Chemotherapy-Resistant Human Acute Myeloid Leukemia Cells Are Not Enriched for Leukemic Stem Cells but Require Oxidative Metabolism

ABSTRACT
Chemotherapy-resistant human acute myeloid leukemia (AML) cells are thought to be enriched in quiescent immature leukemic stem cells (LSC). To validate this hypothesis in vivo, we developed a clinically relevant chemotherapeutic approach treating patient-derived xenografts (PDx) with cytarabine (AraC). AraC residual AML cells are enriched in neither immature, quiescent cells nor LSCs. Strikingly, AraC-resistant preexisting and persisting cells displayed high levels of reactive oxygen species, showed increased mitochondrial mass, and retained active polarized mitochondria, consistent with a high oxidative phosphorylation (OXPHOS) status. AraC residual cells exhibited increased fatty-acid oxidation, upregulated CD36 expression, and a high OXPHOS gene signature predictive for treatment response in PDx and patients with AML. High OXPHOS but not low OXPHOS human AML cell lines were chemoresistant in vivo. Targeting mitochondrial protein synthesis, electron transfer, or fatty-acid oxidation induced an energetic shift toward low OXPHOS and markedly enhanced antileukemic effects of AraC. Together, this study demonstrates that essential mitochondrial functions contribute to AraC resistance in AML and are a robust hallmark of AraC sensitivity and a promising therapeutic avenue to treat AML residual disease.

SIGNIFICANCE: AraC-resistant AML cells exhibit metabolic features and gene signatures consistent with a high OXPHOS status. In these cells, targeting mitochondrial metabolism through the CD36-FAO-OXPHOS axis induces an energetic shift toward low OXPHOS and strongly enhanced antileukemic effects of AraC, offering a promising avenue to design new therapeutic strategies and fight AraC resistance in AML. Cancer Discov; 7(7); 716–35. © 2017 AACR.

See related commentary by Schimmer, p. 670.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by a blockade in differentiation of hematopoietic stem cells and a clonal expansion of myeloid blasts in the bone marrow and peripheral blood. Standard “7 + 3” induction therapy, which combines a nucleoside analogue such as cytarabine (AraC) for 7 days with an anthracycline for 3 days, is highly effective in killing leukemic cells in AML. Despite a high rate of complete remission after these cytotoxic agents, the 5-year overall survival is very poor, especially in patients over 60 years of age. Indeed, most patients relapse and only allologic stem cell transplant is then curative (1, 2). Relapses are caused by tumor regrowth initiated by chemoresistant leukemic cells (RLC). Many hypotheses have been proposed to explain therapeutic resistance (drug efflux, detoxification enzymes, poor accessibility of the drug to the leukemic niche; refs. 3, 4), but none led to a complete understanding of the molecular mechanisms of AML resistance, especially in vivo, nor to new therapies which would effectively eradicate RLCs.

It is also increasingly recognized that the causes of chemoresistance may reside in rare stem cell populations (5, 6). Several laboratories have shown that the presence of high levels of leukemic stem cells (LSC; CD34+CD38−/lo−CD123+ cells) at diagnosis correlates with adverse outcome in patients with AML in terms of response to therapy and overall survival (7, 8). These and other studies support the notion that chemoresistant cells represent LSCs (9, 10), although this hypothesis has never been formally tested in vivo with clinically relevant doses. Recent research in our and other laboratories focus-
ing on the phenotypic characterization of LSCs in highly immunodeficient NSG mice showed that LSCs are phenotypically heterogeneous in AML in vivo (11–14). Moreover, recent data suggested that LSCs are influenced by clonal genetic evolution, epigenetic alterations, and their microenvironment, suggesting that they are themselves heterogeneous, especially with regard to their chemoresistance capacities in vivo (15). AraC is used both in combination regimens for induction and as a single agent for postremission therapy in patients with AML. In cells, AraC is rapidly converted into AraC-triphosphate, which is incorporated into DNA strands during the S-phase of the cell cycle inhibiting further DNA synthesis (16, 17), thereby affecting preferentially rapidly dividing cells. Accordingly, RLCs are thought to be rare, quiescent, and well adapted to hypoxic conditions (18–20).

Here, to exhaustively characterize the response of AML cells to AraC therapy, we treated 25 naïve patient-derived xenografts (PDX) with a clinically relevant sublethal regimen of AraC, also used in previous studies (21, 22). At the nadir of leukemic cell burden, in vivo AraC treatment has a strong cytoreductive effect mediated by death of both proliferating and quiescent AML cells. Surprisingly and as opposed to previous studies (9, 10), this cytoreduction was not associated with any consistent changes in stem cell functions, such as CD34+CD38− phenotype, G0 status, stem cell gene markers/signature, or frequency of leukemia-initiating cells (LIC). Rather, we showed that AraC residual cells have mitochondrial-specific oxidative and bioenergetics features. Furthermore, we identified a specific high oxidative phosphorylation (OXPHOS) gene signature in RLCs that is also predictive for treatment response in PDXs. Accordingly, AML cells with a high OXPHOS energetic phenotype are markedly less sensitive to AraC chemotherapy compared with low OXPHOS AML cells in NSG mice. Finally, modulation of mitochondrial OXPHOS status markedly affected the antileukemic effect of AraC in vitro and in vivo. Together, this study describes a novel model of chemotherapy resistance in AML that provides a better understanding of mechanisms underlying in vitro AraC resistance to new combinatorial therapies.

RESULTS

In Vivo AraC Treatment Induces a Significant Reduction of Tumor Burden in AML-Engrafted Mice

To study the therapeutic response of primary human AML, we used our NSG-based PDX model for AML (14, 23, 24). Twenty-five primary AML patient specimens from two clinical sites were screened for their engraftment capacities in NSG mice and their genetic diversity (Supplementary Table S1; Supplementary Fig. S1A–S1D). Briefly, one million to ten million unsorted AML cells were injected into adult NSG mice after preconditioning with a sublethal treatment of busulfan one day prior to injection (Fig. 1A). Engraftment efficiency was measured in peripheral blood or bone marrow aspirates by flow cytometry analysis of hCD45+CD33+CD44+ cells, starting at 8 weeks after xenotransplantation. Mice showing at least 50% of human AML engraftment were assigned to experimental groups to obtain balanced average engraftment levels in each cohort at initiation of therapy. Preliminary experiments were performed to determine the in vivo AraC regimen (3 or 5 consecutive daily treatments) and the optimal sublethal dose of AraC (10, 30, 60, 90, or 120 mg/kg/day; Supplementary Fig. S2A). Administration of 60 mg/kg/day for 5 consecutive days was determined as the most efficient treatment (Supplementary Fig. S2A–S2C) to observe a significant reduction in total cell tumor burden in bone marrow and spleen (Supplementary Fig. S2D). Tumor reduction was not improved by treating mice for 7 days or with higher doses and led to increased mortality. Analogous to the patient response to chemotherapy, decrease in absolute white blood cell counts, hemoglobin, and platelets was observed in mice one week after treatment with the selected scheme (60 mg/kg/day for 5 days) and resolved by 2 weeks after treatment. Sixty, 90, and 120 mg/kg of AraC induced almost identical effects on blood parameters (Supplementary Fig. S2E). Collectively, these data show that AraC is well tolerated in NSG mice at dose and schedule comparable with those administered to patients with AML (21), allowing us to study mechanisms involved in drug resistance.

These experiments demonstrated that the maximal response to AraC was achieved by day 8 after initiation of chemotherapy. Therefore, response to AraC treatment and various characteristics of RLCs were specifically monitored at day 8 (3 days after the last administration of AraC; Fig. 1A). The total number of circulating human AML cells expressing the cell surface markers CD45 and CD33 in AraC-treated mice was determined by flow cytometry and compared with that of control, PBS-treated mice (Fig. 1B). At that time point, all AraC-treated mice had a significant reduction of human AML peripheral cells that were below the detection threshold (10 events/μL). Total cell tumor burden, for example, total number of viable human AML blasts, was determined in murine bone marrow and spleen (Fig. 1C and D; Supplementary Fig. S3A and S3B). Interestingly, the in vivo cytoreductive effect of this AraC regimen was significant in all patients but heterogeneous, ranging from 4- to 46-fold reduction. On the basis of this response in vivo, we defined low and high responders that exhibited a biological response below or above a 10-fold reduction, respectively (Fig. 1C and D; Supplementary Fig. S3A and S3B). Additional histochemical analyses of tissue sections stained with Goldner trichrome or hematoxylin and eosin demonstrated a marked reduction of human blasts in both the trabecular and cortical zones of murine bone marrow and sternum following AraC treatment (Fig. 1E; Supplementary Fig. S3C and S3D). Of note, we observed a significant increase in adipocyte number after AraC treatment. These data demonstrate a strong cytotoxic effect of AraC on AML cells in this model, regardless of the intramedullary location of leukemic cells.

We studied whether the response to AraC in the PDX model (fold reduction in bone marrow and spleen after AraC treatment) was correlated with clinical characteristics of the corresponding patients (Supplementary Fig. S4). No correlation was observed between AraC response in vivo and either the specimen type (diagnosis vs. relapse, Supplementary Fig. S4A), gender (Supplementary Fig. S4B), the clinical response to intensive “3 + 7” chemotherapy (Supplementary Fig. S4D), the FAB classification (Supplementary Fig. S4E), the expression of CD34 (Supplementary Fig. S4F), the cytogenetic...
Figure 1. In vivo AraC treatment induces a significant reduction of the total tumor cell burden in AML-engrafted mice. A, Schematic diagram of the chemotherapy regimen and schedule used to treat NSG-based PDX models with AraC. Peripheral blood engraftment was assessed between 8 and 18 weeks, and mice were assigned to experimental groups of 4 to 10 mice with similar average engraftment per group. Mice were treated with vehicle (PBS) or 60 mg/kg/day AraC given daily via intraperitoneal injection for 5 days. Mice were sacrificed posttreatment at day 8 to characterize viable residual AML cells in peripheral blood (PB) and in bone marrow and spleen (BM). Total number of human viable AML cells expressing CD45, CD33, and CD44 were analyzed and quantified using flow cytometry in AraC-treated AML-xenografted mice compared with PBS-treated AML-xenografted mice in peripheral blood (PB). B, Graphs of mean Viable hCD45+CD33+CD44+ cells in PB (blasts per μL of mice blood) and in bone marrow and spleen (total cell tumor burden in millions). Fold reduction of total cell tumor burden in AraC-treated mice compared with control-treated mice was calculated individually for each AML patient sample and in the entire PDX cohort. C, Bone marrow and spleen. D, Trabecular zone 325 262 53 973 1956 15 P = 0.0013; ***, P ≤0.0001. Vehicle AraC E, Goldner staining of bone marrow (tibia section at low/×2.5 or high/×20 magnification) shows engraftment and localization of AML cells at the cortical and trabecular region of the bone in vehicle (PBS)- and AraC-treated mice. F, Correlative analysis between the in vivo response to AraC using our PDX model and the overall survival of all matched patients with AML or of those at diagnosis who received intensive induction chemotherapy (Dx + chemo). Graphs of mean ± SEM. P values were determined by Mann-Whitney test. n.s., not significant; n.d., not determined. *, P ≤0.05; **, P ≤0.01; ***, P ≤0.001; ****, P < 0.0001.
risk group (Supplementary Fig. S4G), the FLT3-ITD allelic burden (Supplementary Fig. S4I), or the mutational status (Supplementary Fig. S4J). Strikingly, response to AraC in vivo was decreased in PDXs from older patients (Supplementary Fig. S4C) and correlated with FLT3-ITD mutation (Supplementary Fig. S4H) and with a shorter overall survival (Fig. 1F). Finally, the in vivo cytotoxic response to AraC (as measured as IC_{50} by flow cytometry upon staining with Annexin V at 24 hours or by Cell TiterGlo assay at 48 hours) of cells from these patients with AML did not correlate with their in vivo response to AraC in PDXs (Supplementary Fig. S4K). These results indicate that the analysis of this PDX-based preclinical model can predict patient clinical outcome and is a suitable model to study AraC response and residual disease in AML.

**In Vivo AraC Treatment Affects CD34^{+/-}CD38^{+/-} Phenotypes in AML-Engrafted Mice**

Previous reports showed that immature AML cells that may represent LSCs or LICs are enriched after chemotherapy. Such cells were initially described as CD34^{+}CD38^{+}, although LSCs have also been identified in more mature CD34^{+}CD38^{+} populations *in vivo* (11, 13, 14). We evaluated the diversity of CD34^{+}CD38^{+/-} phenotypes after AraC treatment by analyzing the expression of these two surface markers in cells isolated from the bone marrow of AML PDXs (as described in Supplementary Fig. S5A; representative flow plot in Supplementary Fig. S5B). We observed a significant change in phenotype after AraC treatment with an increase in CD34^{+}CD38^{+} population in only 3 of 22 patients analyzed (Fig. 2A). Of note, four AML PDXs showed a trend of decrease in CD34^{+}CD38^{+} population post-AraC (Ps12, Ps7, Ps18, Ps17; Fig. 2A). A significant increase in the percentage of CD34^{+}CD38^{+} cells after AraC treatment was observed in patients with the lowest level of this immature cell population, whereas patients with the highest basal level of CD34^{+}CD38^{+} cells exhibited a decrease of this subpopulation (Fig. 2A and B). Analysis of the more mature CD34^{+}CD38^{+} cells showed a variable response of this population of cells to AraC treatment that was not statistically different from that of control cells isolated from PBS-treated mice (325, Ps1, Ps2, Ps6, or Ps15; Supplementary Fig. S5C and S5D). CD34 staining alone showed that CD34^{+} cells displayed a variable response to AraC treatment (Ps2, 325 or Ps19, Ps17, Ps15, Ps22; Supplementary Fig. S5E and S5F). Finally, we found that AraC decreased the absolute cell number of both CD34^{+}CD38^{+} (Supplementary Fig. S6A and S6B) and CD34^{+}CD38^{+} cells (Supplementary Fig. S6C and S6D), indicating that AraC treatment affects both mature and immature AML cells in this model.

**In Vivo AraC Treatment Equally Kills Quiescent and Proliferating AML Cells**

Next, we used flow cytometry after Hoechst/Pyronin Y staining (14, 18, 25) to assess the cell-cycle status of bone marrow cells harvested from AML-xenografted mice after AraC. Peripheral blood mononuclear cells stained with Hoechst/Pyronin Y were used to define G0 quiescent cells (Supplementary Fig. S7A). AML cells from 10 AML PDX specimens were analyzed at day 8. No enrichment of quiescent (G0) cells was detected in the residual human AML cell population after AraC-treated compared with PBS-treated AML PDXs in 9 of 10 patient samples (Fig. 2C and D). Moreover, we found no enhancement in quiescent G0 cells in the CD34^{+}CD38^{+} population after AraC treatment (Supplementary Fig. S7B and S7C). Importantly, AraC treatment significantly decreased the absolute number of G0 cells in viable residual human CD45^{+}CD33^{+} cells (Supplementary Fig. S7D and S7E). These results differed from a previous study using a very high dose of AraC (1 g/kg/day for 2 days; ref. 9), and we next compared the two regimes. Consistent with published results, we observed an increase of cells in G0 only with 1 g/kg AraC (Supplementary Fig. S7F). However, the reduction of the total tumor burden with this high dose of AraC was only 4-fold, whereas it reached 33-fold with the regimen of 60 mg/kg/day for 5 consecutive days (Ps8; Supplementary Fig. S7G), suggesting that the duration of treatment might be an important parameter of the AraC response efficacy. Importantly, most of the animals treated with this very high dose of AraC died within the first week of treatment, precluding a follow-up study of disease progression postchemotherapy. The *in vivo* cell-cycle profile of AraC-treated AML cells was confirmed upon staining with an antibody recognizing the cell-cycle marker Ki67 that is expressed during all phases of the cell cycle (G1, S, and G2) but not in quiescent G0 cells. As shown in bone marrow sections prepared from representative control-treated or AraC-treated AML-xenografted mice (Fig. 2E), no significant difference in the number of Ki67^{+} blasts was observed after AraC treatment *in vivo* (Fig. 2F). Moreover, analysis of the mRNA level of cell cycle–related genes did not show any change after AraC in residual human AML cells (n = 3 patients analyzed *in vivo*; Fig. 2G).
**Energetic Control of In Vivo Chemoresistance in AML**

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**Note:**

- **Ps**: *P* values
- **n.s.**: Not significant
- **n.d.**: Not determined
- **Low (<1%)**
- **Dim (1%–9%)**
- **High (>10%)**
together, these results indicated that human AML cells in mice were not highly proliferative and that quiescent G0 cells were not enriched after AraC chemotherapy, although this treatment significantly reduced the total cell tumor burden in vivo. Accordingly, we showed that AraC strongly induced apoptotic cell death in all 18 patient samples analyzed in vivo (ranging from 2- to 13-fold induction; mean = 3; Fig. 2H and I). This suggests that in vivo AraC equally kills quiescent and proliferating AML cells in vivo.

**In Vivo AraC Treatment Does Not Select LSCs but Induces a Specific Gene Signature of Inflammatory and Stress Responses in RLCs**

To determine whether LSCs (functionally defined hereafter as LICs) were enriched in RLCs after AraC treatment, we performed a limited dilution analysis on vehicle- and AraC-treated AML PDXs from the first recipient (Supplementary Fig. S7H). Frequency of LSCs in each group was calculated using regression analysis (L-Calc software). We observed that AraC decreased the LSC frequency in the bone marrow from PDXs (patient 1956) and induced no change in LSC frequency in three other patient samples tested (Fig. 3A; bone marrow, Supplementary Table S2). Moreover, our analyses indicated that AraC did not change the LSC frequency in spleen from these four patient samples (spleen, Supplementary Table S2). These results indicate that some LSCs can be sensitive to AraC treatment in vivo, but LSCs are typically similarly sensitive to AraC as bulk leukemic cells in some patients.

To study the gene expression signature of RLCs in vivo, we performed a full genome transcriptomic analysis on viable human AML blasts purified from the bone marrow of mice treated with PBS or AraC in three different PDX specimens (Fig. 3B; Supplementary Table S3). First, we performed a gene set enrichment analysis (GSEA) with two functionally identified stem cell signatures (13, 26). Expression of these two stem cell signatures negatively correlated with that of residual AraC-resistant AML blasts in vivo (Fig. 3C; normalized enrichment score (NES) = −1.74, FDRq < 0.0001 and NES = −1.70, FDRq = 0.01, respectively, indicating that LSCs, as defined by these specific gene sets, were not enriched in vivo in viable RLCs at day 8. Furthermore, computational analyses of these transcriptomic data identified two subsets of downregulated and upregulated genes (51 and 68 genes, respectively, Fig. 3D) in viable residual post-AraC versus post-PBS human AML blasts (Supplementary Table S3). These sets of genes were enriched in genes associated with immune and inflammatory responses (CXCL10, CXCL11, CXCL7, CCL23), reactive oxygen species (ROS) response (EPX, SESN3, GATM, HGF, HBB), as well as lipid and sterol metabolism (STARD4, STARD6, SLC27A6, SPN53, PLA2G10, PRG2, HPGD, and DHCR24, CYP51A1, INSG1; Fig. 3B and D; Supplementary Table S3). Finally, we studied the prognostic correlation of these upregulated or downregulated gene signatures in transcriptomic datasets from three independently published cohorts of patients with AML [The Cancer Genome Atlas (TCGA), AML cohort; GSE12417, GSE14468; refs. 27–29]. We found that our upregulated gene signature had no significant prognostic value in two of three independent cohorts tested, whereas patients with AML with a signature enriched in our downregulated gene signature had a significant decrease in overall survival in all three cohorts (P = 0.02, 0.005, 0.0001, respectively; Fig. 3E; Supplementary Table S3). This suggests that the genes downregulated by AraC treatment contribute to global chemoresistance mechanism in patients with AML.

**In Vivo RLCs Have Elevated ROS Levels and Active Mitochondria after AraC Treatment**

On the basis of the literature (30, 31) and on our transcriptomic signature of RLCs enriched in inflammatory, stress, and ROS responses, we hypothesized that in vivo AraC chemotherapy could induce redox alteration and ROS production in RLCs. Pimonidazole (PIMO) reacts with free sulfhydryl groups, such as reduced cysteine residues on proteins to form adducts, that can then be detected by antibody staining (32). Thus, PIMO adduct formation can be used as a stable readout of the intracellular redox state in vivo. We injected PIMO in AraC- and PBS-treated AML mice xenografted in bone marrow. RLCs showed higher intracellular PIMO staining than control AML cells in both trabecular and cortical zones of murine femurs (Fig. 4A, representative histochemical slides; Fig. 4B, quantification). AraC induced a significant reduction of human AML cells in the bone marrow of murine femur (Fig. 4C) as previously shown (Fig. 1; Supplementary Fig. S3D and S3E). These data suggest that AraC treatment leads to an enrichment/survival of RLCs in vivo regardless of their localization throughout the femur bone marrow. Consistent with our gene expression profiling showing an increase in ROS-responsive genes, 2′,7′-dichlorofluorescin (DCF) staining by flow cytometry showed a higher total ROS content in RLCs compared with PBS-treated cells in all 15 different PDXs analyzed (Fig. 4D and E). Accordingly, GSEA of an ROS signature generated by Houstis and colleagues (33) identified a positive correlation and an enrichment in ROS markers in RLCs following AraC in vivo (NES = 1.54, FDRq = 0.03; Fig. 4F).

As mitochondria are one of the major sources of intracellular ROS, we next assessed mitochondrial activity by

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**Figure 3.** In vivo AraC treatment does not enrich in LICs in AML-engrafted mice but uncovers a specific gene signature of immune/inflammatory stress response in AraC-resistant cells from AML-engrafted mice. A, The frequency of LICs from four different PDXs was calculated for each treatment (vehicle/PBS vs. AraC) group using regression analysis (L-Calc software). Graph of mean ± range, *P* ≤ 0.05. Mann–Whitney test was performed. MNC, mononuclear cell. B, Uregulated (red) and downregulated (blue) gene signatures were generated from transcriptomes of human residual AML cells purified from AraC-treated AML-xenografted mice or vehicle (PBS)-treated AML-xenografted mice. C, GSEA of stem cell signatures functionally identified by Eppert and colleagues (13) or Ng and colleagues (28) was performed using transcriptomes of human residual AML cells purified from AraC-treated (red) compared with PBS/control-treated (blue) AML-xenografted mice. Kologorov-Smirnov statistical test was performed. D, Gene Ontology (GO) classification of upregulated (red) or downregulated (blue) genes was identified in residual AML cells from AraC-treated compared with vehicle (PBS)-treated AML-xenografted mice by Genomatics software analysis. Fisher exact test was performed. E, Prognostic correlation of the upregulated (red, 68 genes) or downregulated (blue, 51 genes) gene expression signature was performed in three independent AML cohorts of a total of 732 patients. Kaplan–Meier survival curve, number of patients, and *P* values from log-rank tests are displayed.
Energetic Control of In Vivo Chemoresistance in AML

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<th>Gene</th>
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B

Gene expression levels for MMP2, CXCL10, IFI44L, and IGJ.

C

Stem cell signature
114 genes – Eppert et al. (2011)
17 genes – Ng et al. (2016)

D

Upregulated GO-term – 68 genes
Downregulated GO-term – 51 genes

P value
Response to stimulus
Immune response
Cell surface receptor signal
Phosphate metabolism
Cell communication
Ion transport
Inflammatory response
Chemotaxis/angiogenesis
NFKb-IKb signaling
Response to ROS
Immunne response
Lipid transport/storage
Sterol metabolism

E

Cohort TCGA
151 patients with AML

Cohort Verhaak et al.
418 patients with AML

Cohort Metzeler et al.
163 patients with AML

Overall survival (year)

P values:

HR = 1.65; 95 CI, 1.07–2.53; P = 0.02
HR = 1.40; 95 CI, 1.11–1.79; P = 0.005
HR = 2.40; 95 CI, 1.60–3.60; P = 0.0001
Total ROS content in viable hCD45⁺CD33⁺ blasts (MFI)
measuring mitochondrial membrane potential in RLCs using tetramethylrhodamine ethyl ester (TMRE), a cell permeant lipophilic cationic fluorescent dye that concentrates in mitochondria in proportion to the transmembrane potential. Interestingly, mitochondrial membrane potential (MMP), as judged by TMRE labeling, was increased following AraC treatment in 7 PDxS out of 12 tested (Fig. 4G and H), suggesting that RLCs retained active polarized mitochondria with no loss of MMP. Staining with MitoTracker Green, which passively diffuses across the plasma membrane and accumulates in mitochondria regardless of mitochondrial membrane potential, was higher in RLCs from 6 of 7 patients treated with AraC compared with PBS-treated counterparts (Fig. 4I and J), suggesting an increase in mitochondrial mass and activity, underscoring the important role of mitochondrial metabolism that supports residual disease and AraC resistance in AML.

**High OXPHOS AML Cell Lines Are More Resistant to AraC Chemotherapy, and RLCs Exhibit a High OXPHOS Gene Signature In Vivo**

As suggested by our previous data and recently proposed by other groups in solid tumors (34–36), we hypothesized that RLCs responsible for relapse would have mitochondria-driven energetic features modulating their response to AraC in the context of the *in vivo* niche. To analyze whether the bioenergetic status of AML cells might be involved in AraC chemoresistance, we characterized mitochondrial ATP production and oxygen consumption rates (OCR) of six diverse human AML cell lines (molecular and mutational characteristics in *Supplementary Table S1*) and defined their bioenergetic status as low (lowest mitochondrial ATP production and OCR: U937, KG1, KG1a) versus high (highest mitochondrial ATP production and OCR: HL60, MV4-11, MOLM14) OXPHOS activity (low OXPHOS vs. high OXPHOS; *Supplementary Fig. S8A*). In addition, mitochondrial ROS content, mitochondrial membrane potential and mass, and sensitivity to galactose of four human AML cell lines were analyzed to further functionally define low and high OXPHOS status and capacity (*Supplementary Fig. S8B–S8D*). Low and high OXPHOS AML cell lines were next injected into adult NSG mice and treated with AraC (30 mg/kg/day) for 5 days starting 10 days after transplantation. Low OXPHOS AML cells were sensitive to AraC *in vivo* as demonstrated by a significant increase in the overall survival of the AraC-treated compared with PBS-treated mice cohorts (Fig. 5A). In contrast, AML cells with high OXPHOS were less sensitive to AraC chemotherapy *in vivo* compared with low OXPHOS AML cells (Fig. 5A). Furthermore, we observed a significant reduction of total cell tumor burden and a significant increase in apoptotic cell death in low OXPHOS AML U937 cells but no change in total cell tumor burden, apoptosis, and loss of MMP in high OXPHOS AML MOLM14 cells (Fig. 5B–D). Most notably, AraC residual cells have a significant increase in PIMO staining, total and mitochondrial ROS content as well as active MMP in low OXPHOS AML cells, whereas these changes were less pronounced in high OXPHOS AML cells (Fig. 5E–H, respectively).

Finally, as the electron transport chain (ETC) complex I inhibitor metformin induces a well-described energetic shift from high OXPHOS to low OXPHOS (so-called Pasteur effect, *Supplementary Fig. S9A* compared with *Supplementary Fig. S8A*), we performed a comparative transcriptomic analysis of high (HL60, MOLM14) versus low (U937, KG1a) OXPHOS AML cell lines untreated (*Supplementary Table S4*; *Supplementary Fig. S9B*) or treated with metformin (Supplementary Table S4). First, we showed that upregulated genes in untreated high OXPHOS compared with untreated low OXPHOS cell lines were involved in biological pathways, such as ribosome, RNA/DNA metabolism, pyrimidine metabolism (*Supplementary Fig. S9C*), and were specifically encoded for mitochondrial proteins involved in the tricarboxylic acid (TCA) cycle, ETC, ubiquinone Q10 biosynthesis, fatty acid (FA) metabolism, and apoptotic pathways (*Supplementary Table S4*; *Supplementary Fig. S9D*). Using transcriptomes from metformin-treated AML cell lines, we further identified a high OXPHOS gene signature of 221 transcripts (*Supplementary Fig. S9E*; *Supplementary Table S4*) with an enrichment of genes involved in metabolic processes (including ATP, nucleoside, and glycerolipid metabolism), responses to stress, subcellular organization, and cell cycle (*Supplementary Fig. S9F*). Strikingly, this high OXPHOS gene signature was also significantly enriched in the transcriptomes of RLCs from three AML PDxSs (*NES = 1.47; FDRq < 0.0001*; Fig. 5I).

**In Vivo Chemotherapy Sparing Preexisting and Persisting AML Cells with a High OXPHOS Signature**

Next, we performed a transcriptomic analysis of 21 AML primary samples used in this study (before xenotransplantation, *Supplementary Table S1*) comparing low (*n = 10* patients) versus high (*n = 11* patients) responders to AraC *in vivo* in NSG mice (lowR vs. highR; Fig. 5J) and identified
Figure 5. AML cells with high OXPHOS activity are more resistant to AraC chemotherapy in vivo, and residual human AML cells after AraC treatment in AML-engrafted mice exhibit a high OXPHOS gene signature in vivo. A, Kaplan-Meier curves of mice survival were established for high and low OXPHOS AML cell lines engrafted in NSG mice and treated with AraC (30 mg/kg/day) or PBS during 5 days. Significance was determined by log-rank tests. B, Total cell tumor burden of human viable CD45+ CD33+ AML cells was analyzed and quantified in PBS- and AraC-treated AML mice xenografted with high (MOLM14) and low (U937) OXPHOS AML cell lines using flow cytometry. C, Percent of human apoptotic CD45+ CD33+ AML cells was analyzed using Annexin V/7-AAD–based flow cytometric assay in PBS- and AraC-treated MOLM14- or U937-xenografted mice. D, Loss of mitochondrial membrane potential was assessed with fluorescent TMRE probe using flow cytometry in human CD45+ CD33+ AML cells in PBS- and AraC-treated MOLM14- or U937-xenografted mice. E, Intracellular redox status was assessed using PIMO probe and analyzed by flow cytometry in viable human AML cells from MOLM14- or U937-xenografted mice after PBS and AraC treatment. F, Intracellular total ROS levels were assessed using DCF-DA probe and analyzed by flow cytometry in viable human AML cells from MOLM14- or U937-xenografted mice after PBS and AraC treatment. G, Mitochondrial superoxide production was measured using MitoSOX probe using flow cytometry in viable human AML cells from MOLM14- or U937-xenografted mice after PBS and AraC treatment. (continued on following page)

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a signature of 230 upregulated and 151 downregulated genes in lowR compared with highR (Supplementary Table S5). Unexpectedly, we found that high OXPHOS gene signature was also enriched in the transcriptomes of lowR in vivo (Fig. 5K). Altogether, these data strongly suggest that a high OXPHOS phenotype may not only preexist before xenotransplantation and escape from chemotherapy but may also persist and be amplified by AraC treatment in vivo.

To test this hypothesis, we compared the ex vivo chemosensitivity of untreated AML cells and RLCs post-PDX. We observed a significant 5- to 10-fold increase in EC50 of AraC in RLCs compared with post-PBS-treated mice when assayed immediately after sorting (plain symbol) and also after a subsequent 2-week culture (empty symbol; Supplementary Fig. S10A). Consistent with these results, post-AraC sorted RLCs also demonstrated an increased EC50 of idarubicin compared with PBS-matched sorted AML cells from 2 cell lines and 2 patients (MOLM14, U937, Pb19, Pb22; Supplementary Fig. S10B). Furthermore, we also purified high and low ROS fractions of AML cells (MOLM14, U937) in vitro and in vivo using DCF-stained cells and FACs-based sorting (Supplementary Fig. S10C) and showed that high ROS cells exhibited a significantly higher EC50 of AraC compared with low ROS cells in both PBS and RLCs in vivo and in vitro (Supplementary Fig. S10D).

Next, we determined whether RLCs have high ROS content and increased active mitochondria in vivo during AraC treatment (day 3), immediately after the last dose of AraC treatment (day 5), and after the standard day 8 time point in NSG mice. In addition to showing that AraC-induced apoptotic cell death and decreased total cell tumor burden occurred as early as day 3 (Supplementary Fig. S10E and S10F), we also observed the appearance of RLCs with an increased ROS content, mitochondrial mass, and membrane potential starting at day 3 (Supplementary Fig. S10G–S10I). Of note, in this time-course study, RLCs overexpressed CD36, CD44, and not CD123 (Supplementary Fig. S10J–S10L). Selection for high OXPHOS cells occurred without genetic selection, as...
the major founder mutations are present at diagnosis in PDX throughout the same time course (Supplementary Fig. S10M and S10N). All of these data confirmed that in vivo chemotherapy spares preexisting and persisting AML cells with a high OXPHOS signature and CD36** phenotype and enriches them in RLCs (Fig. 5L).

**RLCs Exhibit Increased Mitochondrial Respiration, and Targeting High OXPHOS Enhances AraC Chemotherapy Efficacy in AML**

Overall, our results strongly support the idea that mitochondrial OXPHOS activities greatly influence cytotoxicity of AraC. Interestingly, we showed that RLCs have increased mitochondrial oxygen consumption in vitro and in vivo (Fig. 6A) and an increased concentration of TCA cycle intermediates in vitro (Fig. 6B) compared with PBS-treated cells. Accordingly, we next sought to manipulate the mitochondrial energetic status toward high OXPHOS or low OXPHOS to induce resistance or sensitivity to AraC, respectively. Thus, we first cultured low OXPHOS AML cells (U937) in the presence of medium containing galactose instead of glucose as the sole sugar source, a treatment well documented to shift energetic metabolism from glycolysis to mitochondrial OXPHOS (Supplementary Fig. S11A; refs. 12, 37–39). As expected, we observed an energetic shift toward high OXPHOS with increased mitochondrial oxygen and pyruvate consumption in the presence of galactose and even higher values in combination with AraC (Supplementary Fig. S11B), concomitant with decreased glucose consumption and lactate production (Supplementary Fig. S11C), leading to a higher ATP production by mitochondria in cells grown in galactose medium (Supplementary Fig. S11D and S11E). In addition, the shift to a high OXPHOS phenotype blocked AraC-induced reduction of cell density and viability (Supplementary Fig. S11F and S11G) as well as AraC-induced apoptotic cell death, in a dose- and time-dependent manner (Supplementary Fig. S11H).

Next, we determined whether pharmacologic manipulation of high OXPHOS cells toward a low OXPHOS phenotype would enhance cytotoxic effects of AraC in vitro and in vivo. In high OXPHOS AML (MOLM14) cells, we tested pharmacologic agents well known to inhibit OXPHOS activities through the inhibition of mitochondrial protein synthesis (tigecycline, ref. 40; ethidium bromide, refs. 36, 41), ETCI...
Figure 6. AraC residual AML cells increase mitochondrial respiration, and targeting high OXPHOS with tigecycline (tig) enhances AraC chemotherapy efficacy in AML. A, Rates of oxygen consumption in high OXPHOS MOLM14 cells were measured after PBS and AraC treatment both in vitro and in vivo by ion chromatography coupled to mass spectrometry (IC-MS). B, Schematic diagram of tigecycline-induced effects on shifting high OXPHOS toward low OXPHOS state that leads to a significant abrogation of AraC resistance in high OXPHOS MOLM14 cells. C, Protein expression of mitochondrial transcription factor (mtTFAM) and OXPHOS complexes was assessed by Western blot analysis after 24-hour treatment with AraC (2 μmol/L) in MOLM14 cells in vitro. D and E, Mitochondrial mass (D) and active mitochondrial membrane potential (E) were assessed by flow cytometry using the fluorescent MitoTracker Green (MTG) and TMRE probes, respectively, in MOLM14 cells after PBS, tigecycline, AraC, or tigecycline + AraC treatment. Each parameter was measured relative to PBS-treated samples. F, OCR in MOLM14 cells after PBS, tigecycline, AraC, or tigecycline + AraC treatment. (continued on following page)

(phenformin or metformin, ref. 42; rotenone), or ETCIII (antimycin A or atovaquone, ref. 43). As expected and shown by Jhas and colleagues (44), tigecycline-treated high OXPHOS MOLM14 cells (Fig. 6C) exhibited a significantly decreased level of mitochondrial protein translation of ETCI and ETCIV complexes (Supplementary Fig. S13A–S13K; metformin, Supplementary Fig. S14A–S14I; rotenone, Supplementary Fig. S15A–S15I) or of ETCII (antimycin A, Supplementary Fig. S16A–S16I; atovaquone, Supplementary Fig. S17A–S17I) in high OXPHOS MOLM14 cells yielded similar results.

High OXPHOS Phenotype of RLCs Is Dependent on Mitochondrial Oxidation of FAs and a High CD36 Signature in AML

As shown in Fig. 6A and B, AraC induced a 2.5-fold increase in OCR and increased TCA cycle intermediate concentrations. This OCR enhancement was associated with only a slight increase in pyruvate and glucose consumption and lactate production without any change in glutamine consumption in RLCs (Fig. 7A), suggesting a potential increase in mitochondrial uptake and utilization of other oxidizable substrates, such as amino acids, acetate, ketones, or FAs, which may support mitochondrial OXPHOS and respiration in the presence of AraC. Indeed, we observed an enrichment of genes involved in FA metabolism in transcriptomes
RLCs from AML PDX (Fig. 7B). Among the most differentially expressed genes involved in FA and lipid metabolism, we have identified FA translocase/receptor CD36 in RLCs from AML PDX (Fig. 7C) that is also markedly (21- to 60-fold) upregulated in AML cells either in bulk or in LSCs from patients with AML of two independent AML patient transcriptomic databases, analyzing a total of 1,458 patients, as compared with normal HSCs (Supplementary Fig. S18A). We confirmed that CD36 protein expression at the cell surface was also increased in viable RLCs in vitro and in vivo in two AML cell lines and three primary AML specimens (Fig. 7D). Moreover, patients with AML in the TCGA cohort expressing the highest level of CD36 transcript had a poorer outcome (Supplementary Fig. S18B). From those patients with the highest CD36 expression, we have generated a specific gene set (called high CD36 signature; Supplementary Table S6) and performed a GSEA showing that this gene set is enriched in the transcriptomes of patients with AML used in this study presenting the lower response to AraC in vivo when assayed in NSG mice (NES = 1.45, FDR = 0.04; Fig. 7E, bottom) and in the transcriptomes of RLCs from AML PDX (NES = 3.29, FDR < 0.0001; Fig. 7E, top). Interestingly, transcriptomes of patients with AML with the higher CD36 expression are enriched in our high OXPHOS gene signature (Fig. 7F) and in published gene signatures of OXPHOS, ROS, FA metabolism, and FA oxidation (Supplementary Fig. S18C and S18D) that have an adverse prognostic impact (Supplementary Fig. S18E).

Accordingly, inhibition of FAO should be a promising chemosensitizing intervention in AML. Thus, we tested the effect of the FAO inhibitor etomoxir, which inhibits carnitine palmitoyltransferase 1 (CPT1), the enzyme that catalyzes the rate-limiting step (FA shuttling through the inner mitochondrial membrane) of FAO. First, we showed that AraC alone increased FAO in MOLM14 cells (Fig. 7G), concomitant with OCR enhancement upon AraC treatment in vitro. Etomoxir inhibited FAO and OCR (P = 0.0159, Fig. 7H) induced by AraC in MOLM14 cells, showing that these cells do indeed oxidize mostly FA upon AraC treatment. This treatment led to an energetic shift toward low OXPHOS metabolism (Fig. 7I–K), as described above with other mitochondrial and OXPHOS inhibitors, thereby potentiating the cytotoxicity...
Figure 7. AraC residual cells increase mitochondrial FA oxidation, and targeting high OXPHOS metabolism with etomoxir enhances AraC chemotherapy efficacy in AML. A, Metabolomic profiling of extracellular metabolites was performed to quantify the rate of production and consumption of extracellular glucose, glutamine, pyruvate, and lactate following 24-hour AraC (2 μmol/L) treatment in MOLM14 cells using 1D 1H NMR spectra. B, GSEA of FA metabolism gene signature was performed with transcriptomes of viable human residual AML cells from AraC and PBS-treated AML-xenografted mice. C, Genes involved in lipid metabolism, including CD36, are upregulated in AraC residual AML cells compared with vehicle-treated AML cells in vitro and in vivo, and in three different PDXs using flow cytometry. D, Cell density was determined with Trypan blue count after 24-hour treatment of MOLM14 cells with PBS, AraC, etomoxir, and AraC + etomoxir. E, Enzyme-linked immunosorbent assay (ELISA) of cell surface expression of CD36 was analyzed and quantified in viable human CD45+ AML cells purified from vehicle (PBS)- and AraC-treated xenografted mice (top) and from transcriptomes of patients with AML that are low versus high responder to AraC treatment in NSG mice (bottom). Kolmogorov–Smirnov statistical test was performed. F, GSEA of high CD36 gene signature was performed from transcriptomes of patients with AML that had the highest CD36 mRNA expression compared with those with the lowest expression in TCGA cohort. G, FA oxidation rate was evaluated 24 hours after treatment of MOLM14 cells with PBS, AraC, etomoxir (Ex: 200 μmol/L) and AraC + etomoxir. This assay assessed the labeled 14CO2 after incubation with [1-14C] palmitate acid using scintillation counter and normalized to cell number. H, OCR was measured by Clark electrode at 24 hours posttreatment of MOLM14 cells with PBS, AraC, etomoxir, and AraC + etomoxir in vitro. I, Glucose consumption and lactate production rate in extracellular medium were evaluated using 1D 1H NMR spectra. J, Percentage of glycolytic ATP production was quantified in MOLM14 cells after PBS, AraC, etomoxir, and AraC + etomoxir in vitro treatment by CellTiter-Glo Assay Kit using a plate-reading spectrophotometer. K, Cell density was determined with Trypan blue count after 24-hour treatment of MOLM14 cells with PBS, AraC, etomoxir, and AraC + etomoxir in vitro. M, Percentage of apoptotic cells was measured after 24-hour treatment of MOLM14 cells with PBS, AraC, etomoxir, and AraC + etomoxir using Annexin V(7-AAD)-based flow cytometry. N, Loss of mitochondrial membrane potential was assessed following 24-hour treatment of MOLM14 cells with PBS, AraC, etomoxir, and AraC + etomoxir by fluorescent TMRE probe staining using flow cytometry. O, Schematic diagram of mechanism of resistance of AraC based on FAs as key source for maintaining high OXPHOS metabolism and support cell survival upon AraC treatment. Graphs of mean ± SEM. P values were determined by Mann–Whitney test. n.s., not significant. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
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of AraC in MOLM14 cells (Fig. 7L-N). Altogether, these results suggest a new model of AraC resistance based on the role of the CD36–FAO–OXPHOS axis in AML (Fig. 7O; Supplementary Table S7 and Supplementary Fig. S18F).

DISCUSSION

Prognosis is very poor in patients with AML due to frequent relapse caused by RLCs (1, 2). However, molecular mechanisms of AML resistance are still largely unknown, especially in vivo, and new therapies that effectively eradicate RLCs are an urgent medical need. Previous reports hypothesized that RLCs in AML were enriched in LSCs with an immature cell phenotype and in G0 cells (9, 10). However, these studies used a high dose of AraC, poorly tolerated, or did not formally assess LSC number by secondary transplantation. We have developed a clinically relevant and well-tolerated approach to deliver AraC in NSG mice engrafted with AML. This regimen reduced AML tumor burden in all mice and allows identification of high and low responders to AraC treatment in vivo. Importantly, this PDX model robustly correlates with clinical outcome of respective patients. AraC treatment in this pertinent PDX model induced apoptotic cell death of leukemic blasts. Furthermore, RLCs were not consistently enriched for immature cells, cells in G0 or LSCs as formally assessed by limiting dilution analysis into secondary recipient mice, in agreement with a recent study (22). Compared with normal CD34+ cells, we further show in this study that most leukemic cells are not quiescent (G0-G1: 80%-90% with 0.5%-10% in G0) in PDX and patients with AML, confirming previous studies by us and others in patients with AML (14, 18, 25). However, AraC effectively kills leukemic cells, including CD34+ cells, suggesting that quiescence is not a crucial attribute necessary for AraC sensitivity in vivo. Although we have used AraC as a single agent in our current studies to mimic AraC schedules and doses, most induction treatment regimens used in patients with AML combine AraC with anthracycline. However, the NSG mouse strain has a deficiency in the DNA repair enzyme, and thus NSG mice tolerate DNA double-strand break–inducing agents very poorly (21). Therefore, other mouse models will need to be employed to determine whether anthracyclines spare LSCs or quiescent cells.

We have further shown that, as compared with chemosensitive cells, preexisting and persisting RLCs were distinctly more resistant to drugs (e.g., AraC, IDA), exhibited an enriched high OXPHOS gene signature, displayed high ROS levels with modified intracellular redox status, showed increased mitochondrial mass, and retained active, polarized mitochondria, all features consistent with a high OXPHOS status. Relevant in this regard, a previous publication also showed that AraC enhances mitochondrial activities including mitochondrial respiration and cytochrome C oxidase (45). We also demonstrated that AML cell lines with a high OXPHOS status are resistant to AraC in vitro and that the high OXPHOS gene signature is also enriched at diagnosis in transcriptomes of patients with AML who had a lower response to AraC in NSG mice and a lower overall survival. Interestingly, the gene signature enriched in genes involved in mitochondrial functions strongly correlated with a negative prognosis index in TCGA, was also recently identified in the transcriptomes of AML cell lines less sensitive to AraC in vitro (46). We propose that, in the context of the in vivo bone marrow niche, AraC resistance is likely determined by redox/metabolic/energetic status of AML cells, independent of their stem cell features. In agreement with previously published works (11, 13-15), we can further propose that LSCs are heterogeneous not only in their cell surface immunophenotype, but also in their sensitivity to AraC in vivo. We and others have previously shown that ROS-dependent AMPK or SUMO signaling pathways are strongly involved in AML survival upon metabolic or genotoxic stresses, especially in hypoxic bone marrow, but not in spleen niches (47, 48). Overall, our data and a growing body of evidence in the literature suggest that chemoresistance may be more strongly driven by cellular metabolism rather than by cellular quiescence or stage of maturity of cancer stem cells.

Our results further indicate that mitochondrial and OXPHOS activities greatly influence the sensitivity and in vivo efficacy of chemotherapeutic agents. We observed that RLCs exhibited higher mitochondrial oxygen consumption that is dependent on increased TCA cycle activity and FAO, and not solely on glucose, pyruvate, or glutamine consumption, and is sensitive to FAO inhibitors. A recent publication reported that adipose tissue serves as a reservoir for LSCs in chronic myeloid leukemia (CML), and their respective cross-talk results in lipolysis to fuel FAO of CML cells (49). These authors further observed that in this type of myeloid leukemia, a distinct population of LSCs, which expresses CD36 and higher FAO with a quiescent and drug-resistant phenotype, is protected from chemotherapy by adipocytes (49). As CD36 is also known to have an adverse prognostic impact in patients with AML (50), we found that RLCs overexpressed not only the CD36 gene and cell surface receptor but also genes involved in FA and lipid metabolism. Thus, as shown in carcinoma metastasis (51), cachexia (52), or CML metabolism, increases FAO, maintains OXPHOS state and activity of RLCs by fueling long-chain FA uptake from the extracellular bone marrow matrix and medullar adipocytes, and protects cells from AraC. Adipocytes colonize the empty space of the bone marrow post-AraC as observed in our histological analyses. Bidirectional interplay between bone marrow adipocytes and leukemic blasts is crucial to support energy and cell survival in AML (53, 54). Cachetic-like metabolic reprogramming might occur in the early time points of the postchemotherapeutic residual disease processes in patients with AML to support energy requirements for AML regrowth and relapse. Of note, high expression of CD36 in patients with AML at diagnosis is associated with a poor prognosis in TCGA, and our high OXPHOS gene signature is enriched in transcriptomes of patients with AML who overexpressed CD36 in TCGA. Together, our data suggest that CD36 might be a robust biomarker of residual disease and of this specific metabolic state (Supplementary Fig. S18F).

Interestingly, Sriskanthadevan and colleagues (56) have shown that although AML cells have increased mitochondrial mass and oxygen consumption compared with normal hematopoietic...
cells, they have a lower spare reserve capacity of their respiratory chain that renders them susceptible to oxidative or metabolic (but not genotoxic) stress. Here, we propose that metabolic flexibility as well as mitochondrial dependency are two crucial requirements of AraC resistance of AML cells in vivo. An OXPHOS-dependent energetic flexibility may be responsible for resistance to oncogene ablation or induced senescence, oxidative stress, radiation, and chemotherapeutics (35, 36, 57–60), suggesting that resistance in cancer is associated with a shift toward a high OXPHOS status. Of note, this model may explain several recent studies inducing chemosensitivation in AML cells through modulation of mitochondrial metabolism. For example, inhibition of mitochondrial FA transport and β-oxidation (etomoxir or ST1326; refs. 61–63) and inhibition of the mitochondrial protease ClpP, which interacts with respiratory chain proteins (64), alters chemosensitivity in vitro. Although there is a broad heterogeneity in oxidative and metabolic requirements and capacities as well as mitochondrial energetics in AML cells, targeting mitochondrial OXPHOS presents a promising therapeutic avenue in AML (this study; 42, 56, 65). Furthermore, an important translational result would be to show that treatment of primary AML cells with a drug inhibiting OXPHOS delivered in combination with chemotherapy would increase chemosensitization in vivo and to translate these results to clinical applications. Importantly, initial results suggest that inhibition of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (first-class antimitochondrial TCA cycle agent, CPI-613; ref. 66) may have clinical activity in patients with high-risk AML.

In summary, our data show that mechanisms of resistance of AML to AraC in vivo are not contingent on quiescent LSCs, but rather depend upon both the preexistence of resistant clones and/or the capacity of cells to adapt their energetic metabolism toward a high OXPHOS phenotype in response to AraC. At diagnosis, high and low OXPHOS AML cells coexist in variable proportions, whereas immediately postchemotherapy high OXPHOS cells predominantly persist and survive, especially in poor responders to AraC in vivo. Our data thus show that mitochondrial OXPHOS is a crucial contributory factor in AML chemoresistance, and a high OXPHOS signature and metabolism are for the first time identified as key hallmarks of chemoresistance in vivo. Importantly, our data strongly suggest that an induced shift of human AML cells from a high to a low OXPHOS status would lead to chemosensitization.

**METHODS**

**Primary Cells**

Primary AML patient specimens are from two clinical sites [University of Pennsylvania (UPENN), Philadelphia, PA, and Toulouse University Hospital (TUH), Toulouse, France]. Frozen samples were obtained from patients diagnosed with AML at UPENN in accordance with U.S. Common Rules and at TUH after signed informed consent in accordance with the Declaration of Helsinki, and stored at the HIMIP collection (BB-0033-00060). According to the French law, HIMIP biobank collection has been declared to the Ministry of Higher Education and Research (DC 2008-307, collection I) and obtained a transfer agreement (AC 2008-129) after approbation by the Comité de Protection des Personnes Sud-Ouest et Outremer II (ethical committee). Clinical and biological annotations of the samples have been declared to the CNIL (Comité National Informatique et Libertés ie Data processing and Liberties National Committee). In the United States, AML samples were obtained from the Stem Cell and Xenograft Core Facility at the UPENN School of Medicine. Samples were obtained from patients presenting with AML at the Hospital of the UPENN with informed consent in accordance with institutional guidelines. Peripheral blood or bone marrow samples were frozen in FCS with 10% DMSO and stored in liquid nitrogen. The percentage of blasts was determined by flow cytometry and morphologic characteristics before purification.

**Mouse Xenograft Model**

Animals were used in accordance with a protocol reviewed and approved by the Institutional Animal Care and Use Committee of Région Midi-Pyrénées (France) or at UPENN. NOD/LtSz-SCID/IL-2Rγnull (NSG) mice were produced at the Genotoul Anexplo platform at Toulouse (France) using breeders obtained from Charles River Laboratories. At UPENN, mice were acquired from The Jackson Laboratory and bred at UPENN. Mice were housed and human primary AML cells were transplanted as reported previously (14, 23, 24). Briefly, mice were housed in sterile conditions using HEPA-filtered microisolators and fed with irradiated food and sterile water. Transplanted mice were treated with antibiotic (Baytril) for the duration of the experiment. Mice (6–9 weeks old) were sublethally treated with busulfan (30 mg/kg/day) 24 hours before injection of leukemic cells. Leukemia samples were thawed at room temperature, washed twice in PBS, and suspended in Hank’s Balanced Salt Solution at a final concentration of 1–10 × 10⁶ cells per 200 μL of Hank’s Balanced Salt Solution per mouse for tail vein injection. Daily monitoring of mice for symptoms of disease (ruffled coat, hunched back, weakness, and reduced mobility) determined the time of killing for injected animals with signs of distress. If no signs of distress were seen, mice were initially analyzed for engraftment 8 weeks after injection except where otherwise noted.

**AraC Treatment**

Eight to 18 weeks after AML cell transplantation and when mice were engrafted (tested by flow cytometry on peripheral blood or bone marrow aspirates), NSG mice were treated by daily intraperitoneal injection of 10, 30, 60, 90, or 120 mg/kg AraC for 5 days or 1 g/kg AraC for 2 days; AraC was kindly provided by the pharmacy of the TUIH. For control, NSG mice were treated daily with intraperitoneal injection of vehicle, PBS 1×. Mice were monitored for toxicity and provided nutritional supplements as needed.

**Assessment of Leukemic Engraftment**

Peripheral blood was obtained at the time of first AraC dose (day 0) and at the time of dissection (day 8) 2 days after the last dose of AraC to determine the fraction of human blasts using flow cytometry. NSG mice were humanely killed in accordance with European ethics protocols. Bone marrow (mixed from tibias and femurs) and spleen were dissected in a sterile environment and flushed in Hank’s Balanced Salt Solution with 1% FBS. MNCs from peripheral blood, bone marrow, and spleen were labeled with FITC-conjugated anti-hCD3, PE-conjugated anti-hCD33, PerCP-Cy5.5-conjugated anti-mCD45.1, APC-conjugated anti-hCD45, and PerCy7-conjugated anti-hCD44 [all antibodies from Becton Dickinson (BD), except FITC-conjugated anti-hCD3 from Ozyme BioLegend] to determine the fraction of human blasts (hCD45⁰mCD45.1⁺hCD33⁺hCD44⁺ cells) using flow cytometry. All antibodies used for cytometry were used at concentrations between 1/50 and 1/200 depending on specificity and cell density. Analyses were performed on a Life Science Research II (LSR II) flow cytometer with DIVA software (BD Biosciences) or CytoFLEX flow cytometer with CytExpert software (Beckman Coulter). The number of AML cells/μL peripheral blood and number of AML cells in total cell tumor burden (in bone marrow and spleen) were determined by using CountBright beads (Invitrogen) using described protocol.
Cell Lines and Culture Conditions

Human AML cell lines were maintained in minimum essential medium α supplemented with 10% FBS (Invitrogen) in the presence of 100 U/mL of penicillin and 100 μg/mL of streptomycin, and were incubated at 37°C with 5% CO₂. The cultured cells were split every 2 to 3 days and maintained in an exponential growth phase. All AML cell lines were purchased at DSMZ or ATCC, and their liquid nitrogen stock were renewed every 2 years. These cell lines have been routinely tested for Mycoplasma contamination in the laboratory. The 937 cells were obtained from the DSMZ in February 2012 and from the ATCC in January 2014. MVA-11 and HL-60 cells were obtained from the DSMZ in February 2012 and 2016. KG1 cells were obtained from the DSMZ in February 2012 and from the ATCC in March 2013. KG1 cells were obtained from the DSMZ in February 2016. MOLM14 was obtained from Pr. Martin Carroll (University of Pennsylvania, Philadelphia, PA) in 2011 and from the DSMZ in June 2015. DSMZ and ATCC cell banks provide authenticated cell lines by cytochrome C oxidase I gene analysis and short tandem repeat profiling. Furthermore, the mutation status was also verified by targeted sequencing of a panel of 40 genes frequently mutated in AML as described in Supplementary Methods. Clinical and mutational features of our AML cell lines are described in Supplementary Table S1.

Statistical Analyses

We assessed the statistical analysis of the difference between two sets of data using nonparametric Mann-Whitney test one-way or two-way (GraphPad Prism, GraphPad). P values of less than 0.05 were considered to be significant (*, P < 0.05; **, P < 0.01; and ***, P < 0.001).

For in vitro and in vivo analyses of AraC, residual disease, see Supplementary Methods.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions


Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Aroua, M. Hosseini, H. Boutzen, O. Duchamp, C. Récher, J.-E. Sarry

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REFERENCES


survival in cytogenetically normal acute myeloid leukemia. Onco-
ROS/SUMO axis contributes to the response of acute myeloid leuke-
leukemia-initiating cells in myeloid leukemias from metabolic stress
49. Ye H, Adane B, Khan N, Sullivan T, Minhaujudden M, Gasparetto M,
et al. Leukemic stem cells evade chemotherapy by metabolic adapta-
et al. Adverse prognostic impact of CD36 and CD2 expression in
adult de novo acute myeloid leukemia patients. Leuk Res 2005;29:
1109–16.
51. Pascual G, Avgustinova A, Mejetta S, Martin M, Castellanos A, Atto-
lini CS-O, et al. Targeting metastasis-initiating cells through the fatty
52. Fukawa T, Yan-Jiang BC, Min-Wen JC, Jun-Hao ET, Huang D, Qian
C-N, et al. Excessive fatty acid oxidation induces muscle atrophy in
53. Shafat MS, Oellerich T, Mohr S, Robinson SD, Edwards DR, Marlein
CR, et al. Leukemic blasts program bone marrow adipocytes to gener-
ate a pro-tumoral microenvironment. Blood 2017;129:1320–32.
Survival of acute monocytic leukemia cells is driven by fatty acid
oxidation-mediated activation of AMPK in bone marrow adipocytes.
Cancer Res 2017;77:1453–64.
Impact of obesity in favorable-risk AML patients receiving intensive
56. Sriskanthadevan S, Jeyaraju DV, Chung TE, Prabha S, Xu W, Skrisci
M, et al. LMM cells have low spare reserve capacity in their respiratory
chain that renders them susceptible to oxidative metabolic stress.