

## Establishment of Acute Myeloid Leukemic Xenografts as Preclinical Models of Epigenetic Therapies

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### ABSTRACT

**AIM:** There is currently little information on epigenetic therapy in *in vivo* preclinical models of acute myeloid leukemia. The study was designed to establish a preclinical model for the validation and optimization of epigenetic therapies and other treatment strategies.

**MATERIALS AND METHODS:** We first characterized the response of normal immunodeficient mice to different doses of 5-aza-2'-deoxycytidine valproic acid and all-trans retinoic acid. This allowed us to define an optimized 28-days long protocol showing low toxicity and associated with efficient global DNA demethylation and histone acetylation of bone marrow mononuclear cells.

**RESULTS:** AML xenografts of 5 non-promyelocytic acute myeloid leukemia patients were treated with the defined protocol. One out of 5 demonstrated reduced disease burden, cell differentiation (increase of CD15, CD11b and to some extent CD14 positive cells and decrease of CD34+ population) and reduced capacity in reinitiating leukemia in secondary recipients.

**CONCLUSION:** These findings mimic results of clinical trials and indicate that acute myeloid leukemia xenografts may provide useful preclinical models to optimize epigenetic therapies.

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**Key words:** Acute Myeloid Leukemia; Xenografts; Epigenetic; All-trans retinoic acid; 5-aza-2'-deoxycytidine; Valproic acid; Leukemia initiating cells

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## INTRODUCTION

The majority of acute myeloid leukemia (AML) patients are treated with intensive chemotherapy with serious limiting side effects for older age patients and unfortunately with short complete remissions. The persistence of a subpopulation of cells called leukemia-initiating cells (LIC) resistant to chemotherapy would be responsible for relapses<sup>[1]</sup>. Thus AML treatment still requires improved efficacy while preserving quality of life and alternative approaches and targets are currently being tested.

Aberrant DNA methylation signatures are common in AML and myelodysplastic syndrome (MDS) patients with mutations in genes coding for key proteins such as DNA methyl transferase 3 (DNMT3), Tet methylcytosine dioxygenase 2 (TET2), Isocitrate Dehydrogenase 1/2 (IDH1/2) and Histone deacetylase (HDAC)<sup>[2]</sup>. Unlike deletions or mutations of tumor suppressor genes, altered chromatin modifications are reversible and thus novel approaches such as chromatin remodeling or epigenetic therapy has been investigated as potential anti-cancer therapeutic approaches<sup>[3-6]</sup>.

5-aza-2'-deoxycytidine (AZA), a DNA methyl transferase inhibitor, is currently in clinical practice for patients with MDS<sup>[7]</sup> and with acute and chronic leukemia<sup>[8]</sup>. Of the HDAC inhibitors, the short chain fatty acid, valproic acid (2-propylpentanoic acid; VPA), used since many years in the treatment of epilepsy and mood stabilizer<sup>[9]</sup>, was shown to stimulate differentiation and/or inhibit cell growth of leukemia<sup>[10]</sup>, but has limited clinical activity on its own in MDS<sup>[11]</sup> or AML<sup>[12]</sup> patients. All-trans retinoic acid (ATRA) can induce hypomethylation and its activity is enhanced by AZA<sup>[13]</sup>. PLZF-RARA acute promyelocytic leukemia (APL) variant resistance to ATRA can be reversed by the addition of HDAC inhibitors (Trichostatin A or sodium phenyl butyrate) both *in vivo* and *in vitro*<sup>[14]</sup>. VPA and AZA have been shown to sensitize AML cells for ATRA<sup>[11,12,15]</sup>. The *in vitro* data showed that, the combination had differentiation effect in leukemic cell line (HL-60 and MOLT4)<sup>[16]</sup>. Such combinations are also effective in solid tumors<sup>[17,18]</sup>.

Based on these *in vitro* data, clinical trials have been initiated to investigate the use of HDAC inhibitors, in combination with retinoic acid and/or DNMT inhibitors, for the treatment of solid tumors<sup>[19]</sup> and myeloid malignancies<sup>[20,21]</sup>. We have participated in a phase 2 clinical trial study, where the combination of AZA, VPA and ATRA was studied in 65 patients (median age 72, Ranging from 50-87 years) with high risk AML and MDS<sup>[21]</sup>. Of the 65 patients that received the 6 cycles of the combination AZA, VPA and ATRA, 17 (26%) responded (14 achieved complete remission and 3 partial remission)<sup>[21]</sup>, highlighting both the potential and limitation of these therapies as heterogeneity in the responses are observed.

In order, to increase the number of patients likely to respond to more effective DNMT inhibitor and HDAC inhibitor combinations, preclinical models that reflect patients' responses are mandatory. Indeed, *In vitro* cultures of fresh AML cells are not sufficient, as they do not always predict *in vivo* preclinical efficacy or toxicity. Furthermore, AZA's mechanism of action at the DNA level requires promoter accessibility which is best obtained during DNA synthesis and replication, and difficult to obtain in fresh AML cells in conventional liquid cultures. Considering the important place of AML mouse xenografts to study both human leukemogenesis and leukemic stem cells, we have developed a set of AML xenografts as a preclinical platform to study the efficacy of AZA, VPA and

ATRA combination in AML. Acute promyelocytic leukemia (APL, AML3 subtype) were excluded as ATRA induces leukemic cell differentiation and complete remission in a high proportion of APL patients<sup>[22]</sup>, as well as shown by us in APL xenografts<sup>[23]</sup>.

In this study, we show for the first time that AML preclinical xenografts can be used to identify the AML samples sensitive to the AZA, VPA and ATRA combination and thus providing excellent models for assessing more potent epigenetic drug combinations for all AML patients.

## MATERIALS AND METHODS

### Patient samples

Peripheral blood (PB) and/or bone marrow (BM) samples from AML patients were obtained at diagnosis after informed consent (Table S1). BM and PB mononuclear cells were isolated using density-gradient centrifugation. Cells were injected intravenously into NOD/shi-SCID IL2R $\gamma^{-/-}$  (NOG) mice either fresh or after storage in liquid-nitrogen.

### NOG xenotransplantation assays

NOG mice provided from M Ito<sup>[24]</sup>, were bred in the pathogen-free breeding facility at Gustave Roussy, Villejuif. All animal experiments were performed in accordance with the French Ministry on animal health care regulation. Human CD45 cells ( $1 \times 10^6$  cells) per mouse were transplanted intravenously into 6–8-weeks-old NOG mice, conditioned with either sub-lethal irradiation (3 Gy) or Busulfan treatment (25mg/kg/d twice 24 hours before)<sup>[23,25,26]</sup>. PB samples were taken once a month to validate the engraftment.

### Determination of the maximum tolerable dose (MTD) for AZA and VPA in NOG mice

Eight-week-old NOG mice ( $n=5$  in each cohort) were injected intraperitoneally (300  $\mu$ L per injection) with AZA (0.1 or 0.4 mg/kg) or VPA (125, 250, 400 and 1200 mg/kg) daily over a period of 7 days. Measurements of various hematological parameters of blood were performed using the "Melet Schloesing MS9-5V hematology analyzer (Melet Schloesing Laboratoires, Osny, France)". The MTD was defined as the dose at which all animals survived the 7 days treatment period and the consecutive 21 days of observation and at which overt clinical signs of toxicity, such as body weight loss and mortality were absent. In addition, blood cell counts were measured before treatment, during the treatment, and after the observation period to detect delayed effects of the substances.

### Flow cytometry analysis and cell purification

A panel of FITC, PE, PC7, PerCP or APC-conjugated antibodies was used against mouse CD45, human CD45, CD33, CD38, CD11b, CD15, CD14, CD34, CD44, CXCR4, CD47, CD117, CD123 (Becton Dickinson, Le Pont-De-Claix, France). Human CD45<sup>+</sup> (hCD45<sup>+</sup>) cells from xenotransplanted mice were purified by a double-positive magnetic cell sorting system (AutoMACS, Miltenyi Biotec, France).

### Experiments on AML xenografts

Once AML engraftment was confirmed (hCD45 $>1\%$ ), mice were recruited to the experiments. The experiments comprise 3 groups of xenografts: control group (Placebo), ATRA group and AVA (AZA+VPA+ATRA) group. Mice were bled at day-0, day-8 (before ATRA implantation), day-18 and on day 29. On day 29, bleeding was followed by sacrifice and collection of BM cells and spleen cells. Self-renewal capacity was assessed by secondary transplantations of equal numbers of BM hCD45<sup>+</sup> cells in conditioned mice.

### Experiments on normal human hematopoiesis

For murine hematopoiesis, 8 weeks old C57BL/6 mice were used. For human hematopoiesis, mononuclear cells were separated from human cord blood over a ficoll density gradient. CD34 positive cells were isolated by a double positive magnetic cell sorting system (AutoMACS; Miltenyi Biotec) according to the manufacturer's instructions. CD34 positive cells ( $5 \times 10^4$ ) were transplanted intravenously into 6–8-week-old conditioned NOG mice. After validation of human engraftment, mice were treated with PBS or 0.1mg/kg/day AZA and 250mg/kg/day VPA for 7 days and on day 8 ATRA pellets (21day release) were implanted in treated group and untreated were subjected to the same condition without drugs. At the end of the experiment, mice were sacrificed and processed for various read outs in blood, BM and spleen.

### Western blot

For Western blot, BM cells were lysed in SDS lysis buffer (1% SDS, 10 mmol/L Tris/HCl (pH 7.4), 1 mmol/L vanadate) and proteins were denatured by the addition of SDS-Gel loading buffer containing dithiothreitol (DTT) followed by heating in boiling water for 10 minutes. Equal amounts of proteins were separated on a 10% SDS polyacrylamide gel and blotted onto polyvinylidene difluoride membranes (Millipore). After blocking, the membranes were incubated with rabbit anti-acetylated lysine (Cell signaling technology, Cat No #9441; 1:1000) antibodies diluted in 5% milk in PBST overnight at 4°C. The peroxidase-conjugated Donkey anti-rabbit IgG (Sigma; 1:20,000 in 5% milk in PBST) served as secondary antibody. The bands of acetylated-H3 and acetylated-H4 were found in the range 15KD–20KD. Signals were visualized using the Supersignal West Dura detection system (Thermo Scientific). Signal intensities were measured by densitometric analysis using the Scion Image (Scion Corporation).

### DNA methylation

The global methylated DNA was estimated in the BM hCD45<sup>+</sup> cells of NOG mice according to the manufacturer's instruction {Epigentek [MethylFlash™ methylated DNA Quantification kit (Colorimetric) #P-1034], NY, USA}. Briefly genomic DNA was extracted by Qiagen kit according to the recommendation of the manufacturer. DNA (200ng) was incubated with capture antibody against methylated DNA. Addition of detection antibody was followed by the addition of developing solution and then plate reading was performed in a spectrophotometer. The quantity of methylated DNA was interpolated from the standard curve processed at the same time and the percentage of methylated DNA was calculated and compared to control mice.

### Human myeloid colony assays

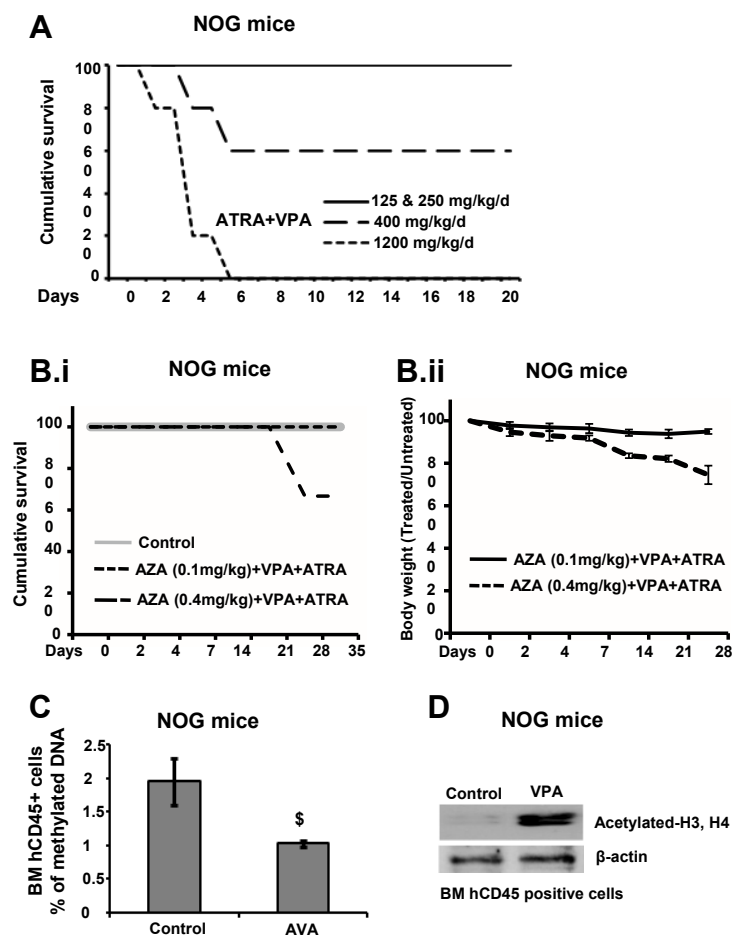
Purified hCD45<sup>+</sup> BM cells ( $10^5$ ) from AML xenograft cells were suspended in 500  $\mu$ l  $\alpha$ -MEM, and cultured in 1.8 mL complete methylcellulose medium allowing the selective proliferation of human progenitor cells (0.8% methylcellulose medium H4100 Stem Cell Technologies, Grenoble, France), 30% FBS, 1% deionized BSA, 10–4 mol/L  $\beta$ -mercaptoethanol) with the addition of 25 ng/mL human Stem Cell Factor (Biovitrum AB, Stockholm, Sweden), 10 ng/mL interleukin-3 (Peprotech, France), 2 U/mL erythropoietin (Amgen, Thousand Oaks, CA), and 10ng/ml rhu-GM-CSF (Peprotech, France). Plates were incubated at 37°C in an air atmosphere supplemented with 5% CO<sub>2</sub> and saturated with humidity. Progenitors were scored at day 14.

### Statistics

Kaplan-Meier analyses were used to assay the survival. Unless otherwise stated, data represent means $\pm$ SEM of at least 3 mice. Statistically significant differences between means were calculated using the non-parametric, unpaired, two tailed, student t-test with a threshold of  $p \leq 0.05$ ,  $p \leq 0.01$ ,  $p \leq 0.001$  designated by \*, \$, # respectively.

## RESULTS AND DISCUSSION

To set up a preclinical model of DNMT inhibitor and HDAC inhibitor combination with AZA, VPA and ATRA used in clinical trials, we first studied, in two different mice strains NOD/shi-SCID IL2R $\gamma^{-/-}$  (NOG) and C57/BL6, the AZA and VPA doses that were well tolerated and effective to induce DNA hypomethylation and histone acetylation (Figure 1). Different doses of AZA (0.1 and 0.4 mg/kg/day) and VPA (125, 250, 400, 1200mg/kg/day) were injected intraperitoneally for 7 consecutive days and mice were monitored for the 21 following days. Based on cumulative survival (Figure 1A and 1Bi) and body weight (Figure 1Bii), the tolerated doses for VPA and AZA in NOG mice and in C57/BL6 (not shown) were identified to be 250mg/kg/day and 0.1mg/kg/day respectively, within the range of reported doses in SCID mice<sup>[27]</sup>. As expected, these doses induced global DNA demethylation (Figure 1C) ( $p \leq 0.01$  compared to the control/untreated) and histone acetylation (Figure 1D) of bone marrow mononuclear cells by spectrophotometric and western blotting assays, respectively.



**Figure 1** Determination of the maximum tolerable dose (MTD) and effective dose of AZA, VPA and ATRA in NOG mice.

**Table 1** Characteristics of the AML patient samples.

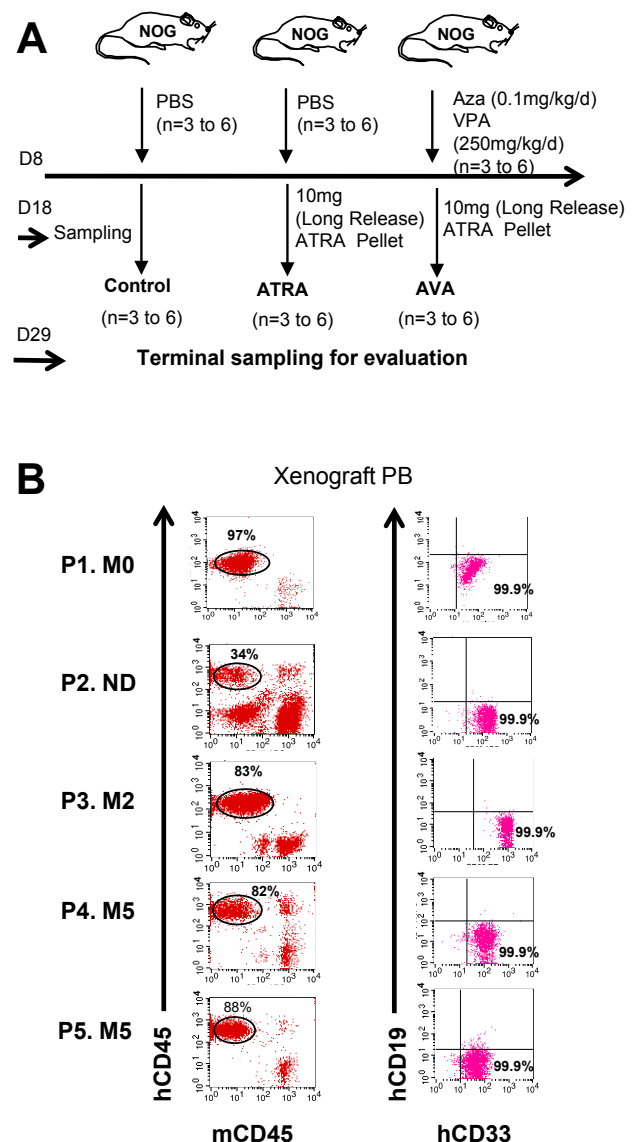
Patient N°	FAB	WBC (x10 <sup>9</sup> )	Flt3-ITD status	Source of Sample (% Blasts)	CD34+ (% )	CD38- (% in CD34+)	CD34+ (% in CD34+)	CD44+ (% In CD34+)	CXCR4+ (% in CD34+)	CD123+ (% in CD34+)	CD47+ (% in CD34+)	CD117+ (% in CD34+)	No of mice positive for engraftment	AML Engraftment (% of hCD45)
P1	0	244	-	Blood (95)	88	18%	86%	100%	100%	88%	100%	23%	5/5	Blood: 63±7% BM: 86±10% Spleen: 75±14%
P2	ND	ND	-	Bone Marrow (ND)	99	ND	ND	ND	98%	95%	100%	7%	2/8	Blood: 76±3% BM: 70±3% Spleen: 30±8% (8 weeks)
P3	5a	84.5	+	Blood (85)	78	8%	92%	100%	2%	81%	100%	81%	5/5	Blood: 28±14% BM: 63±19% Spleen: 22±14% (9 weeks)
P4	2	75	+	Blood (88)	56	27%	73%	100%	46%	56%	100%	56%	5/5	Blood: 4±1% BM: 33±3% Spleen: 2±0.5% (12 weeks)
P5	5	25.5	-	Blood (31)	53	5%	95%	100%	5%	86%	100%	28%	5/5	Blood: 73±15% BM: 97±2% Spleen: 67±13% (15 weeks)

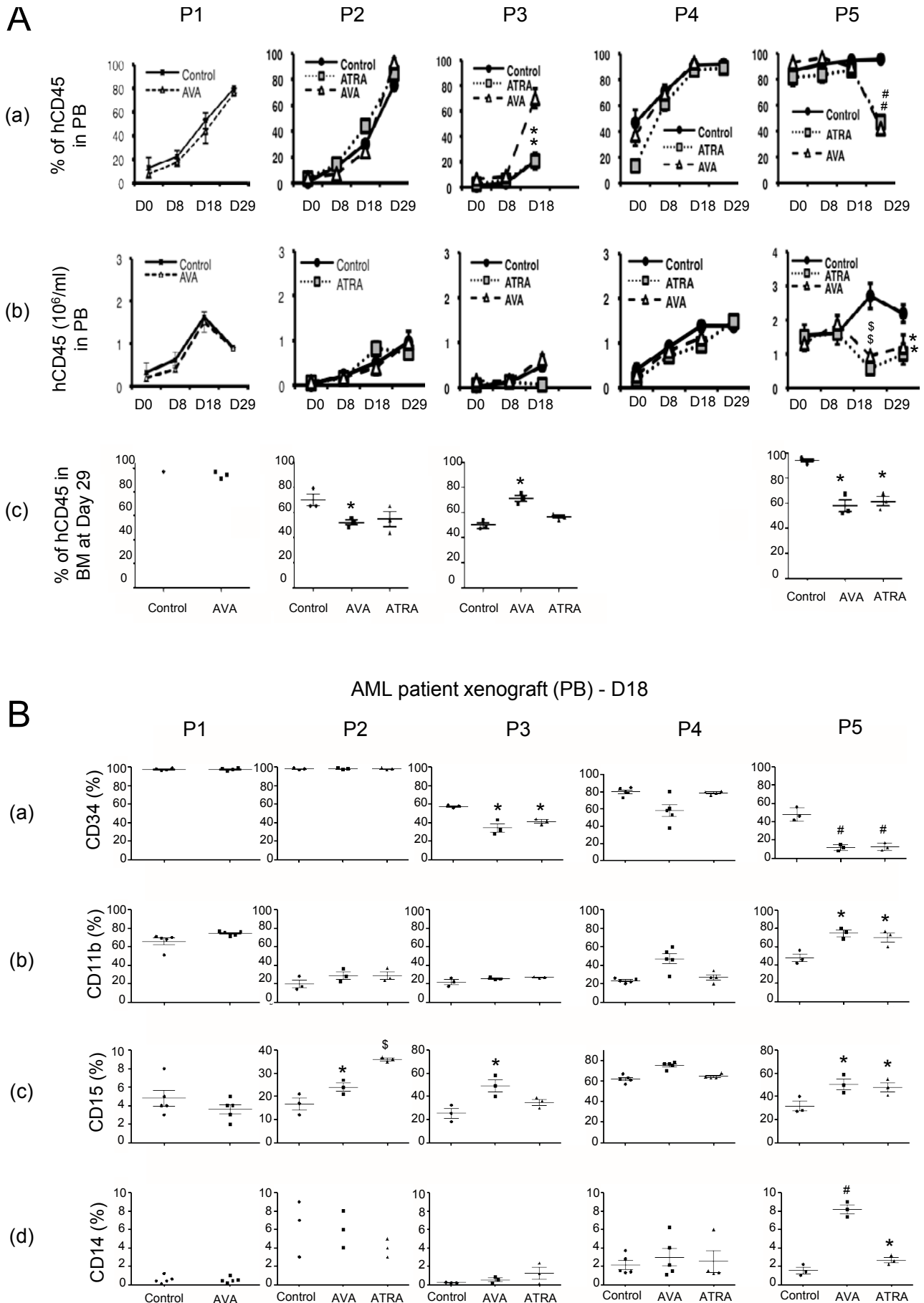
ND: Not done or no data.

We then studied the effects of the AVA combination in 5 different AML xenografts, according to the protocol described in Figure 2A. AML xenografts were obtained as previously described by Patel *et al.*<sup>[23]</sup> and Zhang *et al.*<sup>[28]</sup>. AML cell patients' characteristics at diagnosis are shown in Table 1. Before initiation of treatment, confirmation of leukemia development was determined by flow cytometry. The left panels of figure 2B show the anti human and anti mouse CD45 antibody labeling performed on the PB of xenografts for each patient sample. The lymphoid CD19 and myeloid CD33 marker profile in the hCD45+ population shown in the right panels indicated a robust leukemic engraftment in xenografted mice. Mice were then randomized and injected intraperitoneally with VPA (250mg/kg/d) and AZA (0.1mg/kg/d) for 7 consecutive days followed by sub-cutaneous implantation of one 10mg ATRA 21 daylong releasing pellet. AVA-treated AML xenografts were compared with untreated (Sham-operated) and ATRA treated xenografts. For these comparisons, we analyzed the expression of specific hematopoietic cell surface markers or Cluster of Differentiation (CD) proteins by flow cytometry. CD34 is a surface marker of undifferentiated cells and its down regulation suggests that the cells have progressed down one of the various hematopoietic differentiation cell lineages. As cells differentiate to myeloid lineage, they express various markers of mature cells including CD11b (myeloid marker), CD15 (granulocytic marker) and CD14 (monocytic marker).

Depending on the leukemic aggressiveness and mice survival, the level of activity of each treatment on leukemia development within the peripheral blood and bone marrow was evaluated at either day 18 or day 29. Results are expressed as percentages of hCD45 leukemic cells in PB (Figure 3A, first row, the columns from left to right in each row represent the data of each AML xenograft of the 5 AML patient samples, P1, P2, P3, P4 and P5) and absolute numbers in PB (Figure 3A, second row) and BM (Figure 3A, third row) of AML xenografts. In addition, the percentage of hCD45+ leukemic cells expressing selected differentiation markers was evaluated in PB at day 18 (Figure 3B, rows a, b, c, d correspond to the percentage of CD34, CD11b, CD15 and CD14 positive cells within the hCD45+ population, respectively) and at day 29 (figure 4B). The same assessment was performed on BM hCD45+ leukemic cells at either day 29 or day 18 (Figure 4A) depending on mice survival.

We observed three different groups of responses to AVA or ATRA treatment of AML patient xenografts.

**Figure 2** Schematic diagram of the experimental procedure and validation of AML xenografts.



**Figure 3** Treatment of AML xenografts by AVA combination or ATRA.



Non-responsive group: this group includes AML patient 1 (P1) xenografts ( $n=6$ ) for whom, only minor modifications were detected in the peripheral blood (PB) (Figure 3A P1a, b) and bone marrow (BM) (Figure 3A P1c) in response to treatment whether in the percentage (Figure 3A P1a) or absolute cell counts of hCD45 cells (Figure 3A

P1b). In addition, no significant modifications in the expression of the differentiation markers CD34, CD11b, CD15 and CD14 within the hCD45<sup>+</sup> cells could be detected in the PB at day18 (Figure 3B, a, b, c, d) or at day 29 in BM (Figure 4A) or PB (Figure 4B).

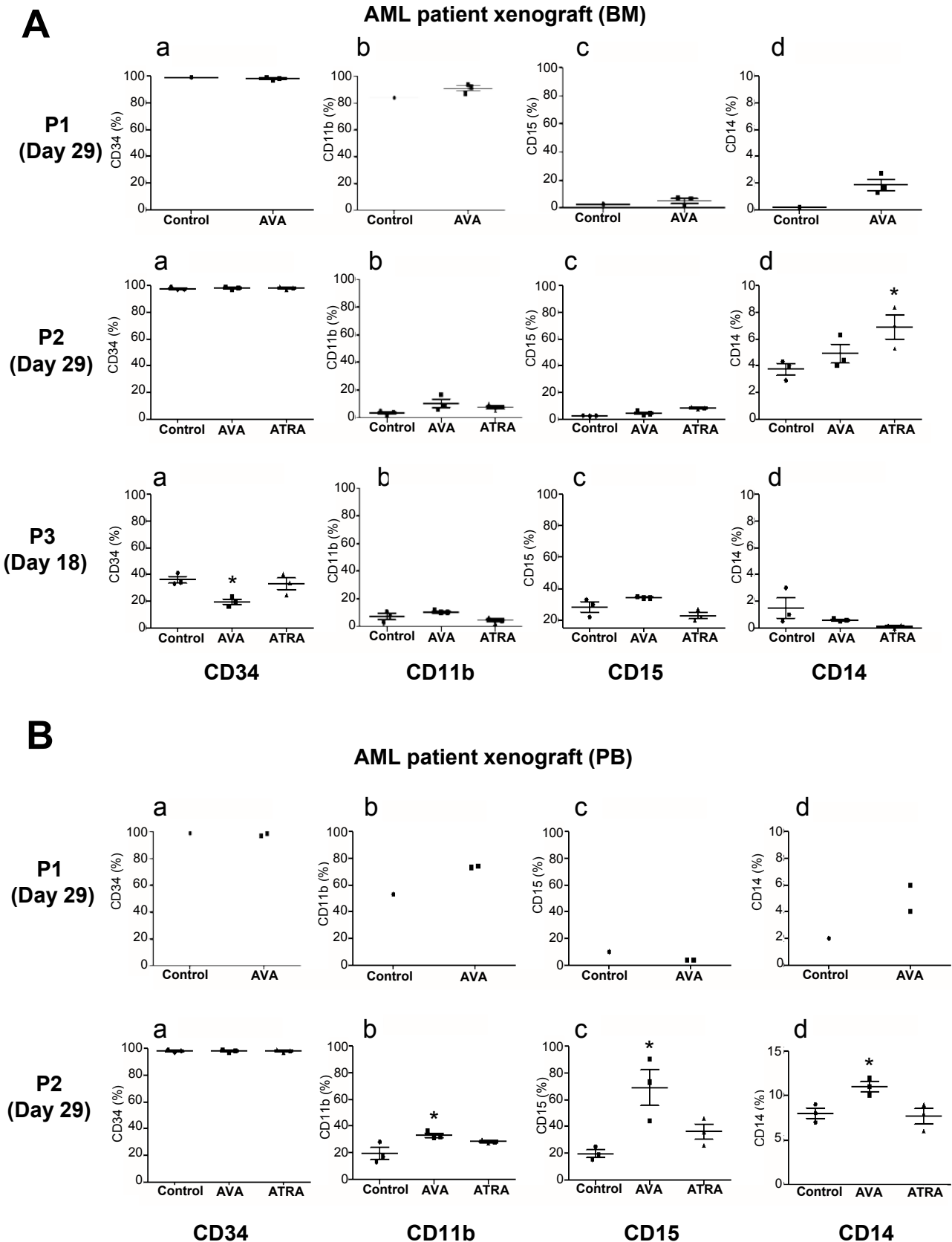


Figure 4 Phenotypic analysis of leukemic cells in the BM (A) and PB (B) of AML xenografts.

Partial responsive group: this group includes AML patient P2, P3 and P4 xenografts for whom partial effects of either AVA or ATRA alone were observed.

P2 AML: while the PB of the treated AML xenografts showed no modification in the percentage (Figure 3A P2a) or cell counts of hCD45 (Figure 3A P2b), the BM cells showed a significant ( $*p \leq 0.05$ ) decrease in the percentages of hCD45 (Figure 3A P2c) and the absolute numbers (data not shown) suggesting a partial anti leukemic

effect of AVA and ATRA treatment. In line with this observation, a close analysis of the phenotype of hCD45+ cells in the PB showed at D18 an increase ( $*p \leq 0.05$  and  $^{\$}p \leq 0.01$  comparing control with AVA and ATRA, respectively) in the percentage of CD15+ (Figure 3B P2c) cells compared to control mice. Similar results were obtained at day 29 in BM (Fig 4A) or PB (Fig 4B). However, the persistence of CD34 expression supports only partial effects of treatment (Figure 3B P2a) and (Fig 4A and B) in this leukemia.

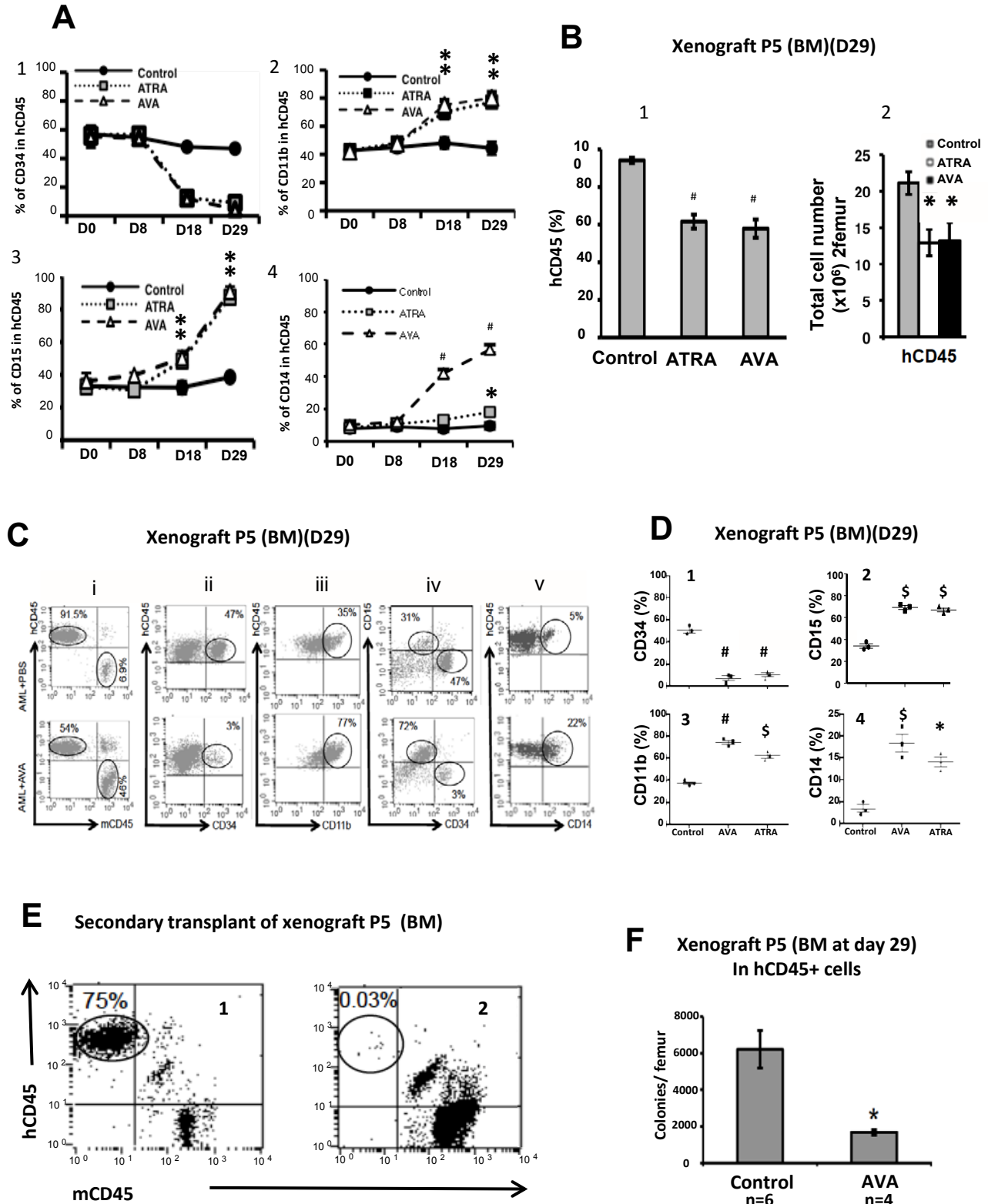
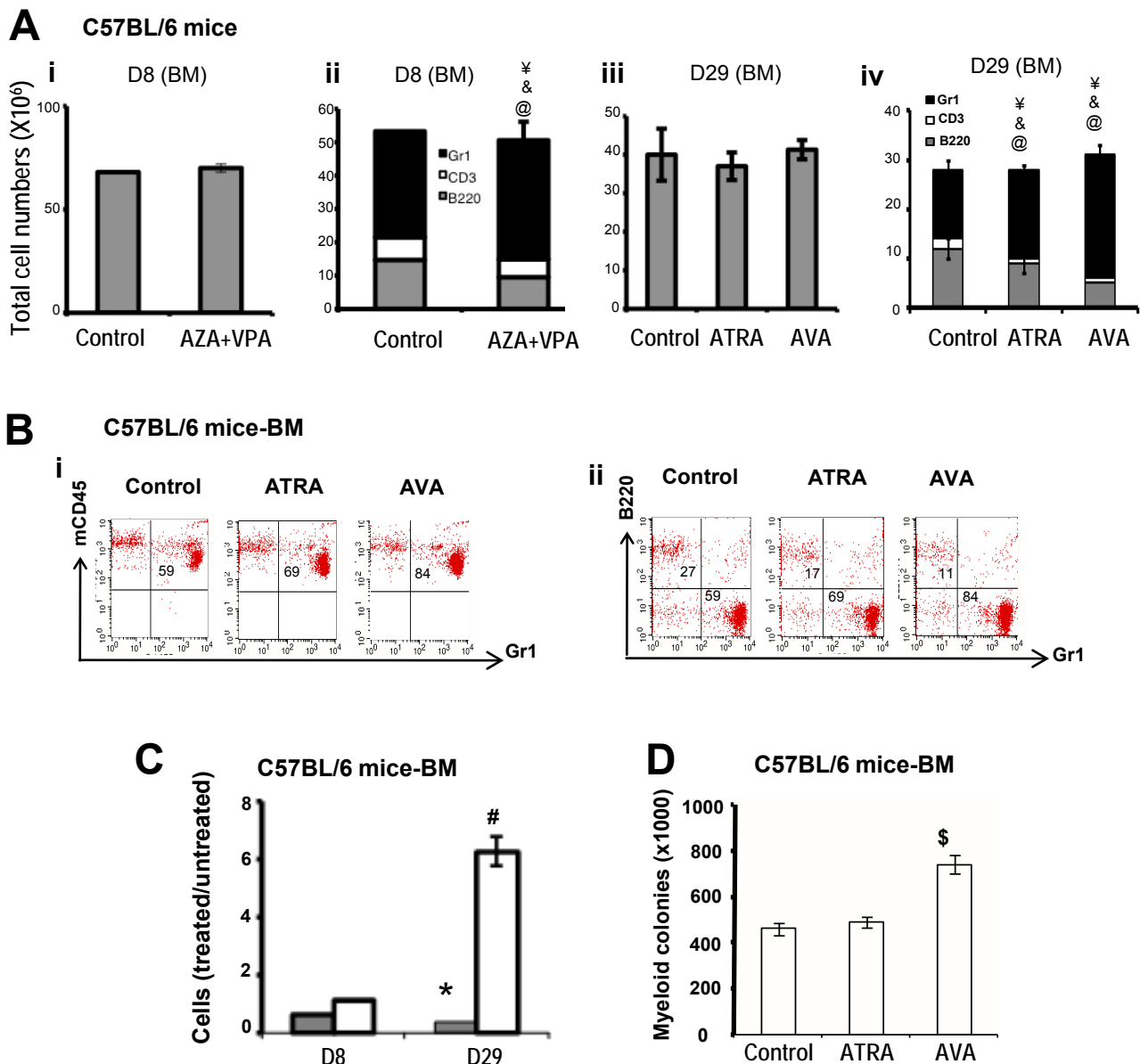


Figure 5 Potential therapeutic effect of AVA combination or ATRA in AML xenotransplantation mouse model (Responder Patient 5 AML xenograft).

**P3 AML:** Similar partial effects of AVA treatment were observed for P3 xenografts ( $n=3$ ). Interestingly, for this patient, a sharp increase in the percentage of hCD45+ cells was observed in the PB (Figure 3A P3a) and BM (Figure 3A P3c) of AVA treated mice, while the percentage of CD34 expressing cells in hCD45+ population showed a decrease in PB (Figure 3B P3a) and BM (Fig 4A) of treated mice ( $*p \leq 0.05$ ). This decrease is associated with an increase ( $*p \leq 0.05$ ) in the percentage of cells expressing CD15 in the PB (Figure 3B P3c) with AVA treatment. These results suggest an increase in leukemic cell differentiation.

**P4 AML:** Partial effects of AVA treatment were also observed for P4 xenografts for which the percentage (Figure 3A, P4a) and the absolute number (Figure 3A, P4b) of hCD45+ cells was not modified in the PB by AVA treatment. However, phenotypic analysis of the hCD45+ cells in PB of treated mice showed a decrease tendency in the percentage of CD34 expressing (Figure 3B, P4a) associated with an increase tendency in the percentage of cells expressing CD11b and CD15 (Figure 3B, P4b, P4c).

**Responsive Group:** one AML xenograft (P5) showed complete antileukemic effects of AVA and ATRA associated with differentiation towards the monocytic lineage. A two fold decreased in the percentages (Figure 3A P5a,  $^{\#}p \leq 0.001$ ) and the absolute numbers (Figure 3A P5b,  $*p \leq 0.05$ ) of hCD45+ cells in PB was detected upon either treatment. The percentage of leukemic cells in the BM (Figure 3A P5c) was correlated to that observed in PB. The anti leukemic effects of AVA and ATRA were accompanied by a parallel decrease ( $^{\#}p \leq 0.001$ ) in the percentage of CD34+ cells (Figure 3B P5a). Concomitantly, we observed an enhancement in the percentages of cells expressing markers of myeloid differentiation including CD11b (Figure 3B P5b), CD15 (Figure 3B P5c) and CD14 (Figure 3B P5d). Of note, an increase in the percentage of cells expressing the monocytic marker was noted with AVA ( $^{\#}p \leq 0.001$ ) and to a minor extent with ATRA ( $*p \leq 0.001$ ) alone. Interestingly, the kinetics of cell differentiation of leukemic cells in the PB showed that changes in percentage of CD34+ (Figure 5A1), CD11b+ (Figure 5A2), CD15+ (Figure 5A3) and CD14+ (Figure 5A4) cells within the



**Figure 6** Effect of AZA, VPA and ATRA (AVA) combination therapy on C57BL/6 mouse normal hematopoiesis.



hCD45 population were detected as soon as day 18 indicating that the antileukemic effects of AVA and ATRA were relatively rapid (Figure 5A 1, 2, 3, 4). The impact of treatments in the BM of treated animals was determined at the time of sacrifice on day 29. We observed that the percentages (Figure 5B1, ( $p \leq 0.001$ )) and the absolute numbers (Figure 5B2,  $*p \leq 0.05$ ) of hCD45+ cells were decreased by almost two fold upon ATRA or AVA treatment. As illustrated in figure 5C, the percentages of leukemic cells (hCD45+) expressing the differentiation markers CD11b (Figure 5Ciii), CD15 (Figure 5Civ) and CD14 (Figure 5Cv) were increased while that of CD34+ cells was decreased (Figure 5Cii, iv). These results were observed for all the mice within each group (Figure 5D) and correlated to those observed in PB (Figure 3B P5). To further document the antileukemic effect of the AVA combination in this AML xenograft, hCD45+ cells from PBS and AVA treated mice were isolated from BM at day 29 and transplanted in secondary recipient mice. The percentage of leukemic cells in the PB (hCD45+) was monitored after 5 weeks. Leukemia engraftment in the secondary transplants was obtained in the recipient of PBS-treated mice (Figure 5E1) while hardly any hCD45+ leukemic cells were detected in the recipient of AVA treated mice (Figure 5E2). These *in vivo* data were corroborated by the reduction of leukemic myeloid colonies obtained from the hCD45 positive cells of AVA treated mice compared to the controls (Figure 5F).

To determine whether normal hematopoiesis could be altered by AVA combination in mice, C57BL/6 mice were treated with either

ATRA or AVA using the same protocol used for AML mice xenografts. As shown in figure 6A, upon 8 days treatment, the total number of BM cells was not modified. However, the distribution of myeloid and lymphoid cells was altered. Indeed, the B-lymphoid (B220<sup>+</sup>) and T-lymphoid (CD3<sup>+</sup>) cell numbers decreased and myeloid cell number (Gr1<sup>+</sup>) increased (Figure 6A,  $^{\dagger}p \leq 0.01$ ,  $^{\&p} \leq 0.01$  and  $^{\textcircled{a}}p \leq 0.01$  for B220, CD3 and Gr1, respectively). At D29, AVA combination effect is even more prominent as illustrated in figure 6B with an important increase in Gr1 positive cells while B220 positive and CD3 positive cell numbers decreased (Figure 6B, 6C). The importance of AVA treatment was evaluated by calculating the AVA treated/untreated ratio of B-lymphoid (B220+) and myeloid (Gr1+). As shown in figure 6C, in AVA treated mice, the ratio for Gr1 was 1.6 at day 8 and reached more than 6 at day 29. To evaluate the effects of these treatments on more immature cells, bone marrow cells from mice treated for 29 days were plated in colony assay. We also observed that the numbers of myeloid colonies were superior ( $^{\dagger}p \leq 0.01$ ) in the AVA group compared to ATRA alone or controls (Figure 6D). Thus, AVA combination alters the myeloid and lymphoid compartment but importantly spares immature progenitor cells.

Finally, the myeloid differentiation effect of the AVA combination was also investigated on human hematopoiesis in chimeric NOG mice (Figure 7). In these experiments, irradiated NOG mice were injected with human CD34<sup>+</sup> cells from cord blood. Three months after injection, chimeric mice fully reconstituted with human hematopoietic cells were treated with AVA according to the protocol

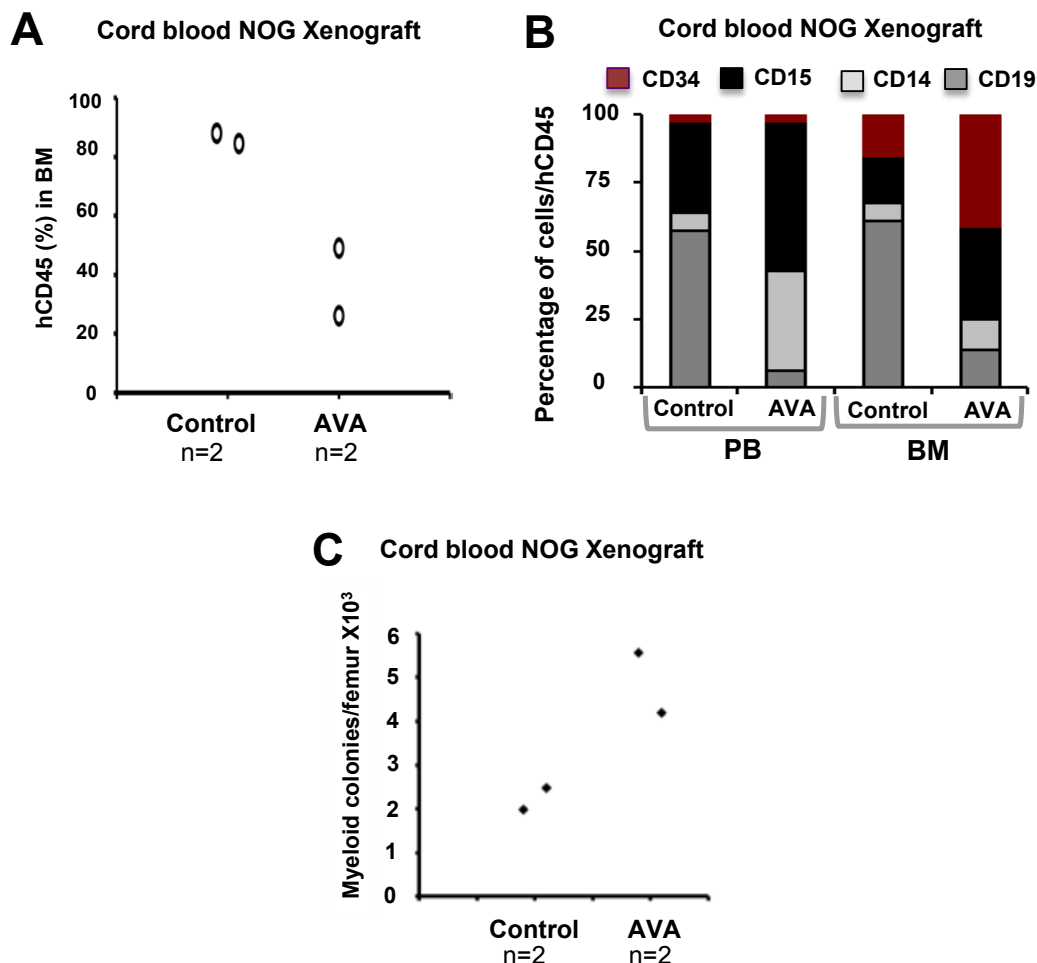


Figure 7 Effect of AVA treatment on normal human hematopoiesis in CD34+ cord blood NOG xenotransplanted mice at day 29.

described in figure 2A. Strikingly, AVA treatment of reconstituted mice resulted in a decrease in the percentage of human CD45 positive cells (Figure 7A). More comprehensive analysis using phenotypic markers of BM cells at day 29 of treatment revealed that AVA modified the distribution of myeloid and lymphoid cells with an decrease in the percentage of B-lymphoid (CD19<sup>+</sup>) and an increase of CD15 and CD14 myeloid cells (Figure 7B). We also observed an increase in the percentage of immature CD34<sup>+</sup> cells in BM treated mice. Consistent with this finding, increased human myeloid colonies were observed (Figure 7C) as already observed in normal C57BL/6 mice. Together, these results suggest that AVA treatment leads to a specific loss of lymphoid cells but spares myeloid cells and maintains immature CD34<sup>+</sup> cells. Altogether, our results indicate that AVA treatment may induce leukemic cell differentiation at least in some selected patients while inducing only minor deleterious effects on normal hematopoiesis further strengthening the potential of these drugs for AML therapy. In the present study, we used drugs whose mechanisms of action are beginning to be known. Indeed, the best known mechanism of action of ATRA is through differentiation<sup>[29]</sup>, AZA is through DNA demethylation<sup>[30]</sup> (Figure 1C) and VPA's action is through histone acetylation<sup>[31-32]</sup> (Figure 1D). These modifications may lead to apoptosis and death of the leukemic cells<sup>[29-32]</sup>. It remains to know why normal and leukemic cells respond differently to the same epigenetic signals.

In conclusion, our study has allowed establishing preclinical models to study combination therapy with epigenetic drugs. Several preclinical models to explore the effects of chemotherapeutic drugs are presently used. So far, most of the studies used well-characterized AML cell lines including OCI-AML2 cells<sup>[33]</sup>, U937 cells<sup>[34]</sup>, the RUNX1-mutated CG-SH AML line<sup>[35]</sup> and AML1/ETO-positive Kasumi-1 cells<sup>[36]</sup>. When inoculated in severe combined immunodeficient (SCID) mice, these cells produced leukemia that can be targeted using chemotherapeutic drugs. These *in vivo* leukemic models are also frequently used to evaluate the effect of gene modification and silencing. In our study, we choose to use primary AML cells from different AML subtypes (AML-M0, M2, M5), which represent the huge heterogeneity of AML disease. In line with this, level of each marker of differentiation is different between AML patient cells. In addition, AML samples are heterogeneous in their ability to engraft in immunodeficient mice with less than 50% of successful engraftment<sup>[23,37]</sup>. This great heterogeneity may explain the variations in sensitivity to drugs. This is further illustrated by the well-known heterogeneity of responses of AML patients in clinical trials (Soriano *et al.*<sup>[20]</sup>, Raffoux *et al.*<sup>[21]</sup>). Strikingly, our small cohort is able to replicate the heterogeneity of responses obtained in clinical trials. Thus, our models should allow testing other combinations of HDAC and DNMT inhibitors and identifying predictive biomarkers of response.

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Authorship: SP, YZ, FL performed research and analysed data; SP wrote the paper; AC, MMD, FZ and BC performed research and analyzed data; FL, CC designed research, analyzed the data and wrote the paper. All authors read, commented on and approved the final version of the manuscript.

## CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare.

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