ and amino acid metabolism¹⁸), shifts in anti-apoptotic dependencies (MCL-1,^{2,19} BCL-xL,²⁰ BCL-2,⁶ and BAX²¹), mutations in apoptotic regulators BAX²² or TP53,²³ changes in calcium and signal transduction pathways (RAS and *FLT3-ITD*),^{17,23} or drug efflux mechanisms.²⁴ These resistance mechanisms often neither consider the hierarchical organization of AML nor the unique disease-driving biology of LSCs.²⁵ Recently, Pei et al. demonstrated the existence of VEN-resistant non-classical LSCs with monocytic features outside the stem/progenitor spectrum.¹⁹ It remains unclear whether the “poor” outcomes of monocytic AMLs proposed by some studies are due to this population.^{2,6,19,26–28} Here, we dissect how LSC genotype and phenotype influence VEN/HMA sensitivity and how LSC subtype-specific vulnerabilities allow biomarker-based personalized precision oncology.

RESULTS

Xenotransplantation of AML samples reveals two engraftment patterns

AML is phenotypically heterogeneous at both the intra-patient and inter-patient levels. Within a single patient, AML cells are composed of various stages of differentiation with highly variable proportions across patients.^{2,6,29,30} To systematically characterize this intra-patient heterogeneity, we divided AML patient samples into three leukemic cell populations: monocytic CD64⁺CD11b⁺ blasts (mature), primitive (*not* CD64⁺CD11b⁺) blasts with LSCs (immature GPR56⁺), and primitive without LSCs (immature GPR56[−]).⁶ We used these subpopulations to classify the cellular heterogeneity between AML patients. Patients in whom mature cells were the largest population were defined as monocytic (mono) AMLs (mean percentage of mature cells: 78%, lowest 34%) (Figure 1A). Meanwhile, patients with mostly immature leukemic cells were defined as either stem (>50% GPR56⁺) or progenitor (<50% GPR56⁺) AMLs (Figure 1A). Immature GPR56⁺ cells were detected in every sam-

ple, albeit at highly variable frequencies (0.06%–92.6%, Figure 1B).

To determine whether this inter-patient heterogeneity is caused by functionally distinct LSC classes, we intra-femur transplanted bulk AML cells from 98 patients spanning all three AML types into NOD.Prkdc^{scid}.Il2rg^{null} (NSG) mice. Patient-derived xenografts (PDXs) could be derived from 59 patients with similar success rates across the three AML classes (Figures 1C and S1A).

Within these PDXs, we observed two distinct engraftment patterns: (1) a classical one in which all three AML subpopulations—mature, immature GPR56[−], and immature GPR56⁺—were re-generated in PDX (Figures 1C–1E), and (2) a non-classical one lacking the immature GPR56⁺ population in PDX (Figure 1F). The classical pattern was detected in 52/59 PDXs (88%), comprising 100% of PDXs derived from stem- or progenitor-AMLs (*N* = 43) and 56% from mono-AML patients (*N* = 16) (Figures 1D, 1E, and S1B). In contrast, 7/16 mono-AMLs (44%) and 7/59 of all AMLs (12%) showed a non-classical pattern lacking engrafted immature GPR56⁺ cells and containing almost exclusively mature leukemic cells in PDX (Figure 1F). We generated secondary PDXs by transplanting bulk AML cells from 43 classical and 3 non-classical PDXs, with 89% (41/46 primary PDX) success. Both classical and non-classical engraftment patterns observed in primary recipients were also maintained in secondary transplantation recipients (Figure S1C).

Classical GPR56⁺ and non-classical GPR56[−] LSCs define distinct engraftment patterns

We hypothesized that these two engraftment patterns corresponded to distinct LSC classes. To identify the leukemic subpopulations containing functional LSCs, we FACS-sorted and transplanted each of the three subpopulations from either stem-, progenitor-, or mono-AML samples (Figure 1G). In AMLs with classical engraftment patterns, only immature GPR56⁺ (LSCs from here on) contained transplantable LSCs (Figures 1H and 1I), supporting the standard concept that LSCs represent an immature cell population in AML.³¹ In contrast, in AMLs with non-classical engraftment patterns, only immature GPR56[−] and mature cells but not GPR56⁺ cells engrafted, suggesting the existence of “non-classical” GPR56[−] LSC subgroup¹⁹ (Figure 1J). Non-classical LSCs neither expressed CD34 nor regenerated CD34⁺ cells in xenografts (Figure S1D). Moreover, non-classical LSCs produced a more aggressive form of AML with a median engraftment time of 10 weeks compared with 28 weeks for classical LSCs (Figure S1E).

In summary, classical LSCs are immature GPR56⁺ AML cells that serially regenerate both immature subpopulations and mature blasts (Figure 1K). Non-classical LSCs are GPR56[−] mature cells and initiate a highly aggressive form of AML almost solely comprised of monocytic AML cells (Figure 1K).

LAMP5 identifies AML patients with non-classical monocytic/dendritic LSCs

To address why mature cells with non-classical LSCs are able to generate PDXs, we performed single-cell RNA sequencing (scRNA-seq) of two mono-AMLs with classical and non-classical LSCs and mapped their cells to a single-cell atlas of healthy

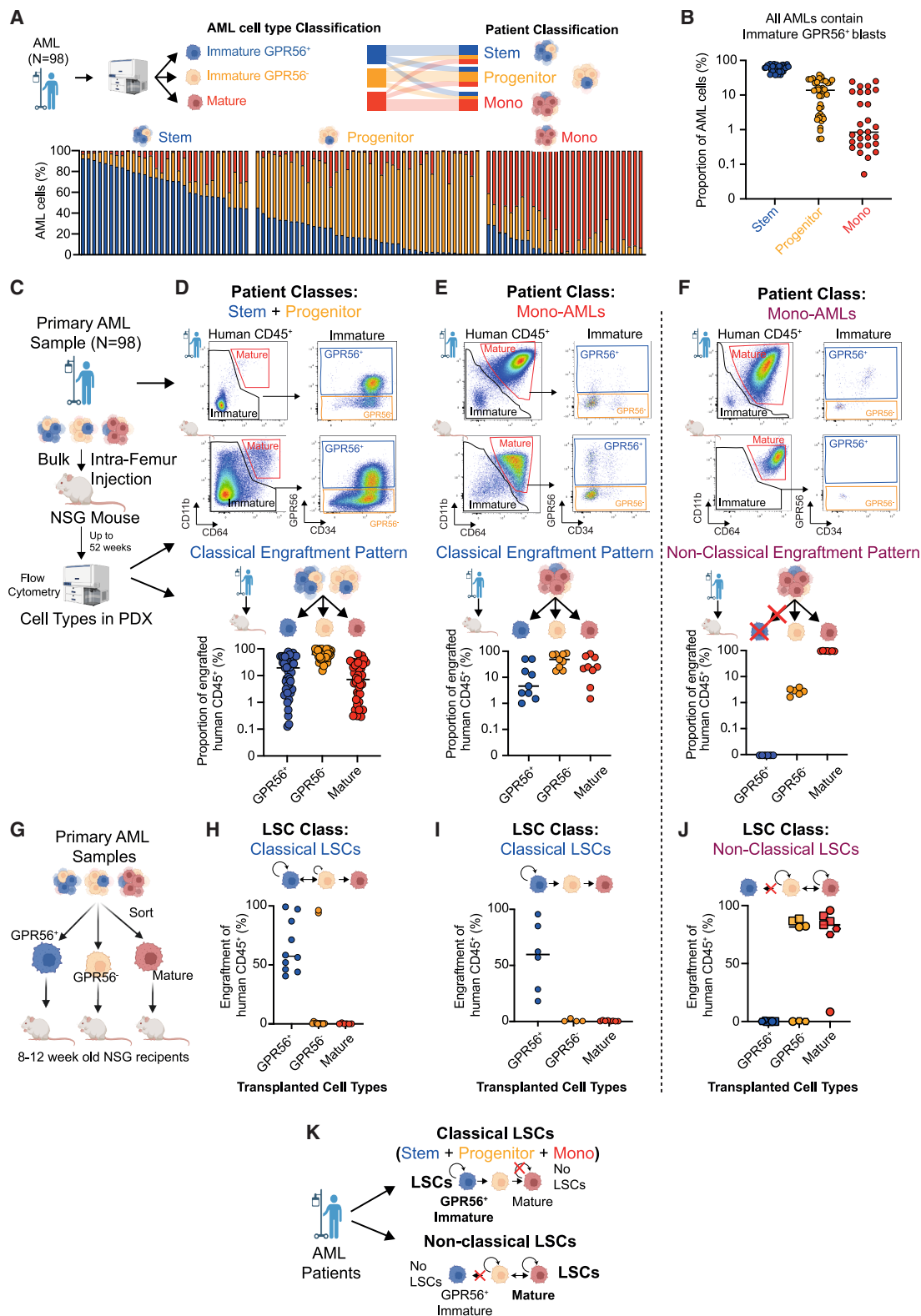


Figure 1. Description of classical and non-classical LSCs in AML

(A) Experimental outline for phenotypic analysis of AML cells of 98 AML patient samples. Patients were classified as stem- (N = 30), progenitor- (N = 40), and mono-AML (N = 28) depending on the proportion of immature GPR56⁺, immature GPR56⁻, and mature cells among total AML cells. Proportions of the cell types are shown for each patient.

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hematopoiesis.³² mature cells from classical LSC patients mostly mapped to mature (CD14⁺) or late-stage (CD16⁺) monocytic cells, whereas mature cells from patients with non-classical LSCs mapped to healthy granulocyte monocyte progenitors (GMPs) and dendritic cell progenitors (ProgDCs) (Figure 2A). This was confirmed using another hematopoiesis single-cell reference²⁹ (Figure S2A).

To further explore this difference, we performed bulk RNA-seq of sorted mature cells from 22 mono-AMLs and mapped their transcriptomes to the healthy hematopoiesis reference.³² Mature cells from classical LSC-AML were linked to CD14⁺/CD16⁺ monocytes, whereas those from non-classical LSC patients resembled GMPs and ProgDCs (Figure S2B). Similarly, cell type deconvolution using CIBERSORTx³³ confirmed an enrichment for a GMP-like state in non-classical LSCs (Figure S2C). Based on these data, we termed AMLs harboring non-classical LSCs with monocytic/dendritic features “monocytic/dendritic (MoDe) AMLs” and their LSCs “MoDe-LSCs.”

To identify biomarkers for MoDe-AML, we assessed differentially expressed genes (DEGs) of mature cells between classical LSCs and MoDe-AML patients for cell surface proteins (Figure 2B). MoDe-AML cells showed high *LAMP5* and *RET* but low *CD52* and *JAML* expression (Figure S2D; Data S1). Next, we measured the cell surface expression of these cell surface proteins in 39 AML samples by flow cytometry and compared them with their engraftment patterns (16 classical LSC-AMLs, 7 MoDe-AMLs, and 16 non-engrafters). This revealed cell surface expression of *LAMP5*⁺ as the best predictor for the presence of MoDe-LSCs (positive predictive value: 78%; negative predictive value: 0%; 7/9 *LAMP5*⁺ samples engrafted) (Figures 2B and S2E). *LAMP5* surface expression was exclusive to mature MoDe cells, and surface levels analyzed by flow cytometry classified MoDe-AMLs better than gene expression (Figures 2B, S2E, and S2F). The recently proposed marker combination CD34⁺CD4⁺CD14⁺CD36⁺CD11b⁺ for mono-LSCs¹⁹ is also associated with MoDe-LSCs but with inferior precision to *LAMP5* (Figure 2B).

In summary, rare AMLs generated by self-renewing mature MoDe-LSCs lack functional classical immature LSCs. MoDe-AML patients can be reliably and quickly identified by surface expression of *LAMP5* via flow cytometry.

LAMP5⁺ MoDe-AMLs represent a subset of VEN/HMA-resistant Mono-AMLs

Our data suggest that mono-AMLs can be driven either by classical, immature LSCs or MoDe-LSCs. The literature remains controversial whether there is an association between monocytic AMLs and increased resistance to VEN/HMA.^{6,26,27} In our cohort, neither response rates nor event-free survival

(EFS) differed among stem-, progenitor-, or mono-AML patients receiving VEN/HMA (*N* = 142) (Figure 2C). However, within mono-AML patients, stratification by LSC type revealed that 11/12 *LAMP5*⁺ but only 4/26 *LAMP5*[−] patients were refractory to VEN/HMA (Figure 2D). *LAMP5*⁺ MoDe-AML patients had a dismal median EFS compared with *LAMP5*[−] mono-AMLs (28 versus 183 days). Other monocytic markers failed to clearly differentiate EFS in response to VEN/HMA (Figures 2D and S2G). Of note, A/C-chemotherapy outcomes were similar between *LAMP5*⁺ and *LAMP5*[−]-AMLs (Figure 2E). To further dissect mechanisms of resistance to VEN/HMA, we compared the MAC-scores between classical LSCs and MoDe-LSCs (Figure 2F). MAC-score predicts the VEN/HMA response and is calculated by the ratio of BCL-2, BCL-xL, and MCL-1 protein expression in GPR56⁺-LSCs.⁶ All MoDe-AMLs showed low MAC-scores, driven by low BCL-2 and high MCL-1 levels, in line with their VEN-resistant phenotype (Figure 2F).

Genetically, MoDe-AML patients did not harbor a unique mutational or cytogenetic feature common to all 31 patients (Figure 2G). MAPK-pathway (*K/NRAS*, *BRAF*, *CBL*, *NF1*, *PTPN11*) and *FLT3* (*FLT3-ITD* and *FLT3-TKD*) mutations were slightly more frequent in MoDe-AMLs than in other monocytic patients (38% versus 26% and 34.5% versus 29%, respectively) (Figure S2H). While *RAS* mutations promote monocytic/dendritic differentiation,³⁴ *RAS*-mutant LSCs retain a primitive CD34⁺ phenotype that is not overlapping and distinct from CD64⁺CD34[−] MoDe-LSCs.^{34,35} The highest relative risk for MoDe-AML was detected in rare *t*(8;16) (5/7 patients *LAMP5*⁺), followed by trisomy 8 (16/47 patients *LAMP5*⁺) and *KMT2A*-rearranged disease (5/13 patients *LAMP5*⁺). Meanwhile, *DNMT3A* and *RUNX1*-mutant AMLs rarely generated MoDe-AML (Figure 2H). Importantly, *LAMP5*⁺ MoDe-AML patients showed poor outcomes even when compared within MAPK-pathway mutated or *FLT3*-mutated patient groups or the 4-gene-score VEN/HMA risk groups (Figures S2I–S2K).⁴

Last, we analyzed the BEAT AML cohort for *LAMP5*^{high}-expressing patients. We detected a similar association of genetic features with *LAMP5*^{high} expression as in our cohort (Figures S3A and S3B). As in our cohort, *LAMP5*^{high}-expressing patients in the BEAT AML cohort did not show inferior outcomes when treated with chemotherapy compared with *LAMP5*^{low} patients, arguing that chemotherapy remains an option to treat this disease subtype³⁶ (Figure S3C).

Together, these data demonstrate that *LAMP5*⁺ MoDe-AMLs, but not other monocytic AMLs, are consistently refractory to VEN/HMA. Our data suggest that the inconsistent findings regarding VEN/HMA response of mono-AMLs in different

(B) Proportion of immature GPR56⁺ cells among total AML cells from 98 AML patients grouped by patient class. The line indicates the median of each group.

(C) Schematic outline of the xenotransplantation assays and subsequent analysis of 98 AML patient samples and their respective PDXs.

(D–F) Phenotypic analysis of representative primary patient samples and respective PDX of (D) stem-/progenitor-AML patients (*N* = 43) and mono-AML patients (E and F) (*N* = 9) (F; *N* = 7) transplanted as bulk. Quantification of immature GPR56⁺, immature GPR56[−], and mature cells in PDX. Each dot represents a patient sample. The line indicates the median of each group.

(G) Schematic outline of the FACS-sorting strategy and injection into mouse recipients.

(H–J) Human CD45⁺ AML cells in mice injected with respective FACS-sorted cell types are shown as a percentage of BM cells. Cell types were sorted from primary samples of stem- and progenitor- (*N* = 5) (H), mono-AML with classical engraftment pattern (*N* = 5) (I), and mono-AMLs with non-classical engraftment pattern (*N* = 3). Each dot represents a mouse. The line indicates the median of each group.

(K) Schematic representation of findings.

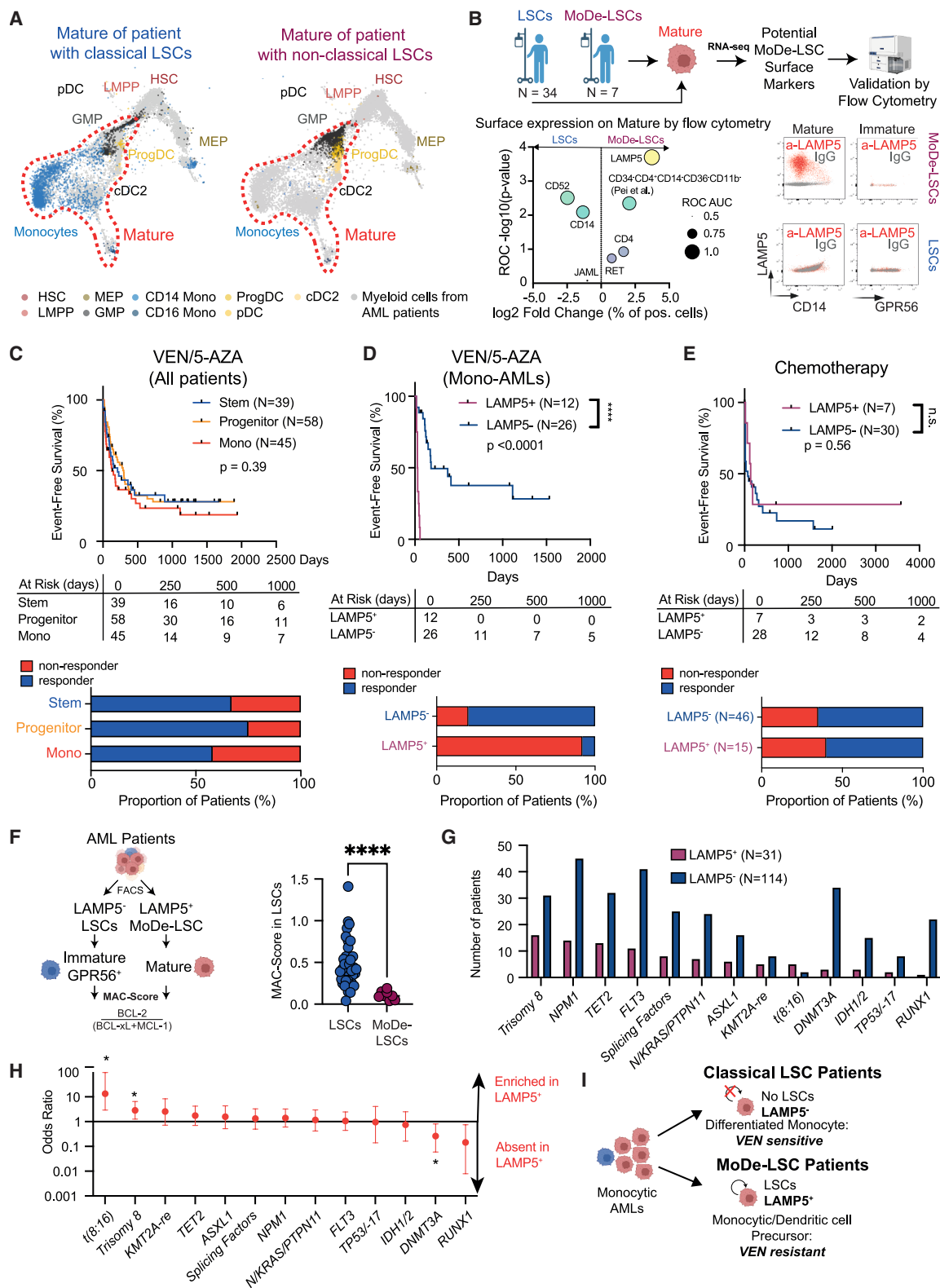


Figure 2. LAMP5⁺ MoDe-AML patients are resistant to VEN/HMA

(A) scRNA-seq data of mature AML cells from a patient sample with classical LSCs (P10) and non-classical LSCs (P16) projected onto a UMAP of healthy adult BM hematopoiesis. Dotted line indicates the mature subpopulation. The color of dots shows the cell type annotation. Gray: myeloid cells from AML patients.

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cohorts may be explained by varying proportions of rare MoDe patients within these cohorts. MoDe-LSCs, easily detectable at diagnosis by surface LAMP5 expression, drive a highly aggressive form of AML with mature LSCs that requires alternative treatment approaches (Figure 2I).

MoDe-AMLs can be targeted using MEK1/2 inhibitors

To determine effective treatment options for MoDe-AMLs, we re-analyzed the *ex vivo* drug response data of the BEAT AML cohort, comprising responses to 122 inhibitors.³⁶ We included 326 AML patients with high-quality RNA-seq and drug profiling data. We first inferred hematopoietic cell type abundances from bulk AML samples using gene expression deconvolution. By correlating the inferred cell type abundances with drug sensitivities, we identified 5 inhibitors whose efficacy correlated with the abundance of monocytic-like cells (Figure S3D). Since 2/5 drugs identified were MEK1/2 inhibitors and monocytic cells have been shown to be sensitive to MEK1/2 inhibition,³⁷ we investigated MEK inhibition in MoDe-AML. After 72 h of *ex vivo* treatment of FACS-sorted immature GPR56⁺ or mature leukemic cells (8 MoDe- and 25 non-MoDe-AMLs), only the MEK1/2 inhibitor binimetinib, but no other AML drug (cytarabine, daunorubicin, 5-AZA, or VEN), reduced the viability specifically of MoDe-AML cells (Figure 3A). Cladribine, a purine analog reported to target monocytic LSCs,¹⁹ was efficient throughout cell types but showed no selective activity in LAMP5⁺ MoDe mature cells (Figure 3A). However, in LAMP5⁺ AMLs, mature cells were sensitive to binimetinib with a median IC50 of 20 nM. This is comparable with the sensitivity observed in binimetinib-treatable melanoma.³⁸

In support of the observed increased dependency of MEK signaling in MoDe-AMLs, MEK activity gene sets³⁹ were upregulated in LAMP5⁺ LSCs compared with classical, GPR56⁺ LSCs (Figure 3B). We observed that *in vitro* treatment with MEK inhibitors caused a selective downregulation of the anti-apoptotic protein and MAPK pathway target gene MCL-1 exclusively in mature cells from MoDe-AML patients, but not in other monocytic AMLs (Figure 3C). These data suggested that binimetinib-treated MoDe cells might become sensitive to VEN. To assess this, we tested binimetinib as monotherapy or in combination with VEN/5-AZA in MoDe-PDX models *in vivo* (Figure 3D). Neither VEN/5-AZA nor binimetinib alone reduced MoDe-AML blasts, whereas the combination of all three induced remission

in this aggressive PDX in the absence of additional hematotoxicity (Figures S3E and S3F). Since cladribine has recently been used as a combination partner for VEN/HMA, we compared the response of 5-AZA/VEN and cladribine or binimetinib in two additional MoDe-PDX models. In both models, cladribine did not deepen responses, whereas binimetinib improved blast clearance, leading to an overall survival benefit for the MEK-inhibitor combination (Figure 3E).

Collectively, we identified LAMP5⁺ MoDe-AML as a novel AML subtype accounting for about one-third of monocytic AMLs. MoDe-AMLs are driven by mature, non-hierarchy-building LSCs that are refractory to VEN/AZA. Notably, MEK1/2 inhibitors such as binimetinib downregulate MCL-1 expression and sensitize MoDe-LSCs to VEN/AZA treatment, providing novel translational therapeutic options for this highly aggressive, VEN-resistant AML subtype (Figure 3G).

Four LSC subtypes are associated with distinct VEN/HMA outcomes

While MEK1/2 inhibitors can suppress the VEN/HMA resistance of MoDe-AML patients, these patients are rare, and most patients contain MEK1/2-resistant GPR56⁺ LSCs. Since previous work suggested significant inter-patient LSC heterogeneity,^{8,40} we sought to understand how LSC heterogeneity contributes to VEN/HMA resistance in patients with GPR56⁺ LSCs to develop personalized treatment strategies also for the main subgroup of patients (Figure 4A). First, we generated RNA-seq data of immature GPR56⁺ cells from 135 AMLs and again projected these data onto a scRNA-seq reference map of healthy hematopoiesis (Figure 4A). In comparison with MoDe-LSCs, immature GPR56⁺ LSCs formed three major clusters within hematopoietic stem and progenitor cells (HSPCs): 53% (72/135) resembled lymphoid-myeloid primed progenitors (LMPP-LSCs), 38% (51/135) megakaryocyte-erythroid progenitors (MEP-LSCs), and 9% (12/135) hematopoietic stem cells (HSC-LSCs) (Figure 4B). Phenotypically, HSC-LSCs showed a higher abundance of CD34⁺CD38[−] cells, whereas MoDe-LSCs were solely composed of CD34[−]CD38⁺ cells, in line with their more differentiated state (Figure S4A). MEP-LSCs and LMPP-LSCs showed more similar profiles with a slight trend toward increased abundance of CD34[−]CD38[−] cells in MEP-LSCs and CD34[−]CD38⁺ cells in LMPP-LSCs (Figure S4A). Analysis of relative transcription factor expression revealed that MEP-LSCs showed high

(B) Flow cytometry-based comparison of marker candidates of MoDe-AML based on DEGs between mature cells of mono-AML patients with MoDe- or classical LSCs ($N = 41$) and surface expression of LAMP5 on mature and immature cells from representative MoDe-LSC and classical LSC patients. Quantification using logistic regression analysis, receiver operator characteristics (ROC) test, and log2-fold-change of the relative % of positive cells.

(C) Event-free survival and response rates of AML patients treated with VEN/HMA stratified by AML class. Stem- ($N = 39$), progenitor- ($N = 58$), and mono-AML ($N = 45$). Log-rank test was used to calculate p values.

(D and E) Event-free survival and response rates of AML patients stratified by LAMP5 expression after VEN/HMA treatment. LAMP5⁺-AML ($N = 12$) and LAMP5[−]-AML ($N = 26$). LAMP5⁺: >0.1% LAMP5⁺ cells in mature cells. Log-rank test was used to calculate p values.

(E) Event-free survival and response rates of AML patients stratified by LAMP5 expression after A/C treatment. LAMP5⁺-AML ($N = 7$) and LAMP5[−]-AML ($N = 30$). Log-rank test was used to calculate p values.

(F) MAC-scores of LSCs from classical LSCs ($N = 37$) and MoDe-AML patients ($N = 8$). For LAMP5[−]-AML patients, immature GPR56⁺ cells, and for LAMP5⁺-AML patients (MoDe-AML), mature cells are used as "LSCs." The Mann-Whitney test was used to calculate p values.

(G) Overview of mutations and cytogenetic aberrations present in LAMP5⁺ ($N = 31$) and LAMP5[−] ($N = 114$) AML samples.

(H) Odds ratio of LAMP5 expression calculated for each mutation and cytogenetic aberration using multiple logistic regression analysis. Error bars indicate upper and lower limits of the odds ratio.

(I) Schematic representation of findings.

* p value < 0.05, ** p value < 0.01, *** p value < 0.001, **** p value < 0.0001, n.s. p > 0.05.

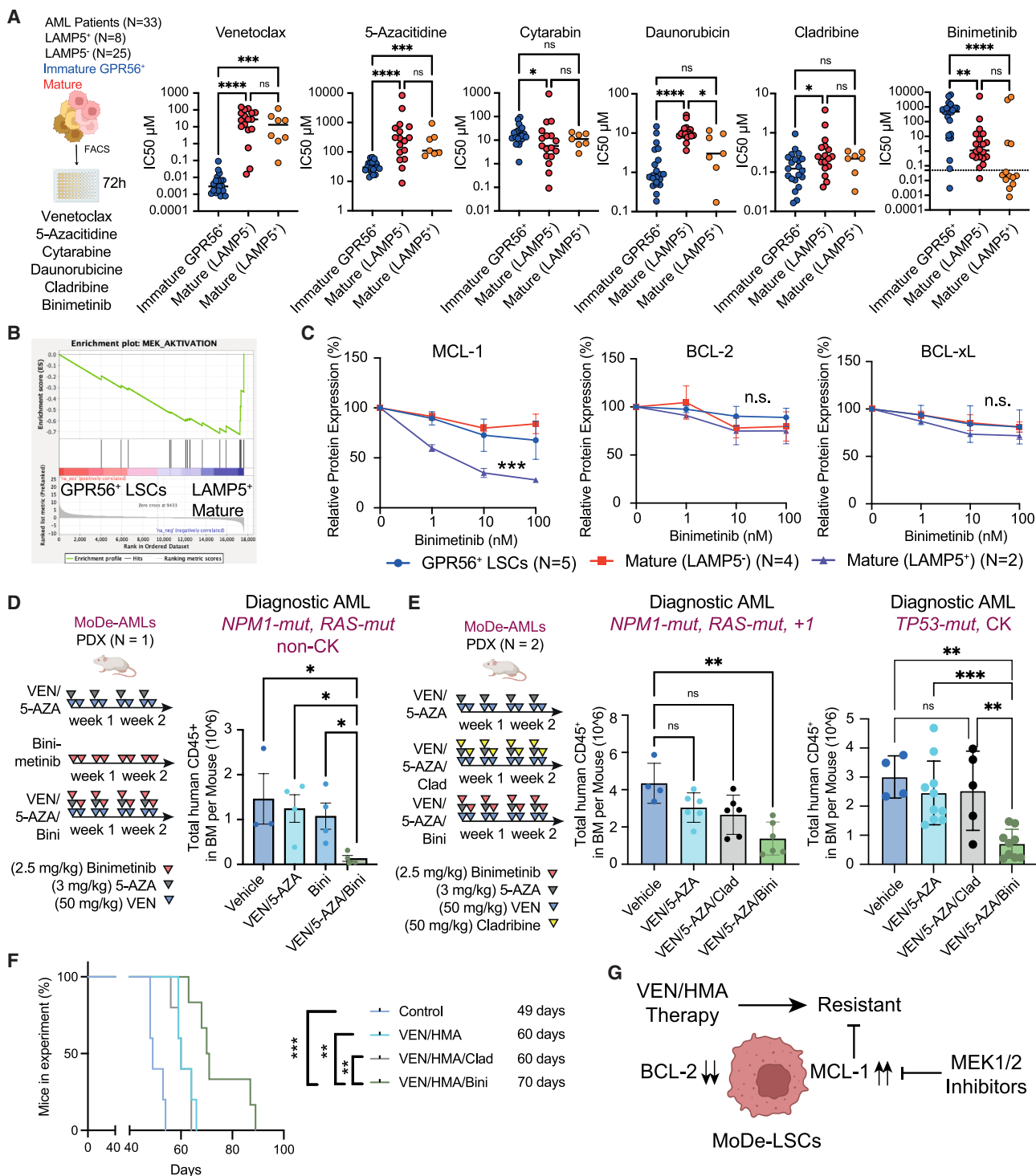


Figure 3. LAMP5⁺ MoDe-AML patients are sensitive to MEK1/2 inhibition

(A) IC50 after 72 h *ex vivo* treatment with indicated compounds of FACS-sorted immature GPR56⁺ (*N* = 20) and mature cells (*N* = 25) from AML patient samples (*N* = 33). For binimetinib: immature GPR56⁺ (*N* = 23) and mature (*N* = 33). The Kruskal-Wallis test was used to calculate *p* values. The line indicates the median of each group.

(B) Gene set enrichment of MEK1/2-activation signature on DEGs between GPR56⁺ LSCs from LAMP5⁻ patients and CD64⁺CD11b⁺ mature from LAMP5⁺ patients.

(C) MCL-1, BCL-2, and BCL-xL protein expression in subpopulations of AML patients treated *in vitro* for 24 h with binimetinib. Groups were compared using two-way ANOVA. Mature cells from LAMP5⁺-AML (*N* = 2), mature cells from LAMP5[−] AML (*N* = 4), and GPR56⁺ LSCs from LAMP5[−] AML (*N* = 5).

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levels of well-documented MEP-transcription factors, such as *KLF1* and *GATA1*, whereas HSC-LSCs showed low levels of myeloid differentiation-promoting transcription factors *CEBPD*/*E* and cell cycle regulators *E2F1/7*, confirming our lineage classification. As expected, MoDe-LSCs showed high levels of macrophage/monocytic regulators such as *MAFB* and *PU.1* (Figure 4C).

Last, we assessed the functionality of these four LSC groups. In PDXs of the sequenced patients, neither the engraftment rates nor their engraftment percentage differed between the four LSC subtypes (Figure S4B). However, in PDXs generated from MEP-LSCs, immature GPR56⁺ cells expressed megakaryocyte-lineage markers, including CD41a, CD42a, CD105, and CD9, suggesting that lineage features are maintained upon transplantation (Figure S4C). Similarly, MoDe-LSCs produced significantly more mature blasts than the other LSC subtypes (Figure S4C). These data reveal that LSCs can be functionally categorized into four subtypes associated with lineage-specific gene programs. Importantly, none of the MEP-LSC patients had M6 or M7 AML, known for extreme shifts toward the MEP lineages.

To link VEN/5-AZA sensitivity to these LSC groups, we performed 24 h *ex vivo* VEN/5-AZA treatment in 59 AML samples. VEN/5-AZA resistance was particularly observed among MEP-LSC and MoDe-LSC samples, which was recapitulated by their low MAC-scores (Figures 4D and S4D). These associations were also observed for *in vitro* drug response data from the BEAT AML cohort, which recapitulated a positive correlation of VEN sensitivity with LMPP-enriched AML and a negative correlation with MEP- and monocyte-enriched AML (Figure S3D). LMPP-LSCs, like their healthy counterparts, expressed the highest levels of the VEN target BCL-2 and displayed the highest MAC-scores across all LSC subtypes. BCL-xL was expressed at the highest levels in MEP-LSCs and MCL-1 in MoDe-LSCs (Figures 4D, S4E, and S4F). In line with this, AML patients with either MEP-, HSC-, or MoDe-LSCs showed lower response rates and shorter EFS to VEN/HMA therapy compared with patients with LMPP-LSCs (Figures 4E and S4G). This association remained significant in multivariate analysis with confounding factors including mutations, age, sex, and treatment (Figure 4F; Table S1).

Combining LSC subtype with genetic profiling improves risk stratification

As cell type compositions have been shown to correlate with genetic lesions in AML,^{30,41} we linked mutational profiles with the three classical immature GPR56⁺ LSC subtypes (see Figure 2 for genetics of the MoDe-LSCs). HSC-LSCs were enriched for complex karyotype (CK), whereas *RUNX1* and splicing factor mutations were associated with LMPP-LSCs. MEP-LSCs were enriched for RAS pathway mutations (*NRAS* and *KRAS*), implying that LSC types are strongly influenced by genetics (Figures S4H and S4I).

To assess whether the predictive value of LSC types depends on mutational status, we analyzed VEN/HMA response rates across mutations and cytogenetics with reported associations to VEN/HMA response. As expected, response rates correlated with specific alterations: CK and *TP53* mutations were associated with poor outcomes, and *NPM1* and *IDH1/2* mutations with more favorable outcomes (Figure 4F). LMPP-LSC patients exhibited higher response rates than HSC/MEP-LSC patients across mutations/cytogenetics, showing that the LSC state provides additional prognostic value (Figure 4G). Consistently, LSC state correlated with EFS across nearly all mutational groups, except in *TP53*/CK and *NPM1*-mutated patients (Figures S4J and S4K). These data support the concept that both genetics and LSC state jointly shape VEN/HMA response. Although certain genetic lesions strongly influence treatment outcomes and can override LSC state effects, in most patients, LSC state, rather than genetics, emerges as the primary determinant of VEN/HMA response.

Lastly, we integrated LSC subtype and genetic profiles into a unified risk stratification model. Patients with LAMP5[−] cells, *NPM1* mutations, or LMPP-LSCs were assigned to the good prognosis group. Patients with LAMP5⁺ MoDe-AML, *TP53*-mut, CK, or *NPM1*-wt non-LMPP-LSCs comprised the poor group (Figure 4H). This risk stratification yielded a median EFS of 1,113 days in the good group and 89 days in the poor group among patients treated with VEN/AZA (Figure 4H). This separation also translated into overall survival and identified poor-outcome patients within the 4-gene higher benefit group and good-outcome patients within the 4-gene lower benefit group (Figures 4I and S4L). Although stratifications based on genetics or MAC-scoring might be easier to implement clinically, our data demonstrate that LSC cell state has a strong influence on response rates and duration to VEN/AZA treatment. Future strategies that combine LSC state with genetics may provide superior patient stratification for VEN/AZA compared with any single variable alone.

Plasticity toward MEP-LSCs drives VEN/HMA resistance via BCL-xL

To investigate why the LSC state is not associated with different EFS in *NPM1*- or *TP53*-mutant patients, we performed scRNA-seq on 8 patients for whom paired diagnostic and relapsed/refractory samples after VEN/HMA treatment were available. First, we mapped their leukemic cells onto the atlas of healthy hematopoiesis. In 5 patients, of whom 3 were not classified as MEP-LSC patients at diagnosis, we found an increased frequency of MEP-like blasts post-VEN/HMA driving a switch to MEP-LSCs in these patients (Figures 5A and 5B). The remaining three patients showed either a shift toward HSC-like cells or contained mostly GMP-like leukemic cells without a lineage shift (Figures 5A and 5B). This MEP-lineage shift post-VEN/HMA

(D) Primary PDX treated with vehicle, VEN/5-AZA, binimetinib, or VEN/5-AZA/binimetinib over two weeks, followed by AML quantification in bone marrow. *N* = 4–6 mice per group. Groups were compared using the Kruskal-Wallis test.

(E and F) Primary PDX treated with vehicle, VEN/5-AZA, VEN/5-AZA/cladribine, or VEN/5-AZA/binimetinib as indicated over two weeks. (E) AML burden in bone marrow and (F) duration of each mouse in the experiment after completion of treatment. *N* = 4–6 mice per group. Groups were compared using the Kruskal-Wallis test and the log-rank test.

(G) Schematic representation of findings.

Data is shown as mean ± SD unless stated otherwise. * *p* value < 0.05, ** *p* value < 0.01, *** *p* value < 0.001, **** *p* value < 0.0001, n.s. *p* > 0.05.

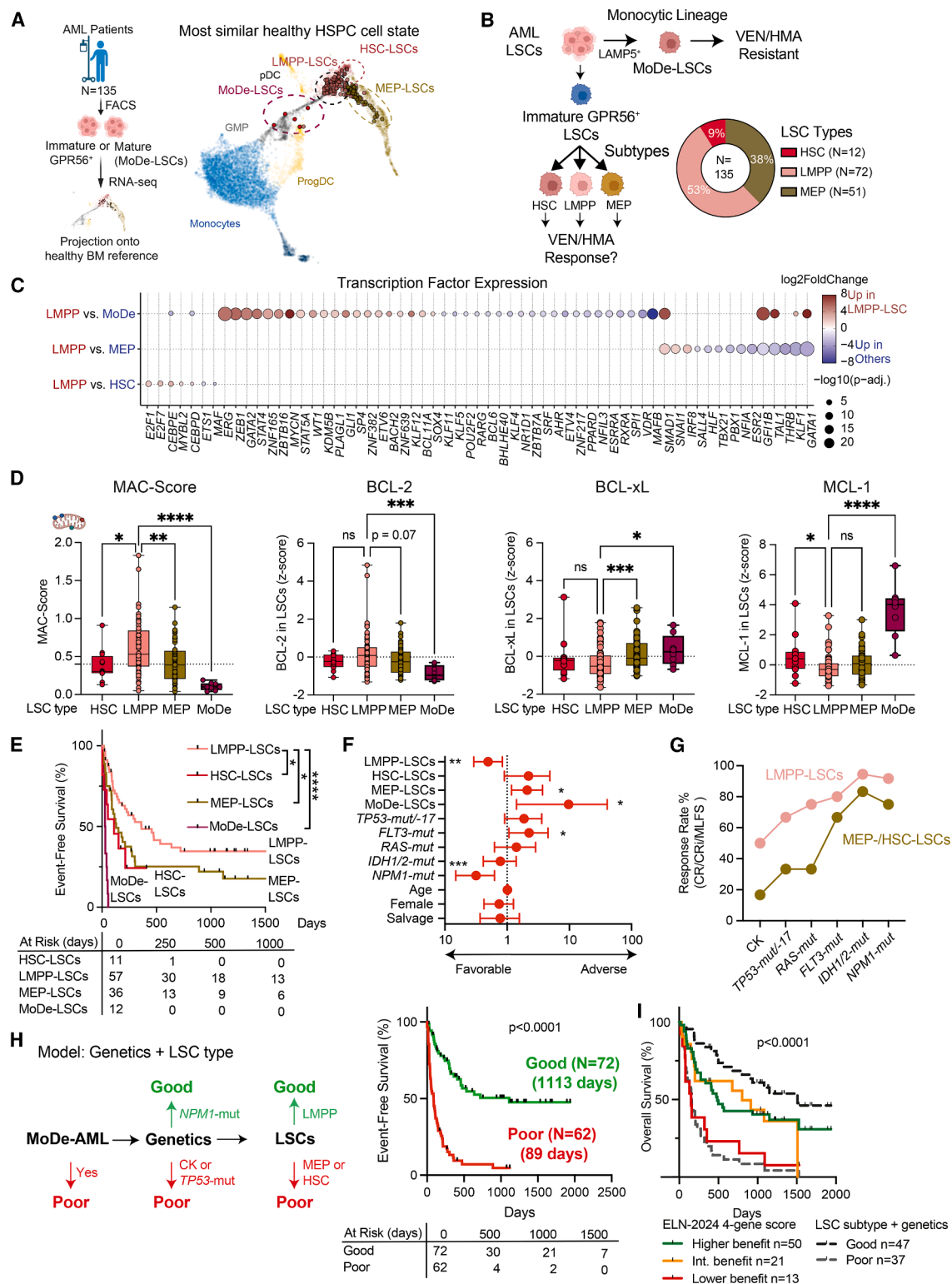


Figure 4. Four LSC subtypes are associated with distinct VEN/HMA outcomes

(A) Projection of the transcriptome of FACS-sorted immature GPR56⁺ cells and LAMP5⁺ mature cells of all 135 AML patient samples onto a CITE-seq reference map of healthy adult BM. Each dot represents a patient.

(B) Schematic of LSC classification into MoDe-LSCs and classical LSCs and subsequent subtyping into HSC-LSCs, LMP-LSCs, and MEP-LSCs. Frequency distribution of LSC types among 135 AML patients.

(C) Differentially expressed transcription factors with adjusted *p* value < 0.05 and log-fold-change > 1.5 across LSC types.

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was observed in all four *TP53*-mutant AMLs using two independent reference atlases for cell type classification (Figures 5B, S5A, and S5B). These data suggest that *TP53*-mutant AMLs can rapidly adapt to VEN/HMA therapy pressure via their high cell state plasticity and provide an explanation for why the pre-therapy LSC states of *TP53*-mutant patients showed different response rates but not different EFS. In line with an MEP-transcriptional program, we observed an increase in BCL-xL protein expression in bulk immature GPR56⁺ and GPR56[−] populations in the 5 patient samples showing the MEP-shift post-VEN/HMA (Figure 5C). This supports the important role of BCL-xL in platelets and increased expression of BCL-xL in healthy MEPs.⁴² These data suggest that the MEP-shift hijacks lineage-associated BCL-xL upregulation to escape BCL-2 inhibition by VEN in LSCs.

To assess whether the MEP shift can also be detected within routine diagnostics, we analyzed clinical flow cytometry data of 40 AML patients with paired diagnosis—VEN/HMA relapse/refractory samples (Table S2). We consistently observed an increase in AML cells expressing the MEP marker CD41a/CD42a by absolute and binned percentages (Figures 5D and S5C). In contrast, paired samples from refractory A/C-treated patients did not upregulate MEP markers (Figure S5D; Table S3). These findings validate the MEP shift and the associated BCL-xL upregulation as a frequent escape mechanism specific to VEN/HMA therapy.

VEN/HMA-resistant AMLs enrich for BCL-xL-dependent MEP-LSCs

Next, we assessed the phenotypic and functional changes associated with the MEP shift in a larger cohort of post-VEN/HMA samples lacking matched diagnostic samples (Figure 5E). All post-VEN/HMA samples contained immature GPR56⁺ cells, and 19/20 re-established a classical engraftment pattern in PDX (Figures 5F and S5E). This implies that immature GPR56⁺ LSCs are not only driving disease, but also VEN/HMA relapse in the majority of patients.

To understand transcriptomic programs driving VEN/HMA resistance, we compared gene expression of 31 VEN/HMA-resistant AML samples (13 relapse/18 refractory) with the 103 diagnostic samples analyzed above (72 responders and 31 non-responders). Again, post-VEN/HMA refractory samples upregulated MEP genes (*GP9/CD42a* and *ITGA2B/CD41*) but reduced expression of myeloid genes (*NFKBID* and *MPO*)

(Figure S5F). Similarly, post-VEN/HMA relapse samples downregulated myeloid (*JUNB* and *FOSB*) but upregulated DNA repair genes (*PARP1* and *RAD1*) (Figure S5G; Data S1). Importantly, *BCL2* levels were down-regulated in both refractory and relapse samples (Figures S5F and S5G). More generally, post-VEN/HMA samples were enriched in gene sets associated with cell cycle, fatty-acid metabolism, glycolysis, and coagulation compared with responders (Figures 5G, 5H, and S5H). Supporting our single-cell data, we detected a doubling of the proportion of AMLs classified as MEP-LSCs in refractory patients (12/18; 67%) compared with pre-treatment samples of patients responding to VEN/HMA (29%; 21/72) (Figure 5I).

Last, we compared the expression of BCL-2 family proteins between time points by flow cytometry. While the MAC score of initially responding patients fell between diagnosis and relapse, non-responding patients generally showed low MAC-scores at both time points (Figure 5J). At relapse or in refractory disease, low MAC-scores were driven by low BCL-2 and high BCL-xL levels, with no significant change in MCL-1 (Figure 5J). Supporting our paired data analysis, BCL-xL increased the most between pre- and post-VEN/HMA LSCs in *TP53*-mut and was also apparent in *NPM1*-wt/*TP53*-wt but not in *NPM1*-mutant patients (Figure S5I). This suggests that the MEP shift is likely not the main driver of resistance in *NPM1*-mutant AMLs. Our data imply that non-responding or relapse-driving LSCs from the majority of AML patients overexpress BCL-xL and escape VEN/HMA therapy through a MEP shift, particularly apparent in initially responding *TP53*-mut AMLs. Of note, MAC-score and the individual BCL-2 family protein levels did not differ between pre- and post-treatment samples from patients receiving standard chemotherapy (Figure S5J), highlighting that the outlined escape mechanisms are highly specific to VEN/HMA therapy.

MEP-LSCs can be targeted using BCL-xL inhibition

To assess how lineage plasticity drives resistance at the cellular level, we subdivided immature GPR56⁺ LSCs into three populations—“MEP-like,” “intermediate,” and “LMPP-like”—based on LMPP-like marker CD44 and MEP-like marker CD41 identified in our scRNA-seq data (Figures 6A and S6A). Although heterogeneous, MEP-like cells were almost exclusively present in MEP-LSC samples and co-expressed other MEP markers, while LMPP-like cells were most abundant in LMPP-LSC samples (Figures 6A, S6B, and S6C). We next performed *ex vivo* VEN or BCL-xL inhibitor⁴³ treatments of the three LSC subpopulations

(D) MAC-score in LSCs and Z scores of BCL-2, MCL-1, and BCL-xL protein expression grouped by LSC subtype. HSC-LSCs (N = 12), LMPP-LSCs (N = 70), MEP-LSCs (N = 49), and MoDe-LSCs (N = 8). Groups are compared using the Mann-Whitney test. Individual data points represent individual patient samples.

(E) VEN/HMA therapy EFS of AML patients grouped by LSC subtype and compared by log-rank test. HSC-LSCs (N = 11), LMPP-LSCs (N = 57), MEP-LSCs (N = 36), and MoDe-LSCs (N = 12).

(F) Cox hazard ratio of VEN/HMA response of LSC types adjusted for age, sex, treatment, genetics, and 4-gene score risk classifications using multivariate Cox regression analysis.

(G) Response rates of LMPP-LSCs and MEP/HSC-LSCs AML patients treated with VEN/HMA. CK: LMPP-LSCs (N = 12), MEP/HSC-LSCs (N = 12); *TP53*-mut/-17: LMPP-LSCs (N = 6), MEP/HSC-LSCs (N = 9); *RAS*-mut: LMPP-LSCs (N = 8), MEP/HSC-LSCs (N = 6); *FLT3*-mut: LMPP-LSCs (N = 15), MEP/HSC-LSCs (N = 5); *IDH1/2*-mut: LMPP-LSCs (N = 17), MEP/HSC-LSCs (N = 13); *NPM1*-mut LMPP-LSCs (N = 12), MEP/HSC-LSCs (N = 12). Patients are grouped by mutation/cytogenetics.

(H) Model considering LSC type and genetics for the prediction of the EFS of AML patients treated with VEN/HMA. LAMP5⁺ MoDe-AML (N = 12), *TP53*-mut or CK (N = 28), *NPM1*-mut (N = 40), LMPP-LSCs (N = 32), and non-LMPP-LSCs (N = 22). Good (N = 72) and poor (N = 62) groups were compared using the log-rank test.

(I) Overall survival of patients treated with VEN/HMA stratified by 4-gene score groups or based on new LSC-type/genetic risk scoring. *p* value was calculated using the log-rank test.

* *p* value < 0.05, ** *p* value < 0.01, *** *p* value < 0.001, **** *p* value < 0.0001, n.s. *p* > 0.05

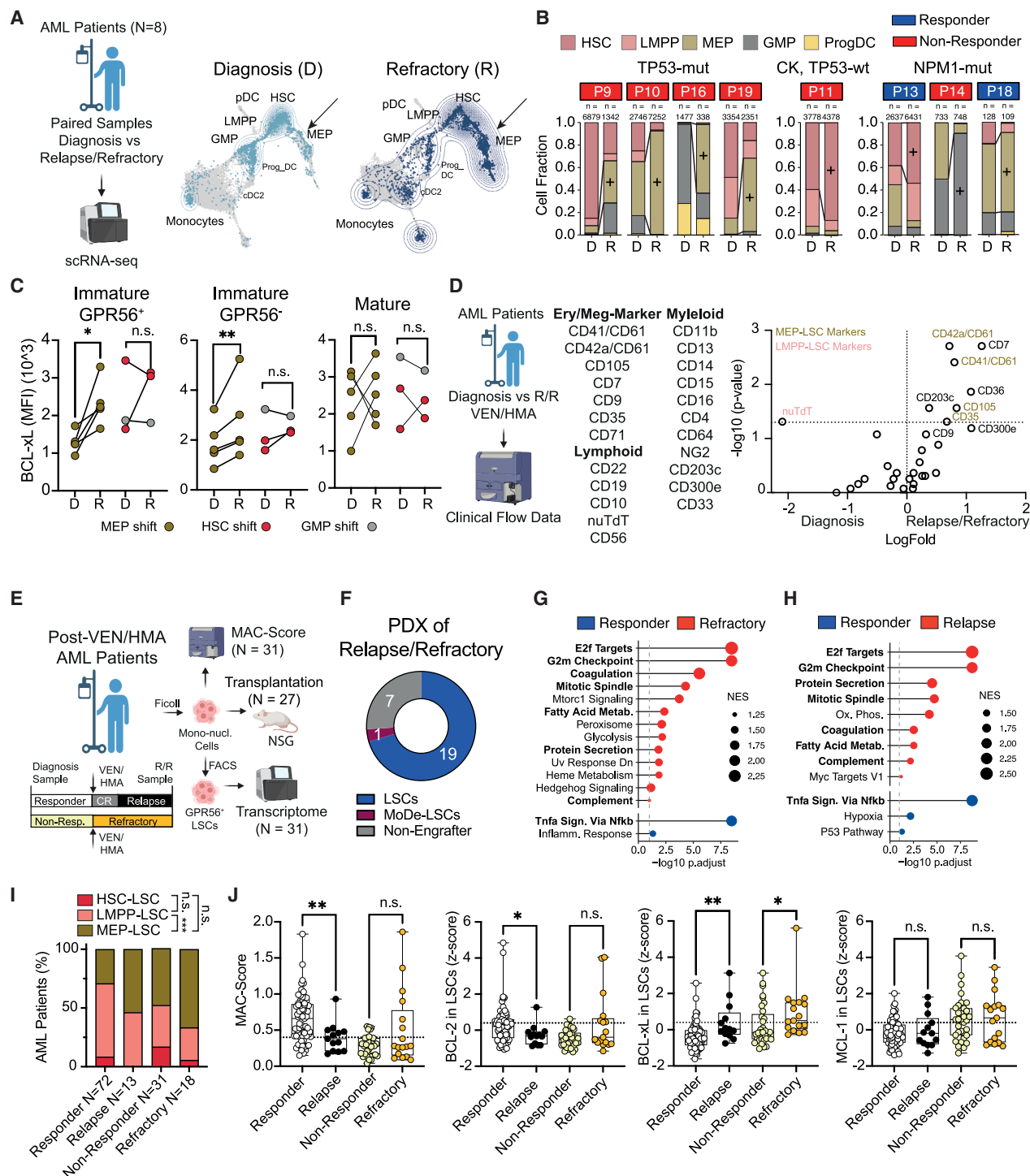


Figure 5. Longitudinal analysis of VEN/HMA therapy reveals MEP-lineage plasticity

(A) Density map of projected scRNA-seq data of four AML patients with paired diagnosis—refractory samples showing MEP-cell enrichment.

(B) Progenitor cell type compositions of paired AML samples (N = 8) across therapy timepoints assessed using scRNA-seq data.

(C) Expression of BCL-xL in AML subpopulations grouped by therapy timepoint in AML patients (N = 8). Lines indicate patient sample pairs compared using the ratio paired *t* test.

(D) Differentially detected AML markers from clinical flow data containing indicated flow parameters of AML cells of VEN/HMA diagnosis—relapse/refractory patient pairs. Data were obtained as absolute percentage values for the initial cohort (N = 10). Therapy timepoints were compared using the Wilcoxon paired signed-rank test.

(E) Experimental workflow for relapse and refractory samples.

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within MEP- or LMPP-LSC AMLs. In all three populations, MEP-LSC AMLs were more VEN resistant and BCL-xL dependent compared with LMPP-LSC AMLs (Figure 6B). This correlated with higher BCL-xL protein levels and BCL-xL/BCL-2 ratio in MEP-LSC patients (Figures 6C and S6D). We termed this ratio the “MAXL-ratio” (mediators of apoptosis BCL-xL-ratio). *Ex vivo* drug treatment of LSCs from VEN/HMA responder and relapse samples showed increased sensitivity to BCL-xL, but not MCL-1 inhibition,⁴⁴ at relapse (Figures 6D and S6E). These data further suggest that the MEP-LSC switch from BCL-2 to BCL-xL dependency is the predominant mechanism driving VEN therapy escape.

Last, we associated the transcriptomes of common AML cell lines with the MoDe-, LMPP-LSC, or MEP-LSC AML subtypes (Figure S6F). Having large numbers of cells available in culture, we assessed the efficiency of targeting LSC subtypes by specific combinations of BCL-2 family inhibitors. In GMP/LMPP- or Mono/ProgDC-like cell lines, MCL-1, but not BCL-xL, inhibition reduced the VEN-IC50 in a dose-dependent manner (Figure S6G). In MEP-like cell lines, BCL-xL inhibition reduced VEN-IC50 even at sub-nanomolar concentrations, whereas high MCL-1 inhibitor concentration was required to achieve significant effects (Figure S6G). These drug sensitivity data in cultured AML cells indicate that MEP-LSCs can be targeted *ex vivo* by BCL-xL inhibition alone or in synergistic combinations with VEN.

TP53 mutant MEP-LSCs can be targeted by BCL-xL inhibition *in vivo*

To assess the therapeutic potential of BCL-xL inhibition *in vivo*, we generated two PDXs from diagnostic samples of high-risk TP53-mutant MEP-LSC patients with low MAC-scores and high MAXL-ratios and one PDX of a VEN/HMA-refractory TP53-mutant MEP-LSC patient. After engraftment, PDXs were treated with VEN/5-AZA with or without BCL-xL inhibitor or left untreated (Figures 6E and S6H).

While VEN/5-AZA treatment reduced overall blasts, persisting AML cells in all three PDXs showed increased expression of the MEP markers CD41a and CD42a compared with vehicle-treated controls (Figures 6E and S6H). This is consistent with the observed patient data and highlights MEP lineage adaptation under VEN/5-AZA pressure as a resistance mechanism. The biweekly addition of a low-dose BCL-xL inhibitor completely prevented the MEP shift and reduced the persisting leukemic burden by up to 10-fold compared with VEN/5-AZA treatment alone (Figures 6E and S6H). To further explore these findings, including the prognostic value of the MAXL-ratio, we established PDX models from two TP53-wt, LMPP-LSC AMLs from VEN/HMA relapsed patients, one with a high and one with a low MAXL-ratio. Although neither of the patients increased MEP-line-

age markers CD41 and CD42a post-treatment, the combination was highly effective in the high MAXL-ratio PDX but failed to reduce blast levels in the PDX with a low MAXL-ratio not overexpressing BCL-xL (Figure 6F). This highlights that the additive benefit of a BCL-xL inhibitor is highly dependent on target expression and may be predicted using a modification of the MAC-score called the MAXL-ratio, which should be further validated in the future.

To prove the lasting benefit of treating MAC-score low, MAXL-ratio high AML with an additional BCL-xL inhibitor, we treated PDXs from a TP53-mutant diagnostic AML with vehicle, VEN/5-AZA with or without BCL-xL inhibitor for 2 weeks, assessing survival. The addition of the BCL-xL inhibitor significantly improved the overall survival (reaching termination criteria) of the animals (Figure 6G). Remarkably, no limiting toxicities besides pronounced but reversible cytopenia were observed (Figures S6I and S6J).

Our *in vivo* data obtained in 5 different PDX models demonstrate that upfront addition of BCL-xL inhibition to VEN/5-AZA therapy not only prevents LSC plasticity and transition toward an MEP-like state but also reduces leukemic burden in PDX models derived from high-risk patients such as TP53-mutant AMLs. These findings identify MEP-like LSCs present at diagnosis or emerging during VEN/HMA treatment as key mediators of VEN resistance, which can be effectively targeted by additional upfront BCL-xL inhibition.

DISCUSSION

Leveraging a cohort of more than 150 AML patients, we describe four functionally distinct LSC subtypes, HSC-, LMPP-, MEP-, and MoDe-LSCs, based on their differentiation state. These subtypes play a critical role in both response and resistance to VEN/HMA therapy. Although mutational profiles are linked to LSC subtypes, our data demonstrate that LSC states provide an additional relevant layer for VEN/HMA response prediction beyond genetics. Integrating genetic and phenotypic LSC features significantly improves patient stratification for VEN/HMA therapy. Distinct LSC states show specific vulnerabilities: MEK inhibition overcomes VEN/AZA resistance in MoDe-LSCs by down-regulating MCL-1, whereas BCL-xL inhibition targets MEP-LSCs in diagnostic or relapsed/refractory patient samples. These insights enable tailored strategies against aggressive, therapy-resistant AMLs, including those with TP53 mutations.

First, we identified a subtype of monocytic AML driven by non-hierarchical, non-classical LSCs that exhibit a monocytic/dendritic transcriptional profile and high resistance to VEN/HMA therapy. These MoDe-LSCs account for approximately 30% of monocytic and 5%–10% of all AML cases. We identified

(F) Distribution of LSC classes among relapse/refractory samples of patients treated with VEN/HMA ($N = 27$). LSC was determined by analysis of cell types in PDX. Non-engrafter: <5% human CD45⁺ in PDX after 52 weeks.

(G and H) Enriched and depleted hallmark pathways in immature GPR56⁺ of unmatched VEN/HMA diagnostic responder samples ($N = 72$) versus (G) refractory samples ($N = 18$) and (H) relapse samples ($N = 13$).

(I) Relative distribution of LSC subtypes across therapy timepoints (responder $N = 72$, relapse $N = 13$, non-responder $N = 31$, refractory $N = 18$). Chi-squared test for trend was used to compare the LSC types.

(J) MAC-score and Z scores of BCL-2, BCL-xL, and MCL-1 protein expression in LSCs grouped by LSC type and compared using the Mann-Whitney U-test. Responder $N = 79$, relapse $N = 13$, non-responder $N = 34$, refractory $N = 19$. Individual data points represent individual patient samples.

* p value < 0.05, ** p value < 0.01, *** p value < 0.001, **** p value < 0.0001, n.s. p > 0.05

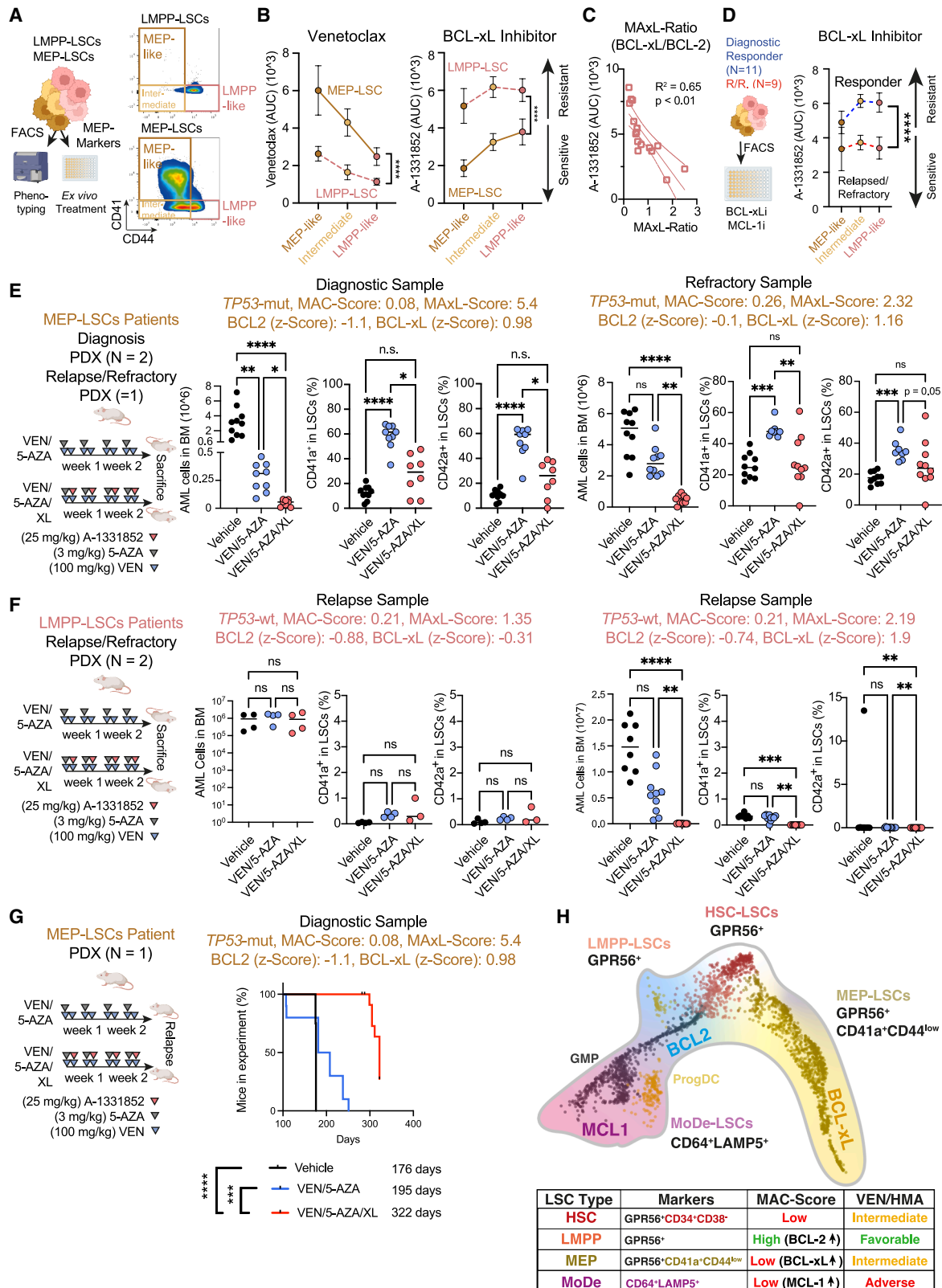


Figure 6. BCL-xL inhibition targets MEP-LSCs

(A) Experimental design and representative LSC heterogeneity across the CD41 and CD44 axes in MEP-LSC and LMPP-LSC patients. LMPP-like: CD44^{high}CD41⁺, MEP-like: CD44^{low}CD41⁺, intermediate: CD44^{low}CD41⁺.

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LAMP5-surface expression as a highly specific marker for MoDe-AMLs, enabling rapid and easy clinical identification via flow cytometry. The frequency of VEN/HMA-refractory MoDe-AML patients in cohorts may explain conflicting outcomes in studies linking monocytic AML to poor prognosis, significantly extending a recent report describing a subpopulation of monocytic LSCs.^{2,6,19,26,27,45} MoDe-LSCs show average outcomes with A/C-based chemotherapy, which may explain why they have remained unrecognized until now. MoDe-LSCs were linked to multiple mutations and aberrations, most notably *t(8;16)* and *KMT2A*-rearranged disease. While these translocations drive MoDe-AML in some patients, most MoDe-AMLs harbor distinct mutational profiles, suggesting a shared cell of origin. MoDe-AML showed a unique sensitivity to MEK inhibition in combination with VEN/HMA, a potential vulnerability for precision medicine in this otherwise treatment-refractory MoDe-AML subtype.

Second, we describe that most resistance to VEN/HMA in patients is unrelated to monocytic differentiation, but with differentiation states within three classical, hierarchy-generating immature GPR56⁺ LSC types: LMPP, MEP, and HSC-LSCs.

No standard AML cell line represents highly immature, poor-prognosis HSC-LSCs, highlighting the importance of primary AML samples. Within all genetic subgroups except for *TP53*- and *NPM1*-mut patients, LSC differentiation states dictated prognosis more precisely than genetic lesions, and the combination of mutations and cell state provided superior outcome predictions to VEN/HMA. It is likely that other mechanisms of VEN resistance, such as *BAX* mutations, are more prevalent in *NPM1*-mut AMLs.²² For *TP53*-mut AMLs, rapid relapse after initial response argues for a fast adaptation to VEN. This high level of plasticity has been described in other *TP53*-mut cancers and contributes to pan-chemoresistance and dismal outcomes across diseases.^{46–48} Our study extends previous studies investigating cell state dependencies in AML under chemotherapy to VEN/HMA.^{30,41} For example, MoDe-AML had worse outcomes with VEN/HMA compared with *TP53*-mut AML, whereas high BCL-2-expressing LMPP-AMLs showed a favorable prognosis. Additionally, we provide a large dataset of transcriptomes of FACS-sorted LSC/non-LSC populations linked to patient outcomes and genetics under VEN/HMA treatment, which will be a helpful resource for the field.

Our study underscores once more that LSCs and their subtypes have high clinical relevance and should be integrated into diagnostic assessments and drug discovery.

Third, our findings demonstrate that VEN/HMA resistance is caused by a switch from BCL-2^{high} LMPP-LSC to a BCL-xL^{high} MEP-LSC subtype. This may be mediated by therapy-stress-induced plasticity or specific selection of resistant states downstream of a plastic LSC. Of note, we did not observe any transition from classical LSCs to MoDe-LSCs. Additionally, MEP-LSCs are not linked to rare FAB M6 or M7 megakaryocyte/erythroblastic morphology known to be BCL-xL-dependent, comprising around 10% of *TP53*-mutant AML patients.^{20,49} Instead, we introduce the concept of lineage bias within morphologically similar diseases. In MEP-LSC patients, more cells aberrantly expressed the megakaryocyte differentiation marker CD41 alongside high BCL-xL and low levels of VEN-target BCL-2, which we quantify as the MAXL-ratio (BCL-xL/BCL-2 in LSCs). Within the GPR56⁺ immature LSC population, we detected additional heterogeneity. Even CD41-negative cells in the same patients overexpressed BCL-xL. These data suggest that the self-renewing population within MEP-LSCs upregulates erythroid/megakaryocytic traits even before expressing MEP surface markers, closely mirroring the biology of healthy HSC differentiation.^{50–52} Similarly, in myelofibrosis, a bias toward megakaryocyte differentiation is known to originate from early stem/progenitor cells.⁵³ Importantly, both diagnostic and relapsed AML patients with MEP-LSCs were highly sensitive to BCL-xL inhibitors in combination with VEN/HMA *in vitro* and *in vivo*, providing a novel route toward clinical translation. This option is available since the BCL-2/BCL-xL inhibitor Navitoclax has a tolerable safety profile in myelofibrosis, inviting further biomarker-guided trials in AML.⁵⁴

In summary, we define four classes of AML LSCs with associated biomarkers, such as LAMP5, enabling rapid detection in clinical flow cytometry (Figure 6H). These biomarkers do not enrich functional LSCs but label their LSC class. This differs from previous studies that described biomarkers to isolate functional LSCs (e.g., GPR56, CD34/CD38, etc.) but did not focus on the inter-patient differences between these LSCs. Upfront resistance and relapse under VEN/HMA are largely driven by plastic LSC states, with transition to the MEP state emerging as a key, targetable mechanism of VEN resistance via upfront BCL-xL inhibition. Importantly, integrating LSC states and plasticity into biomarker-guided strategies may enable the clinical implementation of BCL-xL or MEK inhibitors on a VEN/HMA core to improve patient outcomes.

(B) Drug responses (AUC) to VEN and BCL-xL-I (A-1331852) of LSC subpopulations in LMPP-LSCs (*N* = 9) and MEP-LSCs (*N* = 7). Groups were compared using two-way ANOVA.

(C) Correlation of BCL-xL-I response and MAXL-ratio (BCL-xL/BCL-2-ratio) in LMPP-like cells of both LSC types (*N* = 16). Correlation coefficients were calculated using Pearson correlation.

(D) Experimental design and drug response (AUC) of LSC subpopulations in diagnostic VEN/HMA responders (*N* = 11) and relapse/refractory (*N* = 9) samples treated with BCL-xL-I for 24 h. Groups were compared using two-way ANOVA.

(E) MEP-LSC patient-PDX treated with vehicle, VEN/AZA, and VEN/AZA/BCL-xL-I as indicated over 2 weeks, followed by AML quantification in bone marrow and MEP marker analysis of LSCs. *N* = 5–10 mice/group. Groups were compared using the Mann-Whitney test.

(F) LMPP-LSC patient-PDX treated with vehicle, VEN/AZA, and VEN/AZA/BCL-xL-I over 2 weeks, followed by AML quantification in bone marrow and MEP marker analysis of LSCs. *N* = 5–10 mice/group. Groups were compared using the Mann-Whitney test.

(G) Kaplan-Meier curve of MEP-LSC patient-PDX treated with vehicle, VEN/AZA, and VEN/AZA/BCL-xL-I over 2 weeks. *N* = 5–10 mice/group. Groups were compared using the log-rank test.

(H) Schematic representation of findings

* *p* value < 0.05, ** *p* value < 0.01, *** *p* value < 0.001, **** *p* value < 0.0001, n.s. *p* > 0.05.

Data are presented as mean ± SEM.

Each dot indicates an individual patient sample/mouse

Limitations of the study

We found that specific LSC types are associated with response to VEN/HMA, likely due to lineage-dependent expression of BCL-2 family proteins. Expanding the cohort would help consolidate these findings and strengthen the conclusions with a larger patient population. Furthermore, it is unknown whether our conclusions also extend to VEN/HMA-based triplet (Menin, FLT-3 inhibitors) treated patients.

The combination of VEN/5-AZA with MEK1/2 and BCL-xL inhibitors demonstrated promising activity against LSC types associated with VEN/HMA resistance. However, in most mice, the combinations were not curative, and residual AML cells eventually re-initiated disease in the mice. It remains unclear whether repeated administration of this combination could prolong remission and whether treatment-associated toxicity would permit such an approach. Notably, additional cytotoxicity was observed with the BCL-xL combination therapy, warranting caution in potential clinical translation.

Finally, these therapies represent experimental treatments tested in mice under controlled laboratory conditions and do not guarantee safety or efficacy in human AML patients.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for reagents should be directed to and will be fulfilled by the lead contact, Andreas Trumpp (a.trumpp@dkfz-heidelberg.de).

Materials availability

This paper does not report original materials.

Data and code availability

- RNA sequencing raw data and feature counts can be retrieved from ArrayExpress under accession numbers ArrayExpress: E-MTAB-14142, E-MTAB-14143.
- Re-analyzed bulk RNA-seq data of CD34/CD38 AML populations from Zeng et al.³⁰ were downloaded from Gene Expression Omnibus under accession number GSE199336. BEAT AML⁴¹ RNA-seq and *ex vivo* drug response data were downloaded from <https://biodev.github.io/BeatAML2/>, and CCLE⁵⁵ cell line RNA-seq data from <https://depmap.org/portal/>.
- Code used to generate the figures is available at <https://doi.org/10.5281/zenodo.19552379> and https://github.com/ampleppa/vv-resistance_paper.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.W., A.-M.L., S. Renders, and A.T.; methodology, A.W., A.-M.L., S. Renders, K.S., I.B., B.B., and S.G.; software, A.-M.L. and B.B.; formal analysis, A.W., A.-M.L., and S. Renders; investigation, A.W., A.-M.L., S. Renders, K.S., I.B., B.B., S.G., F.Y.H., M.-E.L., M.K., N.K., M.S., V.T., E.D., H.K., B.H., and M.J.; resources, S. Renders, S.G., H.K., J.M.U., R.S., S. Raffel, S. Richter, M. Heuser, M. Hundemer, T.S., N.C.-W., H.B., C.R., S.R., A.-K.E., D.K., and C.M.-T.; data curation, A.W., A.-M.L., S. Renders, and S.G.; writing—original draft, A.W., A.-M.L., and S. Renders; writing—review & editing, A.W., A.-M.L., S. Renders, C.M.-T., and A.T.; visualization, A.W. and A.-M.L.; supervision, A.W., A.-M.L., S. Renders, T.S., C.M.-T., and A.T.; project administration, A.W., A.-M.L., S. Renders, C.M.-T., and A.T.; funding acquisition, A.W., C.M.-T., and A.T.

DECLARATION OF INTERESTS

A.W., A.-M.L., S. Renders, C.M.-T., and A.T. are holders of a patent application related to MAC-Scoring published under WO2023237236A1.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human GPR56 -PE/Cyanine7	Biologend	Cat#358206; RRID:AB_2562090
Anti-human CD64 -Brilliant Violet 785	Biologend	Cat#305044; RRID:AB_2800780
Anti-human CD11b -Brilliant Violet 711	Biologend	Cat#301344; RRID:AB_2563792
Anti-human CD45 -Brilliant Violet 421	Biologend	Cat#304032; RRID:AB_2561357
Anti-human CD3 - Brilliant Violet 510	Biologend	Cat#317332; RRID:AB_2561943
Anti-human CD20 -Brilliant Violet 510	Biologend	Cat#302340; RRID:AB_2561941
Anti-human CD235a -Brilliant Violet 510	BD Biosciences	Cat#740174; RRID:AB_2739927
Anti-human CD34 -BUV395	BD Biosciences	Cat#563778; RRID:AB_2738424
Anti-human CD38 -BUV496	BD Biosciences	Cat#564657; RRID:AB_2744376
Anti-human BCL-2 -Alexa Fluor® 647	Cell Signaling	Cat#82655; RRID:AB_2799997
Anti-human MCL-1 -Alexa Fluor® 488	Cell Signaling	Cat#58326; RRID:AB_2799546
Anti-human BCL-xL -PE	Cell Signaling	Cat#13835S; RRID:AB_2798326
Mouse IgG1 - Alexa Fluor® 647	Cell Signaling	Cat#4843; RRID:AB_1281292
Rabbit IgG -Alexa Fluor® 488	Cell Signaling	Cat#2975S; RRID:AB_10699151
Rabbit IgG -PE	Cell Signaling	Cat#5742S; RRID:AB_10694219
Anti-mouse CD45 -Alexa Fluor® 700	Biologend	Cat#103128; RRID:AB_493715
Anti-human CD19 - FITC	Biologend	Cat#302256; RRID:AB_2564143
Anti-human CD33 -PE/Cyanine7	Thermofisher Scientific	Cat#25-0338-42; RRID:AB_1907380
Anti-human GPR56 – PE	Biologend	Cat#358204; RRID:AB_2562084
Anti-human CD64 – APC	Biologend	Cat#305014; RRID:AB_1595428
Anti-human CD45 – FITC	Biologend	Cat#304006; RRID:AB_314394
Anti-human CD56 -Brilliant Violet 785	Biologend	Cat#362550; RRID:AB_2566059
Anti-human CD45 - Brilliant Violet 711	BD Biosciences	Cat#564357; RRID:AB_2744404
Anti-human CD41 - Brilliant Violet 421	Biologend	Cat#303729;RRID:AB_2629626
Anti-human CD44 - Brilliant Violet 650	BD Biosciences	Cat#743665; RRID:AB_2871540
Anti-human CD9 - FITC	Biologend	Cat#312104; RRID:AB_2075894
Anti-human CD7 - BUV737	BD Biosciences	Cat#753774; RRID:AB_3687447
Anti-human CD36 - Alexa Fluor® 700	Biologend	Cat#336236; RRID:AB_2890767
Anti-human CD105 -PE/Dazzle™ 594	Biologend	Cat#323223; RRID:AB_2832634
Anti-human CD41a -BUV395	BD Biosciences	Cat#740295; RRID:AB_2740034
Anti-human CD42a - PE	BD Biosciences	Cat#558819; RRID:AB_397130
Anti-human CD61 - Brilliant Violet 510	BD Biosciences	Cat#563303; RRID:AB_2738128
Anti-human CD35 - BUV496	BD Biosciences	Cat#750521; RRID:AB_2874675
Anti-human CD71 - BUV805	BD Biosciences	Cat#749294; RRID:AB_2873669
Anti-human LAMP5 – APC	Miltenyi	Cat#130-109-204; RRID:AB_2652590
Anti-human RET – PE	Cell Signaling	Cat#68897; RRID:AB_2799756
Anti-human CD52 - PerCP-Vio® 700	Miltenyi	Cat#130-128-781; RRID:AB_2921941
Anti-human JAML - Alexa Fluor® 488	R&D Systems	Cat#FAB34491G; RRID:AB_3649344
Anti-human CD14 - BUV805	BD Biosciences	Cat#612903; RRID:AB_2870190
Anti-human CD45 - Brilliant Violet 421	Biologend	Cat#304032; RRID:AB_2561357
Anti-human CD4 - Brilliant Violet 480	BD Biosciences	Cat#566104; RRID:AB_2739506
Human IgG - PerCP-Vio® 700	Miltenyi	Cat#130-113-453; RRID:AB_2733067
Human IgG – APC	Miltenyi	Cat#130-113-446; RRID:AB_2733446
Mouse IgG2A - Alexa Fluor® 488	R&D Systems	Cat#IC003G; RRID:AB_10718683

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-human CD38 - BUV496	BD Biosciences	Cat#564657; RRID:AB_2744376
Anti-human CD123 - PE/Cyanine7	BD Biosciences	Cat#560826; RRID:AB_10563898
Anti-human CD25 - BUV737	BD Biosciences	Cat#564386; RRID:AB_2916878
Anti-human CD90 - Brilliant Violet 510	Biolegend	Cat#328125; RRID:AB_2562199
Anti-human CD45RA -Brilliant Violet 421	BD Biosciences	Cat#562885; RRID:AB_2737864
Anti-human CD34 - PE	BD Biosciences	Cat#555822; RRID:AB_396151
Anti-human CD45RA - FITC	BD Biosciences	Cat#983002; RRID:AB_2650650
Anti-human CD38 - Alexa Fluor® 700	ThermoFisher Scientific	Cat#56-0389-42; RRID:AB_10804040
Anti-human CD90 - PerCP 5.5	BD Biosciences	Cat#561557; RRID:AB_10712762
Anti-human Ki67 - APC	ThermoFisher Scientific	Cat#17-5698-80; RRID:AB_2688056
Anti-human CD14 - PE/Cyanine7	Biolegend	Cat#325617; RRID:AB_830690
Anti-human FcR Blocking Reagent	Miltenyi	Cat#130-059-901; RRID:AB_2892112

Chemicals, peptides, and recombinant proteins

GM-CSF	Peptrotech	Cat# 300-03;
Venetoclax	MedChemExpress	Cat#HY-15531
A-1331852	MedChemExpress	Cat#HY-19741
MIK-665	MedChemExpress	Cat#HY-112218
Zombie NIR Fixable Viability stain	Biolegend	Cat#423105
7-AAD	BD Biosciences	Cat#559925
Absolute Counting Beads	ThermoFisher Scientific	Cat#C36950
SMART First Strand Buffer	Takara Bio Clontech	Cat#639538
5-Azacytidine	MedChemExpress	Cat#HY-10586
Cytarabine	MedChemExpress	Cat#C3350000
Cladribine	MedChemExpress	Cat#HY-13599
Doxorubicin	MedChemExpress	Cat#S1208
Binimetinib	MedChemExpress	Cat#HY-15202
Ficoll Paque Plus	GE Healthcare	Cat#GE17-1440-03
True-Stain Monocyte Blocker™	Biolegend	Cat#426103

Critical commercial assays

CellTiter-Glo Luminescent Cell Viability Assay	Promega	Cat#G7571
Picopure RNA-Extraction kit	Thermo Fisher	Cat# KIT0214
Fixation/Permeabilization Solution Kit	BD Biosciences	Cat#554714
RevertAid First Strand cDNA Synthesis Kit	ThermoFisher Scientific	Cat# K1622
SMARTScribe	Takara Bio Clontech	Cat#639538
Nextera XT DNA Library Preparation Kit	Illumina	Cat#FC-121-1030
Chromium Single Cell 3' Library and Gel Bead Kit	10X Genomics	Cat#1000128
RNasin Plus RNase Inhibitor	Promega	Cat#N2615
PowerUp™ SYBR™ Green Master Mix	ThermoFisher Scientific	Cat#A25777

Deposited data

RNA-seq of LSC-like and mature populations from human AML patient samples	This paper	ArrayExpress:E-MTAB-14142
Single-cell RNA-seq pre- and post-venetoclax therapy of AML patients	This paper	ArrayExpress:E-MTAB-14143
RNA-seq data of CD34/CD38 AML populations	Zeng et al. ³⁰	GEO: GSE199336
CITE-seq of human bone marrow mononuclear cells	Stuart et al. ³²	SeuratData: bmcite

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Single-cell RNA-seq of human bone marrow reference atlas	Zeng et al. ²⁹	https://github.com/andygxzeng/BoneMarrowMap
BEAT AML RNA-seq and ex vivo drug response data of AML patients	Bottomly et al. ⁴¹	https://biodev.github.io/BeatAML2/
CCLL RNA-seq data	Broad Institute DepMap; Ghandi et al. ⁵⁵	https://depmap.org/portal/
Experimental models: Cell lines		
MV4-11	DSMZ	Cat#ACC-102; RRID:CVCL_0064
Nomo1	DSMZ	Cat#ACC 542; RRID:CVCL_1609
OCI-AML3	DSMZ	Cat#ACC 582; RRID:CVCL_1844
THP-1	DSMZ	Cat#TIB-202; RRID:CVCL_0006
Molm-13	DSMZ	Cat#ACC 554; RRID:CVCL_2119
HL-60	DSMZ	Cat#ACC-3; RRID: CVCL_0002
Kasumi-1	DSMZ	Cat#ACC-220;RRID:CVCL_0589
GDM-1	DSMZ	Cat#ACC-11; RRID:CVCL_1230
KG-1	DSMZ	Cat#ACC-14; RRID:CVCL_0374
SKNO-1	DSMZ	Cat#ACC-690; RRID:CVCL_2196
Experimental models: Organisms/strains		
NOD. <i>Prkdc</i> ^{scid} . <i>Il2rg</i> ^{null}	Jackson Laboratory	Cat# JAX #005557; RID:IMSR_JAX:005557
Oligonucleotides		
Primer: CGGCTACAGCTTCACCACCACG – bActin Rev	Sigma	N/A
Primer: TCTTGCTCGAAGTCCAGGGCGA – bActin Fwd	Sigma	N/A
Primer: TGCGGGGAAAATGGGACGACGTG – LAMP5 Fwd	Sigma	N/A
Primer: CCGGGTCAATGCGATATCGGCC – LAMP5 Rev	Sigma	N/A
Software and algorithms		
GraphPad Prism 10	GraphPad software	https://www.graphpad.com/
Flowjo Software (v10.9.0)	Flowjo	https://www.flowjo.com/
R	R Core	https://www.r-project.org/
GSEA (v3.0)	Subramanian et al. 2017	https://www.gsea-msigdb.org/gsea/index.jsp
Cell Ranger (v6.1.1)	10x Genomics	https://www.10xgenomics.com/support/software/cell-ranger/latest
Seurat (v4.3.0.1)	Hao et al. ⁵⁶	https://satijalab.org/seurat/
SeuratData (v0.2.2)	GitHub; Satija et al.	https://github.com/satijalab/seurat-data
glmGamPoi (v1.13.2)	Ahlmann-Eltze and Huber. ⁵⁷	https://bioconductor.org/packages/glmGamPoi
sctransform (v0.4.1)	Hafemeister and Satija ⁵⁸	https://github.com/satijalab/sctransform
DESeq2 (v1.42.1)	Love et al. ⁵⁹	https://bioconductor.org/packages/DESeq2
dplyr (v1.1.4)	CRAN; Wickham et al.	https://cran.r-project.org/package=dplyr
tidyverse (v2.0.0)	Wickham et al. (2019)	https://tidyverse.tidyverse.org/
ggplot2 (v3.5.1)	Wickham (2016)	https://ggplot2.tidyverse.org/
ggbeeswarm (v0.7.2)	CRAN; Clarke et al.	https://cran.r-project.org/package=ggbeeswarm
ggrastr (v1.0.2)	CRAN; Petukhov et al.	https://cran.r-project.org/package=ggrastr

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
ggh4x (v0.2.8)	CRAN; van den Brand	https://cran.r-project.org/package=ggh4x
ggnewscale (v0.4.9)	CRAN; Campitelli	https://cran.r-project.org/package=ggnewscale
rstatix (v0.7.2)	CRAN; Kassambara	https://cran.r-project.org/package=rstatix
Matrix (v1.6-5)	CRAN; Bates et al.	https://cran.r-project.org/package=Matrix
GSEABase (v1.62.0)	Bioconductor; Morgan et al.	https://bioconductor.org/packages/GSEABase
Rcpp (v1.0.12)	Eddelbuettel and François (2011)	https://cran.r-project.org/package=Rcpp
RcppAnnoy (v0.0.22)	CRAN; Eddelbuettel and Bernhardsson	https://cran.r-project.org/package=RcppAnnoy
BoneMarrowMap (v0.1.0)	Zeng et al. ²⁹	https://github.com/andygxeng/BoneMarrowMap
clusterProfiler (v4.8.2)	Wu et al. ⁶⁰	https://bioconductor.org/packages/clusterProfiler
leiden (v0.4.3)	CRAN; Kelly	https://cran.r-project.org/package=leiden
uwot (v0.1.16)	CRAN; Melville	https://cran.r-project.org/package=uwot
dorothea (v1.14.1)	Garcia-Alonso et al. ⁶¹	https://bioconductor.org/packages/dorothea

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human patient samples

AML samples were collected from diagnostic peripheral blood (PB) or BM aspirations at the University Hospitals in Heidelberg, Hannover, Frankfurt and the Finnish Hematology Registry and Clinical Biobank (FHRB) in accordance with the Declaration of Helsinki and based on institutional approvals after obtaining written informed consent from each patient. The project was approved by the Ethics Committee of the Medical Faculty of Heidelberg (S-169/2017 and S-648/2021), the local Ethics Review Committee of Hannover Medical School (ethical vote No.7972_BO_K_2018) and Ethics Committee of Goethe University Medical Center (vote #329/10). Pseudonymized left-over samples from healthy bone marrow donors (rinseback from bone marrow filters) were used with written informed consent in accordance with vote #329/10 (Ethics Committee of Goethe University Medical Center).

Mononucleated cells (MNCs) were isolated by density gradient centrifugation using Ficoll Paque Plus (GE Healthcare, cat #GE17-1440-03) followed by red-cell lysis. Prior to freezing, MNCs were resuspension in FBS supplemented with 10% DMSO and aliquoted in cryotubes. For the first 24h, samples were stored in Mr. Frosty Freezing contains at -80 ° C then transferred for long-term storage in liquid nitrogen until further use.

Detailed patient and specimen characteristics are listed in the Table S4 and are based on the clinical diagnostic reports. An experienced hematologist categorized all patients by retrospectively reviewing all pathology and flow cytometry reports in accordance with ELN 2017. Patients achieving complete response (CR), CR with incomplete recovery (CRI), complete remission with partial hematologic recovery (CRh) and morphological leukemia-free state (MLFS) at any time during induction therapy were defined as responders, and patients achieving only partial response (PR), stable disease (SD), or progressive disease (PD) as non-responders. For event-free survival (EFS) calculations, time to proven molecular, flow cytometry-based, or cytologic relapse from start of therapy or death of any cause was calculated, whatever occurred first. VEN/HMA protocols can contain both decitabine (5 days or 10 days)/vVEN (14–28 days) or 5-AZA/vVEN (14–28 days). Induction chemotherapy is 7+3, CPX-351, +/- gemtuzumab-ozagomycin, +/- FLT-3 inhibitor (midostaurin).

Mice

NOD.*Prkdc*^{scid}.*Il2rg*^{null} (NSG) mice were obtained from Jackson Laboratory. Breeding was performed under specific pathogen-free conditions at the central animal facility of the German Cancer Research Center (DKFZ). Female mice between 8 to 12 weeks of age were sublethally irradiated (175 cGy) 24 hours before xenotransplantation assays. Animal experiments were conducted in compliance with all relevant ethical regulations. All animal experiments were approved and performed in accordance with all regulatory guidelines of the official committee at the Regierungspräsidium Karlsruhe (G-140-21 and G42/18).

Cell lines

MV4-11, Nomo1, OCI-AML3, THP-1, Molm-13, HL-60, Kasumi-1, GDM-1 and KG-1 and SKNO-1 were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). SKNO-1

was additionally supplemented with 10 ng/mL of GM-CSF (PeproTech). AML cell lines were cultivated at 37 °C in a humidified incubator with 5% CO₂. Authenticated and *Mycoplasma*-screened cell lines were received from the German Collection of microorganisms and Cell Cultures (DSMZ).

METHOD DETAILS

In vitro drug screening in leukemia cell lines

0.1 × 10⁵ cells/well from each cell line were seeded in flat-bottom 96-well plates following the German Collection of microorganisms and Cell Cultures (DSMZ) culture recommendations. Cells were treated with increasing concentrations of vVEN (Medchemexpress cat#HY-15531) (0, 1, 10, 100, 1000, 10000 nM) by column and BCL-xL inhibitor (A-1331852, Medchemexpress, cat#HY-19741) or MCL-1 inhibitor (MIK665, Medchemexpress, cat#HY-112218) (0, 0.0256, 0.128, 0.64, 3.2, 16, 80, 400 nM) by row to obtain a drug matrix. After 72 hours, viability was assessed using CellTiter-Glo luminescent Cell Viability Assay (Promega, cat #G7571). IC₅₀ of VEN was calculated for each concentration of BCL-xL/MCL-1 inhibitor using PRISM v.9. To obtain viability curves to calculate VEN IC₅₀ at each incremental concentration of BCL-xL/MCL-1 inhibitor each column was normalized using the corresponding 0 nM VEN (top row) as control.

Processing of cryopreserved samples

Cryopreserved MNCs were thawed at 37 °C in a waterbath and slowly resuspended with Roswell Park Memorial Institute 1640 (RPMI-1640) medium containing 10% FBS and 1% penicillin/streptomycin (P/S). MNCs were pelleted by standard centrifugation (5 min, 300 RCF) and incubated for 15 min at 37 °C in RPMI-1640 supplemented with 10% FBS, 1% P/S and 100 µg/mL DNase I. Afterwards MNC suspension was stored on ice until further processing.

Surface staining with fluorophore-conjugated antibodies

Mononucleated cells were resuspended at a maximum cell density of 3 × 10⁶ cells/25 µL antibody staining mix. Staining mix was composed of Cell Staining buffer (BioLegend, cat. Nr. 420201) containing fluorophore-conjugated antibodies at indicated dilutions (Table S5), FcR Blocking Reagent (Miltenyi, cat. Nr. 130-059-901, 1:50 dilution) and True-Stain monocyte Blocker™ (BioLegend, cat. Nr. 426103, 1:50 dilution). Incubation was carried out at room temperature for 15 min followed by washing with PBS. Cells were resuspended in PBS and kept on ice until further processing.

For MoDe-LSCs, samples were considered LAMP5⁺ when the percentage was above 0.1% among AML blasts, an IGG control was always measured in parallel to exclude imprecise gating due to unspecific binding.

Fluorescent-activated cell sorting (FACS)

Antibody panels are described in Table S5. Primary AML cells were stained with cell-surface antibodies as described above. Before sorting 7AAD (BioLegend, 1:70) was added to the PBS-cell suspension. Prior to acquisition the cell suspension was filtered through 40 µm FACS tube strainers. Upon acquisition, dead cells were excluded using 7AAD followed by doubled exclusion. AML cells were selected using CD20, CD3 and CD235a lineage exclusion followed by selection of AML cells using CD45 and SSC-A. For LSC sorting CD33 and CD56 were additionally used to eliminate NK cells. For LSC/mature subpopulations AML cells were sorted according to CD11b, CD64, and GPR56 expression as shown in Figure 1C. For MEP/LMPP subpopulations (MEP-like, intermediate, LMPP-like) GPR56⁺ LSCs were sorted into quadrants based on CD44 and CD41 expression as shown in Figure 5C. For bulk RNA-sequencing 10,000 cells from each population were sorted directly into RNA extraction buffer (Picopure RNA-Extraction kit, Thermo Fisher, cat #KIT0214), snap-frozen, and stored at –80 °C until RNA extraction. For xenotransplantations, cells from each population were sorted into PBS.

Intracellular staining for BCL-2 family members

Thawed cells were stained with Zombie NIR Fixable Viability stain in PBS (BioLegend, cat #423105), followed by staining with cell-surface antibodies as described above. Stained cells were fixed and permeabilized using the Fixation/permeabilization Solution Kit (BD Biosciences, cat #554714) according to the manufacturer's instructions. For BCL-2 family members, fixed and permeabilized cells were stained for 1h with anti-human-BCL2-AF647 (clone 124, Cell Signaling Technology, cat #82655), anti-human-MCL1-AF488 (clone D2W9E, Cell Signaling Technology, cat #58326) and anti-human-BCL-xL-PE (clone 54H6, Cell Signaling Technology, cat #13835S). Samples were analysed with BD LSRFortessa Cell Analyzer.

MAC-score calculation

To ensure consistent and comparable mean fluorescence intensity (MFI) measurements of samples processed and analyzed on separate days, a reference AML sample was processed and run along with each cohort. Detector voltages were adjusted to keep MFI for each BCL-2, MCL-1, and BCL-xL of the GPR56⁺ LSC population in the reference sample constant. Small fluctuations of reference sample MFIs were adjusted by normalizing the measurement day's reference sample to match previous reference sample measurements. For each sample, normalized MFI for each BCL-2, MCL-1, and BCL-xL of the LSC population were divided by the respective median MFI of patients with AML classified as responders within the cohort to obtain relative MFI values (rel.MFI). Afterward, MAC-score was calculated using the following formula: BCL-2/(MCL-1+BCL-xL).

Ex vivo drug treatment of immature GPR56⁺ subpopulations

For drug assay in Figure 3D, 0.5×10^5 AML cells/well were seeded in flat-bottom 96-well plates in RPMI-1640 + 10% FBS + 1% P/S, and cells were treated with 1.5 $\mu\text{mol/L}$ of 5-AZA and 100 nmol/L of VEN for 24 hours. After 24 hours, nonadherent cell fraction was collected, and any adherent cells were trypsinized and scraped off the well bottom using a pipette tip. Lastly, both fractions were combined before the cells were stained with cell-surface antibodies. 10 μL CountBright Absolute Counting Beads (Thermo Fisher Scientific, cat #C36950) together with 7-AAD (BD Biosciences, cat #559925) was added to each sample prior to analysis with BD LSRFortessa Cell Analyzer. LSC cell counts were calculated using (Number of Absolute Counting Beads in 10 μL /Recorded number of Absolute Counting Beads)*Recorded number of LSC. Relative viability of LSCs was calculated using matched vehicle-treated cells as normalization controls.

For drug assay in Figure 5A-D, primary AML samples were FACS-sorted for CD44^{high}CD41⁻ (LMPP-like), CD44^{low}CD41⁻ (Intermediate) and MEP-like (CD44^{low}CD41⁺) and seeded at cell densities ranging from 1,000–10,000 cells per well in RPMI-1640 + 10% FBS + 1% P/S. Cells were treated with either VEN (0.1, 1, 10 and 100 nM), BCL-xL inhibitor (A-1331852) (0.1, 1, 10 and 100 nM) or MCL-1 inhibitor (MIK665) (10, 100, 1000 nM). After 24 hours, viability was assessed using CellTiter-Glo (Promega, cat #G7571) luminescent Cell Viability Assay. Relative viability was calculated using matched vehicle treated cells as normalization controls. AUC was calculated using PRISM v.9.

In vivo treatments

Once tumor burden in the bone marrow was determined to be above 5% in sentinel mice the treatment was started. Mice were treated with various *in vivo* regimens as follows. All treatment regimens included vVEN given at 50mg/kg via oral gavage, four days/week for two weeks; 5-AZA was given at 3mg/kg via intraperitoneal injection, two days/week for two weeks. MEP-LSC treatment regimen additionally included BCL-xL inhibitor (A-1331852) at 25mg/kg via oral gavage, two days/week for two weeks and MoDe-AML treatment regimen included Binimetinib at 2.5 mg/kg via oral gavage, four days/week for two weeks. All treatments were given in the same two-week time window when stated together. For oral gavage the inhibitors were in a solution containing 60% Phosal, 28% PEG-400, 10% ethanol and 3% DMSO. For intraperitoneal injections 5-AZA was dissolved in PBS. For survival analysis mice were sacrificed when termination criteria were reached.

Xenotransplantations and analysis of leukemic engraftment

Female mice 8 to 12 weeks of age were sublethally irradiated (175 cGy) 24 hours before xenotransplantation assays. Primary AML samples (2×10^6 bulk per mouse) were injected into the femoral BM cavity of sublethally irradiated mice. For transplantation of unsorted mononucleated cells of AML patients, samples were treated prior to injection with anti-CD3 antibody (OKT3 clone) for 15 minutes in PBS. Mice were monitored daily for a period of 52 weeks unless defined termination criteria were reached before. BM cells were harvested from tibiae, femurs, and iliac crests by bone crushing. Following filtration and red blood cell lysis, cells were resuspended in FBS + 10% DMSO (Sigma, cat #D2650-100) and stored in liquid nitrogen until further use. Human leukemic engraftment in mouse BM was evaluated by flow cytometry using anti-human-CD45-FITC (clone HI30), anti-human-CD34-BUV395 (clone 581), anti-human-GPR56-PE (clone CG4), anti-human-CD19-FITC (clone HIB19), anti-human-CD33-PE-Cy7 (clone WM53), CD64-APC (clone 10.1), CD11b-BV711 (clone ICRF44), and anti-mouse-CD45-Alexa700 (clone 30-F11). AML engrafters were defined as hCD45⁺CD33⁺ > 5% and absence of hCD45⁺CD19⁺CD33⁻ or hCD45⁺CD3⁺CD33⁻ cells at endpoint.

RNA isolation and qRT-PCR

Total RNA was isolated from 2×10^5 FAC-Sorted AML cells using the RNeasy® Mini Kit (Qiagen) according to the manufactures protocol and quantified using NanoDrop™ Spectrophotometer (Thermo Fisher Scientific). For cDNA generation, the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was used. qRT-PCR was performed in a QuantStudio5 (applied bio systems by Thermo Fisher Scientific) using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) with 5ng of cDNA per reaction. Primer sequences used for the study are *ActinB* Fwd-CGGCTACAGCTTACCACCACG, Rvs-TCTTGCTCGAAGTCCAGGGCGA and *LAMP5* Fwd-TGCGGGAAAATGGGACGACGTG and Rvs-CCGGGTCAATGCGATATCGGCC. Mean cycle threshold (Ct) values from triplicate were calculated for each gene using the housekeeping gene *ActinB*.

RNA sequencing of AML populations

RNA extraction and purification of FACS-sorted cells were done using the PicoPure RNA Isolation Kit according to the manufacturer's instructions (Thermo Fisher, cat #KIT0214). RNA quality assessment and quantification were performed with Bioanalyzer using Agilent RNA 6000 Pico Kit (Agilent, cat #5067-1513). Whole-transcriptome amplification was performed using a modified Smart-seq2 protocol, with 5 μL of a modified RT Buffer containing 1 $\times$ SMART First Strand Buffer (Takara Bio Clontech, cat #639538), 1 mmol/L dithiothreitol (Takara Bio Clontech), 1 $\mu\text{mol/L}$ template switching oligo (IDT), 10 U/ μL SMARTScribe (Takara Bio Clontech, cat #639538), and 1 U/ μL RNasin Plus RNase Inhibitor (Promega, cat #N2615). Tagmentation of cDNA was done using Nextera XT DNA Library Preparation Kit (Illumina, cat #FC-121-1030). All RNA libraries were pooled and sequenced together on an Illumina NextSeq 550 high-output sequencer (1.4 pmol/L with 1% PhiX loading concentration, single-end 75 bp read configuration).

Raw data processing of bulk RNA sequencing data

Reads were demultiplexed, and STAR aligner v. 2.5.3a was used to align FASTQ files containing reads for individual samples by two-pass alignment, as previously described.⁶ Reads were aligned to a STAR index generated from the 1000 Genomes Project human genomes assembly (hs37d5), using GENCODE v.19 gene models. Gene-specific gene counting over exon features based on GENCODE v.19 gene models was performed using featureCounts v.1.5.1. Quality threshold was set to 255, which indicates that STAR found a unique alignment. Strand-unspecific counting was used.

Differential gene expression and gene-set enrichment analysis of bulk RNA sequencing data

DESeq2 was used for statistical analysis of the read counts to identify differentially expressed genes between various groups.⁵⁹ Gene-set enrichment analysis based on a log fold-change order-ranked gene list using gsea function from clusterProfiler was performed to model gene expression changes between groups across the Hallmark modules from MSigDB⁶².

Single-cell RNA-sequencing in primary leukemia cells

For single-cell RNA sequencing (scRNA-seq) analysis, recovered cells were sorted for live CD45⁺ cells. scRNA-seq library preparation was performed using the Chromium Single Cell 3' Library and Gel Bead Kit (10x Genomics, cat # 1000128), according to the manufacturer's instructions. Five to ten thousand cells were targeted for each sample and processed according to the 10x Genomics Single Cell 3' v3.1 protocol to generate the transcriptome libraries. scRNA-seq libraries were sequenced on an Illumina NovaSeq 6000 S1 (300pM with 1% PhiX loading concentration, 28+94 bp read configuration).

Pre-processing and dimensional reduction of scRNA-seq data

Cell Ranger v.6.1.1 (10x Genomics) was used to align the sequencing reads to the GRCh38 human reference genome build, distinguish cells from the background, and generate a unified feature-barcode matrix that contains gene expression counts for each cell barcode. The R package Seurat v.4.3.0 was used to calculate the quality control metrics.⁶³ Cells with fewer than 200 or more than 8,000 distinct genes, fewer than 1,000 counts, less than 0.8 log10GenesPerUMI (genes detected per UMI) or more than 25% of reads mapping to mitochondrial genes were removed from the analysis. Gene counts from the scRNA-seq data were normalized using log transformation (Seurat NormalizeData), followed by scaling (Seurat ScaleData) and principal component analysis (PCA) with highly variable genes as input. k-nearest neighbors were computed using FindNeighbors function. Downstream analysis including uniform manifold approximation and projection (UMAP) visualization of the data.

"Reference-based" mapping of leukemic cells

Transcriptomes of sorted mini-bulk populations (LSC and mature or CD34/CD38 populations from Zeng et al.³⁰), AML cell lines and AML single cells were assigned to corresponding healthy counterparts by determining similarity to reference BM cell types. Previously published CITE-seq dataset, which consists of 30,672 scRNA-seq profiles measured alongside a panel of 25 antibodies from BM, was used as the reference BM atlas.³² "Supervised principal component analysis" (sPCA) was applied to the transcriptome measurements in the reference dataset using the RunSPCA function. After computing the transformation, it was projected onto each dataset. Anchors between the query datasets and the multimodal BM reference dataset were computed using the FindTransferAnchors function, followed by individual mapping of the dataset using the MapQuery function.³² The resulting supervised annotations were leveraged for downstream analyses.

AML single cells were additionally assigned to corresponding healthy counterparts using a second scRNA-seq reference dataset of human BM hematopoiesis, consisting of 263,159 BM cells.²⁹ Symphony was used to map the query dataset using the map_Query() function.⁶⁴

Differential expression analysis of scRNA-seq data

Differential expression analysis was performed between diagnosis and relapse/refractory samples showing MEP-bias, using the reference-based cell type annotations as groups. Expression values of cells from each group from the same patient were aggregated to create pseudobulk samples. Differential gene expression analysis on the pseudobulk samples was done using DESeq2 and gene set enrichment analysis was done using ClusterProfiler.

Deconvolution of bulk RNA sequencing data

Gene expression counts from single-cell myeloid cells belonging to the seven leukemic populations (HSC-like, LMPP-like, MEP-like, GMP-like, CD14 Mono-like, CD16 Mono-like, ProgDC-like, pDC-like, cDC2-like) from 16 AML samples were used as input generate a signature matrix with CIBERSORTx.³³ The dataset was downsampled to 10,000 cells. Default settings were used, with the exception of the minimum expression parameter, which was set to 0.25. Deconvolution was performed on TPM-normalized bulk RNA-seq data using S-mode batch correction and Absolute mode. Estimated abundances from leukemic populations were normalized to 1, so that each cell type's value reflects its proportion of the total leukemic cells. Deconvolution was done on FACS-sorted LSC and mature samples as well as patients from BEAT AML.³⁶

Drug response analysis of BEAT AML

Ex vivo drug response in AML patient samples from BEAT AML cohort³⁶ was measured through the AUC metric where low AUC score represents sensitivity and high score resistance. AUC values were multiplied by -1 to represent sensitivity in each treatment condition. Acute promyelocytic leukemia samples were excluded from the analysis. Pearson correlation was used to measure association between drug sensitivities and cell type abundance, based on deconvolution results. For visualization purposes, only drugs showing a p -value < 0.05 and $|\text{pearson correlation } r| > 0.3$ with any cell type abundance were plotted.

Linear regression analysis of LAMP5 expression in BEAT AML

Association between clinical and genomic variables and LAMP5 expression was assessed using multiple linear regression. The following covariates were included in the model: sex, age at specimen acquisition, cytogenetic features (duplication of chromosome 8, translocation $t(8;16)$, and loss of $17p13/TP53$), gene rearrangements (*KMT2A-re*), and mutations in *IDH1/2*, *ASXL1*, *RUNX1*, *DNMT3A*, *TET2*, *FLT3-ITD*, *NPM1*, splicing factors, and Ras pathway.

Ex vivo drug response of FACS-sorted cell types

Primary AML samples were FACS-sorted into immature GPR56⁺ and CD64⁺CD11b⁺ (mature) cells and seeded in a density of around 5×10^4 (immature GPR56⁺) and 1×10^5 (mature) cells per well in flat-bottom 96-well plates in RPMI-1640 supplemented with 10% FCS and 1% P/S. Cells were treated with the following drugs and the respective concentration ranges via serial dilution: vVEN (500 μ M–5nM), 5-AZA (1mM–10nM), cytarabine (500 μ M–5nM), cladribine (500 μ M–5nM), doxorubicin (250 μ M–2,5nM) or binimetinib (500 μ M–5nM). After 72 hours, viability was assessed using CellTiter-Glo (Promega, cat #G7571) luminescent Cell Viability Assay. IC50 of each drug was calculated using PRISM v.9. To obtain viability curves to calculate IC50, values were normalized to untreated controls.

QUANTIFICATION AND STATISTICAL ANALYSIS

All the experiments were repeated with at least three biological replicates. Results are shown with each dot representing a biological replicate and the line indicating the median unless specified otherwise in the figure legend. PRISM software version 9 and R version 4.3.0 were used to generate graphs and to perform data analysis and statistical tests. P -values were calculated using a two-tailed unpaired t -test or the appropriate statistical methods as mentioned in the figure legends. The Statistical tests used, exact value of n , what n represents, definition of center, and dispersion and precision measures are provided in the figure legends. P -values are represented with asterisks: * p -value < 0.05 , ** p -value < 0.01 , *** p -value < 0.001 , **** p -value < 0.0001 n.s. $p > 0.05$. Survival durations were analyzed using the Kaplan–Meier method and compared using the log-rank test. Flow cytometry data analysis was done using FlowJo v.10.5.3. TSNE plots for BCL-2 family members and monocytic AML markers were done using the FlowJo TSne plugin v.2.0.0.