Extended exposure to low doses of azacitidine induces differentiation of leukemic stem cells through activation of myeloperoxidase

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Received: May 2, 2023.
Accepted: October 30, 2023.
Early view: November 9, 2023.

https://doi.org/10.3324/haematol.2023.283437

Abstract

Oral azacitidine (oral-Aza) treatment results in longer median overall survival (OS) (24.7 *vs.* 14.8 months in placebo) in patients with acute myeloid leukemia (AML) in remission after intensive chemotherapy. The dosing schedule of oral-Aza (14 days/28-day cycle) allows for low exposure of Aza for an extended duration thereby facilitating a sustained therapeutic effect. However, the underlying mechanisms supporting the clinical impact of oral-Aza in maintenance therapy remain to be fully understood. In this preclinical work, we explore the mechanistic basis of oral-Aza/extended exposure to Aza through *in vitro* and *in vivo* modeling. In cell lines, extended exposure to Aza results in sustained DNMT1 loss, leading to durable hypomethylation, and gene expression changes. In mouse models, extended exposure to Aza, preferentially targets immature leukemic cells. In leukemic stem cell (LSC) models, the extended dose of Aza induces differentiation and depletes $CD34^{+}CD38^{-}$ LSC. Mechanistically, LSC differentiation is driven in part by increased myeloperoxidase (MPO) expression. Inhibition of MPO activity either by using an MPO-specific inhibitor or blocking oxidative stress, a known mechanism of MPO, partly reverses the differentiation of LSC. Overall, our preclinical work reveals novel mechanistic insights into oral-Aza and its ability to target LSC.

Introduction

Acute myeloid leukemia (AML) is a disease of the elderly with a median age at diagnosis >68 years. Unfavorable cytogenetics and associated comorbidities are among the factors that make elderly patients unfit/ineligible for intensive chemotherapy, known as the 7+3 regimen.[1-3] As a result, targeted and less intensive treatments are used as first line therapy for many of these patients. Venetoclax/azacitidine (Ven/Aza) is one such treatment regimen that has improved outcomes in chemotherapy-ineligible newly diagnosed AML patients.[4,5] The backbone of this regimen, Aza, is a hypomethylating agent that incorporates into RNA and DNA. DNA-incorporated Aza leads to loss of DNMT1 and therefore, genome-wide hypomethylation.[6] Through hypomethylation-dependent/independent effects, Aza impacts multiple cellular processes such as apoptosis, DNA damage response, tumor suppressor re-expression, immune modulation, and more.[7] Given the genomic and epigenomic dysregulation and heterogeneity in AML, the broad hypomethylating effect and multitude of mechanisms impacted by Aza could be one of the keys to its clinical efficacy. Venetoclax selectively inhibits the anti-apoptotic protein, BCL-2.[8] On the other hand, many AML patients eligible for intensive chemotherapy achieve complete remission with the 7+3 regimen.[3] However, most of these

patients on chemotherapy as well as Ven/Aza therapy eventually relapse.[9-11]

A multicenter study identified longer relapse-free survival following first remission as one of the key parameters for better prognosis.[12] Thus, better maintenance therapy is critical for AML patients. The QUAZAR AML-001 study demonstrated that maintenance therapy with oral-Aza significantly prolonged overall and relapse-free survival.[13,14] Interestingly, single agent injectable-Aza did not provide a significant overall survival benefit in maintenance therapy in a similar patient population.[15,16] The key translational question posed by this observation is, what specific mechanisms are associated with the oral formulation that may contribute to the observed clinical benefit in AML maintenance. In order to further explore this, we modeled injectable- and oral-Aza in our preclinical studies and explored the mechanism of action of oral-Aza. Equally important is to identify similarities between the two dosing regimens for potential future investigations of oral-Aza with agents that could combine well with injectable-Aza.

Methods

Human and mouse cell lines

AML cell lines were cultured in RPMI1640 (SKM1), Iscove's Modified Dulbecco's Medium (IMDM; OCI-AML2, OCI-AML20, MV411) or α-MEM (OP9, OCI-AML3, OCI-AML5) supplemented with 10% or 20% fetal bovine serum, sodium pyruvate, glutamine, and penicillin-streptomycin. OP9 cells were further supplemented with β-mercaptoethanol (β-ME) and OCI-AML20 with β-ME and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Flow cytometry

For OCI-AML20, CD33, and CD45, stains were used to distinguish OP9 cells (mouse) and OCI-AML20 cells (human). OCI-AML20 were then assessed for differentiation and changes in the leukemic stem cell (LSC) population with the following markers: live/dead stain, CD34 and CD38. Flow cytometry was performed in a BD LSRFortessa™ X-20.

Myeloperoxidase inhibition

OCI-AML20 cells were treated with Aza (conventional or extended dose) alone or in combination with 10 μM myeloperoxidase (MPO)-IN-28 (dosed on days 1 and 4). Flow analysis was done on day 7.

Cytokine array

OCI-AML20 cells were seeded at 0.5x10^6 cells/mL and treated with Aza, conventional or extended dosing. Supernatant from the cells was collected on days 3, 5, and 7. Cytokine array was performed using Proteome Profiler Human XL Cytokine Array Kit (biotechne, ARY022B), according to the manufacturer's recommendation except that membranes were incubated with IRDye 800CW Streptavidin (Licor, 926-32230) to allow fluorescent detection of the cytokines. Membranes were imaged with Odyssey CLx (Licor).

Primary acute myeloid leukemia cell culture and CD34$^+$ cells enrichment

Frozen primary patient samples (mutations in *BCOR*, *BCORL1*, *CDKN2A*, *IKZF1*, *NOTCH1 RUNX1*, *SF3B1*, *STAG2*, *TET2*, *TP53*) were quickly thawed and followed by CD34$^+$ cells isolation (Stemcell Technologies, 17856) according to the manufacturer's protocol. Flow cytometry was used to verify enrichment of the CD34$^+$ population before treatment initiation. Cells were resuspended to a concentration of 0.5x10^6 cells/mL in X-VIVO 10 medium (Lonza, 04-380Q) supplemented with 20% BIT 9500 serum substitute (STEMCELL Technologies, 09500) and cytokines: interleukin-6 (IL-6) (10 ng/mL; PeproTech, 200-06), IL-3 (10 ng/mL; PeproTech, 200-03), stem cell factor (50 ng/mL; Peprotech, 300-07), FLT3-ligand (50 ng/mL; PeproTech, 300-19), granulocyte colony-stimulating factor (10 ng/mL; PeproTech, 300-23), and TPO (25 ng/mL; PeproTech, 300-18). The cells were dosed with Aza (extended or conventional) and flow analysis was performed on day 7 day with live/dead stain, CD34, and CD38 antibodies.

In vivo studies

For i*n vivo* intrafemoral engraftment studies, we modeled injectable-Aza and oral-Aza doses with 3 mg/kg once daily for 5 days (QDx5) and 1 mg/kg QDx15, respectively (intraperitonially in both cases, modified from Vu *et al.*)[13] Twelve-week female immunodeficient NOD.CB17-Prkdc^{scid}/J (NOD-SCID)[17] mice used for the transplantation of primary AML cells were obtained from the Ontario Cancer Institute (Toronto, Ontario, Canada). All animal studies were performed in accordance with the Ontario Cancer Institute Animal Use Protocol (AUP): # 1251.33 (NOD-SCID).

For the syngenic mouse model: C1498 (ATCC® TIB-49™) murine acute leukemia cells (American Tissue Culture Collection) expressing firefly luciferase and GFP (designated C1498-Luc3-GFP) were developed at Charles River Laboratories. Mice were injected with 1x10^6 C1498-Luc3-GFP tumor cells via the tail vein. Animals were randomized into treatment groups based on body weight on day 1 post-injection. All drug treatments were initiated on day 5 and were administered QD. Vehicle (phosphate-buffered saline [PBS]) and Aza were administered intraperitoneally.

For the chimeric AML model: *FLT3*-internal tandem duplication (ITD), *TET2*, and *LysM-cre* mice were bred to generate a heterologous genotype of *FLT3*-ITD$^{-/+}$*TET2*$^{-/+}$*LysM-cre*$^{-/+}$. Bone marrow chimeras were generated by irradiating mice in an X-ray irradiator at a lethal dose of 9 Gy. Bone marrow from *FLT3*-ITD$^{-/+}$*TET2*$^{-/+}$*LysM-cre*$^{-/+}$ (CD45.2) was adoptively transferred via intravenous injection into 8-week-old CD45.1 congenic disparate recipient mice. Mice were monitored for chimerism after week 8 post-adoptive transfer. Aza was administered intraperitonially.

Results

Extended-dose Aza leads to durable hypomethylation and sustained gene expression independent of the integrated stress response pathway

While both injectable- and oral-Aza have the same active ingredient, their clinical dose and schedules are different, and they do not have similar bioavailability, pharmacokinetic (PK) or pharmacodynamic (PD) profiles.[18,19] In a phase I study, compared with non-responders, patients who achieved clinical response with oral-Aza had greater hypomethylation as measured by global demethylation scores.[19] This indicated that the dosing schedule might impact the hypomethylative effect of Aza. In order to test this hypothesis, we modeled injectable- and oral-Aza based on a recent study by Vu and colleagues.[20] They modeled the clinical exposures of injectable- and oral-Aza using a "high exposure, limited duration" (HE-LD) regimen mimicking injectable-Aza and a "low exposure, extended duration" (LE-ED) mimicking oral-Aza hereafter referred to as conventional and extended dose respectively.

In order to understand the mechanistic basis of greater hypomethylation by oral-Aza, we treated three different AML cell lines with the *in vitro* modeling of the conventional- *versus* extended-dosing regimens. In these Aza-treated samples, genome-wide methylation was measured by whole-genome bisulfite sequencing and gene expression changes by RNA sequencing (RNA-seq) (Figure 1A) on days 3 and 5. While day 3, showed greater genome-wide hypomethylation in the conventional dose in two of the three cell lines (Figure 1B-D, upper panels), by the end of treatment (day 5), the extended-dose Aza regimen demonstrated durable hypomethylation in all three cell lines relative to the conventional Aza (P<0.001) (Figure 1B-D, lower panels, based on β score greater than 0.7; *Online Supplementary Figure S1A*; *Online Supplementary Table S1*). This suggested progressive hypomethylation in the extended dose but not in the conventional dose. Similarly, gene expression changes were greater/progressive in the extended dose at the end of treatment (day 5) (Figure 1E). Intrigued by the sustained effects of the low-dose Aza, we explored the status of DNMT1, which is degraded through entrapment by Aza thereby facilitating hypomethylation.[6] Biochemical analysis revealed loss of DNMT1 (90% reduction) at early time points (Figure 1F). On day 7, DNMT1 levels recovered to nearly 90% with the conventional dose (Figure 1F) but not in the extended dose. Thus, the extended dose, albeit at lower exposures of Aza, facilitates prolonged loss of DNMT1 and thereby durable hypomethylation. Of note, while up to day 10, DNMT1 levels continue to be low in extended Aza treatment, there is about 20-50% cell death at these time points raising the possibility that at time points beyond day 7, cell death could contribute to loss of DNMT1 (*data not shown*).

In order to further understand the sustained hypomethylation of the extended-dose Aza, we analyzed the PD basis in an *in vivo* study measuring [^{14}C] Aza incorporation in peripheral blood mononucelar cells (PBMC) after intraperitoneal administration to male C57BL/6 mice. These mice were treated with the two dosing regimens to mimic the conventional dose (3 mg/kg, QDx5) and extended dose (1 mg/kg, QDx15) regimens of Aza (*Online Supplementary Figure S1B*) with both regimens receiving the same cumulative dose of Aza. Results showed that the relative incorporation of Aza into DNA was greater in the extended dose based on relative area under the curve (AUC) (Figure 1G). Thus, the continued exposure of Aza resulted in a sustained therapeutic effect through greater nucleic acid incorporation thereby explaining the prolonged loss of DNMT1.

RNA incorporation of Aza is a potential mechanism through which, integrated stress response (ISR), one of the known mechanisms of action of Aza,[21] is activated. In a recent study, this has been postulated as a mechanistic basis for synergy with venetoclax.[22] In order to investigate if the extended dose also activates ISR, we treated MV-4-11, SKM-1, and AML-193 cell lines with 0.2 μM or 1 μM Aza in short-term (24 hours) experiments (Figure 1H). As expected, 1 μM Aza, mimicking conventional Aza, resulted in increased levels of eIF2α phosphorylation as well as ATF4, the core and effector of ISR, respectively.[23] With the 0.2 μM dose, we observed slightly lower levels of phospho-eIF2α and ATF4. In addition, treatment with ISRIB,[24] a known inhibitor of ISR, reversed ATF4 expression without impacting DNMT1 loss. In order to investigate if ISR could be activated at later stages of the extended dose, we performed a time-course experiment over a period of 7 days (Figure 1I). While the extended dose was able to induce ATF4 expression, it did not result in activation of C/EBP Homologous Protein (CHOP), a downstream effector of ATF4 that leads to cell death[25] which was observed in the 1 μM dose at 24 hours. These results indicate that while both the conventional and extended dosing trigger a stress response, the extended dose is not robust enough to mediate cell death through the ISR pathway.

Taken together, the above-stated observations indicate that while conventional-Aza works through activation of stress response and hypomethylation mediated changes, the extended dosing of oral-Aza results in sustained gene expression driven temporal effects that could be a key differentiating factor between injectable- and oral-Aza.

Extended dosing of Aza preferentially targets bone marrow blasts than conventional-Aza

In order to further investigate the therapeutic impact of the above-observed differences, we investigated the effect of the two dosing regimens in an *in vivo* syngeneic and patient-derived xenograft (PDX) model of AML. C1498-Luc3-GFP cells were injected into the tail vein of

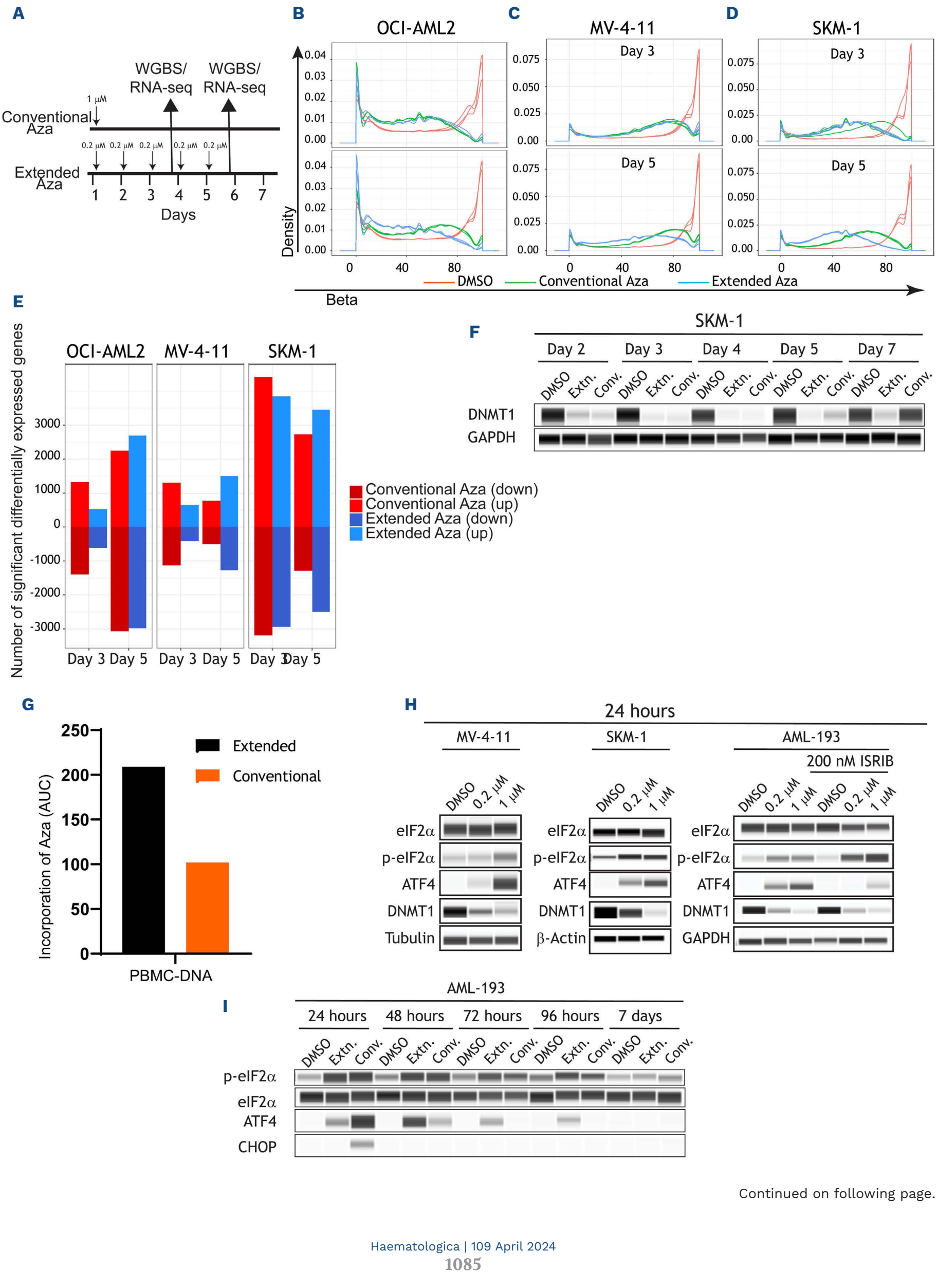

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Figure 1. Extended-dose Aza leads to a more sustained hypomethylation and gene expression. (A) Schematic of experimental design of (B) OCI-AML2, (C) MV-4-11, and (D) SKM-1 cell lines DNA methylation profiles as measured by whole-genome bisulfite sequencing (WGBS) in response to the 2 dosing regimens. Compared to day 3, in day 5 extended dose results in a progressing/sustained hypomethylation. (E) Barplots indicating the total number of significantly differentially expressed genes in all 3 cell lines at the 2 time points (day 3 and day 5). Three replicates per cell line, per time point, per gene was used to generate the barplots using the R limma package. Note the increase in the number of genes in extended dose but not in conventional dose azacitidine (Aza) at the later time point ("down" indicates downregulated and "up" indicates upregulated). (F) DNMT1 levels were measured by protein simple western blotting at the indicated times in SKM-1 cell line. (G) Area under the curve (AUC) barplots representing the amount of Aza incorporation in peripheral blood mononucear cells (PBMC) using the conventional or extended dose of Aza. AUC data was generated from 3 animals and 5 time points for each dosing regimen (H) Biochemical analysis was performed in MV-4-11, SKM-1, and AML-193 cells treated with low (0.2 μM) or high (1 μM) dose of Aza for 24 hours. Protein lysates were prepared and assessed for treatment-mediated activation of ISR and loss of DNMT1. AML-193 experiment was duplicated (right panel) to assess the effect of ISRIB, an ISR inhibitor, in ablating Aza-induced ISR activation. Tubulin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (I) AML-193 cells were treated with Aza in conventional or extended regimen and protein lysates were prepared from days 1-7 and assessed for DNMT1 expression and ISR activation markers. EIF2α was used as a loading control. RNA-seq: RNA sequencing.

syngeneic mice which were then treated with the two dosing regimens of Aza. Ventral bioluminescence imaging (BLI) of luciferase-expressing tumor cells demonstrated that both regimens of Aza significantly decreased tumor burden at 15, 18 and 21-days post-injection (*Online Supplementary Figure S2A*). At day 25 post-injection, flow cytometry analysis demonstrated that both dosing regimens significantly decreased the percentage of GFP^+ C1498 tumor cells in peripheral blood (*Online Supplementary Figure S2B*). Both Aza regimens similarly increased survival of tumor-bearing mice, compared to vehicle controls (Figure 2A) suggesting that both regimens are equally effective in reducing disease burden.

In order to further validate these *in vivo* observations, we used an IDH1R132H PDX AML model, CTG-2227.[26] Cells were injected into the tail vein of sublethally irradiated, immunocompromised mice (NOG-EXL) and the animals were treated with the two dosing regimens. Tumor burden was assessed by measuring spleen weight at the end of the study. Similar to the syngenic model, both dosing regimens caused significant and comparable decreases in spleen weight compared to vehicle-treated animals (Figure 2B). Taken together, the syngeneic and PDX models indicate that despite the difference in dosing schedule, both the Aza dosing regimens are equally effective against bulk AML cells and contribute to lowering disease burden.

Given that oral-Aza is effective in maintenance therapy where leukemic stem/progenitor cells are one of the drivers of relapse, we tested the two dosing regimens in a chimeric AML model. Bone marrow from *FLT3*-ITD; *TET2*; *LysM-cre* compound heterozygous mice on a CD 45.2 background was adoptively transferred to CD45.1 wild-type (WT) recipient mice. Again, the extended-Aza dosing was as effective as the conventional dosing at resolving splenomegaly (Figure 2C), a strong indicator of AML progression; However, the extended-Aza dosing significantly decreased blasts in the bone marrow, whereas conventional dosing did not (Figure 2D). These data suggest that the extended dosing regimen more effectively controls bone marrow disease in this model.

Extended-dose Aza induces differentiation/elimination of leukemic stem cells

LSC are primitive hematopoietic cells related to the origin and relapse in AML.[27,28] In order to investigate the impact of the oral-Aza/extended dosing on the LSC compartment, we utilized an *in vitro* LSC model, OCI-AML20.[29] Extended-Aza dosing resulted in depletion of LSC ($CD34^+/CD38^-$ or CD38 low) and concomitant enrichment of $CD34^+/CD38^+$ cells at day 7 (Figure 3A, representative flow plot; *Online Supplementary Figure S3A*, replicate data). While the conventional-Aza dosing also resulted in depletion of $CD34^+CD38^-$ cells, the extended-Aza dosing had greater depletion of LSC in response to the extended-dose Aza. Notably, no overt cell death/apoptosis observed in these cells (*Online Supplementary Figure S3B*). In order to further validate the differentiation phenotype, we treated a $CD34^+$ cell-enriched primary AML sample (TP53- and TET2-mutated) with both dosing regimens and identified differentiation of $CD34^+$ cells to $CD34^-$ cells (Figure 3B; *Online Supplementary Figure S3C*). In order to further substantiate these observations, we performed single cell RNA-seq in OCI-AML20 cells at different time points (3, 5, and 7 days). Data were analyzed using a previously described classifier to identify leukemic myeloid cell lineages.[30] Compared to untreated cells, the extended dosing resulted in an increase of granulocyte-monocyte progenitor (GMP) and myeloid cells indicating induction of differentiation in immature cells. While the conventional dosing also resulted in similar change in cell type composition, the extended-dosing regimen was associated with significantly greater differentiation compared with the conventional regimen (Figure 3C).

In order to test the effects of oral-Aza like dosing on leukemic progenitors *in vivo*, we leveraged a mouse xenograft model (NOD/SCID). Following injection of AML patient-derived PBMC into the right femur, the mice were treated with Aza regimens mimicking the injectable/conventional dose (3 mg/kg x5 days) or the oral/extended dose (1 mg/kg x15/21 days) (Figure 4A). At day 42, animals were sacrificed, and primary engraftment was measured in the left femur/distal site. Both conventional and extended dosing

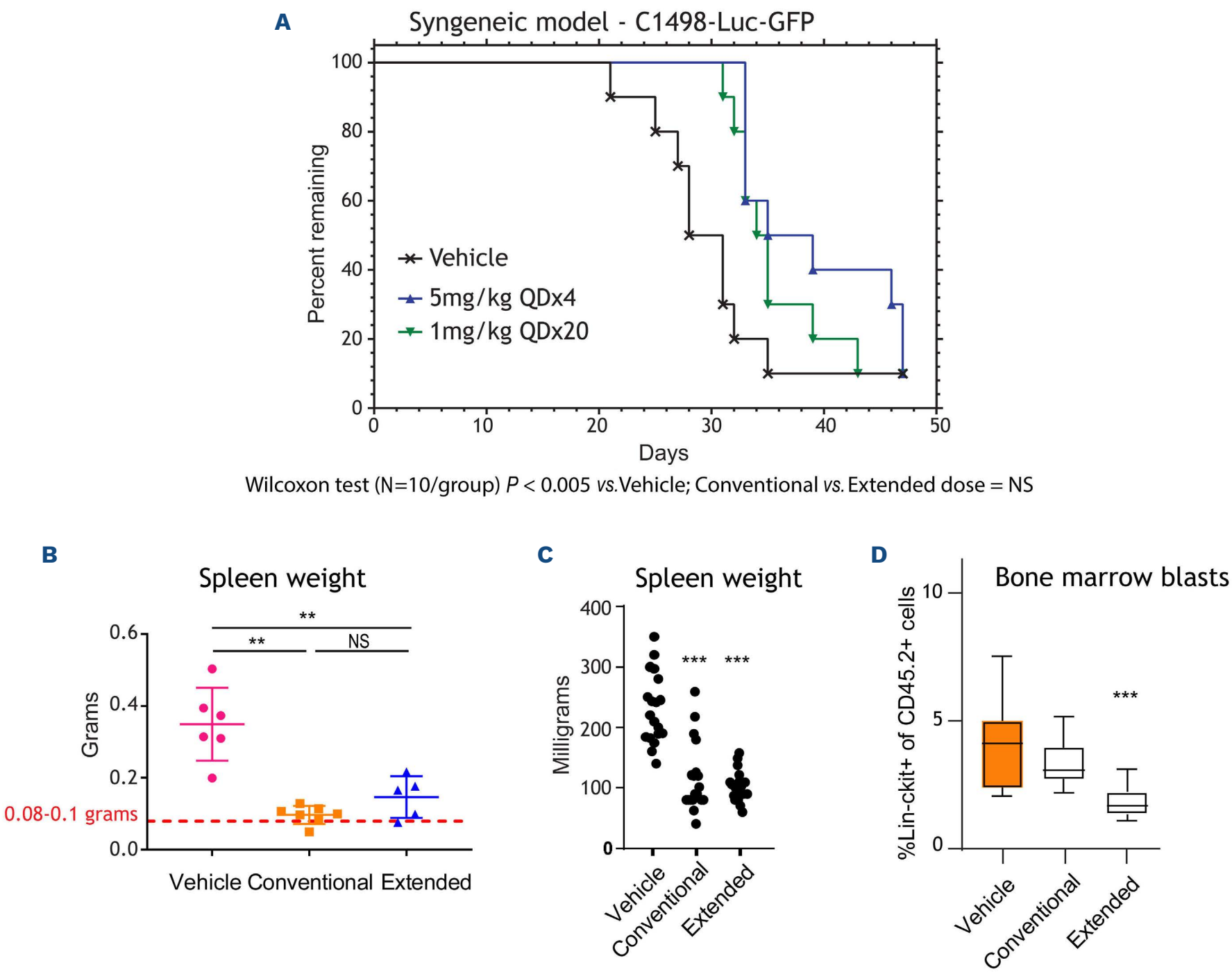

Figure 2. Extended dosing of Aza preferentially targets bone marrow blasts. (A) Survival of B6 albino mice injected with C1498-Luc3-GFP$^+$ cells and treated with vehicle, conventional-azacitidine (Aza) (5 mg/kg once daily for 4 days [QDx4]) or extended-Aza (1 mg/kg QDx20); N=10 per group. Representative bioluminescence imaging (right panel) of mice are shown (N=1 representative mouse/group). Blue to red color represents low to high intensity of bioluminescence. (B) Mean spleen weights measured at the end of the study for the patient-derived xenograft (PDX) acute myeloid leukemia (AML) mice treated with vehicle, conventional-Aza (5 mg/kg QDx5) or extended-Aza dosing (1 mg/kg QDx25) are shown as group mean ± standard deviation (N=5 to 7/group). Red dotted line indicates normal C57BL/6J mouse spleen weight. **P≤ 0.01. (C) Median spleen weights from GEM model of AML harboring *FLT3*-ITD and *TET2* loss (N=17-19/group) (normal spleen weight of wild-type mice is 72 milligrams). *P≤0.05; **P≤0.01, ***P≤ 0.001 for Aza relative to vehicle/isotype control using non-parametric one-way ANOVA. (D) Flow cytometry was performed on cells collected at the end of week 4 and cell percentages are presented as group medians (N=12/group). ***P≤0.001 for Aza relative to vehicle/isotype control. P values were calculated using non-parametric one-way ANOVA. NS: not significant.

reduced engraftment by about 10-fold relative to the control treatment (Figure 4B).

In order to further validate the differentiation phenotype observed with the extended dose, we analyzed the human PBMC that remained at the site of injection (right femur) in the primary engraftment experiment. Similar to the engraftment, the degree of differentiation induction on progenitors was similar in both regimens. Most of the cells in the sample tested were arrested in the GMP-like phase and both regimens of Aza induced the differentiation of >50% of these cells into a common myeloid progenitor (CMP) or megakaryocyte-erythrocyte progenitor (MEP) like phenotype (Figure 4C-E). There was also reduction of CD33$^+$CD123$^+$ stem/progenitor cells at the site of injection (Figure 4F). Notably, there was no overt loss of weight in the animals (Figure 4G).

Taken together, our data based on an *in vitro* LSC model suggest that the efficacy of oral-Aza in AML maintenance is in part linked to the ability of the extended dosing schedule to target LSC. In addition, the *in vivo* model demonstrates

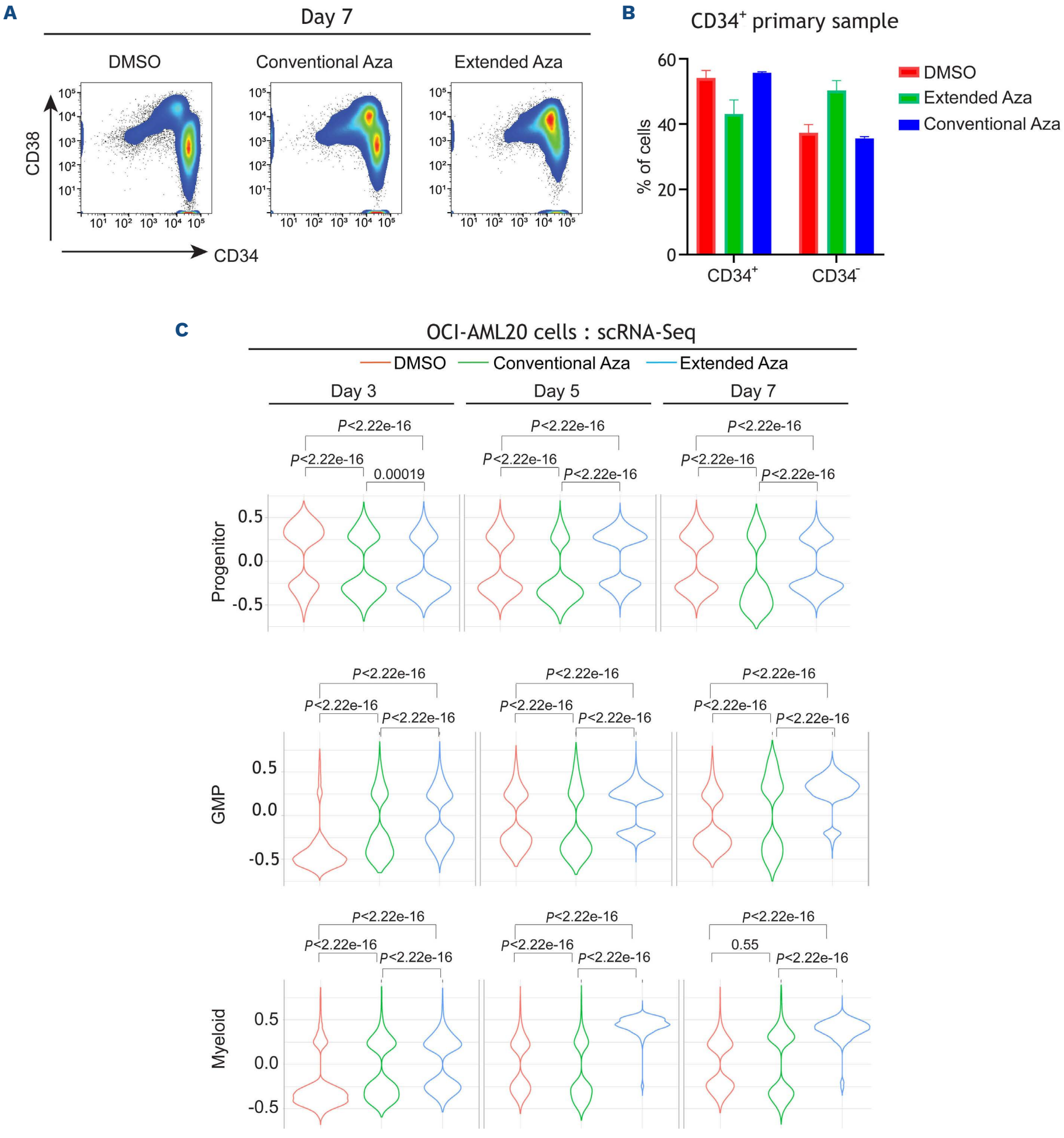

Figure 3. Extended dosing of Aza induces greater differentiation of leukemic stem cells. (A) OCI-AML20 cells were cultured on OP9 feeder layer and the cells were treated with dimethyl sulfoxide (DMSO) or azacitidine (Aza) (extended or conventional dosing). At day 7, all cells were collected and subjected to flow cytometry. After discriminating live/dead cells and singlets, human cells (to differentiate from mouse OP9 cells) were identified by $CD33^+/CD45^+$ staining. Within that population the profile of CD34/CD38 was identified. (B) $CD34^+$-enriched primary acute myeloid leukemia (AML) sample was treated with both dosing regimens. Flow cytometry was performed on day 7 to identify CD34 and CD38 population (C) Samples from the above-described experiment were collected and subjected to single-cell RNA sequencing (scRNA-seq) as described in the methods. Violin plots indicating the treatment-specific distribution and the Wilcoxon *P* value of the GSVA enrichment on Van Galen signatures at days 3, 5, and 7. GMP: granulocyte-monocyte progenitors.

that oral-Aza induces differentiation of LSC and leukemic progenitor cells.

Extended-dose Aza induces differentiation through increased myeloperoxidase activity

In order to understand the molecular determinants of the greater degree of differentiation occurring in the LSC compartment in the extended dose, we sorted $CD34^+CD38^-$ cells from OCI-AML20 cells (Figure 5A) and treated them with the two Aza dosing regimens and performed bulk RNA-seq at day 5. As expected, pathway analysis (GO process) revealed that the most significantly upregulated pathway was cellular differentiation in the $CD34^+CD38^-$ cells upon 5 days of extended dosing but not in the conventional dosing (Figure 5B).

In order to investigate drivers of the upregulated cell differentiation pathway, we analyzed secreted factors from cell culture supernatants of OCI-AML20 cells treated with both the Aza-dosing regimens in a feeder layer-free culture using the Proteome Profiler™ antibody array. Among the differentially secreted factors, MPO, a hallmark enzyme of the myeloid lineage was the secreted factor that was also the most upregulated by gene expression analysis in LSC that were treated with the extended-dose Aza (*Online Supplementary Figure S4A*). In addition, western blot analysis confirmed that MPO is upregulated in OCI-AML20 cells as well as in $CD34^+$ cells from a primary sample (TP53- and TET2-mutated) in response to the extended dose (Figure 5C). Given MPO's association to myeloid differentiation, we hypothesized that upregulation of MPO could be implicated in extended Aza-induced LSC differentiation. In order to test this hypothesis, we used an inhibitor of MPO, MPO-IN-28[31] and treated OCI-AML20 cells with MPO-IN-28 and the both the Aza doses. Inhibition of MPO countered the differentiation induction by extended-dose Aza (Figure 5D; *Online Supplementary Figure S4B*) by more than 20% reduction in the non-LSC population ($CD34^+CD38^+$ or $CD34^-$). Being a peroxidase, MPO has been shown to induce reactive oxygen species (ROS) generation in myeloid cells,[32] a potential mechanism that has been shown to induce differentiation of LSC.[33,34] In order to investigate if MPO-induced ROS could facilitate differentiation in LSC, OCI-AML20 cells were concurrently treated with N-acetylcysteine (NAC) and the two Aza dosing regimens. ROS scavenging with NAC, blocked differentiation of LSC with the extended dose but not with the conventional dose further validating our hypothesis (Figure 5E; *Online Supplementary Figure S4C*). Taken together, our results suggest that extended dose Aza induces differentiation of LSC through upregulation of MPO and ROS.

Discussion

In AML maintenance therapy, the primary goal is to eliminate residual leukemic cells in the bone marrow thereby eliminating minimal residual disease (MRD) and reducing the risk of relapse. However, rare therapy-resistant cells that reside within the MRD, also known as LSC, can drive clonal leukemic repopulation resulting in relapse.[28,35] Thus, understanding the biology of these LSC and identifying therapeutic agents that effectively target LSC during induction or maintenance therapy would be important in reducing rates of relapse in AML. While oral-Aza is effective in AML maintenance therapy, the mechanistic basis was not clear. Our preclinical work, for the first time demonstrates oral-Aza's ability to target LSC.

Even though both injectable- and oral-Aza have the same active ingredient, the dosing schedule results in varying PK and PD profiles as well as therapeutic effects. Based on the preclinical data presented here, oral-Aza acts primarily through sustained hypomethylation-mediated gene expression changes and injectable-Aza acts through stress response as well as hypomethylation (not as sustained as oral-Aza). Consistent with our observations, a study with low-risk MDS patients reported sustained hypomethylation imparted by oral-Aza over the entire treatment cycle (up to 21 days). In addition, patients administered with oral-Aza and with resultant reduction in DNA methylation had better hematologic response.[36] Similarly, sustained hypomethylation with oral-Aza were observed in another independent study.[19]

Given the biochemical differences observed (Figure 1) between the two dosing regimens, our *in vivo* studies yielded interesting similarities and differences (Figure 2) highlighting the functional impact of the two dosing regimens based on differentiation state of the cell. Both oral- and injectable-Aza were equally efficacious in targeting bulk AML cells in the syngeneic and the PDX model, both of which used bulk AML cells. However, when we looked at less differentiated bone marrow blast cells in the *FLT3*-ITD; TET2 model, the extended dose was effective in targeting this less differentiated population. When we applied this finding to an *in vitro* LSC model (OCI-AML20), we observed similar results and demonstrated that oral-Aza is more effective in eliminating immature myeloid cells and inducing differentiation of leukemic stem cells through upregulation of MPO. MPO, being a ROS generator, in turn likely acts through ROS to induce differentiation of LSC. Inhibition of MPO activity as well as ROS scavenging both rescue/block oral-Aza-mediated differentiation of LSC. Thus, a key understanding from our work is that the impact of oral-Aza is cell type specific.

Aza in combination with other agents has been shown to target LSC and induce differentiation.[37,38] However, the specific Aza-associated mechanism was not clear. A key understanding from our work is the differential effect of oral-Aza on LSC *versus* non-LSC. In the *in vitro* model (OCI-AML20) enriched in LSC, the extended dose was more effective than the conventional dose. In order to recapitulate

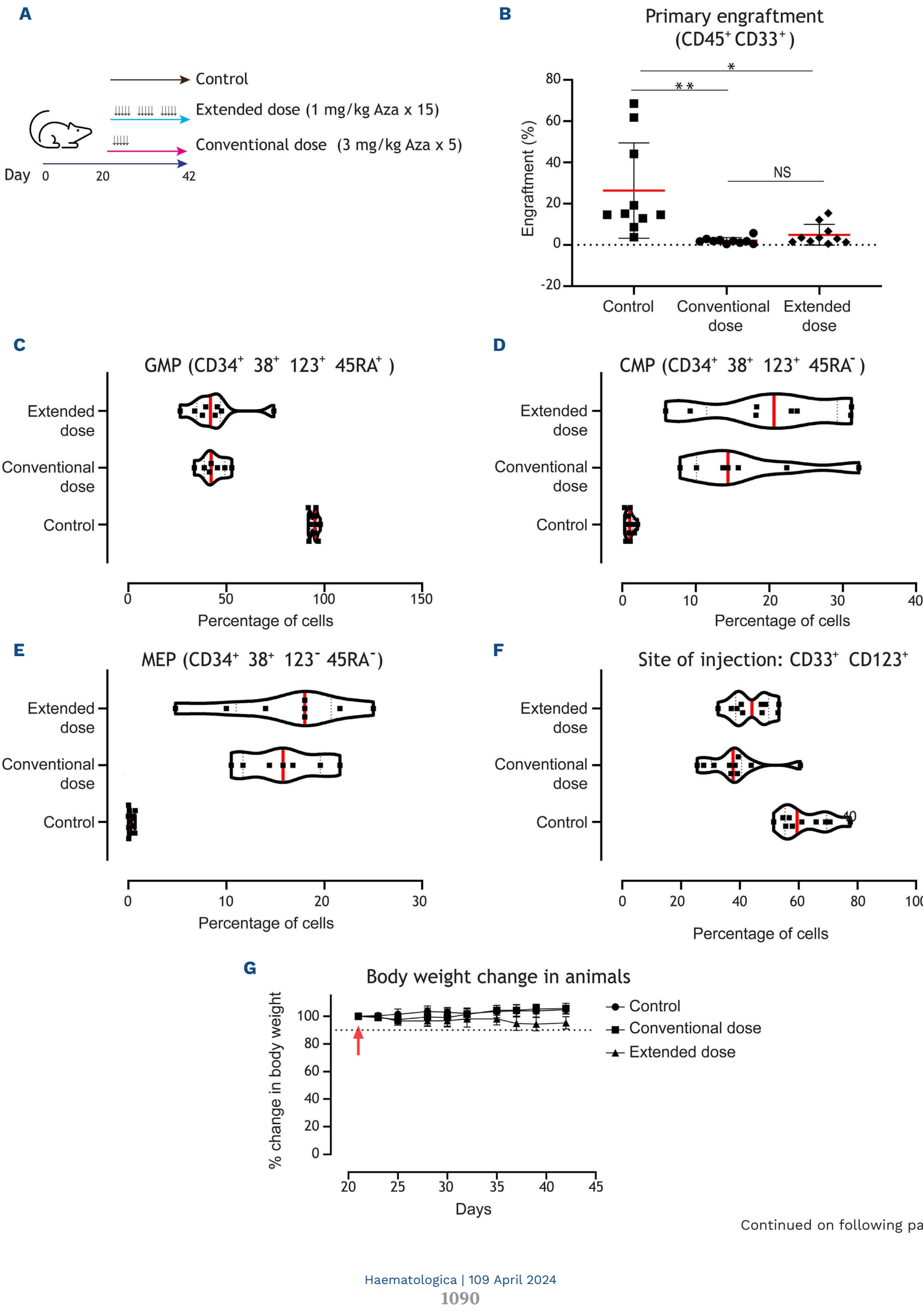

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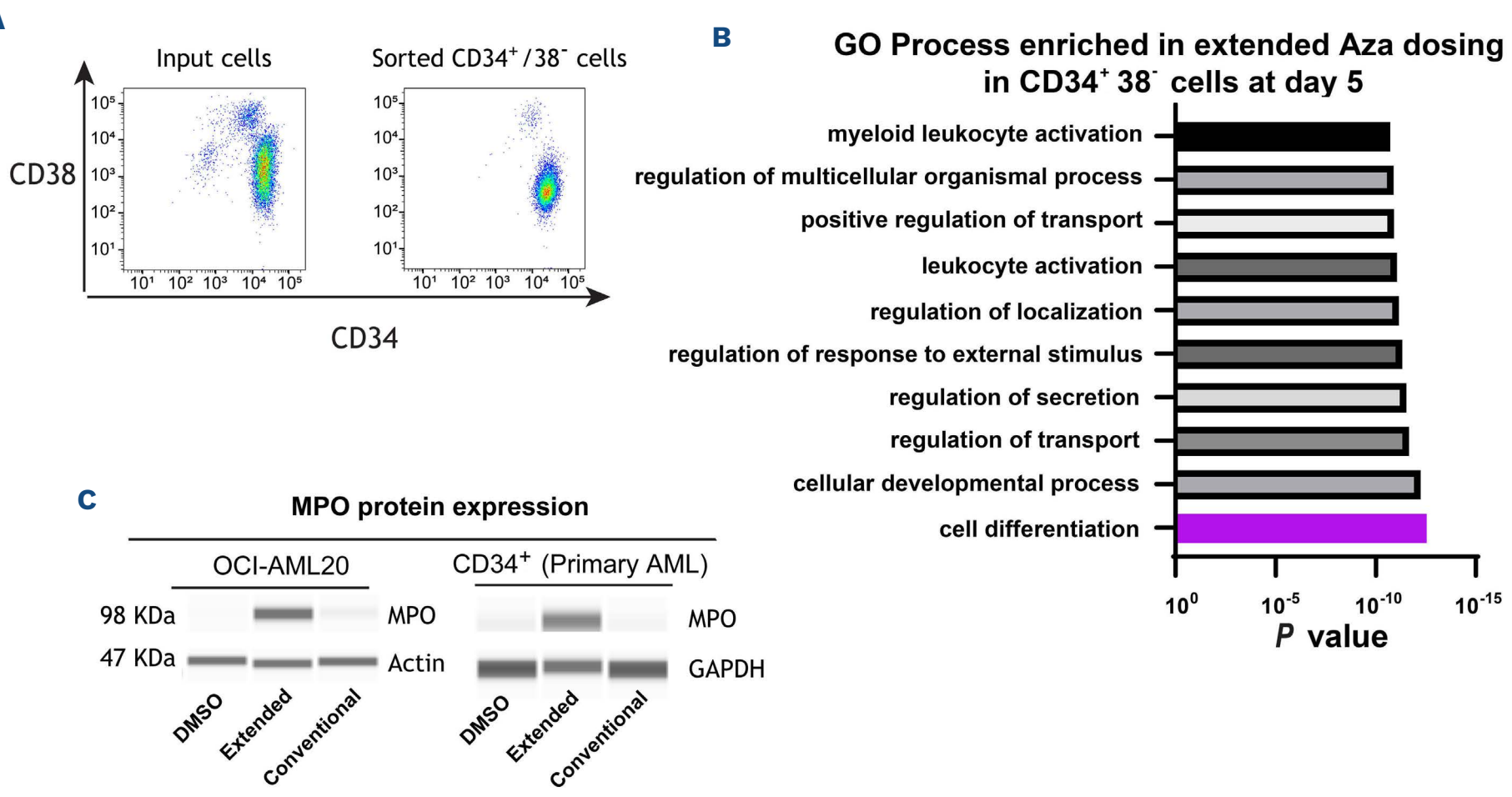
Figure 4. Extended-dose Aza is equally effective as conventional dosing in targeting acute myeloid leukemia cells and stem/progenitor cells *in vivo*. (A) Schematic of experiment. Primary acute myeloid leukemia (AML) cells were injected into the right femurs of sub-lethally irradiated NOD/SCID mice. Twenty days after injection, mice (10 animals per group) were treated with vehicle or 2 different doses of azacitidine (Aza) mimicking conventional/injectable dosing (3 mg/kg 5 doses for 5 consecutive days) or extended dosing (1 mg/kg 15 doses over 21 days [5/7 days per week]). At the end of treatment (42 days from day of injection), flow cytometry was performed in both right and left femurs to measure engraftment. (B) Plots indicate the percentage of human cells that engrafted in the left femur (distal site). The red bar indicates the median values, and each data point indicates a mouse. In order to measure the ability of Aza to target the persistence of leukemic cells at the site injection, human cells in the right femur (site of injection) were assessed with the indicated surface markers to identify (C) granulocyte-monocyte progenitors (GMP), (D) common myeloid progenitors (CMP) and (E) megakaryocyte-erythrocyte progenitors (MEP) (F) CD123 (IL-3 receptor α chain), a marker for AML stem and progenitor cells, was also reduced at the site of injection demonstrating equivalent efficacy for the conventional dosing and the extended dosing of Aza. (G) Animal body weight was monitored during treatment as a proxy for animal health. The dotted line indicates 90% (10% change). Data represent median ± standard deviation. **P*<0.05 and ***P*<0.01 by unpaired *t* test.

the *in vitro* findings, we performed engraftment studies of primary AML samples using bulk mononuclear cells which predominantly includes progenitors and blasts. The two dosing regimens performed similarly in reducing disease burden. Notably, conventional Aza is administered for 7 days in the clinic while our animal models received Aza only for 5 days based on our modeling. While we attempted to perform secondary engraftment, a measure of the capacity of re-initiation of leukemia by LSC when serially transplanted into mice, we did not achieve any engraftment in the conventional or extended-dose regimens even at the site of injection in the secondary mice likely because of the robust effect of both dosing regimens in the primary engraftment (*data not shown*). Thus, we could not leverage our transplant models to interrogate effects on LSC *in vivo*. Multiple studies have demonstrated that LSC rely on oxidative phosphorylation, a process that could generate more ROS and result in loss of stemness.[33,39,40] However, LSC have developed unique mechanisms to maintain stemness by countering increased ROS such as increased antioxidant enzymes, increased levels of mitophagy and others.[41-44] Thus, when oxidative stress/damage/signaling increases, stem cells tend to differentiate.[45,46,33] MPO is a hallmark enzyme of the myeloid lineage and patients with high MPO have been shown to have superior overall survival[47] and has been shown to be expressed at high levels in cells committed to granulomonocytic differentiation and activation of MPO facilitates oxidative damage.[48] In



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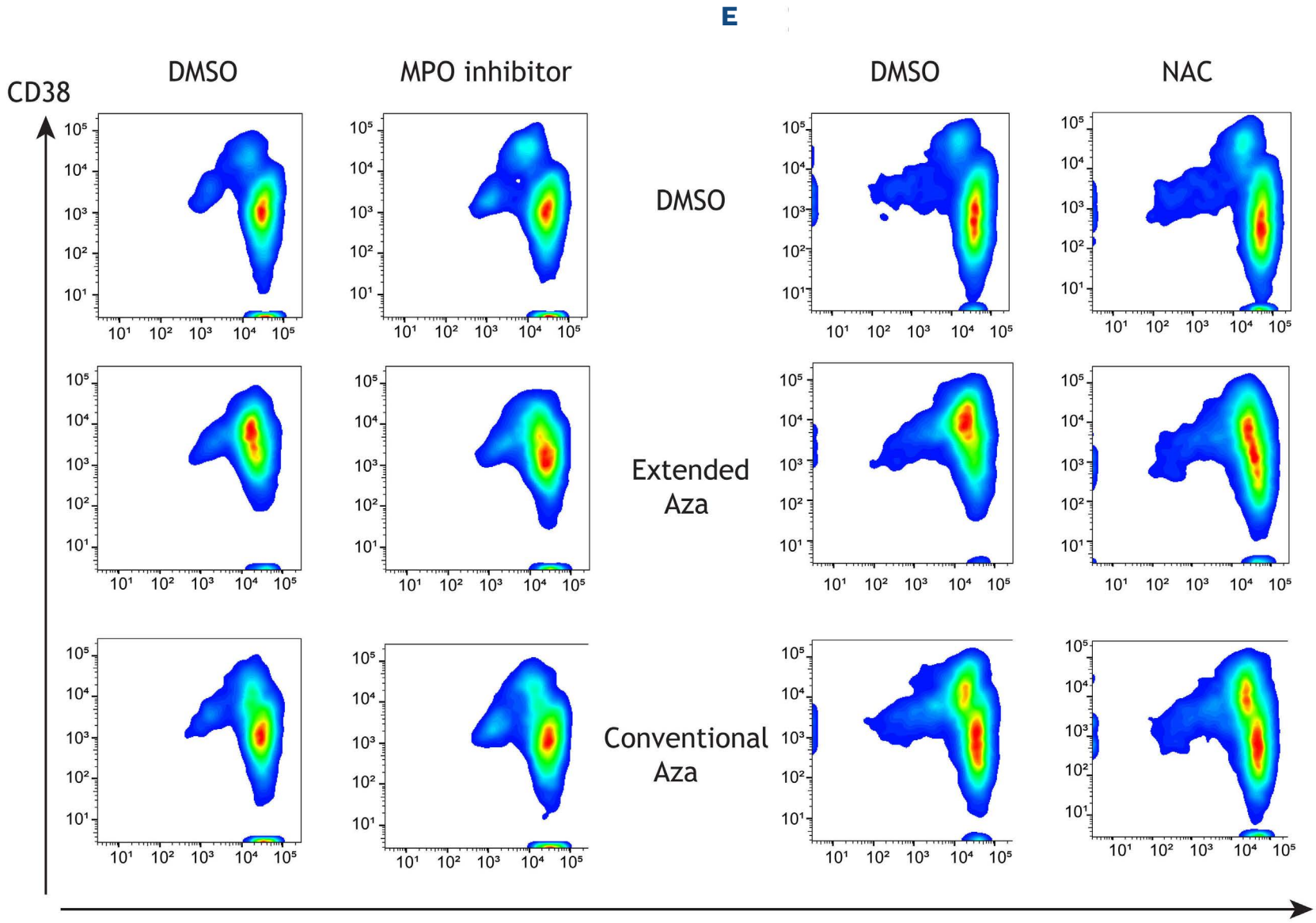

Figure 5. Extended-dose Aza upregulates myeloperoxidase expression to induce leukemic stem cell differentiation, which can be partly rescued by myeloperoxidase inhibition or reactive oxygen species scavenging. (A) OCI-AML20 cells were sorted to retrieve the $CD34^{+}38^{-}$ population. (B) Sorted cells were treated with azacitidine (Aza) (extended or conventional dose) and bulk RNA sequencing was performed at day 5 followed by pathway enrichment analysis (gene ontology [GO] process) to assess Aza-induced pathways. (C) OCI-AML20 cells were treated with Aza (conventional or extended regimen) and myeloperoxidase (MPO) expression was assessed by capillary electrophoresis on day 7, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (D) Representative flow data from 1 experiment with OCI-AML20 cells were treated with extended or conventional regimen of Aza with or without 10 μM MPO-IN-28 or (E) N-acetylcysteine (NAC) on days 2 and 4 and flow cytometry assessment was conducted on day 7 using live/dead, CD45, CD34 and CD38 staining. DMSO: dimethyl sulfoxide.

addition, MPO has been shown to be increased in response to Aza in a few cases through promoter demethylation by DNMT-inhibitors.[49,50] Our work uniquely demonstrates MPO upregulation in the LSC compartment upon Aza treatment which induce differentiation through oxidative stress as evidenced by a block in differentiation through ROS scavenging. A limitation of our study is that the cell lines and the LSC model that we have used are not truly representative of *de novo* AML and do not carry the common molecular mutations observed in elderly AML patients. To this end we attempted to replicate our key findings in commercially available multiple primary AML samples. However, we were successful in culturing $CD34^{+}$ cells from only one primary sample (TET2- and TP53-mutated and not the commonly observed *DNMT3A/NPM1/FLT3*-ITD mutation) for 7 days and replicated our key findings.

Overall, we have highlighted mechanistic insights of oral-Aza that make it an effective maintenance therapy. Aza is known to have additional mechanisms of action beyond the ones described here including immune modulation, viral mimicry, and others. While we have primarily focused on tumor intrinsic mechanisms as part of this work, our data warrant exploring other mechanisms where Aza is implicated and identify further unique properties of oral-Aza.

Disclosures

DVJ, MA, AP, AR, PS, AA, KG, CK, DH, DLM, PRH and AG are current employees of Bristol Myers Squibb and received equity compensation. ADS has received research funding from Takeda Pharmaceuticals, BMS and Medivir AB, and consulting fees/honorarium from Takeda, Novartis, Jazz, and Otsuka Pharmaceuticals; and is named on a patent application for

the use of DNT cells to treat AML. AGT and AA are former employees of BMS and received equity compensation. EFL received research funding from Celgene/BMS.

Contributions

DVJ, MA, AP, AR, PS, CK, DH, RH, XW, MG, AA, GT and HC designed and performed the research, analyzed data, and wrote the paper. AA, EFL, KG, DLM, ADS, PRH, AG and AGT designed the research, analyzed data and wrote the paper.

Data-sharing statement

Original data are available upon request to the first and corresponding authors.

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