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A novel vitamin D gene therapy for acute myeloid leukemia

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ABSTRACT

Current treatment approaches for older adult patients with acute myeloid leukemia (AML) are often toxic and lack efficacy. Active vitamin D3 ($1,25(OH)_2D3$) has been shown to induce myeloid blast differentiation but at concentrations that have resulted in unacceptable, off-target hypercalcemia in clinical trials. In our study, we found that the combination of $1,25(OH)_2D3$ and the hypomethylating agent (HMA) 5-Azacytidine (AZA) enhanced cytotoxicity and differentiation, and inhibited proliferation of several AML cell lines (MOLM-14, HL60) and primary AML patient samples. This observation was corroborated by our RNA sequence analysis data in which VDR, CD14, and BAX expression were increased, and FLT-3, PIM1 and Bcl-2 expression were decreased. To address the hypercalcemia issue, we genetically engineered MOLM-14 cells to constantly express CYP27B1 (the VD3 activating enzyme, 1-α-hydroxylase-25(OH) D3) through lentiviral transduction procedures. Subsequently, we used these cells as vehicles to deliver the CYP27B1 enzyme to the bone marrow of AML mice. We observed that AML mice with CYP27B1 treatment had longer overall survival compared to no treatment and displayed no significant change in calcium level.

Introduction

Acute myeloid leukemia (AML) is a bone marrow (BM) malignancy in which immature hematopoietic myeloid precursors have lost their ability to differentiate and are locked in a highly proliferative state [1,2]. Standard treatment for AML consists of induction chemotherapy with a combination of anthracyclines and cytarabine. An important factor in AML treatment is the patient's performance status (PS). Many elderly patients cannot tolerate intensive regimens because of co-morbidities and poor PS [3,4]. As a result, outcomes for this group, who actually constitute the majority of AML, are extremely poor [5]. Recently, with the introduction of hypomethylating agents (HMAs) and venetoclax, we observed longer progression-free survival for this population; however, most patients will eventually succumb to the disease and toxicity remains a concern [6]. Further research on less toxic and more effective therapeutic modalities for older AML patients is necessary [7].

Vitamin D is a group of fat-soluble secosteroids responsible for essential roles in bone metabolism and calcium homeostasis [8]. An equally important but less known function of active $1,25(OH)_2D3$ is its autocrine/paracrine effect on local tissues (*i.e.* skin, placenta, colon, pancreas, and macrophage) through extra-renal expression of CYP27B1. The CYP27B1 gene encodes a member of the cytochrome P450 superfamily of enzymes, which hydroxylases 25-hydroxyvitamin D3 at the 1-alpha position, resulting in active $1,25(OH)_2D3$. Ogunkolade et al. found that the expression of CYP27B1 mRNA in healthy colon tissue surrounding cancerous tissue in patients with colorectal cancer was significantly lower compared to colon tissue of healthy patients, suggesting that the tumor could have secreted endocrine/paracrine factors that could affect the enzyme level [9]. We have limited knowledge of CYP27B1 expression in the BM of AML patients.

There is considerable interest in using $1,25(OH)_2D3$ to treat cancer due to its ability to inhibit proliferation and induce differentiation in many types of

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cancer cells (*i.e.* colon, breast, prostate). As a secosteroid hormone, 1,25(OH)$_2$D3 binds to the nuclear vitamin D receptor (VDR), which heterodimerizes with the retinoid X receptor (RXR) and attaches to the promoter regions of target genes [10–14]. It has been reported that 1,25(OH)$_2$D3 induces differentiation of monoblast cells through interactions with major molecular pathways such as MAPKs, JAK/STAT, and PI3K/Akt [15–19]. 1,25(OH)$_2$D3 stimulated monocytic differentiation was associated with increased ERK and JNK activity and was augmented by p38 MAPK inhibition [20]. Prior studies demonstrated that increased PI3K/Akt activity promotes neutrophil and monocyte development and is critical to induction of monocytic differentiation and protection from apoptosis [15,17,18]. In addition to the two aforementioned pathways, 1,25(OH)$_2$D3 also causes phospholipase A2-mediated release of arachidonic acid from leukemic cells, which in turn increases protein kinase C activity, leading to monocytic differentiation [21,22]. However, the concentrations of 1,25(OH)$_2$D3 required to induce differentiation *in vitro* are typically in the range of 100–1000 nanomolar (nM) [23], and such a serum level would result in serious hypercalcemia in humans (typical concentration around 0.1 nM). Thus, many clinical trials failed because they could not achieve therapeutic levels of 1,25(OH)$_2$D3 without systemic hypercalcemia [23–25].

There is a mutual interaction between the 1,25(OH)$_2$D3 system and epigenetic mechanisms. 1,25(OH)$_2$D3 has been reported to be able to alter methylation of DNA in the promoters of some genes. Conversely, 1,25(OH)$_2$D3 insensitivity is related to methylation of the VDR promoter, which impairs 1,25(OH)$_2$D3 regulation of tumor suppressor genes [26–28]. Epigenetic modification drugs have the potential to reverse 1,25(OH)$_2$D3 insensitivity. VDR reactivation can be induced by HDAC inhibitors in combination with 1,25(OH)$_2$D3, which has been shown to upregulate a unique group of suppressed gene targets in control of proliferation and induction of apoptosis [29,30]. Other study also indicates a synergistic role of DNA demethylation in 1,25(OH)$_2$D3 metabolism and enhancement of 1,25(OH)$_2$D3 efficiency for monocytic differentiation by a DNA hypomethylating agent [31]. VDR interacts with chromatin modifiers and remodelers directly or indirectly to fine-tune gene expression, whereas VDR and 1,25(OH)$_2$D3 target genes can be silenced by DNA methylation or histone modification [26–28,32,33]. Because both 5-Azacytidine (AZA), a hypomethylating agent approved by the FDA for palliative use in AML patients and 1,25(OH)$_2$D3 demonstrate the ability to affect gene expression and induce leukemic differentiation, it is possible that their combined treatment might result in enhanced anti-leukemic effects. Here, we study the anti-leukemia effects of combining 1,25(OH)$_2$D3 and AZA on AML cells *in vitro*, and *ex vivo*. Our studies also address the issue of systemic hypercalcemia through the CYP27B1 gene therapy. We use of vehicle cells to carry the CYP27B1 gene to the BM to produce local, high concentration 1,25(OH)$_2$D3, thus limiting systemic hypercalcemia.

Materials and methods

The list of reagents including manufacturers and catalogues of antibodies and kits are found in the Supplementary data (Table S1). Detailed information on cell lines, primary leukemic samples, mice and IACUC approved protocols can be found in Supplementary materials and methods.

Combination of 1,25(OH)$_2$D$_3$ and AZA in vitro and ex vivo

1×10^6 cells/ml AML primary cells and cell lines were plated into 12-well plates for 2 days in the presence of different concentrations of 1,25(OH)2D3, or AZA, or combination of 1,25(OH)2D3 and AZA. The cells were then harvested and examined for the expression of CD14 or phenotypic blasts by flow cytometry. Each experiment was replicated a minimum of three times for reproducibility.

Preparation of plasmid constructs and lenti-viruses

The lenti-CYP-GFP plasmid was used in this study, which carried the cytochrome P450 family 27 subfamily B member 1 (CYP27B1) gene for encoding the 25-hydroxyvitamin D 1α-hydroxylase (1α-hydroxylase) (Fig. S3). The detailed protocol for generating lentivirus can be found in Supplementary materials and methods.

RNA-sequencing and data processing

AML blast cell samples pretreated for 48 h with 1,25(OH)2D3, or AZA, or combination of 1,25(OH)2D3 and AZA were collected and sent to BGI Hong Kong for RNA preparation and sequencing. RNA-seq libraries were prepared by BGI Hong Kong. Expression data was compared between both samples by the analysis of individual selected genes for differential expression. Plots represent differentially expressed genes in fragments per kilobase of exon per million fragments mapped (FPKM).

Establishment of xenograft mouse model and combination treatment

To generate the MOLM-14/NRG xenograft AML mouse model, on day −15, NRG mice were injected with intra-peritoneal (IP) AZA (10 mg/kg), and 5×10^5 cherry-MOLM-14 cells were injected on day −14 intravenously (IV) through tail vein. AZA was used to deplete BM cells *in vivo*. Some mice were sacrificed at day 0 for histological analysis to confirm the establishment of the AML model. Treatments were given on day −1 and day 0 based on study group (see specific description in Fig. 5A). The mortality and body weights of experimental mice were recorded daily.

Statistical analysis

Statistical significance was assessed by ANOVA or by independent student "t" test for comparison between two groups. All values were presented as mean ± SEM. In comparing the survival time of the mice, all times were measured from the day of BM transplant, estimated by the method of Kaplan and Meier. Results were considered significant when the p value was <0.05.

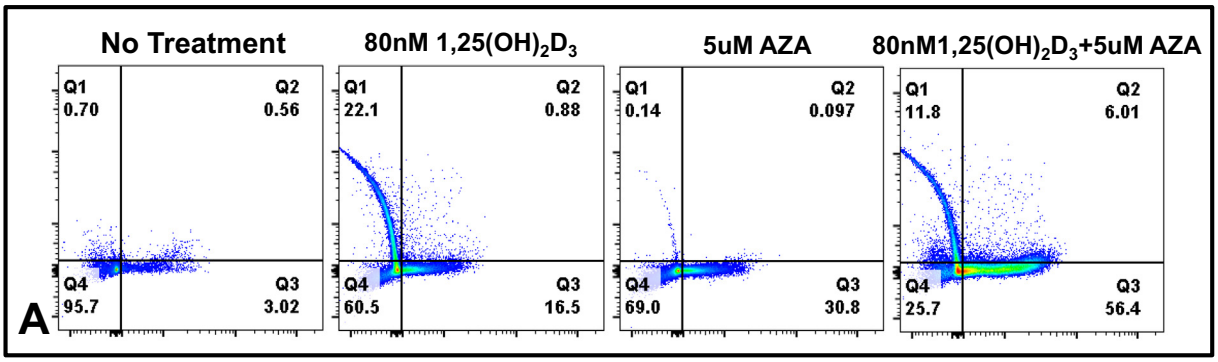
For original data, please contact hcao@llu.edu.

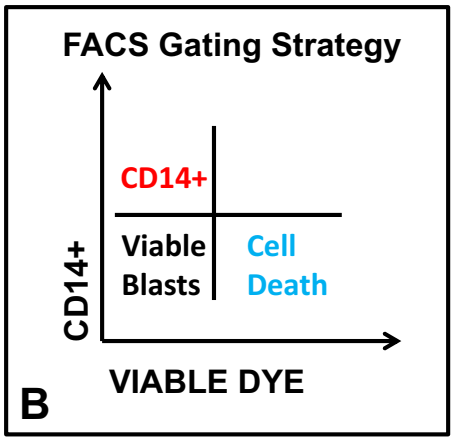
Results

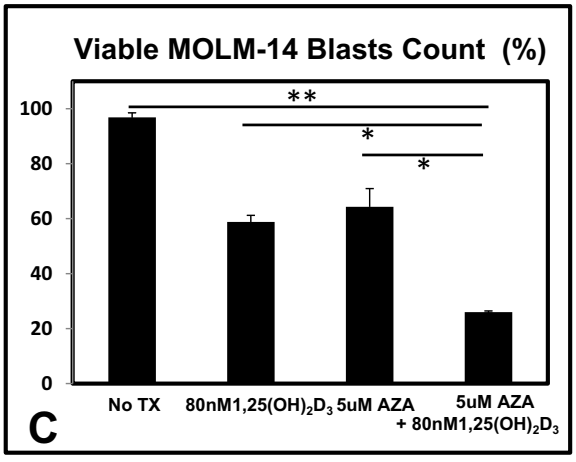
Enhanced in vitro anti-leukemic effects of 1,25(OH)2D3 in combination with AZA on AML cell lines

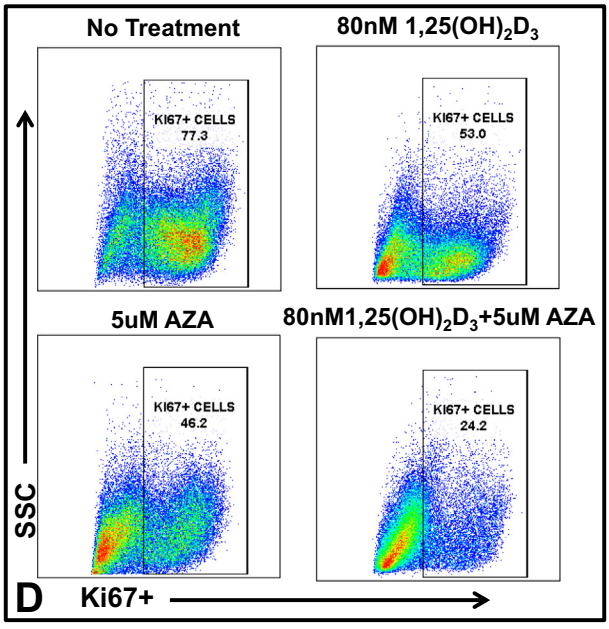
We tested the combination of AZA and 1,25(OH)2D3 for anti-leukemia effects on MOLM-14 cells, a monoblast cell line harboring a FLT3 internal tandem duplication mutation (FLT3-ITD). We treated MOLM-14 cells with different concentrations of 1,25(OH)$_2$D$_3$ and AZA alone and in combination for 48 h. Cytotoxic effects were determined by flow cytometry (FACS) to detect CD14 and viability dye (Fig. 1A, see Fig. 1B for gating strategy). Combination therapy significantly reduced the percentage of viable blasts (Viable/CD14-cells) compared to 1,25(OH)$_2$D3 alone and AZA alone (25.7% *vs.* 60.5% and *vs.* 69.0%, respectively) (Fig. 1A, C). Similar results were obtained with the HL60 AML cell line; however, combination therapy was not effective for the THP-1 AML cell line (Figs. S1, S2). Further FACS analysis showed that 1,25(OH)$_2$D3 and AZA in combination significantly reduced the blast populations through increased cell death (62.4% combination, *vs.* 17.4% 1,25(OH)$_2$D3 alone, and 30.9% AZA alone).

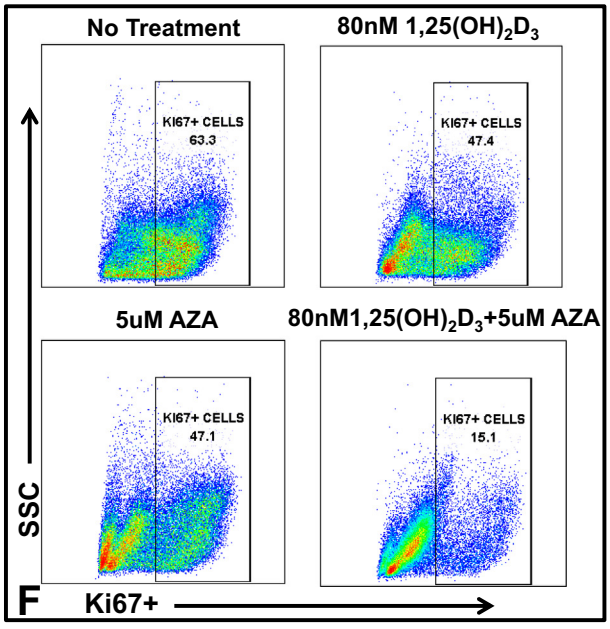
In addition to enhanced cytotoxicity, combination therapy also significantly reduced the proliferation of viable MOLM-14 blasts, as indicated by Ki-67 staining (see Fig. S3 for gating strategy), when compared to no treatment, 1,25(OH)$_2$D3 alone and AZA alone (25.2% *vs.* 73.6%, 55.5% and 49.0%, respectively) (Fig. 1D, E). Similar results were obtained (Fig. 1F, G), with MOLM-14-TKIR, an acute myeloblastic leukemia cell line resistant to midostaurin (see Supplementary materials and methods for generation of this cell line). Collectively, our data suggest that the combination of 1,25(OH)$_2$D3 and AZA is effective in enhancing cell death and inhibiting the proliferation of AML blasts *in vitro*.

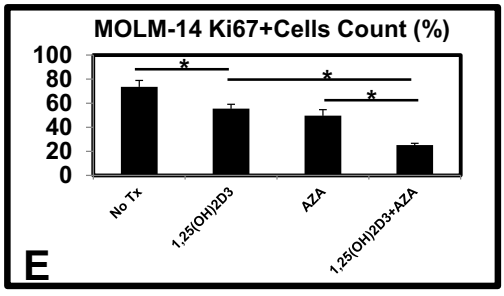


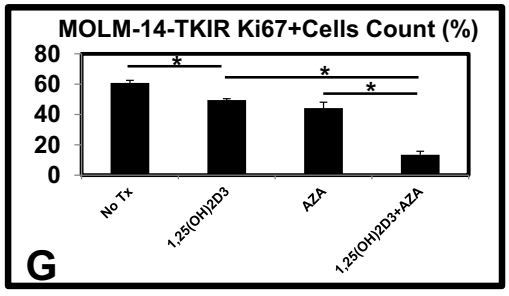












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Effects of combined treatment with 1,25(OH)$_2$D$_3$/AZA on primary AML and CD34+ hematopoietic stem cells (HSCs)

Next, we tested the combination therapy on primary AML blasts. Blasts were treated with different concentrations of 1,25(OH)$_2$D$_3$, AZA, and their combination for 48 h. FACS analyses demonstrated that combination therapy consistently reduced blasts more than either single therapy alone in patient A (Fig. 2A and B). Data from 7 primary leukemia patient samples and their mean reduction in blast counts are shown in Table 1 and Fig. 2D. Combination therapy was effective against patient samples (obtained from BM marrow aspirates) with different mutations, particularly the FLT-3 mutation (more than 3-fold reduction in blast counts, AZA *vs.* combination therapy, $p < 0.05$) (Fig. 2D, Table 1). Importantly, when using this combination to treat patient samples (initial blast population negative for CD34), we noticed that while the therapy suppressed the blast population, it did not affect the CD34+ population (Fig. 2C). It would be interesting in future experiments to determine whether this population is actually CD34 + HSCs or leukemic stem cells.

Mechanism of action of combined treatment with 1,25(OH)$_2$D3 and AZA

To understand the mechanism of 1,25(OH)$_2$D$_3$ based treatment for AML, in context of combination with AZA, we performed the RNA-Seq transcriptome profiling of different AML cells lines, which were pre-treated with either 1,25(OH)$_2$D$_3$ alone, AZA alone or their combination *in vitro* for 48 h (Fig. 3). Here, we report the RNA-Seq results from MOLM-14, MOLM-14-TKIR and THP-1.

THP-1 had the lowest baseline expression of human VDR with FPKM (detailed descriptions in methods) approximately 3 times lower than MOLM-14 and MOLM-14-TKIR. In addition, THP-1's VDR expression did not change significantly after treatment with 1,25(OH)$_2$D$_3$ or combined 1,25(OH)$_2$D$_3$ and AZA. This was consistent with our FACS assay, showing that THP-1 had minimal therapeutic response (CD14+ differentiation) in response to 1,25(OH)$_2$D$_3$ or the combination of 1,25(OH)$_2$D$_3$ and AZA treatment *in vitro* (Fig. S1).

The combination of 1,25(OH)$_2$D$_3$ and AZA was most effective in up-regulating baseline VDR expression in both MOLM-14 cells (100% *vs.* 45% for AZA treatment alone) and MOLM-14-TKIR cells (87% *vs.* 24% for AZA treatment alone) (Fig. 3A). This result was confirmed by quantitative PCR (qPCR) experiments (2.4 fold increase, combination *vs.* no treatment, $p < 0.05$) and Western blot assays (6.6 fold increase, combination *vs.* no treatment, $p < 0.05$) (Fig. 3G, H).

RNA-seq data were consistent with our FACS assays, confirming that the combination of 1,25(OH)$_2$D$_3$ and AZA has enhanced efficacy against MOLM-14 and MOLM-14-TKIR cells.

We identified 3 major transcriptome changes that could explain the mechanisms of enhanced anti-leukemic activity of combination therapy: 1) Differentiation of blasts into mature monocytes with CD14+ expression (200 fold up-regulation for MOLM-14 cells, and even higher in MOLM-14-TKIR cells, Fig. 3B); 2) suppression of blasts proliferation by inhibiting FLT-3 expression (45% reduction in 1,25(OH)$_2$D$_3$, 134% reduction in the combination of 1,25(OH)$_2$D$_3$ and AZA for MOLM-14 cells, Fig. 3C) and a reduction in downstream PIM1 expression (up to 689% with the combination of 1,25(OH)$_2$D$_3$ and AZA *vs.* no treatment for MOLM-14 cells, Fig. 3C, D); 3) enhancing apoptosis of blasts by down-regulation of BCL-2 (484% reduction in the combination of 1,25(OH)$_2$D$_3$ and AZA for MOLM-14 cells, Fig. 3E) and up-regulation of BAX (56% in the combination of 1,25(OH)$_2$D$_3$ and AZA for MOLM-14 cells, Fig. 3F).

Collectively, our *in vitro*, *ex vivo* and RNA-seq data indicate that combining 1,25(OH)$_2$D$_3$ administration with AZA reduces the percentage of AML blast cells, through proliferation suppression, enhanced differentiation and cell death. It is important to recognize that 1,25(OH)$_2$D$_3$ based treatment will likely not be effective in blast cells with low baseline expression of VDR, as demonstrated in THP-1 cells.

Deficient expression of the CYP27B1 enzyme in the BM of MDS/AML patients

Given that CYP27B1 can be downregulated in tissue surrounding tumors [9], we asked whether CYP27B1 expression is also affected in BM of AML patients. We collected BM aspirates from both non-AML/MDS patients, and AML/MDS patients. Western blot data showed that CYP27B1 protein levels were significantly lower in AML/MDS patients' BM, as compared to controls (>2 fold, $p < 0.05$) (Fig. 4A). Next, we addressed whether suppressed expression of CYP27B1 is corrected after AML treatment. We found that induction chemotherapy treatment augmented the expression of CYP27B1 in day 30 BM when the patient achieved remission (Fig. 4B).

CYP27B1-MOLM14 cells show stable transgene expression and function in vitro and in vivo

FACS was performed to confirm the generation of a CYP27B1-MOLM-14 cell line by analyzing the GFP expression, which displayed 98.8% GFP+ in the CYP27B1-MOLM-14 cell line comparing to the non-GFP+ naïve MOLM-14 (Fig. S4). We confirmed the function of CYP27B1 in generating 1,25(OH)$_2$D$_3$ *in vitro* by measuring 1,25(OH)$_2$D3 level after adding 25 (OH)D3 substrate into cell cultures with CYP27B1-GFP-MOLM-14 for three days (Table S2). We determined that CYP27B1-transfected cells generated more 1,25(OH)$_2$D$_3$ than GFP-MOLM-14 by adding 50 nM of 25(OH)D3 into cell cultures of MOLM-14-CYP27B1 and GFP-MOLM-14 (Fig. S5).

The first question is where these vehicle cells will localize after engraftment and the second question is whether they will continuously express the CYP27B1 transgene *in vivo*. To address these questions, we transplanted MOLM-14 cells engineered to express CYP27B1, and followed these engrafted cells *in vivo*. For this AML xenograft model, a dose of AZA was injected IP into NRG mice on day 0 to ablate the BM. On day 1, half a million of either GFP-MOLM-14 cells or CYP27B1-GFP-MOLM-14 cells were injected into mice *via* tail vein. GFP-labeled MOLM-14 cells served as the vector control group. On day 14, mice were euthanized and histology was performed on femur BM (Fig. 4C). Under fluorescent microscopy, femur BM was occupied with GFP positive MOLM-14 cells, which were also stained with CYP27B1 antibodies (Fig. 4D). Immunohistochemistry showed that many co-localized cells stained positively for both green (GFP) and red (CYP) in the CYP27B1-GFP-MOLM-14 group. As expected, GFP-MOLM-14 control cells on the bottom panels only stained for green (GFP). These data demonstrated that CYP27B1-MOLM-14 cells are functional in localizing to the BM and in persistently expressing 1α-hydroxylase to generate active Vitamin D.

In vivo effects of CYP27B1

Above we established proof of principle for delivery of CYP27B1 *via* transduced MOLM-14. Next, we determined whether expressing the transgene CYP27B1 in MOLM-14 blasts improves the survival of MOLM-14

← **Fig. 1.** Combined treatment with 1,25(OH)$_2$D$_3$ and AZA significantly reduces human AML blasts *in vitro*. Human AML patient-derived cell lines MOLM-14 cells and MOLM-14-TKIR were cultured with 1,25(OH)$_2$D$_3$ and AZA alone and in combination, or left untreated (control). 48 h later, cells were harvested and analyzed by FACS for expression of CD14 and viability dye. A Representative FACS plots of MOLM-14 cells show the viable blast population (CD14– viability dye–) under the indicated conditions. B FACS gating strategy for MOLM-14 cells. C Percentage of blasts cells within the MOLM-14 cells under indicated conditions. D, E MOLM-14 cells were collected after 48 h of treatment and analyzed by FACS for the expression of Ki67. Representative FACS plots show the viable Ki67+ blasts under indicated conditions. F, G MOLM-14 TKIR cells were collected after 48 h of treatment and analyzed by FACS for the expression of Ki67. Representative FACS plots show the viable Ki67+ blasts under indicated conditions. Where applicable, data are means ± SEM from each group and were analyzed by Student *t*-test. *$p < 0.05$; **$p < 0.01$; $N = 6$.

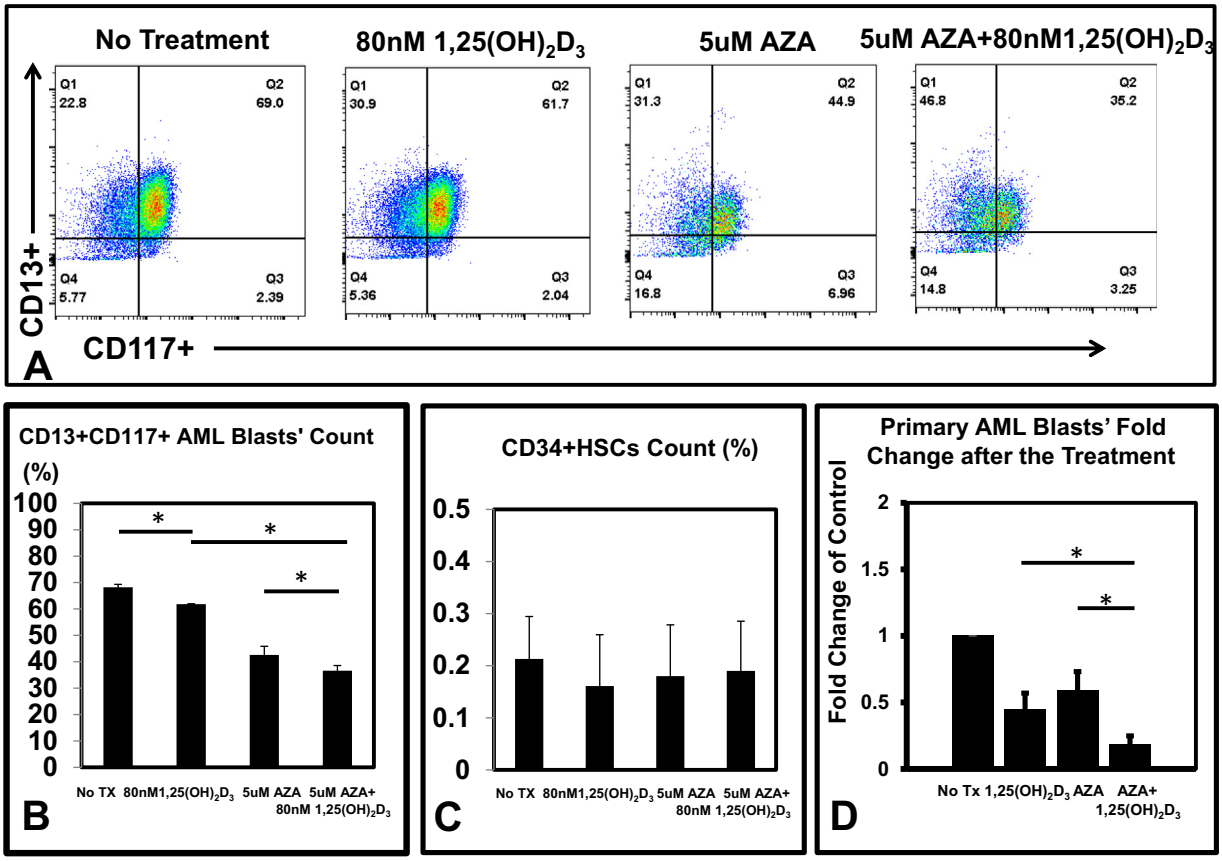

Fig. 2. Combined treatment with $1,25(OH)_2D_3$ and AZA significantly reduces primary AML blasts *ex vivo*. A Human AML patient BM cells were cultured with $1,25(OH)_2D_3$ and AZA as single agents and in combination. 48 h later, the cells were harvested and analyzed by FACS for expressions of CD117 and CD13 among viable cells. Representative FACS plots show viable blasts (CD13 + CD117 + viability dye–) under the indicated conditions. B Percentages of CD13 + CD117 + AML blast cells under indicated conditions. C Percentages of CD34 + HSCs under indicated conditions (N = 3). D Therapeutic effect of $1,25(OH)_2D_3$, AZA and their combination on AML patient samples after 48 h. Where applicable, data are means ± SEM from each group and were analyzed by Student *t*-test. *$p < 0.05$.

xenograft mice. In our first survival preclinical model, we performed IP injections on 4 groups of mice with AZA on day 0 to condition the BM and improve engraftment, and on day 1 they were treated as follows: 1) 5×10^5 GFP-MOLM-14 cells, 2) 5×10^5 CYP27B1-GFP-MOLM-14 cells, 3) 2×10^6 CYP27B1-GFP-MOLM-14 cells, and 4) 4×10^6 CYP27B1-GFP-MOLM-14 cells. We observed mice for physiologic changes and their median overall survival was compared between the four treatment groups using Kaplan Meier curve. BM cells harvested from freshly sacrificed mice were analyzed by FACS. Of AML xenografts in group 1, 90% experienced lower body paralyses, and significant weight loss, in contrast to the healthy CYP27B1-treated group 2 (Fig. S6 and videos in Fig. S7). Median survival for group 2 was significantly prolonged compared to group 1 (29.4 days *vs.* 24.75 days, $p < 0.01$) (Fig. 5A). While injecting more engineered MOLM-14 cells would lead to increase local expression of CYP27B1, it would certainly increase the leukemic burden in the BM and eventually would lead to early demise of AML mice as observed in group 3 and 4.

Next, we examined the fate of transplanted AML blasts inside the mouse BM. FACS showed that cells collected from the BM of group 2 at day 14 after transplantation had increased expression of CD14 compared to group 1 (7.44% *vs.* 0.92%, $p < 0.05$) (Fig. 5B). IHC demonstrated that some transplanted GFP + blasts had started to express CD14 (red color, Fig. 5C).

To assess for hypercalcemia, we obtained peripheral blood from each group of mice at baseline, day 7 and day 14, and measured calcium levels using the Calcium Colorimetric Assay Kit. No significant changes were observed in calcium levels in any of the groups at day 7 or 14 (Fig. 5D).

Table 1
Primary blast percentage after AML treatment.

Demographic	AML subtype Cytogenetic/FISH	Molecular marker	Initial blast count (%)	Blast count (%) after 1,25-VD3 (80 nM)	Blast count (%) after 5-Azacytidine (5 μM)	Blast count (%) after combination
30y F	M1, normal	+ NPM1, + FLT3-ITD	14%	6.88%	14%	2.44%
47y M	M4, normal	+ NPM1	36%	2.56%	19.8%	0.91%
50y F	M5, normal	+ NPM1, + FLT3-ITD	19.3%	3.85%	21.8%	2.35%
32y F	M4, inv.(16)	Normal	71.2%	17.1%	28.2%	11.0%
53y F	M2, t(8;21)(q22;q22); RUNX1-ETO	+ FLT3-TKD	20.8%	8.44%	1.3%	0.8%
A.2568		+ FLT3-ITD	69.6%	58.7%	23.6%	19%
A.2431		+ FLT3-ITD	69%	61.7%	44.9%	35.2%

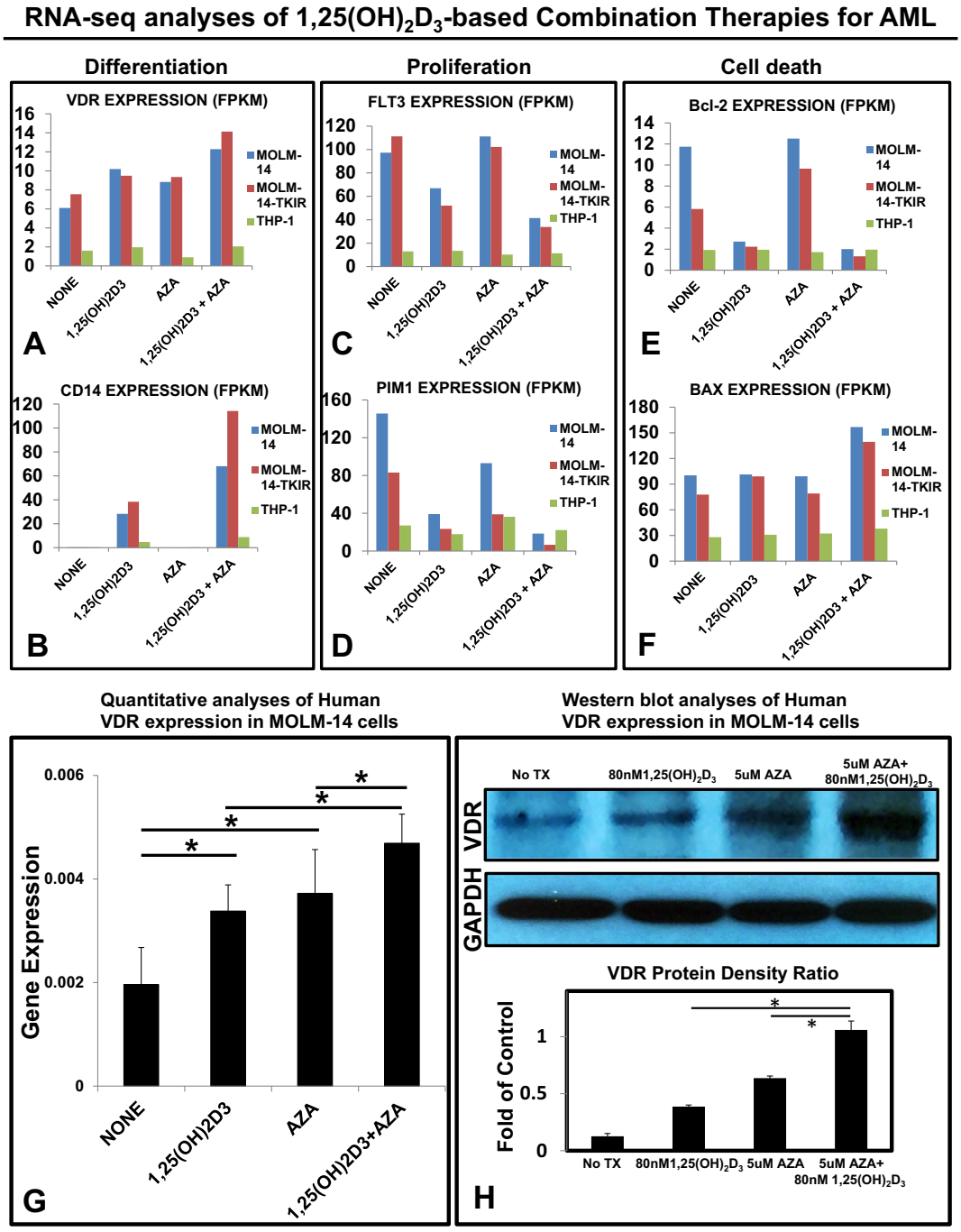

Fig. 3. RNA-seq analysis of AML cells treated with combination therapy and single agents. A–F Selected RNA seq results were segregated into 3 categories, genes primarily acting on: 1) differentiation, 2) proliferation and 3) cell death. G, MOLM-14 cells were cultured with 1,25$(OH)_2D_3$ or AZA or a combination of 1,25$(OH)_2D_3$ and AZA for 48 h then harvested and analyzed G by RT-qPCR for expression of human VDR and H By Western blot for protein expression of human VDR (N = 3). Where applicable, data are means ± SEM from each group and were analyzed by Student *t*-test. *p < 0.05.

Discussion

Here we provide evidences for the use of gene therapy to deliver 1,25$(OH)_2$D3 therapy while overcoming systemic hypercalcemia. We have demonstrated that although a high concentration of 1,25$(OH)_2$D3was effective at inducing MOLM-14 and HL-60 to undergo differentiation, cells with low baseline VDR (THP-1) did not respond to 1,25$(OH)_2$D3. Mutations leading to AML are heterogeneous, thus this could possibly explain why 1,25$(OH)_2$D3 is effective against some AML cell lines and patient samples, but not others.

Low expression of VDR mRNA before and after treatment with 1,25$(OH)_2$D3correlated with a lack of downstream expression of genes that control proliferation and cell death. This could possibly be a potential reason for mixed responses to 1,25$(OH)_2$D3treatment for AML in many clinical trials due to the varying expression of baseline VDR of leukemic blasts [12,34,35]. Interestingly, with combination treatment of 1,25$(OH)_2$D3and AZA we noticed a significant decrease in mRNA expression of the FLT-3 and PIM-1 genes in both MOLM-14 and MOLM14-TKIR. Constitutively active FLT3 signaling up-regulates PIM-1 expression *via* the STAT5 pathway resulting in phosphorylation of BAD protein. Silencing PIM-1

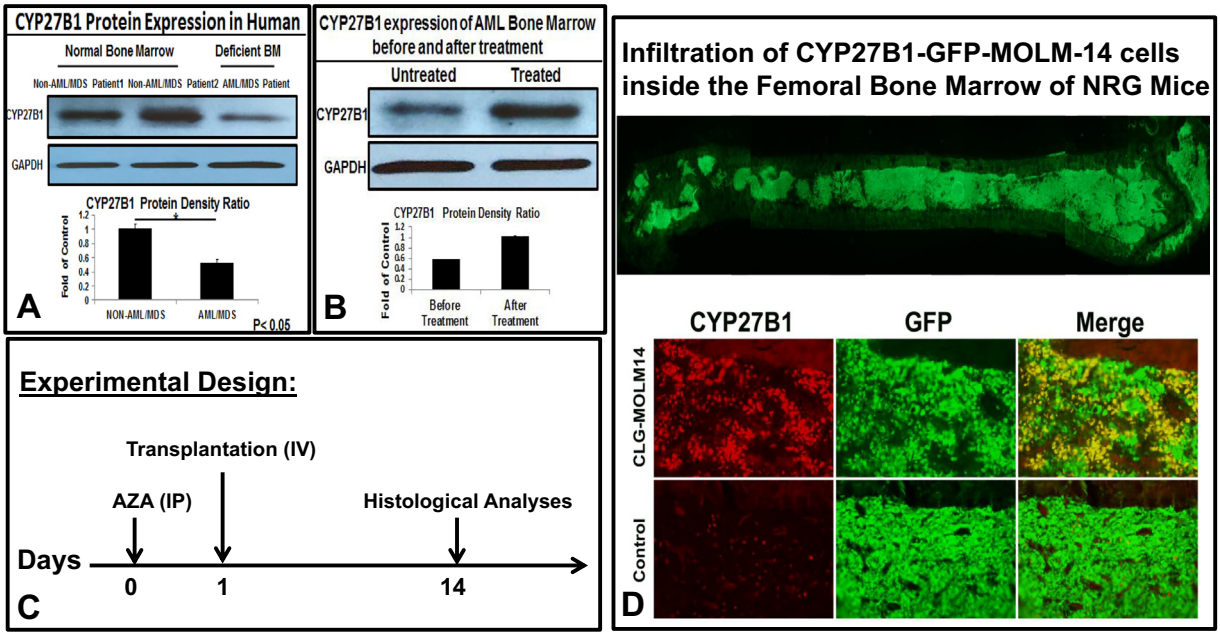

Fig. 4. Endogenous and ectopically expressed CYP27B1 enzyme in Human AML BM. A Protein levels of CYP27B1 in human BM samples were determined by Western blot analyses. Levels from non-AML/MDS patients (left), and AML/MDS patients (right) are shown (N = 5 in AML/MDS patients, and N = 8 in control patients). Data are means ± SEM from each group and were analyzed by Student t-test. *p < 0.05 *vs.* control (normal). B CYP27B1 protein levels in AML patient BM before and after chemotherapy induction. C Schematic of experimental design. On day −1, NRG mice were intraperitoneally injected with AZA. On day 0, 1 × 10^6 CYP27B1-MOLM-14 cells were transplanted through tail vein injection into the experimental group. In the control group, NRG mice received 1 × 10^6 GFP-MOLM-14 on day 0. On day 14, mice were euthanized for histological analyses. The CYP27B1-GFP lenti-viral vector and virus used in this study and stable expression in the CYP27B1-MOLM-14 cell line are shown in Fig. S3. D Immunohistology was performed to confirm homing of GFP+ engrafts inside the femur BM (upper image). Double staining shows co-localization of CYP27B1 expression (red color) and GFP+ engrafts (lower images). Where applicable, data are means ± SEM from each group and were analyzed by Student t-test. *p < 0.05; (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sensitizes resistant cells to FLT3 inhibitors [36]. This has important clinical implications because treating resistant FLT-3 cells with 1,25(OH)$_2$D3could potentially resensitize them to TKI. Another important implication is that 1,25(OH)$_2$D3can potentially be combined with commercially approved FLT-3 and Bcl-2 inhibitor for synergistic effects in AML treatment. The use of 1,25(OH)$_2$D3should be selective and will likely not work for AML cells with low baseline expression of VDR as demonstrated above. Our finding is consistent with recent publication from Paubelle et al. that VDR is important for myeloid progenitor differentiation and is a prognostic factor in AML. Further, mice with deficient VDR have increased numbers of hematopoietic and leukemia stem cells [37].

This report is the first to identify a significant decrease in CYP27B1 protein levels in the BM aspirate of MDS/AML patients *vs.* non-MDS/AML patients and to show that it increases in the BM aspirate in an AML patient after 7 + 3 induction chemotherapy.

As mentioned before, supra-physiologic treatment with 1,25(OH)$_2$D3results in systemic hypercalcemia. Gene therapy allows us to bypass this limitation without compromising treatment efficacy. Further, CYP27B1 gene therapy also serves to replenish the depleted 1-alpha-hydroxylase in the bone marrow of AML mice. We demonstrated that treatment for gene therapy experimental arms were not limited by systemic hypercalcemia, but were limited by the number of engineered AML cells we can inject due to concern for increased blast burden. Another important factor to be considered in future experiments is 25(OH)D3 substrate, which can be depleted with repeated, interval gene therapy treatment. Future experiments will involve a more clinically suitable vehicle cell such as HSCs. Our lab has successfully generated CYP27B1-GFP CD34 + HSC cells that constantly expressed CYP27B1 gene *ex vivo* (data not shown). Without concern for hypercalcemia or increase in AML burden by using the appropriate vehicle cells, we can repeatedly inject engineered HSCs on a regular interval. Oral supplement with 25 (OH)D3 would also be considered. We envision a clinical scenario, where an older patient will receive a dose of autologous, engineered HSCs after several doses of palliative chemotherapy to reduce blast burdens.

In summary, our data provides strong *in vitro* and *ex vivo* evidence to support combination therapy of 1,25(OH)$_2$D$_3$ and AZA for AML treatment. For future experiments, we will utilize a more suitable vehicle such as CD34 + HSC and add AZA, a hypomethylating agent, for potential synergistic effect.

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CRediT authorship contribution statement

Huynh Cao: conceptualization, methodology, supervision, writing – review and editing; Yi Xu: methodology, validation, investigation, formal analysis, writing – original draft; Linh Pham: investigation, writing – original draft; Park Eunwoo: investigation, writing – original manuscript; Jeffrey Xiao – investigation; David Chi: investigation; Justin Lyu: investigation; Rosalia Campion: formal analysis, investigation; Samiksha Wasnik: investigation, resources; Il Seok Jeong: software; Xiaolei Tang: conceptualization; David Jeston Baylink: conceptualization, supervision, funding acquisition; Chien Shing Chen: conceptualization, supervision; Mark Reeves: supervision, writing – review and editing; Kimberly Payne: supervision, writing – original draft; Mojtaba Akhtari: writing – review and editing; Saied Mirshahidi: resources; Guido Marcucci: supervision, writing – review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

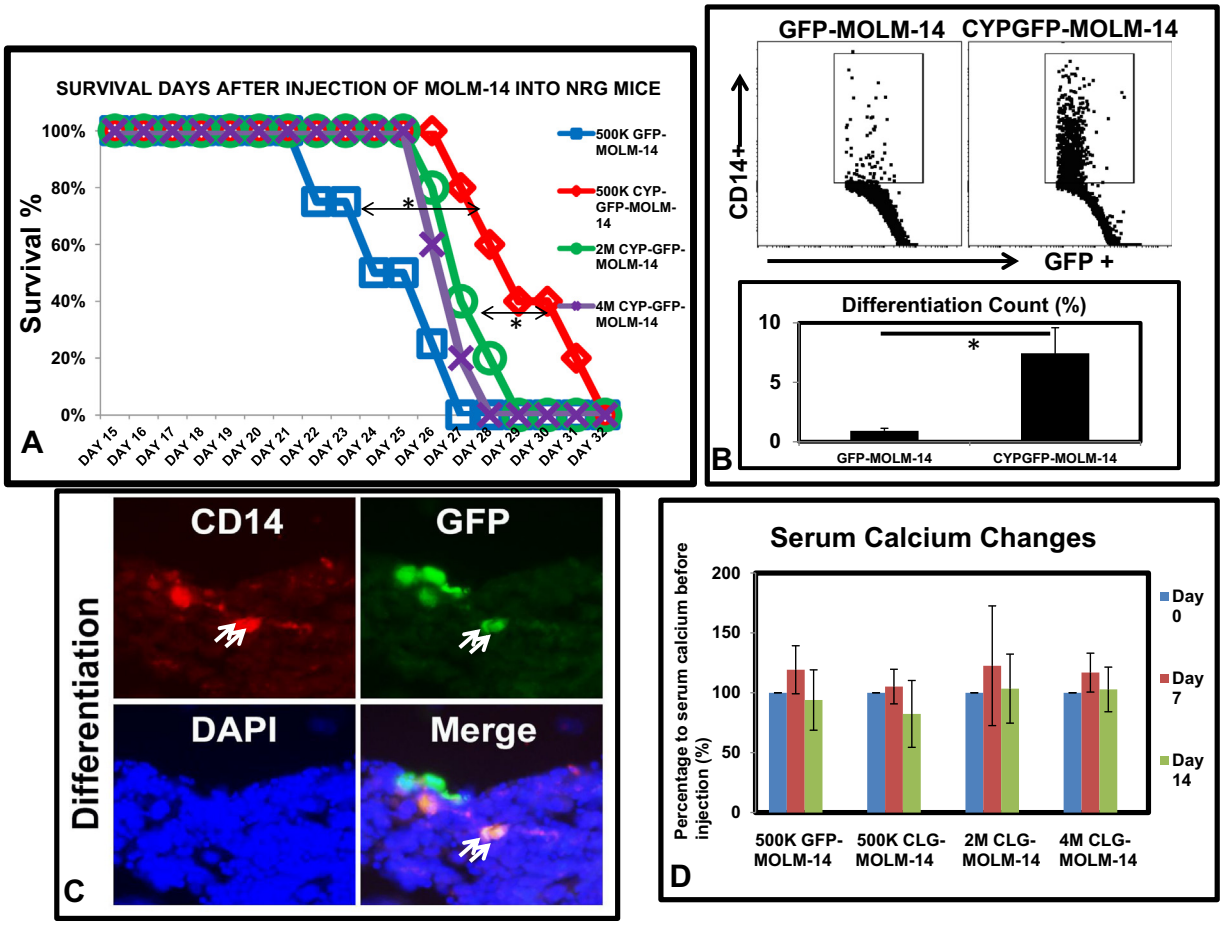

Fig. 5. Enhanced cellular differentiation and survival of mice engrafted with CYP27B1-MOLM14 without hypercalcemia. A The survival curve of engrafted NRG mice is shown. On day −1, NRG mice were intraperitoneally injected with AZA. Then mice were separated into four groups to receive intravenously one of the following MOLM-14 engraftments: 1) Control (injection of 5×10^5 GFP-MOLM-14 on day 0), 2) low CYP27B1 arm (injection of 5×10^5 CYP27B1-GFP-MOLM-14 (CLG-MOLM-14) on day 0), 3) middle CYP27B1 arm (injection of 2×10^6 CLG-MOLM-14 on day 0), 4) high CYP27B1 arm (injection of 4×10^6 CLG-MOLM-14 on day 0). B The femur BM cells of experimental mice were isolated and evaluated by FACS. Representative FACS plots show expression of CD14. C Immunohistolochemistry was performed to display co-localization of CD14 expression and GFP + MOLM-14 cells inside the BM of experimental animals on day 14. D Calcium levels measured on day 7 and 14 were reported as percentage changes compared to baseline values for each group. Where applicable, data are means ± SEM from each group and were analyzed by Student t-test. *$p < 0.05$; $N = 5$.

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