

Research Article

Valproic acid affects the engraftment of TPO-expanded cord blood cells in NOD/SCID mice

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ABSTRACT

Hematopoietic stem and progenitor cells (HSPC) can improve the long-term outcome of transplanted individuals and reduce the relapse rate. Valproic acid (VPA), an inhibitor of histone deacetylase, when combined with different cytokine cocktails, induces the expansion of CD34 + cell populations derived from cord blood (CB) and other sources. We evaluated the effect of VPA, in combination with thrombopoietin (TPO), on the viability and expansion of CB-HSPCs and on short- and long-term engraftability in the NOD/SCID mouse model. *In vitro*, VPA + TPO inhibited HSPC differentiation and preserved the CD34 + cell fraction; the self-renewal of the CD34 + TPO + VPA-treated cells was suggested by the increased replating efficiency. *In vivo*, short- and long-term engraftment was determined after 6 and 20 weeks. After 6 weeks, the median chimerism percentage was 13.0% in mice transplanted with TPO-treated cells and only 1.4% in those transplanted with TPO + VPA-treated cells was three times more effective than that induced by TPO alone, and over ten times more effective compared to the short-term engraftment induced by the TPO + VPA-treated cells. The *in vivo* results are consistent with the higher secondary plating efficiency of the TPO + VPA-treated cells *in vitro*.

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Introduction

The *ex vivo* expansion of hematopoietic stem and progenitor cells (HSPC) has been used to increase the number of cells capable of rapid repopulation *in vivo* and to improve the kinetics of

hematopoietic recovery after cord blood (CB) transplant [1]. HSPC expansion strategies based solely on the use of cytokines have not resulted in a significant expansion of repopulating cells, in that the cytokines increase the rate of differentiation and decrease the quantity of primitive cells. Given that the primitive cells are

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considered to be responsible for improving the long-term outcome of transplanted patients and for reducing the relapse rate, these strategies have not translated into improved engraftment in clinical trials [2,3].

Of the cytokines that have been evaluated for HSPC expansion, thrombopoietin (TPO) [4,5] has been widely used in combination with early acting growth factors (i.e., Flt3 ligand, c-kit ligand and IL3) both in mice and humans [6,7]. When used alone, TPO has been shown to maintain *in vitro* the viability of primitive CD34 + CD38 – HSPC [6,8]. In a study using non-obese diabetic (NOD) / severe combined immunodeficient (SCID) mice, the percentage of engrafted TPO-amplified CB-CD34 + cells was high beginning 4 weeks after transplantation, and these cells maintained their capacity for self-renewal and multilineage differentiation, as demonstrated after secondary transplantation [9]. Another study has shown that the CD34 + CD38 – cell fraction seems to contain the majority of long-term engrafting cells [10].

A role in hematopoiesis was recently demonstrated for valproic acid (VPA), an inhibitor of histone deacetylase (HDAC) [11-13]. In the presence of different cytokine cocktails, VPA induces the expansion of CD34 + cell populations derived from CB and other hematopoietic sources [14-17], and it inhibits differentiation, limiting the transition from the common myeloid progenitor to the committed lineage-specific precursors [16,18]. These results suggest that VPA could be a useful reagent for improving the *ex vivo* culture of HSPCs and long-term engraftment. The objectives of the present study were to evaluate the effect of VPA, in combination with TPO, on the viability and expansion of HSPCs and on engraftability in the NOD/SCID mouse model.

Materials and methods

CB units

CB units judged inappropriate for banking, according to transfusion regulations, were obtained from the Cord Blood Bank of the "Azienda Policlinico Umberto I" of Rome. CB units from the placenta of full-term newborn infants were collected after having obtained informed consent approved by the Ethics Committee of the "Azienda Policlinico Umberto I", in accordance with the Declaration of Helsinki.

CB-CD34 + cell purification and culture

Mononuclear cells were cryopreserved and maintained at a concentration of 5×10^7 cells/mL in liquid nitrogen until use. CD34 + progenitor cells were purified according to Mattia et al. [9] and grown (75×10^3 cells/mL) in clinical-grade HP01 (Macopharma, Mouvaux, France) serum-free medium with 100 ng/mL TPO (Peprotech EC, London UK), with or without 0.25 mM VPA (Sigma-Aldrich), for 7 days.

Flow cytometry analysis

Monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) [anti-CD34, -CD38, -CD45, -CD61 (Becton Dickinson, San Jose, CA, USA)], and with phycoerythrin (PE) [anti-CD34, -CD38, -CD90, -CD33, -CD19, -CD71 (Becton Dickinson), anti-CD235a (Glycophorin A) (Dako S.p.A.)] were used to characterize cell populations. Isotype

controls were mouse immunoglobulin G. A minimum of 50,000 events were acquired for each sample by a FACScan flow cytometer (Becton Dickinson) using CellQuest software (Becton Dickinson) for data acquisition and analysis.

Cell cycle

The analysis was performed with a DNAcon3 kit (Dako, Italy). Briefly, 1 mL propidium iodide was added to each DNAcon3 test-tube containing dehydrated buffer mixture. After 5 min, cells were added to each tube and incubated at 4 °C for 1 h until analysis. Flow cytometry was performed with a FACScan flow cytometer, and the cell-cycle distribution was analyzed using the ModFit LT software (Becton Dickinson).

Apoptosis assay

Cell viability and apoptosis were measured by flow cytometry using the APOTEST-FITC kit (Dako, Italy). Cells were centrifuged at $300 \times g$, and washed twice with ice-cold PBS. The final cell pellet was resuspended in ice-cold binding buffer at 5×10^5 cells/mL. Ninety-six microliters of cell suspension were incubated with 1 µL of Annexin V-FITC and 2.5 µL of propidium iodide and incubated on ice in the dark for 10 min. Following the incubation, samples were diluted with binding buffer to 250 µL and run on FACScan flow cytometer to distinguish viable, apoptotic and necrotic cells.

Western blot

Cells were lysed in 2% SDS containing phosphatase and protease inhibitors. Proteins from an equal amount of cells (2×10^5) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Schleicher & Schuell, BioScience GmbH, Germany) by electroblotting. Immunoblotting was performed with the following antibodies: cyclin D1 (sc–718), cyclin D3 (sc-182), cyclin B1 (sc-7393), cyclin A (sc-751), GAPDH (sc-51907) (all provided by Santa Cruz Biotechnology, Inc., Santa Cruz, CA., U.S.A.), anti-histone H3 (05-499), and anti-acetyl histone H4 (06–866) (both provided by Upstate, Lake Placid, NY). Peroxidase-conjugate anti-mouse or anti-rabbit immunoglobulin G (Santa Cruz Biotechnology) was used for enhanced chemiluminescence detection. ECL blotting reagents were provided by Amersham (GE Healthcare, UK).

Gene expression analysis

Total RNA, from 1×10^5 cells, and reverse-transcribed-RNA were prepared as described elsewhere [19]. Real time RT-PCR (gPCR) was performed with a "BioRad iCycler® IQ Real-Time PCR Detection System" using the "iQ SYBR[®] Green Supermix" (BioRad), following the manufacturer's instructions. cDNA (100 ng) was amplified over 40 cycles (15 s at 95 °C, 30 s at 60 °C, and 30 s at 55 °C). In all cases, fewer than 30 calculated quantification cycles (Cp) were performed. The results were normalized using GAPDH as reference gene, and the changes in gene expression were calculated using the Pfaffl method. An additional melting curve was recorded for each gene to confirm the specificity of the reaction. Primers were designed using the OligoPerfect™ Designer online tool, and the selected sequences were as follows: GATA1 (NM_002049.3) fw 5'-TCTACCCTGCCTCAACTGTG-3' and rev 5'-TGGGGACAGGGAGTGAT-3' (amplicon length = 174 bp); GATA 2 (NM_032638.4) fw 5'-GCATGGACAGTTGTTTGGAG-3' and

rev 5'-ACCACCAAGTCTCCAAGTCC-3' (amplicon length = 157 bp); GAPDH (NM_002046.3) fw 5'-GAGTCAACGGATTTGTCGT-3' and rev 5'-GACAAGCTTCCCGTTCTCAG-3' (amplicon length = 185 bp). The primer specificity was validated using the Basic Local Alignment Search Tool (BLAST). The primer structure was controlled on the Promix website to avoid the use of primers with stem and loop secondary structure.

Migration assay

Cells collected from cultures at day 7 (1×10^5), suspended in 200 µL serum-free HP01 medium, were loaded in the upper chamber of Transwell (Costar, Cambridge, MA) and induced to migrate through 5-µm pore filters in the lower chamber, which contained SDF-1 α at a concentration of 200 ng/mL. After 5 h of incubation at 37 °C in 5% CO₂, cell migration was calculated by counting the cells recovered from the lower chamber. The viability of the cells was assessed by trypan blue dye exclusion. The efficiency of the migratory response is expressed as the percentage of the loaded CD34 + cells that migrated.

Clonogenic assay

Cells collected from cultures at day 7 (10^3 cells) or from bone marrow (BM) (5×10^4 cells), suspended in 1 mL of methylcellulose medium (Methocult GFH4434, Stem Cell Technologies, Vancouver, British Columbia, Canada), were plated in duplicate in 35-mm culture dishes. The number of colonies was determined after 14 days of culture.

Replating test

The ability of primary GM-CFU, BFU-E, and GEMM-CFU to give rise to secondary colonies was evaluated by picking up individual colonies (about 30): each colony was dispersed to a single cell suspension in 200µL of Methocult and seeded into one well. The percentage of positive wells (i.e., with one or more colonies) was established after 14 days of culture and represents the replating efficiency. The number of all secondary colonies from the positive wells was also determined.

NOD/SCID mice transplantation

NOD/SCID mice, purchased from Harlan Laboratories, Inc., UK, were maintained in sterile microisolator cages at the Department of Quality and Security of Animal Experimentation of the *Istituto Superiore di Sanità*, Rome, Italy. Twelve hours prior to transplantation, 8- to 10-week-old mice were sub-lethally irradiated with 300 cGy. The mice were injected intravenously with amplified CD34 + cells suspended in 100 µL medium. In each series of experiments, one mouse was irradiated but not transfused and considered as a negative control.

Mice were sacrificed by cervical dislocation either 6 or 20 weeks after transplantation (to evaluate short- and long-term engraftment, respectively), and BM cells from both femurs and both tibias were harvested, pooled and analyzed by flow cytometry for the presence of human CD45 + cells [9].

Homing analysis

Amplified CD34 + cells collected at day 7 of culture were labeled with PKH-26, in accordance with the manufacturer's (Sigma)

instructions. The labeled cells were then injected into the tail vein of NOD/SCID mice. The mice were sacrificed 16 h after transplantation. Mononuclear cells obtained from BM were analyzed for PKH-positive cells by flow cytometry, and at least 500,000 events were acquired and analyzed using Becton Dickinson Cell-Quest Software. The results of the homing analysis are expressed as the percentage of PKH-positive cells. BM from non-transplanted mice was considered as controls. All experiments were conducted in accordance with the Italian Legislative Decree no. 116 of 27/01/1992 (adoption of the European Directive 86/609/EEC on "Protection of animals used for experimental and other scientific purposes").

Statistical analysis

Descriptive statistics are reported as the mean \pm SEM. Student's *t* test was used to test the probability of significant differences between samples. Differences were considered significant if the *p* value was ≤ 0.05 .

Results

VPA modulates the proliferation, differentiation, and clonogenic potential of TPO cultured CB cells

Of the cells purified from human CB, $97.8 \pm 1.1\%$ (mean \pm SEM) were characterized as CD34+, based on cytofluorimetric analysis. CD34 + cells were grown for 7 days in liquid suspension culture in a serum-free medium in the presence of 100 ng/mL TPO or TPO + VPA. To identify the dose of VPA that preserves the highest cell viability and the highest number of cells, we performed experiments with 0.25, 0.5 and 1 mM VPA. The cell growth and viability of the cultures are shown in Fig. 1A. The VPA concentration of 0.25 mM was found to preserve the highest viability (90%) of cultured cells without inducing apoptosis ($4.3 \pm 0.3\%$ TPO + VPA vs. $6.8 \pm 0.4\%$ TPO) and to induce HDAC inhibition, as shown by the increased acetylation of histone H4 (Fig. 1B).

The growth rate of cells treated with TPO + VPA for seven days was about 2.5 times lower than that of cells treated with TPO alone (Table 1). In TPO + VPA-treated cells, the cell-cycle profile indicated a decreased proportion of cells that were in the S-phase $(22.0 \pm 6.8\% \text{ TPO} + \text{VPA} \text{ vs. } 34.7 \pm 5.1\% \text{ TPO})$ and in the G2/M-phase $(1.7 \pm 1.1\% \text{ TPO} + \text{VPA} \text{ vs. } 2.7 \pm 1.8\% \text{ TPO})$, and an increased proportion of cells in the G1 phase $(76.3 \pm 7.4\% \text{ TPO} + \text{VPA} \text{ vs. } 63.5 \pm 7.8\% \text{ TPO})$ (Fig. 1C). Accordingly, VPA reduced the expression of cyclin D1 and cyclin D3, which are responsible for the progression along the G1 phase, and of cyclin A and cyclin B1, which are responsible for the progression along the S-phase and G2/M-phase, respectively (Fig. 1D).

After 7 days of exposure to TPO + VPA, the CD34 + cell population constituted $85.6 \pm 8.9\%$ of the cells, compared to $30.8 \pm 8.8\%$ after exposure to TPO alone (Table 1). Almost all of the CD34 + cells were also CD38-negative. The earlier subtype CD34 + CD90 + population was 4.5 times higher in TPO + VPA-treated cells, compared to the cells treated with TPO alone, and the earlier subtype CD34 + CD33 - fraction was 9.3 times higher. Multipotent myeloid early progenitors (CD34 + CD33 +) and MK precursors (CD34 + CD61 +) were also present at high levels (Table 1).

The expression level of the transcription factors GATA1 (which plays a major role in erythroid and megakaryocytic differentiation)



Fig. 1 – A) Cell growth (left panel) and cell viability (right panel) of CB cells cultured at various concentrations of VPA, compared to TPO alone. Viability was determined using 7-ADD exclusion and annexin expression. The data shown are the mean of three independent experiments. B) Immunoblot analysis of acetylated histone H4 (ac H4) after 15 h of VPA treatment; histone H3 expression showed equal loading. C) Pie chart of a representative experiment showing the percentages of cells in different cell-cycle phases at day 7; a total of three experiments, with similar results, were performed. D) Immunoblot analysis of different cell-cycle markers. GAPDH expression showed equal loading. A representative experiment is shown; a total of three experiments, with similar results, were performed. In (B) and (D) the difference between the effect induced by TPO alone and TPO + VPA was evaluated by densitometric analysis of bands and is expressed as the ratio of the quantity of proteins for TPO to the quantity for TPO + VPA (on the right).

and GATA2 (which plays a role in early HSPC proliferation/ differentiation) is shown in Fig. 2. At day 3, the GATA1 and GATA2 expression levels were similar when comparing TPO + VPA and TPO-treated cells. By contrast, at day 7, the expression level of GATA2 was significantly higher in the TPO + VPA-treated cells, whereas the expression level of GATA1 was significantly higher in the cells treated with TPO alone.

At day 7 of culture, the plating efficiency, which represents the clonogenic potential of CD34 + cells, was significantly different between TPO + VPA and TPO-treated cells. The percentage of total colony forming cells (CFC) was $31.3 \pm 11.2\%$ in TPO + VPAtreated cells and $83.2 \pm 32.5\%$ in cells treated with TPO alone; a difference was observed in all of the lineages (Fig. 3A). A mean of 27 primary CFC (GM, BFU-E and GEMM) were reseeded and assayed for their capacity to form secondary CFC (replating efficiency), in nine matched independent experiments: the replating efficiency was significantly higher for the TPO + VPA-treated cells, compared to those treated with TPO alone (Fig. 3B). The mean of the secondary colonies derived from TPO + VPA primary colonies was 25% higher than that of the secondary colonies derived from TPO primary colonies, although the difference was not statistically significant.

VPA affects the BM engraftment of NOD/SCID mice

Sublethally irradiated NOD/SCID mice were injected intravenously with TPO- and TPO + VPA-amplified cells from 75×10^3 CB CD34 + cells after 7 days of expansion (see also Table 1). Engraftment was determined after 6 weeks as the percentage of human CD45 + cells out of the total number of BM cells from 11 mice transplanted with TPO-treated cells and 13 mice transplanted with TPO + VPA-treated cells. Mice with > 0.1% human CD45 + cells were considered as positive (Fig. 4). All of the

Table 1 – Immunophenotypic features of CB-CD34 + cells cultured with TPO with or without VPA. Data indicate percentage \pm SE of the expression of different cell surface markers evaluated by flow cytometry before culture (day 0) and after 7 days of culture (day 7). The data represent the summary of at least 6 independent experiments.

	-			-	-				
	$\frac{\text{Cells/ml}}{(\times 10^3)}$	Fold increase	%CD34+	%CD34+/ CD38-	%CD34+/ CD90+	%CD34+/ CD33-	%CD34+/ CD33+	%CD34+/ CD61+	%CD34+/ CXCR4+
Day 0 Tpo Day 7 Tpo + VPA Day 7	$75459.7 \pm 118165.2 \pm 84.6$	- 5.7±1.8 2.2±1.1	$\begin{array}{c} 98.2 \pm 0.9 \\ 30.8 \pm 8.8 \\ 85.6 \pm 8.9 \end{array}$	60.2 ± 18.8 27.3 ± 7.4 83.1 ± 8.2	$\begin{array}{c} 14.2 \pm 6.1 \\ 2.1 \pm 0.6 \\ 9.6 \pm 2.9 \end{array}$	$- \\ 0.36 \pm 0.03 \\ 3.35 \pm 0.24$	$\begin{array}{c} 82.2 \pm 21.4 \\ 28.0 \pm 8.0 \\ 73.7 \pm 10.2 \end{array}$	- 4.1±1.7 11.4±7.5	$\begin{array}{c} 86.2 \pm 7.0 \\ 7.8 \pm 1.8 \\ 36.7 \pm 14.9 \end{array}$



Fig. 2 – The gene expression levels of GATA-1 and GATA-2. Quantitative real-time PCR analysis of GATA-1 and GATA-2 gene expression was performed after culture of CD34 + cells with TPO alone (\blacksquare) and in the presence of VPA (\Box) for 3 and 7 days. GATA-1 and GATA-2 expression levels are expressed as relative expression with respect to the value obtained in TPO treated cells at day 3. The data shown are the mean of three independent experiments. *p < 0.05.

mice were positive, independently of the transplanted cells. The median chimerism percentage was 13.0% in mice transplanted with TPO-treated cells and 1.4% in those transplanted with TPO + VPA-treated cells.

Differences in homing capability were demonstrated by tracking functional progenitors using an *in vivo* homing assay. Cultured CB cells were labeled with PKH26 dye and injected $(1 \times 10^6 \text{ cells/} \text{mouse})$ into sub-lethally irradiated NOD/SCID mice (n=4); after 16 h, BM cells were analyzed by cytofluorimetric analysis. In the BM of mice that had been transplanted with TPO + VPA and TPOtreated cells, $0.2 \pm 0.06\%$ and $0.4 \pm 0.15\%$ PKH26 + cells were recovered, respectively (Fig. 5A).

TPO + VPA treated cells were found to have migrated less efficiently than TPO cultured cells also in *in vitro* experiments (n = 4), as shown by an SDF-1 α transwell migration assay. After 5 h, the efficiency of the migratory response of CD34 + cells was $8.9 \pm 1.7\%$ for TPO + VPA and $20.2 \pm 4.7\%$ for TPO alone (Fig. 5B), although the fraction of CD34+/CXCR4+ cells was 4–5 times higher for TPO + VPA-treated cells, as reported in Table 1.

The FACS analysis of BM obtained from mice 6 weeks after transplantation showed that all of the human hematopoietic lineages were represented (data not shown). Furthermore, the percentage of HSPCs was higher for mice transplanted with TPO + VPA-treated cells, compared to mice transplanted with TPO-treated cells: the mean percentage of CD34 + cells was $33.1 \pm 12.0\%$ for TPO + VPA and $21.1 \pm 10.6\%$ for TPO. The clonogenic potential of BM cells, expressed as the plating efficiency of CD45 + CD34 + cells,

was significantly higher for mice transplanted with TPO-treated cells, compared to mice transplanted with TPO + VPA-treated cells (Fig. 6A). Although the replating efficiency of primary CFC obtained from BM cells was similar in both conditions (Fig. 6B), the total number of CFC secondary colonies was significantly higher in BM cells of mice transplanted with TPO + VPA cells (p = 0.02) (Fig. 6C). This difference was mainly given by the primary GEMM colonies (29.9 ± 18.4 TPO + VPA vs. 5.4 ± 3.8 TPO).

To assess the long-term repopulation capacity of the transplanted cells, the percentage of human CD45 + cells was evaluated in the BM of three mice at 20 weeks post-transplantation (Fig. 4). The median chimerism percentage was 8% (min. value = 6%; max. value = 12%) in mice transplanted with TPO-treated cells and 28% (min. value = 15%; max. value = 42%) in mice transplanted with TPO + VPA-treated cells. FACS analysis revealed a multilineage engraftment in the BM of mice 20 weeks after transplantation (Fig. 7).

Discussion

The results of this study indicate the ability of VPA to enhance the long-term engraftment of HSPCs in the BM of immunodeficient mice. *In vitro*, VPA combined with TPO inhibited the differentiation of HSPCs and preserved the CD34 + cell fraction after several days of culture, which resulted in an increase in the percentage of the more immature populations, CD34+/CD90+ and CD34+/CD33-.



Fig. 3 – Comparative effect of TPO (\blacksquare) and TPO + VPA (\Box) on the clonogenic potential of CB cells. (A) Plating efficiency of CB cells after 7 days of amplification; error bars represent SEM between experiments; n = 9 (*p < 0.01). (B) Replating efficiency of primary CFC colonies (27 ± 3 CFC), collected at day 14 and replated in the same combination of factors as the primary culture (Methocult); n = 9 (p < 0.05).



Fig. 4 – Bone marrow repopulating potential of TPO and TPO + VPA treated cells following *ex vivo* culture for 7 days. A) Percentage of human CD45 + cells in individual NOD/SCID mice; the horizontal bars represent the median values. Engraftment was evaluated after 6 weeks [TPO (\bullet) and TPO + VPA treated cells (\bigcirc)] and 20 weeks [TPO (\bullet) and TPO + VPA treated cells (\Box)].

Several studies have demonstrated the capacity of VPA combined with different cocktails of cytokines to stimulate the selfrenewal of normal HSPCs *in vitro* [14,15,20]. We chose to use TPO because, compared to cocktails of cytokines, it allows the culture conditions to be better controlled and efficient engraftment to be obtained after transplantation in NOD/SCID mice [9].

In our study, the cells treated with TPO alone showed a higher proliferation rate than the TPO + VPA-treated cells. The self-renewal of the TPO + VPA-treated cells was demonstrated, using clonogenic assay, by the increased replating efficiency of the CD34 + cells. Thus it is conceivable that the cells treated with TPO alone, compared to TPO + VPA-treated cells, more rapidly lose the ability to give rise to cells with replating CFC. Given that the population of quiescent cells that would potentially sustain self-renewal is limited, the cells treated with TPO alone lose this capacity over time, resulting in high proliferation and differentiation. By

contrast, when VPA is added to the cultures, the quiescent cells are relatively slow-cycling cells, so that they maintain the longterm capacity to proliferate.

In our model, GATA2 expression was higher in TPO + VPAtreated cells, compared to cells treated with TPO alone, indicating that VPA can retain this trait of stemness in CB-CD34 + cells, whereas GATA1 expression was higher in the cells treated with TPO alone. These findings are consistent with reports that GATA2 plays a major role in early HSPC proliferation/differentiation and that GATA1 is involved in erythroid differentiation and terminal MK maturation [21,22].

The negative effect of VPA on proliferation may have resulted from the down-regulation of cyclins D, B and A, which are responsible for the reduced percentage of the S and G2/M cycling cells and for the accumulation of G0/G1 cells (76%), which confer characteristics of quiescence to HSPCs. The importance of the non-cycling cells in long-term engraftment was recently reported by Shima et al. [23].

The engraftment of HSPCs into BM first requires the active migration of HSPCs to hematopoietic niches. In our model, VPA interfered with the homing capability, as shown by the finding that the quantity of transplanted labeled cells recovered from the BM of NOD/SCID mice was 2 times lower in mice receiving cells treated with TPO + VPA, though this does not fully explain the poor short-term engraftment. The reduced homing is consistent with the findings of our *in vitro* experiments using the SDF-1 α gradient, which showed a poor migratory response. However, CD34+/CXCR4+ expression was high. Thus it is more likely that the poor migratory response and homing capability were related to the greater fraction of immature cells, which migrate less efficiently [24], though this would need to be further investigated.

Based on the functional role of the HSPCs in the recovery of BM following NOD/SCID transplantation, repopulation is considered to occur in two phases (short-term and long-term), with the long-term phase depending greatly on the short-term phase [25]. In our study, the short-term (6 weeks) engraftment induced by the TPO + VPA-treated cells was five times less effective than that induced by TPO alone, in contrast to the findings of a previous study [18], in which VPA improved engraftment, though in different culture conditions. Conversely, the long-term engraftment (20 weeks) induced by the TPO + VPA-treated cells was three times more effective than that induced by TPO alone, and over ten times more effective compared to the short-term



Fig. 5 – (A) Homing capability of TPO (\blacksquare) and TPO + VPA treated cells (\Box). The results are expressed as percentage recovery (\pm SEM), within the BM after 16 h, of the cells labeled with PKH26 dye; n = 4. (B) Chemiotaxis induced by SDF-1 α , added in the low chamber of transwell. CB cells cultured for 7 days in the presence of TPO, with or without VPA, were plated on the upper chamber of transwell in serum free medium. The total cells are represented with white bars; the dotted bars represent the number \pm SEM of CD34 + cells (TPO \boxtimes ; TPO + VPA \boxtimes); n = 4.



Fig. 6 – Clonogenic potential of CFC from BM of mice inoculated with TPO (\blacksquare) and TPO + VPA treated cells (\Box). (A) Plating efficiency of CFC from BM cells, error bars represent SEM between experiments; n = 7 (* $p \le 0.05$). (B) Replating efficiency of primary CFC colonies collected at day 14 and replated in the same combination of factors as the primary culture (Methocult). (C) Secondary CFC colonies per 100 replated primary colony (*p = 0.02).

engraftment induced by the TPO + VPA-treated cells, suggesting a relationship between the more immature HSPC fraction (CD34+/ CD90+ and CD34+/CD33-) transplanted and the long term engraftment. This result is consistent with the significantly higher secondary plating efficiency of the TPO + VPA-treated cells.

We hypothesize that the TPO + VPA-treated cells reach the niche yet their low proliferation potential *in vitro* is maintained also *in vivo*. The cells could remain in this state and not proliferate actively. The microenvironment, with the release of cytokines and growth factors and the activation of specific pathways, could stimulate the cycling activity, resulting in long-term engraftment. By contrast, in cells treated with TPO alone, the higher proliferation and the higher number of committed progenitors result in short-term engraftment, although the immature population that is still present also allows long-term engraftment to occur.

Conflict of interest disclosure

All authors declare no conflicts of interest.

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Fig. 7 – Multilineage engraftment in BM at 20 weeks for representative NOD/SCID mice. FACS analysis of BM from mice inoculated with TPO treated cells (A) and TPO + VPA treated cells (B).

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