Acute myeloid leukemia (AML) is a genetically heterogeneous disease characterized by the accumulation of immature myeloid blasts in the bone marrow. While conventional chemotherapy usually results in initial reduction of leukemic blasts in the majority of patients, disease relapse is frequent, especially in elderly patients. Disease relapse is likely driven by leukemia stem cells that are not affected by chemotherapy and therefore retain their disease-initiating properties. Further characterization of this leukemia stem cell population is therefore highly relevant.

In hematopoietic cells, the stage of differentiation and metabolic properties are closely linked. Hematopoietic stem cells characteristically have low levels of mitochondrial oxidative metabolism and consequently low levels of reactive oxygen species (ROS), which is relevant for...
their function. A similar concept seems to apply to leukemic cells: within the total mononuclear AML cell population, leukemia stem cells were shown to be predominantly in the cell fraction with relatively low levels of ROS.

While previous studies highlighted that leukemia stem cells have lower ROS levels compared to the remaining mononuclear AML cells, in the present study we investigated how distinct ROS levels within the stem-cell-enriched CD34⁺ AML cell fraction correlate with cellular characteristics such as morphology, gene expression, metabolic activity and drug responsiveness. We found that CD34⁺ AML cells with low ROS levels are smaller, have a significantly increased expression of genes associ-

Figure 2. CD34⁺ acute myeloid leukemia cells with low or high levels of reactive oxygen species show altered metabolic activity. (A) Electron microscopy (EM) images of acute myeloid leukemia (AML) cells with low or high levels of reactive oxygen species (ROS-low and ROS-high, respectively). Samples from two AML patients are shown. Mitochondrial structures are outlined in red. (B) Number of mitochondrial structures in ROS-low and ROS-high cells quantified from EM images. Per sample, 25-72 cells were scored (n=4). Two-way analysis of variance revealed a significant impact of ROS levels on the number of mitochondrial structures (P=0.0001). (C) Mitochondrial copy number was calculated by comparing levels of mitochondrial and nuclear DNA measured by quantitative reverse transcriptase polymerase chain reaction. Relative fold-change between ROS-low and ROS-high CD34⁺ AML cells is shown (n=6). (D) Cytoplasm surface area of ROS-low and ROS-high CD34⁺ AML cells on EM images was determined using ImageJ software. Per sample, 25-72 cells were scored (n=4). (E) The ratio calculated from average cytoplasm surface area and average number of mitochondrial structures for the ROS-low and ROS-high cell fraction is shown. (F) Western blots indicating TOM20 and β-ACTIN protein levels in ROS-low and ROS-high sorted CD34⁺ AML cells (left panel). Numbers indicate relative TOM20 levels normalized to β-ACTIN, and a summary of this quantification is shown in the right panel (n=5). (G) ATP levels of ROS-low and ROS-high sorted CD34⁺ AML cells are shown (n=9). (H) Measurement of mitochondrial membrane potential using the fluorescent dye tetramethylrhodamine methyl ester (TMRM). Mean fluorescence intensity (MFI) of ROS-low and ROS-high sorted CD34⁺ AML cells is shown (n=8). (I) Fluorescence intensity of staining with Rhodamine 123 (Rh123) in ROS-low and ROS-high sorted CD34⁺ AML cells is shown. The staining was performed in the presence of cyclosporine A to prevent dye efflux (n=5). (C-I) Error bars indicate standard deviation. *P<0.05; **P<0.01; ***P<0.001; ns=not significant.
ated with stemness (e.g., ABCB1, MEIS1, CD109) and negative regulation of signaling (e.g., SPRY1, DUSP10, PIK3IP1), and show increased sensitivity to the BCL2 inhibitor venetoclax, an effect which was not linked to an increased expression of BCL2, but which might be related to an increased expression of the MCL1 regulator NOXA and the BCL2L1 repressor ATF3.

We compared morphological characteristics of CD34+ AML cells with low or high ROS levels, which were defined as cell fractions with the 15% lowest and 15% highest signal intensity for a fluorescence-based ROS dye (Figure 1A). Among AML patients with various genetic backgrounds, ROS-low CD34+ cells were consistently smaller than their ROS-high counterparts (Figure 1B, C). ROS-low and ROS-high sorted CD34+ cells maintained a distinctive ROS-state for up to 1 week in culture (Online Supplementary Figure S1A, B), indicating that ROS levels in CD34+ AML cells do not fluctuate greatly and that cell sorting based on ROS levels separates two distinct cell entities. Notably, we confirmed the presence of leukemia-associated mutations in both the ROS-low and ROS-high fractions. In some samples, the co-mutational pattern was different between the two fractions, likely reflecting different leukemic subclones (Online Supplementary Figure S1C, D and Online Supplementary Table S1).

RNA sequencing was used to gain insight into transcriptional differences between ROS-low and ROS-high CD34+ AML cells (n=4). Due to high biological variance, principal component analysis showed that samples clustered based on the patients’ samples and not on ROS levels (Online Supplementary Figure S2A). Differential expression analysis identified 58 high confidence genes that were commonly upregulated and 148 that were commonly downregulated in the ROS-low AML CD34+ cell fraction compared to the ROS-high AML CD34+ fraction (Figure 1D, Online Supplementary Figure S2B, C). Gene ontology analysis revealed that genes upregulated in the ROS-low fraction were associated with negative regulation of signaling, whereas downregulated genes were associated with increased cell differentiation (Figure 1E). Validation of RNA-sequencing data by quantitative real-time polymerase chain reaction in additional AML samples (n=8) demonstrated that ROS-low CD34+ AML cells

Figure 3. CD34+ acute myeloid leukemia cells with low levels of reactive oxygen species have increased sensitivity to the BCL2 inhibitor venetoclax. (A) Western blots indicating protein expression of BCL2; HDAC1 and β-ACTIN served as loading controls; Numbers indicate relative BCL2 levels normalized to HDAC1 or β-ACTIN. (i) Expression of the indicated proteins is shown for the fractions of cells with low or high levels of reactive oxygen species (ROS-low and ROS-high, respectively) sorted from CD34+ acute myeloid leukemia (AML) cells. (ii) Expression of the indicated proteins is shown from the ROS-low and ROS-high fractions of the total mononuclear cell population (MNC) or CD34+ AML cells. (B) ROS-low and ROS-high sorted CD34+ AML cells were treated for 24 h with 100 nM of the BCL2 inhibitor venetoclax. The reduction of viability compared to dimethylsulfoxide-treated cells (indicated by 4′,6-diamidino-2-phenylindole staining) is shown. (C) Cells were treated, as in (B), for 24 h with various concentrations of the MCL1 inhibitor S63845. (D) ROS-low and ROS-high sorted CD34+ AML cells were treated for 24 h with a combination of 100 nM venetoclax and increasing concentrations of the MCL1 inhibitor S63845. One-way analysis of variance revealed a dose-dependent effect of addition of MCL1 on the viability of CD34+ ROS-high but not of ROS-low AML cells. (B-D) Error bars indicate the standard deviation.

*P<0.05; **P<0.01; ***P<0.001; ns=not significant.
have significantly increased expression of stemness-associated genes (ABCB1, MEIS1, CD109, GFI1B), genes related to negative regulation of signaling (SPRY1, DUSP10, PIK3IP1, DDIT4) and apoptosis (NOXA, ATF3) (Figure 1F, Online Supplementary Figure S3). High expression of the drug-efflux transporter ABCB1 is associated with therapy resistance and poor prognosis in AML, and functional validation of ABCB1 activity by transporter activity assays showed almost exclusive activity in the ROS-low CD34+ AML cells (Figure 1G, Online Supplementary Figure S4A). In line with the increased expression of genes related to stemness, we observed an increased percentage of CD34+CD38- cells in the ROS-low fraction (Figure 1H).

Notably, the observed transcriptional differences between ROS-low and ROS-high CD34+ cells were not AML-specific, since increased expression of most of these genes could also be found in the ROS-low fraction of normal CD34+ cells isolated from cord blood or mobilized peripheral blood mononuclear cells (Online Supplementary Figures S3 and S5A). Further similarities between normal and leukemic CD34+ ROS-low cells were their small size (Online Supplementary Figure S5B, C) and a higher frequency of CD34+CD38- cells (Online Supplementary Figure S5D). To functionally confirm the relevance of increased stemness-related gene expression, long-term culture-initiating cell assays were performed on three distinct cell fractions from cord blood and peripheral blood mononuclear cells: ROS-low/CD34+/CD38-, ROS-low/CD34+/CD38- and ROS-high/CD34+ (Online Supplementary Figure S5E). For both cord blood and peripheral blood mononuclear cells, a significantly higher frequency of long-term culture-initiating cells was found in the ROS-low cells compared to ROS-high cells (Online Supplementary Figure S5F), with the highest frequency observed in ROS-low/CD34+/CD38- cord blood cells.

Although ROS production is strongly correlated with mitochondrial activity, our RNA-sequencing data did not show differentially expressed genes related to mitochondrial energy metabolism in ROS-low versus ROS-high CD34+ AML cells. Analysis of mitochondrial characteristics by electron microscopy revealed fewer mitochondrial structures in ROS-low CD34+ AML cells (Figure 2A, B), which was in line with a lower mitochondrial DNA copy number (Figure 2C). However, ROS-low CD34+ AML cells had a smaller cytoplasm area (Figure 2D), and the ratio calculated from cytoplasm area and number of mitochondria was similar in both fractions (Figure 2E). This suggests that ROS-low cells contain a lower absolute number of mitochondria compared to ROS-high cells, but their mitochondrial content relative to their cell size is the same. Consistent with that, we observed comparable TOM20 protein expression in both fractions when the signal was normalized to β-ACTIN (Figure 2F). ROS-low AML CD34+ cells had significantly lower amounts of ATP (Figure 2G) and the fluorescence of the tetramethylrhodamine indicated lower levels of mitochondrial membrane potential in this fraction (Figure 2H). To exclude that ROS-low and ROS-high cells might metabolize fluorescence dyes differentially, mitochondrial membrane potential was also measured using Rhodamine 123 in the presence of cyclosporine A, which blocks drug efflux transporters. These measurements yielded similar results (Figure 2I).

It has been shown that ROS-low AML cells from the mononuclear cell fraction are more sensitive to inhibition of the anti-apoptotic mitochondrial regulator BCL2 compared to ROS-high cells, which was linked to increased BCL2 expression at both RNA and protein levels. Furthermore, combination of the BCL2 inhibitor venetoclax and azacitidine was shown to target leukemia stem cells in AML patients. Notably, our RNA-sequencing data did not reveal increased BCL2 expression in ROS-low CD34+ AML cells compared to ROS-high CD34+ AML cells, nor did we observe a consistent overexpression of BCL2 protein (Figure 3A). Despite this, we observed that the ROS-low CD34+ AML cells were significantly more sensitive to venetoclax treatment compared to their ROS-high counterparts (Figure 3B, Online Supplementary Figure S6A), whereas ROS-low CD38 and CD38 cells were equally efficiently targeted (Online Supplementary Figure S6B). This seems to be a venetoclax-specific effect, since treatment of ROS-low and ROS-high CD34+ AML cells with the MCL1 inhibitor S63845 only mildly affected the viability of both cell fractions (Figure 3C). While combined treatment with both venetoclax and S63845 had no additional effect on the AML CD34+ ROS-low fraction, it eventually also eliminated ROS-high cells in a dose-dependent manner (Figure 3D). This supports the notion that the anti-apoptotic proteins BCL2 and MCL1 are both involved in regulating AML cell survival, but demonstrates that ROS-low cells have increased BCL2 dependency. Considering the role of BCL2 as a mitochondrial regulator, we wondered if ROS-low cells are generally more sensitive to a direct block of mitochondrial activity. However, treatment of ROS-low and ROS-high CD34+ AML cells with the mitochondrial uncoupler FCCP targeted both cell fractions (Online Supplementary Figure S7).

In summary, our data highlight that CD34+ AML cells with low ROS levels are smaller, have stemness-related features and thereby most likely coincide with the leukemia stem cell population. ROS-low CD34+ AML cells showed increased expression of genes previously linked to stress- and drug-resistance and poor prognosis in AML patients (e.g., CD109, MEIS1, ABCB1), highlighting that targeting this population is crucial for successful AML therapy. We show that ROS-low AML CD34+ cells are highly sensitive to BCL2 inhibition by venetoclax, which could not be explained by higher BCL2 expression in this fraction, but is potentially related to increased expression of additional apoptosis regulators such as NOXA or ATF3, which are involved in inactivation of the anti-apoptotic proteins MCL1 and BCL2L1, respectively. Notably, MCL1 expression was shown to correlate inversely with sensitivity to BCL2 inhibition in AML, whereas recently impaired ATF3 expression was associated with resistance to the combination of venetoclax and ibrutinib in mantle cell lymphoma.


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