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Phenotype-based drug screening reveals association between venetoclax response and differentiation stage in acute myeloid leukemia

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ABSTRACT

Ex vivo drug testing is a promising approach to identify novel treatment strategies for acute myeloid leukemia (AML). However, accurate blast-specific drug responses cannot be measured with homogeneous "add-mix-measure" cell viability assays. In this study, we implemented a flow cytometry-based approach to simultaneously evaluate the *ex vivo* sensitivity of different cell populations in 34 primary AML samples to seven drugs and 27 rational drug combinations. Our data demonstrate that different cell populations present in AML samples have distinct sensitivity to targeted therapies. Particularly, blast cells of FAB M0/1 AML showed high sensitivity to venetoclax. In contrast, differentiated monocytic cells abundantly present in M4/5 subtypes showed resistance to Bcl-2 inhibition, whereas immature blasts in the same samples were sensitive, highlighting the importance of blast-specific readouts. Accordingly, in the total mononuclear cell fraction the highest *BCL2/MCL1* gene expression ratio was observed in M0/1 and the lowest in M4/5 AML. Of the seven tested drugs, venetoclax had the highest blast-specific toxicity, and combining venetoclax with either MEK inhibitor trametinib or JAK inhibitor ruxolitinib effectively targeted all venetoclax-resistant blasts. In conclusion, we show that *ex vivo* efficacy of targeted agents and particularly Bcl-2 inhibitor venetoclax is influenced by the cell type, and accurate blast-specific drug responses can be assessed with a flow cytometry-based approach.

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Introduction

The treatment of AML with high-dose cytarabine and anthracycline-based intensive chemotherapy has remained the standard of care for the last four decades.[1] Despite the increase in overall survival, only 35 to 40% of adult patients under 60 years are cured with chemotherapy and allogeneic stem cell transplantation.[2] A number of novel targeted agents have been investigated in AML, but have usually generated clinical responses only in small patient subsets. Currently, genetic profiling is used for patient stratification and determination of treatment, evident by the recent approvals of midostaurin/gilteritinib and ivosidenib/enasidenib for treating AML patients with *FLT3* or *IDH1/IDH2* mutations, respectively.[3–5] Furthermore, the Bcl-2 inhibitor venetoclax combined with a hypomethylating agent has recently been approved for AML with increased efficacy in patients with *IDH1/2* and *NPM1* mutations.[6,7] However, the majority of AML patients lack actionable mutations and our understanding of the relationship between the cancer genotype, phenotype and drug function remains limited. *Ex vivo* drug testing with primary patient samples

may help to identify novel treatment options and patient subgroups with sensitivity to a specific targeted therapy.

AML is diagnosed when the bone marrow (BM) contains at least 20% of myeloid lineage blast cells, and hematological relapse is defined when the BM exceeds 5% of blasts. The non-blast cells of the AML BM are comprised of other cell types, mainly lymphocytes and more mature leukemic cells (monocytes, granulocytes) or healthy cells. The BM content and the maturity level of leukemic cells is reflected in the French-American-British (FAB) subtypes.[8] In FAB M0/1 subtypes, the differentiation blockade occurs at the early myeloid progenitor stage,

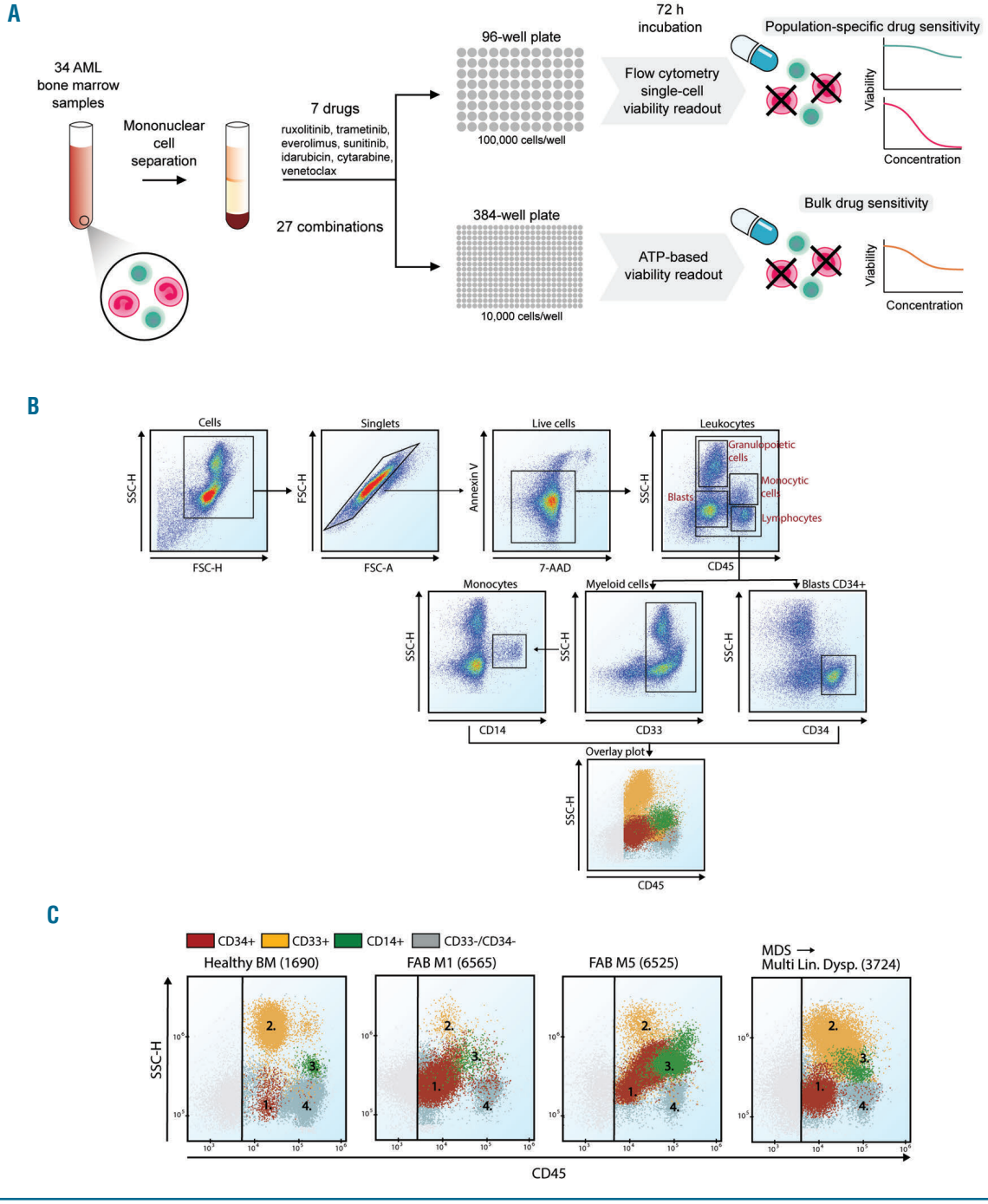

Figure 1. Study outline and gating strategy. (A) Schematic outline of the experimental setup. (B) Gating strategy of cell populations. Dead and apoptotic cells were stained with 7-AAD and Annexin V, respectively, and cells negative to these markers were gated as live cells. $CD45^{dim}/SSC^{low}$ and $CD34^{+}$ population was used as the standard gate for acute myeloid leukemia (AML) blast cells. For samples with blast cells negative for CD34, $CD45^{dim}/SSC^{low}$ and CD33 positivity was used to identify blasts. Lymphocytes were gated based on $CD45^{bright}/SSC^{low}$ and were confirmed to be CD33 negative. Immature granulocytes (present after Ficoll gradient centrifugation) were gated based on $CD45^{dim}/SSC^{high}$, $CD33^{+}$ and $CD34^{-}$. Monocytes were identified based on CD14 positivity. Clinical immunophenotype data were obtained for all samples to validate the gated cell populations. The illustration shows patient sample 6323 at day 0. (C) Illustration of the immunophenotypic profiles of AML samples with different French-American-British (FAB) subtypes and healthy bone marrow (BM) samples represented by CD45 *versus* SSC plots at day 0.

whereas in FAB M4/5 subtypes the differentiation blockade is "leaky". In addition to immature blasts in FAB M4/5 samples, leukemic cells often show myelomonocytic or monocytic differentiation, respectively. To achieve optimal response in patients, the drugs should target the less differentiated leukemic blasts.[9] However, due to cellular heterogeneity, blast-specific drug responses are challenging to measure with conventional cell viability assays such as CellTiter-Glo (CTG) or tetrazolium reduction assays (MTT/MTS).[10] Although enrichment of blasts is possible,

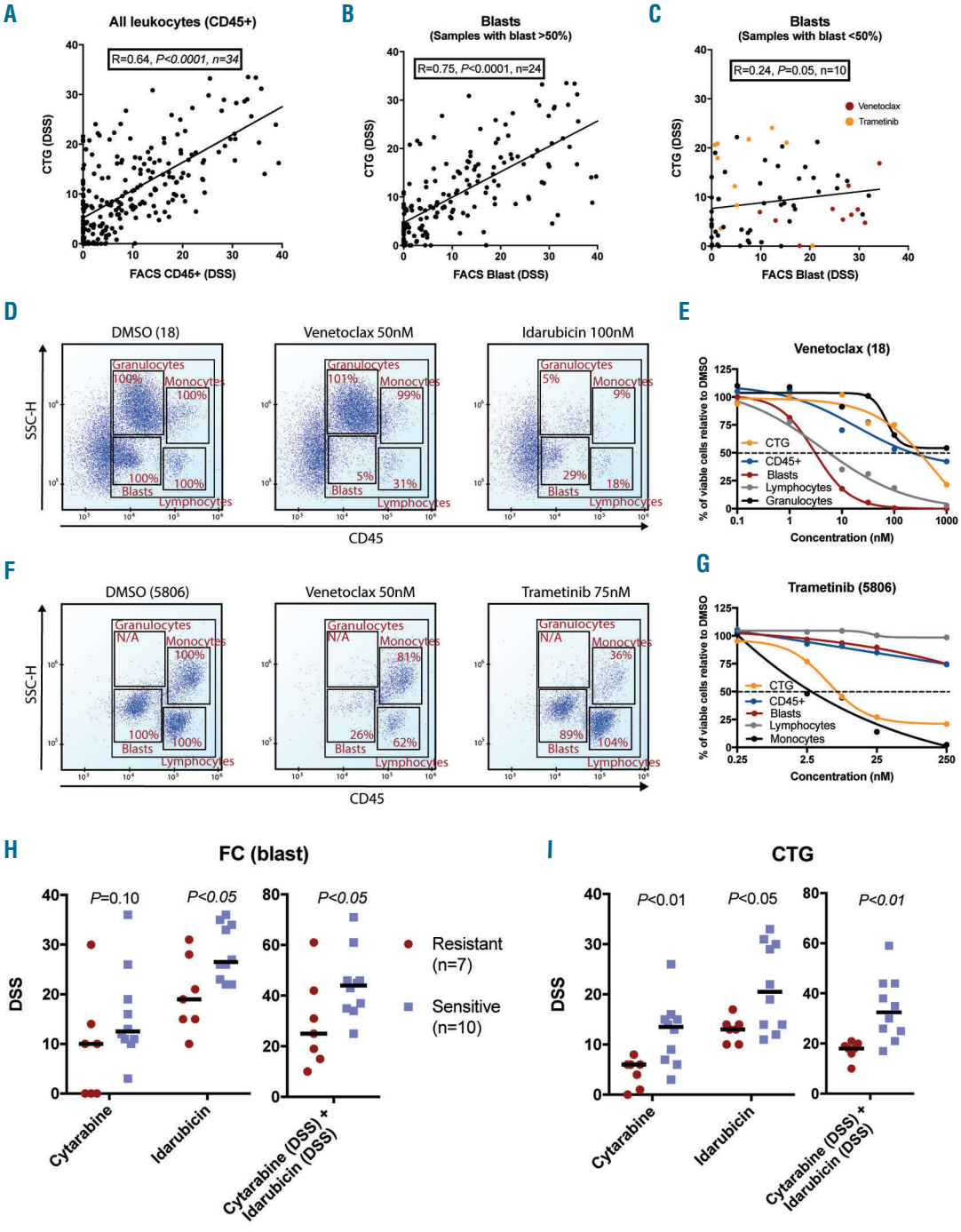

Figure 2. Comparison of flow cytometry (FC) and CellTiter-Glo (CTG) based drug screening approaches. (A) Spearman's correlation between CTG and FC-based cell viability assays with CD45$^+$ leukocytes as the FC readout, or (B) blasts in samples with clinical blast count >50%, or (C) blasts in samples with clinical blast count <50% as the FC readout. (D) Representative FC scatter plots of drug effects on different cell populations in acute myeloid leukemia (AML) sample 18 with low blast count (20%). Absolute cell counts inside the gates were calculated after 72h drug treatment and normalized to the cell counts in the DMSO-treated wells (represented as percentages). (E) Venetoclax dose response curves of different cell populations present in acute monocytic leukemia (FAB M5) sample 18 assessed by FC and overall BM sensitivity with the CTG-based cell viability assay. (F) Representative FC scatter plots of drug effects on patient sample 5806 with FAB M5. (G) Dose response curves of different cell populations after MEK inhibitor trametinib treatment calculated with FC or overall sensitivity calculated with CTG. (H) Comparison of the drug sensitivity score (DSS) values for idarubicin, cytarabine and idarubicin+cytarabine combination in blasts between induction treatment resistant and sensitive patient samples using FC. (I) DSS measured with CTG from the same cohort. *P*-values calculated with Mann-Whitney U test.

this can be time consuming and enrichment might deplete cell populations such as monocytes that secrete cytokines important for blast cell survival and drug responses.[11–13]

To evaluate the *ex vivo* sensitivity of AML patient samples at a cell population level, we applied a multiplexed, 96-well format flow cytometry (FC)-based drug sensitivity assay. We compared this approach with the CTG-based cell viability assay to study potential inconsistencies between these two methods. Furthermore, we aimed to identify drugs and drug combinations that could effectively target leukemic blasts in physiologically relevant concentrations. In addition to standard of care drugs, cytarabine and idarubicin, we selected five Food and Drug Administration approved targeted small molecule inhibitors that have shown AML-selective responses in our earlier studies:[14,15] MEK inhibitor (trametinib), JAK1/2 inhibitor (ruxolitinib), mTORC1 inhibitor (everolimus), FLT3/broad range tyrosine kinase inhibitor (TKI, sunitinib) and Bcl-2 inhibitor (venetoclax). Most importantly, we demonstrate that targeted agents, particularly venetoclax, have different efficacies towards AML cells at distinct stages of myeloid differentiation.

Methods

Methods are described in more detail in the *Online Supplementary Material and Methods.*

Patient samples

BM samples from 34 AML patients and three healthy volunteers were obtained from the Helsinki University Hospital Comprehensive Cancer Center after informed consent (*permit numbers 239/13/03/00/2010, 303/13/03/01/2011, Helsinki University Hospital Ethics Committee*) and in compliance with the Declaration of Helsinki. The patient characteristics are presented in the *Online Supplementary Table S1.*

Preparation of drug plates

The compounds (*Online Supplementary Table S2*) were dispensed on 96-well V-bottom plates (Thermo Fisher Scientific, Carlsbad, CA) and 384-well plates (Corning, Corning, NY, USA) using an acoustic liquid handling device Echo 550 (Labcyte, Sunnyvale, CA). Drug plate layouts and concentrations are presented in *Online Supplementary Figure S1*. BM mononuclear cells (BM-MNC) were isolated using Ficoll-Paque Premium (GE Healthcare, Little Chalfont, Buckinhamshire, UK) density gradient centrifugation. Fresh or frozen BM-MNC were suspended in mononuclear cell medium (MCM; PromoCell, Heidelberg, Germany) supplemented with 10 μg/mL gentamicin and 2.5 μg/mL amphotericin B and plated in parallel on pre-drugged 96-well plates (100,000 cells/well in 100 μl) for FC analysis and 384-well plates (10,000 cells/well in 25 μl) for CellTiter-Glo® (CTG)-based cell viability assay. The cells were incubated with the drugs for 72 hours at 37°C and 5% CO_2.

FC-based readouts

Following the 72-hour incubation with the drugs, cells were stained with an antibody mix (CD33, CD45, CD14, CD38 and CD34) followed by apoptosis (Annexin-V) and dead (7-AAD) cell staining. A detailed description of the methods is presented in *Online Supplementary Material and Methods* and the gating strategy is illustrated in Figure 1B.

Cell viability analysis using CellTiter-Glo®

Parallel to FC analysis, cell viability was measured with CellTiter-Glo® (CTG; Promega, Madison, WI) in 384-well plates as described earlier.[14] After the 72-hour incubation with the drugs, 25 μL CTG was added to each well. The luminescence signal was measured using a PHERAstar plate reader (BMG LABTECH, Ortenberg, Germany).

Calculation of the drug sensitivity (DSS) and drug combination scores

Ex vivo drug sensitivity of AML and healthy BM cells to the tested drugs was calculated using a DSS as previously described.[16] Drug combination efficacies were calculated as the difference between observed and expected values. The expected value is computed using the Bliss independence model[17] as reference, which assumes that two drugs exhibit their effect independently.[18]

Gene expression and pathway analysis

Publicly available microarray data from the Hemap data set[19,20] (*http://hemap.uta.fi/*) and RNA-seq data (RSEM values) from the TCGA Research Network[21] (*http://cancergenome.nih.gov/*) also included in the Hemap resource were used for gene expression and pathway analysis. Beat AML data[22] was used to assess the correlation between venetoclax drug sensitivity and *BCL2* family and monocytic/granulocytic differentiation marker gene expression. For the analysis of gene expression in healthy hematopoietic cell types Differentiation Map data was used.[23] Detailed methods are described in the *Online Supplementary Material and Methods*.

Statistical analysis

Statistical analysis was conducted with Graph Prism version 7.0 (GraphPad Software, San Diego, CA). Differences between drug responses were analyzed by Mann-Whitney U test, and for multi-

Table 1. Median drug sensitivity score (DSS) and IC50 values of the seven tested drugs against different cell populations.

	Blasts (n=33)		Monocytes (n=18)		Lymphocytes (n=31)		Granulocytes (n=5)	
	DSS Median (Range)	IC50 (nM) Median (Range)	DSS Median (Range)	IC50 (nM) Median (Range)	DSS Median (Range)	IC50 (nM) Median (Range)	DSS Median (Range)	IC50 (nM) Median (Range)
Venetoclax	27.1 (0-43)	3.0 (1-1000)	7.1 (0-29)	122.0 (1-1000)	18.1 (9-30)	20.3 (2-84)	5.7 (0.3-9)	113 (11-220)
Idarubicin	22.0 (0-40.0)	28.7 (2-212)	16.1 (6-34)	78.7 (13-390)	16.5 (9-28)	84.0 (18-227)	19.0 (12-24)	41.1 (26-154)
Cytarabine	9.7 (0-36)	894.2 (50-10000)	7.5 (0-23)	1071 (20-10000)	4.8 (0-10)	2550 (43-10000)	9.5 (4-19)	953 (305-1189)
Ruxolitinib	5.0 (0-32)	302.7 (50-3000)	17.2 (0-37)	93.3 (60-2896)	0 (0-8)	2511 (99-10000)	0 (0.0-7)	2476 (246-3 000)
Trametinib	3.0 (0-27)	18.9 (1-250)	25.9 (0-42)	2.4 (1-250)	0 (0-1)	> 250 (7-250)	1.1 (0.0-7)	165 (15-250)
Sunitinib	1.0 (0-17)	321.1 (8-1000)	5.7 (3-22)	223.7 (71-423)	0 (0-4)	> 1000 (6-492)	4.4 (1-11)	352 (92-434)
Everolimus	0.0 (0-19)	55.6 (1-100)	4.6 (0-28)	7.5 (3-28)	0 (0-10)	> 100 (2.5-100)	0 (0.0-3)	> 100 (33-100)

ple *t*-tests *P*-values were adjusted using the Benjamin-Hochberg method ($P<0.10$ used to determine significance). The Kruskal-Wallis test was used when more than two groups were tested and significant comparisons were validated with *post-hoc* analysis (Dunn's test). Statistical dependence between two variables was assessed by Spearman's rank correlation.

Results

Analysis of the AML bone marrow compartment

To measure blast-specific drug responses in mononuclear cell (MNC) enriched BM AML samples, we tested 34 AML samples collected at diagnosis or relapse with seven drugs. Following a 72-hour drug treatment we analyzed the samples by both FC and CTG-based cell viability assays (Figure 1A). With the CTG assay we measured the overall BM-MNC sensitivity, while with the FC analysis the number of viable cells in different cell populations was measured. We used four cell surface markers (CD45, CD34, CD33, CD14) to identify the major leukocyte populations present in the AML BM: leukemic blasts, immature granulocytes, promonocytes/monocytes and lymphocytes (Figure 1B). In the studied samples, the fraction of blasts out of $CD45^+$ positive leukocytes varied between 17-92% and the lymphocyte population ranged from 1-49% (*Online Supplementary Table S3*). As expected, we observed high numbers of monocytic cells in FAB M4/5 samples, whereas M0/1 samples mainly consisted of blasts and lymphocytes (Figure 1C). After 72-hours in culture, we observed monocytic maturation in several M5 samples,[24] and in many samples the granulopoietic cell population diminished or was completely lost (*Online Supplementary Figure S2*).

FC *versus* homogeneous cell viability assay-based drug sensitivity profiling

In order to determine the correlation between drug sensitivity of the samples measured by FC or CTG-based methods, we converted the cell viability readouts from

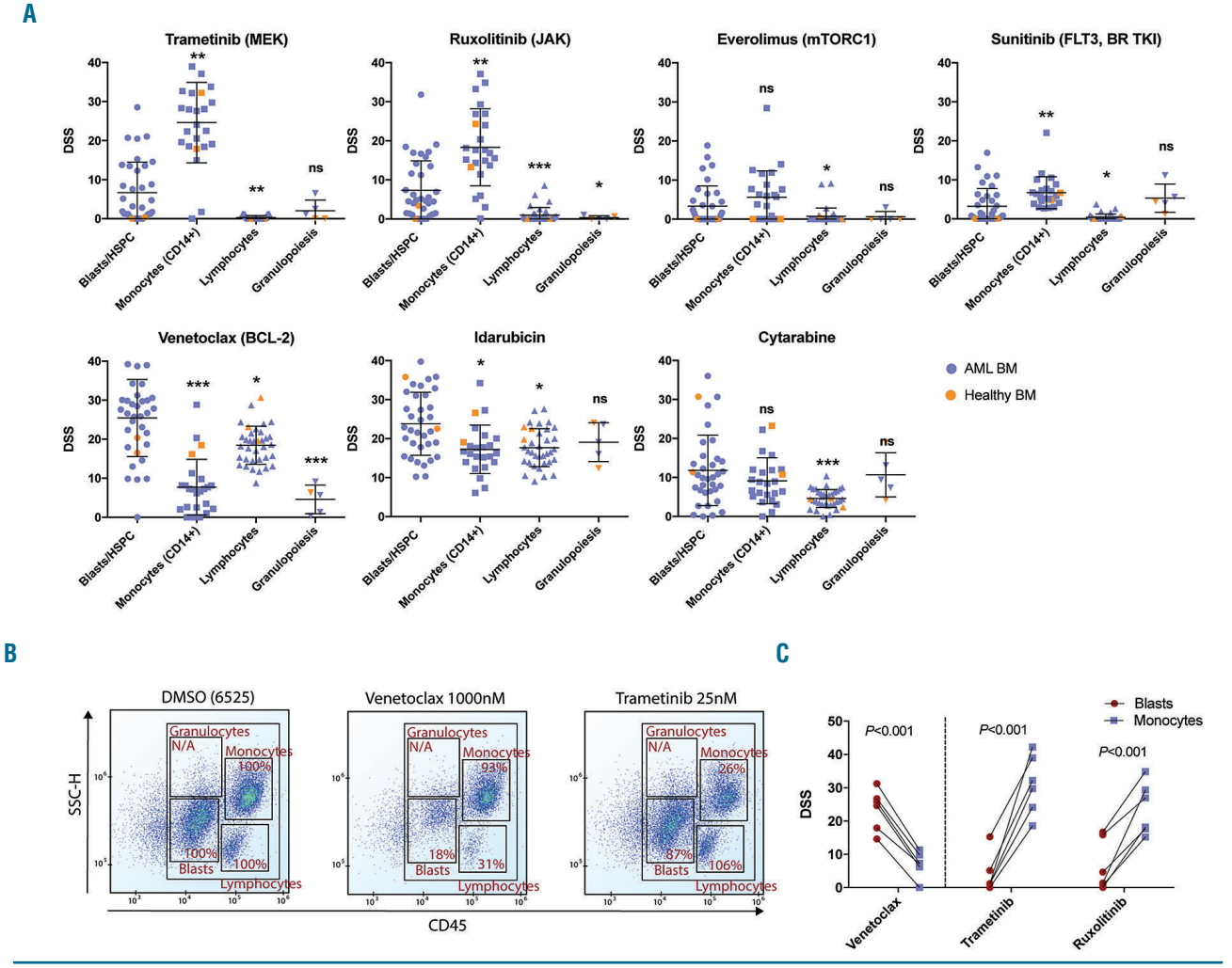

Figure 3. Maturation stage of acute myeloid leukemia (AML) cells affects drug sensitivities. (A) Drug sensitivity score (DSS) values for distinct cell populations in 33 AML samples (blue) and 2-3 healthy controls (orange). Cell population means were compared against blasts with Kruskal-Wallis test (Dunn's test, $*P<0.05$, $**P<0.001$, $***P<0.0001$). (B) Representative flow cytometric (FC) scatter plots of the effects of venetoclax and trametinib on blasts, monocytic cells ($CD14^+$) and lymphocytes after 72h drug treatment with the indicated concentrations. Absolute cell counts inside the gates were calculated after drug treatment and normalized to the cell counts in the DMSO-treatment wells (represented as percentages). (C) Inter- and intra-patient comparison of the DSS values in blasts and monocytic cell fraction calculated with Mann-Whitney U test. HSPC: healthy hematopoietic stem/progenitor cells.

each assay to DSS (a drug sensitivity metric based on area under the dose-response curve, higher DSS indicates higher sensitivity).[16] We observed a strong correlation between CTG and FC viability derived DSS when all live CD45$^+$ leukocytes were used as the FC readout (R=0.64, P<0.0001, Figure 2A), and when the blast-specific drug responses were exclusively taken as the FC readout from samples with blast counts over 50% (R=0.75, P<0.0001, Figure 2B). However, we observed poor correlation between the FC and CTG results in a sample cohort with blast counts below 50% (R=0.24, P=0.05, Figure 2C). The most prominent differences were seen in the response to trametinib and venetoclax (*Online Supplementary Figure S3*). The poor correlation was partly due to highly different drug sensitivities of the non-blast cell populations compared to blasts as demonstrated in two samples with low blast counts (Figure 2D-G). Our data shows that AML BM subpopulations have heterogeneous drug responses that confound the assessment of blast specific drug sensitivities when using homogenous cell viability assays in unsorted BM-MNC samples.

Ex vivo drug screening predicts induction therapy response

Next, we evaluated whether incomplete BM blast clearance at day 14 and day 28 after induction treatment was associated with decreased *ex vivo* drug sensitivity. We evaluated BM samples from 15 patients collected prior to anthracycline+cytarabine induction chemotherapy. Amongst these patients, five had >10% blast cells at day 14 and/or day 28 and were defined as chemoresistant as described in the *Online Supplementary Table S1*. Additionally, we included samples from two patients resistant to induction (collected at the time of resistant disease) in the chemoresistant group. A combined DSS of cytarabine and idarubicin showed significantly lower values for the resistant patients both with FC and (P<0.05, 2H) and CTG (P<0.01, Figure 2I). Furthermore, we observed a significant difference between responders and non-responders when blast-specific idarubicin response was measured with FC (P<0.05, Figure 2H) or total sample sensitivity was measured with CTG (P<0.05, Figure 2I). These results are in line with a recent study demonstrating that a similar FC-based platform can predict induction therapy response in a larger AML cohort.[25]

Blasts are highly sensitive to Bcl-2 inhibition whereas monocytes and granulocytes are resistant

Using the FC approach, we were able to evaluate blast-specific drug responses and compare them to other cell types within the same or between samples. Amongst the seven tested drugs, venetoclax (IC50=3.0nM) and idarubicin (IC50=28.7nM) showed the highest toxicity against blasts (Table 1). However, between these two drugs venetoclax showed the most selective efficacy against blasts when compared to other cell populations and healthy CD34$^+$ cells (Figure 3A, IC50 values in the *Online Supplementary Figure S4*). Moreover, venetoclax was also effective against CD34$^+$CD38$^-$ cells, which suggests activity against leukemic stem cells (*Online Supplementary Figure 5*). Compared to blasts, monocytic cells (CD14$^+$) were highly resistant to Bcl-2 inhibition (P<0.001, Mann-Whitney U test), but sensitive to MEK and JAK inhibition (P<0.001, Figure 3A). The phenomenon was clearly observed in samples from patients diagnosed with acute monocytic leukemia (M5) that contained substantial fractions of both cell types (Figure 3B-C).

Overall BM AML sample sensitivity to venetoclax is associated with FAB subtype

To follow-up on our findings, we hypothesized that AML samples with a high monocytic cell content should have a distinct drug response profile when overall BM-MNC sensitivity is measured with the CTG assay. We re-analyzed our earlier published CTG-based drug sensitivity data of 37 AML samples comprised of FAB M1, M2, M4 and M5 samples that were screened with 296 compounds.[14,15] Amongst the 296 compounds, venetoclax showed the largest drug sensitivity difference between M1 and M5 AML (P<0.001, *Online Supplementary Table S4*, Figure 4A). Similarly, the CTG-based sensitivity of the AML sample cohort studied here showed a gradual decrease in venetoclax sensitivity from M0 towards M5 subtype (Figure 4B). When we limited our FC analysis to diagnostic samples, a significant but smaller difference in blast-specific venetoclax sensitivity was also associated with FAB subtype (P<0.05, Figure 4C). This significance was not observed when we also included relapse and chemorefractory samples in the analysis (Figure 4D) largely due to a high number of chemorefractory M1/2 samples in our cohort that were more resistant to venetoclax (P<0.001, Figure 4D-E). Taken together, monocytic cells blur the high blast specific venetoclax effect in Ficoll-enriched M4/5 samples when measured with CTG but FAB subtype still has a significant effect on venetoclax response in blasts in our diagnosis AML sample cohort.

FAB subtype is associated with *BCL2* and *MCL1* gene expression

Anti-apoptotic Mcl-1 and Bcl-2 are considered the most important pro-survival factors in AML.[26,27] Furthermore, their expression and phosphorylation has been shown to be regulated through the Ras/Raf/MEK/ERK, PI3K/PTEN/AKT and JAK/STAT signal transduction pathways in different leukemias.[28–31] To study whether the expression of *BCL2* family members and activity of signal transduction pathways is associated with FAB subtypes, we analyzed gene expression data of MNC of diagnosis AML samples using publicly available microarray and RNA-seq data. *BCL2* was highly expressed in M0/1 AML and gradually decreased towards M5 samples and healthy monocytes (Figure 5A, *Online Supplementary Figure S6*). Notably, *MCL1* showed an opposite trend in expression and was most highly expressed in healthy monocytes (Figure 5A). We also detected higher expression of *BCL2A1, BCL2L11 (BIM), BID* and *JAK2* in M4/5 AML. A more detailed analysis of the healthy myeloid compartment revealed that *BCL2* family expression is highly dependent on differentiation stage, which likely also influences the expression patterns seen between the different FAB subtypes (Figure 5B). Interestingly, high *BCL2* and low *MCL1* expression was also observed in FAB M3 AML and their healthy counterparts, colony forming unit (CFU) granulocytes (Figure 5A-B). High *BCL2/MCL1* expression ratio in CFU granulocytes might explain the neutropenia seen in venetoclax treated patients.

Next, we investigated whether common cytogenetic abnormalities (*RUNX1-RUNX1T1, CBFB-MYH11, MLL, PML-RARA*) or mutations (*FLT3, NPM1, RUNX1, CEBPA*) explain some of the variations we observed in *MCL1*,

BCL2 or *BCL-xL* gene expression within FAB subgroups (*Online Supplementary Figure S7*). AML samples with *RUNX1T1-RUNX1T1* fusions showed significantly different gene expression exclusively in the M2 subgroup while samples with *MLL* or *CBFB-MYH11* fusions showed significantly different gene expression exclusively in the M4 subgroup (Figure 5C, *Online Supplementary Table S5*). Particularly, M4 samples with MLL fusions had high *BCL2* but low *MCL1* expression levels compared to other M4 samples. To assess whether major signal transduction pathways are differentially active in FAB subtypes, we performed gene set enrichment analysis (GSEA). The analysis revealed significant enrichment of gene sets associated with inflammatory signaling and IL6/JAK/STAT pathway in M4/5 AML (Figure 5D-E, *Online Supplementary Table S6*).

To study whether *ex vivo* venetoclax response is associated with differentiation markers and *BCL2* family expression, we analyzed the published Beat AML data set which includes data from 562 AML patients.[22] Supporting our previous findings, samples that had high expression of monocytic/granulocytic cell markers (CD14, CD11b, CD86, CD68) were resistant to venetoclax (Figure 5F). High *BCL2* expression was associated with venetoclax

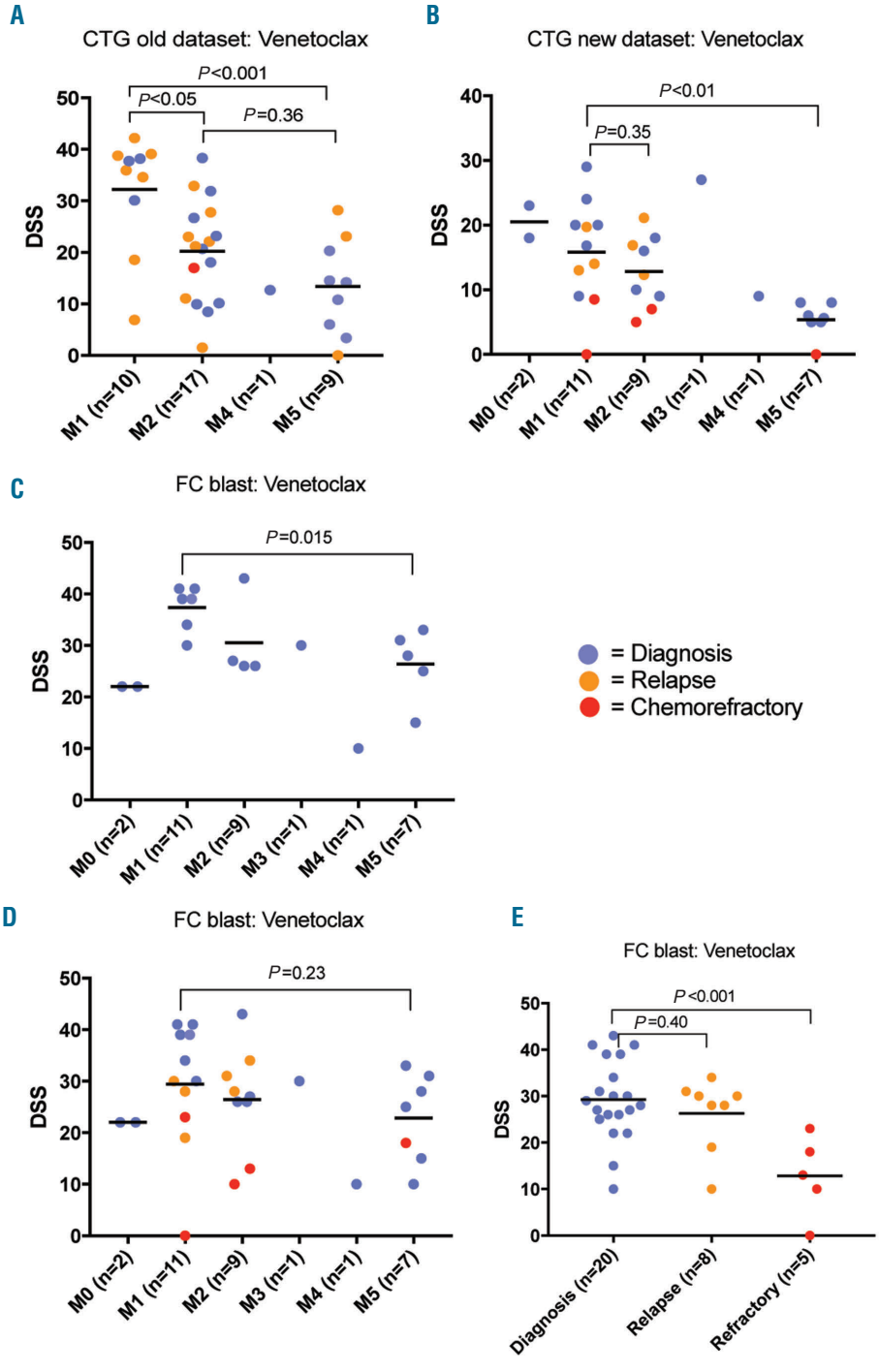

Figure 4. Mononuclear cell (MNC) fraction sensitivity to venetoclax is dependent on FAB subtypes. (A) Venetoclax drug sensitivity score (DSS) values of AML samples with different French-American-British (FAB) subtypes from an earlier published data set, and (B) from the present data set both measuring MNC fraction sensitivity with CTG based cell viability assay. (C) Blast-specific venetoclax sensitivity of diagnosis samples in FAB subgroups measured by FC from the present data set. (D) Blast-specific venetoclax sensitivity in different FAB subgroups measured by FC including chemorefractory and relapse samples. (E) Comparison of venetoclax DSS values between diagnosis, relapse and chemorefractory samples (induction resistant n=3, azacytidine resistant n=2). Black lines represent the mean of each subgroup. *P*-values calculated with the Kruskal-Wallis (and Dunn's) tests. FC: flow cytometric.

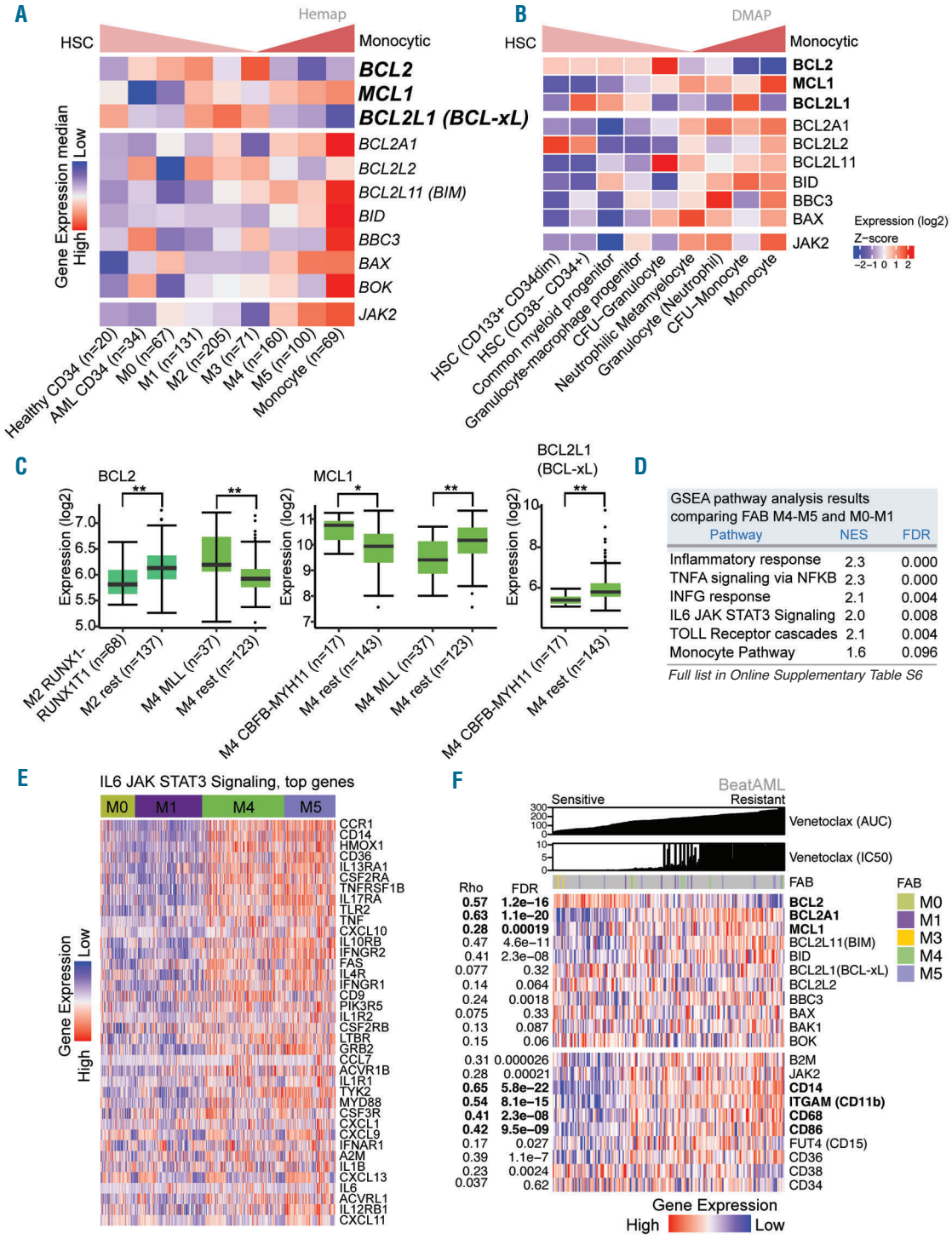

Figure 5. Cell differentiation is associated with low *BCL2* expression and venetoclax *ex vivo* resistance. (A) Heatmap of the median gene expression for each French-British-American (FAB) class and control samples are shown for *BCL2* family genes in the Hemap acute myeloid leukemia (AML) data set. Sample groups are ordered based on the differentiation state between HSC and healthy monocytes. *Z*-scores were used to define high and low expression categories. Z-scores were further discretized to low and high categories, defined as having *Z*-score cutoff over 2 for high and less than -2 for low expression. *P*-values for FAB subgroup comparisons are presented in the *Online Supplementary Table S5*. Similar analysis for TCGA data set is presented in the *Online Supplementary Figure S6*. (B) Heatmap of the median gene expression of *BCL2* family genes for healthy hematopoietic cell types using Differentiation Map data set. (C) Significant *BCL2*, *MCL1* or *BCL-xL* gene expression differences between samples with *MLL*, *CBFB-MYH11* or *RUNX1-RUNX1T1* fusion genes when compared to non-fusion gene containing samples in FAB M2 and M4 groups. Values obtained from the Hemap data set. **P*-value<0.05, ***P*-value<0.001. (D) Pathway enrichment results with normalized enrichment score (NES) and significance as false discovery rate (FDR) *q*-value are shown for pathways upregulated in M4 and M5 samples when compared to M0 and M1 samples. Pathways consistently enriched in both Hemap and TCGA data sets are shown here, while full results are shown in the *Online Supplementary Table S6*. (E) Heatmap of IL6-JAK-STAT3 signaling pathway leading edge gene expression Z-scores using the Hemap data set. Z-scores were further discretized to low and high categories, defined as having Z-score cutoff over 2 for high and less than -2 for low expression. Samples are ordered based on FAB type. (F) Venetoclax drug response AUC and IC50 profiles, *BCL2* family genes and differentiation marker gene expression value Z-scores and FAB subtypes are shown as a heatmap. Samples are ordered based on drug sensitivity with sensitive samples on the left and resistant on the right. Pearson correlation Rho and FDR value is shown for each gene.

sensitivity whereas high *MCL1* and *BCL2A1* expression was associated with resistance (Figure 5F). These findings were also presented earlier by two different research groups.[32,33]

Taken together, the gene expression data of mononuclear cell enriched AML samples indicate that M4/5 AML have low *BCL2* but high *MCL1* and *BCL2A1* expression and increased inflammatory signaling. Thus, the data support the decreased venetoclax sensitivity we observed with the total mononuclear cell fraction of M4/5 samples.

MEK and JAK inhibitors sensitize venetoclax-resistant blast cells to venetoclax

Next, we studied whether mutations might explain the observed differences in blast specific venetoclax responses, but no significant correlation between genetic lesions and venetoclax response in our limited patient cohort was found (*Online Supplementary Table S7*). However, as demonstrated earlier, we detected decreased venetoclax sensitivity in chemorefractory and M5 samples (*Online Supplementary Table S7*, Figure 4A-E). When we divided the AML samples into two subgroups (sensitive DSS 21-43, IC50<20nM and resistant DSS 0-21, IC50>20nM) from the mid-point of the venetoclax response range, we noticed that resistant blasts were sensitive to either MEK and/or JAK inhibitors (Figure 6A). This finding suggests that venetoclax resistant blasts rely on either JAK/STAT and/or MAPK pathways. Furthermore, venetoclax sensitive blasts were enriched for *NPM1* (8 of 25, 32% in sen-

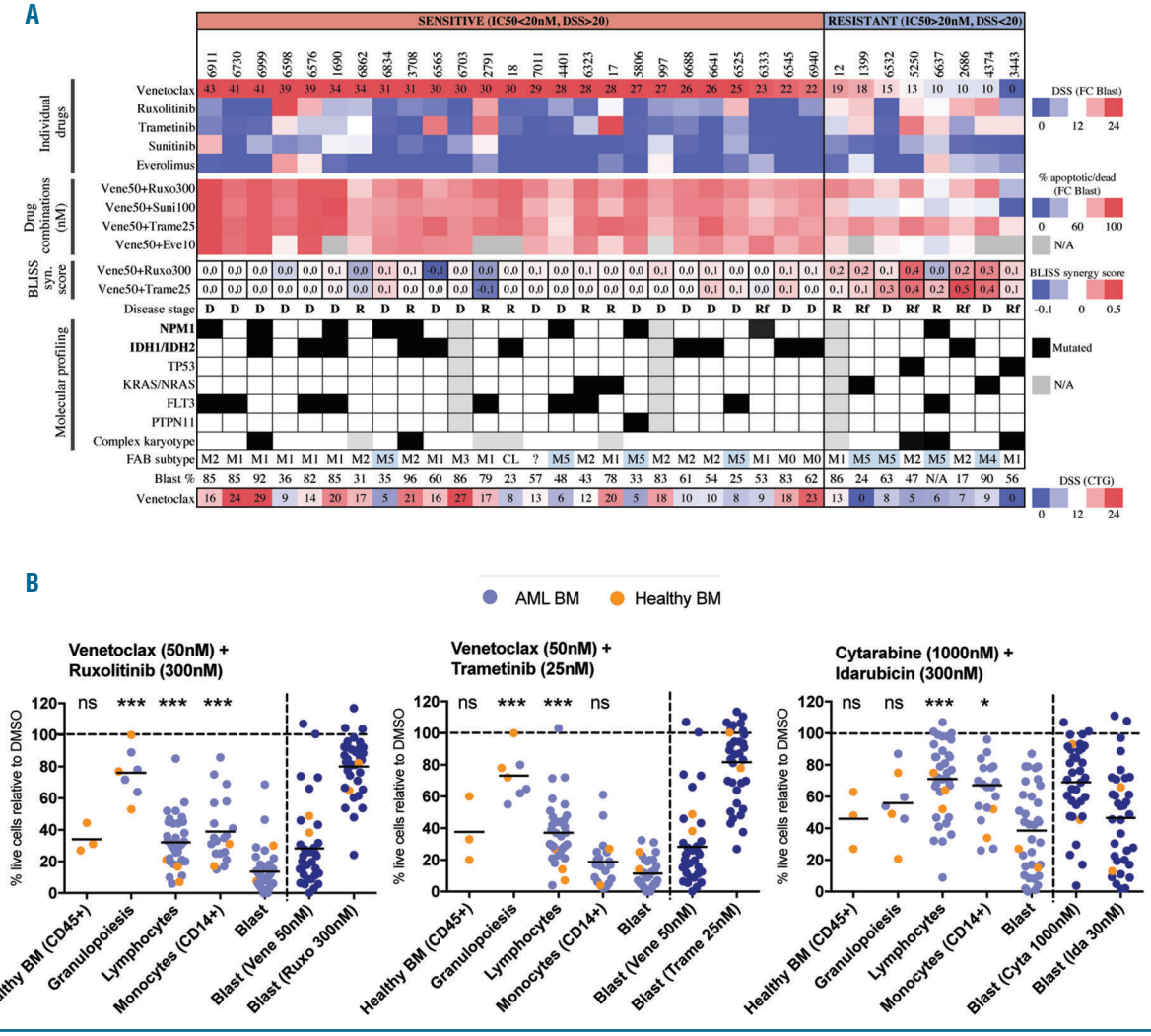

Figure 6. Inhibition of MEK and JAK pathways can overcome venetoclax resistance. (A) Heatmap showing characteristics of venetoclax sensitive (IC50<20nM, DSS>20) and resistant blasts (IC50>50nM, DSS<20) based on single agent venetoclax response measured by flow cytometry (FC) (top row). Blast-specific response of individual drugs is highlighted according to drug sensitivity score (DSS) values with red corresponding to high DSS value and blue to low DSS value. Blast-specific response to venetoclax combinations is highlighted according to percentage of apoptotic/dead cells with red corresponding to high percentage and blue to low percentage of apoptotic/dead cells. The synergistic effect of the drug combination was assessed based on the BLISS synergistic score and is shown in the graph. Other characteristics covered include disease stage, molecular profiling, French-American-British (FAB) subtype with M4 and M5 highlighted blue and FC-determined blast percentage. Overall BM venetoclax sensitivity measured with CellTiter-Glo (CTG) (bottom row) is used to demonstrate how low blast cell percentage affects DSS values when compared to blast-specific DSS values. (B) Dot scatter plots of venetoclax (50nm) + ruxolitinib (300nM), venetoclax (50nM) + trametinib (25nM), and cytarabine (1000nM) + idarubicin (30nM) responses in healthy CD45$^+$ leukocytes, granulopoietic cells, lymphocytes, monocytes and blasts. Orange dots represent healthy BM samples and light blue dots acute myloid leukemia (AML) samples. Dark blue dots represent single agent toxicity to blasts. Cell population means were compared against blasts with the Kruskal-Wallis test (Dunn's test, *P<0.05, **P<0.001, ***P<0.0001). R: relapse; Rf: refractory; CMML: chronic myelomonocytic leukemia; CL: CMML transitioned to AML.

sitive and 1 of eight, 12.5% in resistant) and *IDH1/IDH2* (10 of 25, 40% in sensitive and 1/8, 12.5% in resistant) mutations supporting the good clinical activity of venetoclax seen in this patient group (Figure 6A, *Online Supplementary Table S7*).

To assess the efficacy and clinical relevancy of 27 drug combinations against blasts, we used concentrations achieved in patients' plasma during treatment. The results demonstrated prominent inter-patient variability with the most synergistic drug combinations when blast-specific drug responses were measured by FC (*Online Supplementary Figure S8*). Of the 27 tested drug combinations, venetoclax plus kinase inhibitors showed the highest average synergistic and blast killing effect (Table 2, higher BLISS score and lower mean % live blasts). Importantly, blasts were highly sensitive to single-agent venetoclax in 76% (25 of 33) of the samples with IC50<20nM. Thus, we did not observe synergy in the majority of the samples with a single venetoclax concentration of 50nM as this concentration alone was sufficient to kill the blasts (Figure 5A). To study the drug combination effect in more detail, we conducted additional drug testing of venetoclax with a more detailed concentration range on four AML samples. We observed that with lower venetoclax concentrations (10nM) a synergistic effect with MEK and/or JAK inhibitors was also detected in samples that were sensitive to single agent 50nM venetoclax treatment (*Online Supplementary Figure S9*).

Importantly, venetoclax (50nM) plus ruxolitinib (300nM) showed high efficacy (apoptosis/death>70%) and synergism in 6 of eight venetoclax resistant samples (Figure 5A-B). Strikingly, by combining venetoclax (50nM) with trametinib (25nM), all venetoclax resistant blasts were effectively targeted (Figure 6A-B). Although the combinations showed substantial toxicity to healthy $CD34^+$ cells, they targeted most effectively leukemic blasts (Figure 6B). As a comparison, a drug combination used during induction treatment (cytarabine+idarubicin) showed remarkable inter-patient differences in blast toxicity and it was also toxic to healthy $CD34^+$ cells (Figure 6B). Furthermore, the broad-spectrum tyrosine kinase and FLT3 inhibitor sunitinib (100nM) or mTOR inhibitor everolimus (10nM) were not as effective when combined with venetoclax (Figure 6A, Table 2). Our data demonstrate that by simultaneously inhibiting JAK and/or MEK signaling and Bcl-2, blast cells involving chemorefractory AML cells, can be effectively targeted *ex vivo* in physiologically relevant concentrations.

Table 2. Drug combination synergism and combination sensitivity in blasts.

Drug I	Drug II	Drug III	Mean BLISS score*	Mean % of live blasts**
Venetoclax 50nM	Trametinib 25nM		0.083	11.3
Cytarabine 300nM	Trametinib 25nM		0.076	56.7
Trametinib 25nM	Everolimus 10nM		0.075	62.9
Venetoclax 50nM	Ruxolitinib 300nM		0.068	13.4
Idarubicin 10nM	Trametinib 25nM		0.057	51.3
Trametinib 25nM	Ruxolitinib 300nM		0.046	59.7
Venetoclax 50nM	Everolimus 10nM		0.040	18.6
Sunitinib 100nM	Trametinib 25nM		0.034	69.0
Venetoclax 50nM	Sunitinib 100nM		0.030	21.8
Idarubicin 10nM	Ruxolitinib 300nM		0.029	61.8
Venetoclax 50nM	Cytarabine 300nM		0.023	19.6
Sunitinib 100nM	Ruxolitinib 300nM		-0.004	73.3
Sunitinib 100nM	Everolimus 10nM		-0.001	80.8
Everolimus 10nM	Ruxolitinib 300nM		-0.018	73.9
Cytarabine 300nM	Sunitinib 100nM		-0.020	75.9
Idarubicin 10nM	Cytarabine 300nM		-0.026	64.9
Idarubicin 30nM	Cytarabine 1000nM		-0.029	40.1
Idarubicin 10nM	Everolimus 10nM		-0.038	69.5
Cytarabine 300nM	Everolimus 10nM		-0.046	80.1
Everolimus 10nM	Ruxolitinib 300nM	Trametinib 25nM	-0.051	52.4
Cytarabine 300nM	Ruxolitinib 300nM		-0.058	72.2
Sunitinib 100nM	Everolimus 10nM	Ruxolitinib 300nM	-0.067	63.8
Idarubicin 10nM	Ruxolitinib 300nM	Trametinib 25nM	-0.073	39.6
Idarubicin 10nM	Sunitinib 100nM		-0.088	74.7
Cytarabine 300nM	Ruxolitinib 300nM	Everolimus 10nM	-0.123	62.5
Cytarabine 300nM	Ruxolitinib 300nM	Trametinib 25nM	-0.129	54.4
Idarubicin 10nM	Ruxolitinib 300nM	Everolimus 10nM	-0.146	57.3

*Synergism calculated using BLISS score **Normalized to DMSO treated cells.

Discussion

With FC-based drug testing we were able to simultaneously measure drug sensitivities of different cell populations in primary AML BM samples. Monocytic cells abundantly present in FAB M4/5 AML were markedly resistant to the Bcl-2 inhibitor venetoclax, while less differentiated blast cells in the same M4/5 samples or in M0/1/2 samples were sensitive. Accordingly, the overall BM-MNC sensitivity to venetoclax was strongly influenced by FAB subtype. Our study shows that FC-based, phenotypic drug testing can improve the current understanding of *ex vivo* drug effects and may help to identify blast-specific treatments for AML patients.

Along with our previous studies, several other groups have evaluated *ex vivo* drug responses of Ficoll-enriched AML mononuclear cells using high-throughput CTG or MTS based cell viability assays.[14,34–36] While these assays provide fast and robust readouts they fail to accurately measure blast specific drug responses. By using more accurate microscopy based screening, Snijder *et al*. have recently demonstrated that blast specific or relative blast fraction-based readouts increase predictive accuracy to treatment outcome.[37] Similarly, Martinéz-Cuadrón *et al*. showed that a FC-based platform measuring blast specific effect in whole BM without MNC enrichment, predicted clinical response to induction therapy.[25] We also showed earlier that in chronic myeloid leukemia, CD34-depleted cells (mature granulopoietic cells) were insensitive to BCR-ABL-1 inhibitors *ex vivo* whereas $CD34^+$ progenitor cells showed good sensitivity.[38] In accordance, we demonstrate here with a FC-based approach that blasts differ in their drug sensitivities in comparison to other cell populations in the same AML samples. The highest blast-specific efficacy was observed with venetoclax, whereas ruxolitinib and trametinib showed increased activity towards monocytic cells. Importantly, we demonstrate that in samples with a low blast count, the overall mononuclear cell fraction sensitivity does not correlate well with the blast-specific drug sensitivity.

Consistent with our results, earlier studies have shown that primary AML samples are sensitive to venetoclax *ex vivo*.[15,39,40] Most of the studies have used mononuclear cell fractions to assess cell viability and to measure protein and gene expression levels. We observed that mononuclear cells of M0/1 samples that mainly consisted of blasts, were sensitive to venetoclax compared to mononuclear cells of M4/5 samples when using a homogenous CTG-based cell viability assay. Earlier, high *ex vivo* sensitivity to Bcl-2 inhibition has been associated with M3 AML in a study by Niu *et al*., whereas Pan *et al*. found no associations with FAB subtypes.[39,40] Importantly, both study cohorts lacked comprehensive spectra of different subtypes, with none or only one M0/1 AML case. To support our observation, mononuclear cells of M0/1 samples had a high *BCL2/MCL1* gene expression ratio whereas M4/5 samples had a low ratio. Increased Bcl-2 protein expression has also been reported in M0/1 AML,[41] and increased Mcl-1 expression in M4/5 AML[26] of which the latter has been linked to elevated Mcl-1 expression in differentiating monocytes.[42] Accordingly, we observed high *MCL1* and *BCL2A1* but low *BCL2* expression in healthy monocytic and granulocytic cell populations.

By using a FC-based approach, we observed that several M5 samples contained venetoclax-sensitive blasts and a resistant monocytic cell fraction. This observation raises the question whether drug sensitivity profiling and gene/protein expression studies should focus on the immature blast cells and not the total MNC fraction especially in in M4/5 samples and samples with low blast count. When we compared the FC measured blast-specific venetoclax response between FAB subtypes, we observed a smaller but still significant difference between diagnosis M1 *versus* M5 subgroups. In clinical trials, *NPM1, IDH1/2* and *RUNX1* mutations have shown to be promising biomarkers for venetoclax+HMA treatment.[7,43] Based on a study analyzing genotype and FAB subtype-specific patterns of 4,373 adult *de novo* AML cases,[44] both *IDH1/2* and *RUNX1* mutations are enriched in M0/1/2 AML whereas *NPM1* mutations are common in FAB M1/2/4/5 subtypes. Therefore, patient cohorts with mutated *IDH1/2* or *RUNX1* may be skewed to contain larger numbers of FAB M0/1/2 samples. To identify responders, it might be useful to evaluate the combined genetic and cell phenotype/FAB subtype information in a clinical setting.

With the FC method we also looked for effective combinations, since an overall response rate of only 19% was observed with venetoclax monotherapy in patients with high-risk relapsed/refractory (R/R) AML.[6] In our study, all venetoclax-resistant blasts showed sensitivity to MEK and/or JAK inhibitors suggesting that JAK/STAT and MAPK pathways play a major role in venetoclax resistance. We showed earlier that stromal cell secreted cytokines such as GM-CSF mediate resistance to venetoclax, which can be counteracted by JAK inhibition.[45] Moreover, the MAPK pathway plays a critical role in resistance through the proposed upregulation of *MCL1*.[28] Both of these studies also demonstrated remarkable antileukemic activity in murine xenograft models when inhibiting JAK or MEK kinases together with *Bcl-2*. In agreement with the good synergism between ruxolitinib or trametinib with venetoclax observed here and in a recent study by the Beat AML study group,[46] Kurtz *et al*. additionally showed that several different kinase inhibitors exhibited good synergism with venetoclax in AML samples.[47] However, a recent clinical study with MEK inhibitor cobimetinib and venetoclax in R/R AML was closed due to limited clinical activity demonstrating that *ex vivo* drug screening results might not directly translate into a clinical setting.[48]

Inflammatory pathways are more active in M4/5 AML based on GSEA, consistent with the observed high sensitivity of monocytic cells to ruxolitinib and trametinib. Earlier studies have demonstrated that leukemic cells of patients with M4/5 AML produce IL1/IL6[13] and have a higher proliferative activity in cytokine-free medium.[49] Thus, secreted cytokines and culturing conditions may have a big impact on the drug sensitivity profiles. While further investigation is warranted, results suggest that the JAK/STAT and MEK pathways are more active in differentiated monocytic cells as well as in venetoclax resistant blasts.

In summary, we show that *ex vivo* sensitivity of AML patient samples to venetoclax is associated with cell composition. Furthermore, we demonstrate that FC-based drug screening could be implemented to identify effective targeted drugs and drug combinations against immature blasts, accelerating drug discovery and individualizing therapy for AML patients.

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