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Nicotinamide, a SIRT1 inhibitor, inhibits differentiation and facilitates expansion of hematopoietic progenitor cells with enhanced bone marrow homing and engraftment

Tony Peled[a], Hadas Shoham[a], Dorit Aschengrau[a], Dima Yackoubov[a], Gabi Frei[a], Noga Rosenheimer G[a], Batya Lerrer[b], Haim Y. Cohen[b], Arnon Nagler[c], Eitan Fibach[d], and Amnon Peled[e]

[a]*Gamida Cell Ltd., Jerusalem, Israel;* [b]*The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel;* [c]*Hematology Division, BMT&CBB, Chaim Sheba Medical Center, Tel-Hashomer, Israel;* [d]*Department of Hematology;* [e]*Goldyne Savad Institute of Gene Therapy, Hadassah-Hebrew University Medical Center, Jerusalem, Israel*

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Strategies that increase homing to the bone marrow and engraftment efficacy of ex vivo expended CD34$^+$ cells are expected to enhance their clinical utility. Here we report that nicotinamide (NAM), a form of vitamin B-3, delayed differentiation and increased engraftment efficacy of cord blood–derived human CD34$^+$ cells cultured with cytokines. In the presence of NAM, the fraction of CD34$^+$CD38$^-$ cells increased and the fraction of differentiated cells (CD14$^+$, CD11b$^+$, and CD11c$^+$) decreased. CD34$^+$ cells cultured with NAM displayed increased migration toward stromal cell derived factor–1 and homed to the bone marrow with higher efficacy, thus contributing to their increased engraftment efficacy, which was maintained in competitive transplants with noncultured competitor cells. NAM is a known potent inhibitor of several classes of ribosylase enzymes that require NAD for their activity, as well as sirtuin (SIRT1), class III NAD$^+$-dependent-histone-deacetylase. We demonstrated that EX-527, a specific inhibitor of SIRT1 catalytic activity, inhibited differentiation of CD34$^+$ cells similar to NAM, while specific inhibitors of NAD-ribosylase enzymes did not inhibit differentiation, suggesting that the NAM effect is SIRT1-specific. Our findings suggest a critical function of SIRT1 in the regulation of hematopoietic stem cell activity and imply the clinical utility of NAM for ex vivo expansion of functional CD34$^+$ cells.

Strategies to expand hematopoietic progenitor cells (HPC) in vitro are of clinical importance to improve the outcome of cord blood transplantations. Exposure of HPC to different combinations of cytokines promotes their exit from the G_0 phase of the cell cycle and enables extensive proliferation. Nonetheless, in vitro proliferation is tightly coupled with a commitment to differentiation and reduced self-renewal [1]. Although early-acting cytokines induce robust in vitro expansion of CD34$^+$ cells, expansion of engraftable progenitors is modest [2]. This phenomenon could be explained, at least in part, by an acquired defect in the bone marrow (BM) homing capacity of ex vivo–expanded HPC [3,4], which is primarily attributed to their active cycling [5], accompanied by alterations in adhesion and chemokine receptor expression or functionality [6]. Therefore, strategies to augment BM homing and engraftment efficacy are particularly important to increase clinical applicability of ex vivo–expanded CD34$^+$ cells [7].

Nicotinamide (NAM), a form of vitamin B-3, serves as a precursor of nicotinamide adenine dinucleotide (NAD$^+$) [8]. NAM is also a potent inhibitor of enzymes that require NAD$^+$ for their activities [9,10], such as mono-ADP-ribosyltransferases, poly-ADP-ribose polymerases, CD38, and cyclic ADP ribose/NADase [11]. In addition, NAM is a well-established potent inhibitor of the sirtuin family of histone/protein deacetylases, the NAD-dependent class III histone deacetylase (HDAC) [12]. SIRT1, one of the mammalian sirtuins, catalyzes the deacetylation of acetyl-lysine residues by a mechanism whereby NAD$^+$ is cleaved. The reaction results in the release of NAM, which acts as an end-product noncompetitive inhibitor of SIRT1 by binding

Offprint requests to: Tony Peled, Ph.D., Gamida Cell Ltd., Cell Therapy Technologies, P.O. Box 34670, Jerusalem 91340, Israel; E-mail: Tony@gamida-cell.com; http://www.gamida-cell.com

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to a conserved pocket adjacent to NAD(+), thereby blocking NAD(+) hydrolysis [13]. It was reported that hematopoietic cells derived from SIRT1-deficient mice ($SIRT1^{-/-}$) display increased in vitro proliferation activity, although their self-renewal and in vivo function were not addressed [14]. Having multiple effects on numerous cells, NAM is implicated in the regulation of cell adhesion, polarity, migration, proliferation, and differentiation [15]. NAM was shown to modulate the fate of embryonic stem cells [16] and primary nonhematopoietic cells [17–19]. With regard to hematopoietic cell lines, NAM was reported to inhibit HL-60 cell differentiation mediated by retinoic acid [20], and other studies reported that NAM enhances HL-60 cell differentiation [21].

Here we studied the effect of NAM on primary cultures of umbilical cord blood (UCB) $CD34^+$ cells. Our results demonstrate that NAM delayed differentiation and enhanced migration, homing, and engraftment of $CD34^+$ cells expanded ex vivo with cytokines. The SIRT1-specific inhibitor, EX-527 [22,23], exhibited an effect similar to that of NAM on cultured $CD34^+$ cells, while NAM-related [24–26] and nonrelated [27–29] NAD-dependent ADP-ribosyltransferase inhibitors were not effective. Based on these findings, we propose SIRT1 deacetylase as a target accountable for NAM modulating $CD34^+$ cell differentiation in ex vivo cultures.

Materials and methods

CB samples

Cells were obtained from UCB samples harvested from consenting mothers after normal full-term deliveries (Sheba Medical Center, Tel-Hashomer, Israel). Samples were collected and frozen according to Rubinstein et al. [30]. Before use, cells were thawed, the mononuclear cells purified on a Ficoll-Hypaque gradient, and $CD34^+$ cells were isolated using a MidiMACS CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Bergisch, Gladbach, Germany) as described previously [31].

Ex vivo expansion cultures

Purified $CD34^+$ cells were cultured in culture bags (American Fluoroseal Co., Gaithersburg, MD, USA) at 1×10^4 cells/mL (at least 8 mL/bag) in minimum essential medium–α, 10% fetal bovine serum (FBS), and cytokines: thrombopoietin, interleukin (IL)-6, fms-like tyrosine kinase–3 ligand, and stem cell factor, each at a final concentration of 50 ng/mL (Pepro Tech, Inc., Rocky Hill, NJ, USA), with or without NAM (Sigma Aldrich, Milwaukee, WI, USA; catalog number N5535 and Vertellus, Indianapolis IN, USA; catalog number 100547) and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Until week 3, the cultures were topped weekly with the same volume of fresh medium. For long-term experiments, the cultures were weekly demi-depopulated. The number of total nucleated cells (TNC) in culture was determined after dilution (1:10) with phosphate-buffered saline (PBS) by CEDEX, an automatic cell counter (Innovatis AG, Bielefeld, Germany). The CEDEX is an automated cell counting system based on the well-established Trypan Blue exclusion method for determining cell viability. The number of cells in culture was determined by multiplying the number of cells/mL by the culture volume. Fold-expansion was calculated by dividing total number of cells in culture with the culture input number of cells. Number of $CD34^+$ cells and $CD34^+38^-$ cells were calculated by multiplying percentages with total number of cells in culture/100. Fold-expansion was calculated by dividing the calculated number of $CD34^+$ and $CD34^+CD38^-$ cells obtained following culture with the number of culture seeded $CD34^+$ and $CD34^+$CD38– cells. For the colony-forming unit (CFUc) assay, 1000 $CD34^+$ cells before culture and 1500 cells after culture were added per 3 mL MethoCult (MethoCult GF+H4435 complete methylcellulose medium with recombinant cytokine and erythropoietin for colony assays of human cells; StemCell Technologies, Vancouver, BC, Canada). After stirring, the mixture was divided into two 35-mm dishes and incubated for 14 days at 37°C in a humidified atmosphere of 5% CO_2 in air. At the end of the incubation period, colonies (both myeloid and erythroid) were counted under an inverted microscope at 40× magnification. The CFUc content/mL culture was calculated as follows: number of scored colonies per two dishes × total cell number/1500 or 1000. Fold-expansion was calculated by dividing total number of colonies in culture (number/mL × culture volume) with the culture input number of colonies.

Immunophenotyping

Cultured and noncultured cells were washed with PBS containing 1% bovine serum albumin and double-stained (at 4°C for 30 minutes) with phycoerythrin (PE) or fluorescein isothiocyanate–conjugated antibodies to CD45, CD34, CXCR4, very late antigen 4 and lymphocyte function–associated antigen 1 (Becton Dickinson, Erembodegem, Belgium) or with differentiation antigens CD38, CD33, CD14, CD15, CD11b, and CD11c (DAKO, Glostrup, Denmark). Cells were then washed in the buffer and analyzed using a flow cytometry (Becton Dickinson, San Jose, CA, USA). The emission of 10^4 cells was measured using logarithmic amplification and analyzed using CellQuest software (Becton Dickinson, Belgium).

PKH26 labeling

Freshly purified $CD34^+$ cells (2×10^6) were incubated at room temperature for 5 minutes with 4 μM PKH26 (PKH26–PE-Cell Linker Kit; Sigma Aldrich, Milwaukee, WI, USA) according to manufacturer's instructions. Then, an equal volume of 1% FBS was added for 1 minute, and the labeled cells were washed three times in PBS supplemented with 5% human serum albumin.

2′, 7′-Bis-(carboxyethyl)-5(6′)-carboxyfluorescein acetoxymethyl ester (BCECF/AM) labeling

Briefly, cells were washed and resuspended at $<10^7$ cells/mL in serum-free medium, and BCECF/AM at a final concentration of 5 μg/mL was added for 10 minutes at 37°C. Uptake of the dye was stopped by the addition of FBS (to give a final concentration of 10%). After labeling, cells were washed three times in PBS with 10% FBS and analyzed by flow cytometry for fluorescence intensity [32].

Tracking of cell-division history

Purified $CD34^+$ cells were labeled with PKH2. An aliquot was analyzed with flow cytometry for PKH2 intensity ($t = 0$) and the rest was cultured with cytokines (i.e., stem cell factor, thrombopoietin, fms-like tyrosine kinase–3, and IL-6), with or without

5 mM NAM. On day 7, cells were harvested and $CD34^+$ cells were reisolated using the MiniMACS CD34 progenitor cell isolation kit and double-stained for CD34 and CD38. CD34 cells and the gated $CD34^+CD38^-$ cells were analyzed with flow cytometry for PKH2 fluorescence intensity.

In vitro migration assay

Minimum essential medium–α plus 1% FBS and 100 ng/mL stromal cell derived factor–1 (SDF-1; R&D Systems Inc, Minneapolis, MN, USA) was placed into the lower chamber of a Costar 24-well Transwell (Corning, Corning, NY, USA). Cells (2×10^5) in 100 μL medium were placed into the upper chamber over a porous membrane (pore size, 5 μm). After 4 hours, cells were collected from the lower chamber and counted. Spontaneous migration was evaluated without SDF-1 in the lower chamber.

In vivo homing assay

NOD/LtSz- (nonobese diabetic/severe combined immunodeficient [NOD/SCID]) mice (8–10 weeks old) (Harlan Biotech Israel Ltd., Rehovot, Israel) were sublethally irradiated (with 375 cGy at 67 cGy/min) and 24 hours later inoculated via the tail vein with 10 to 20 million cells stained with BCECF/AM (Calbiochem, Darmstadt, Germany). The experiments were approved by the Animal Care Committee of the Hadassah, Hebrew University Medical Center. Mice were sacrificed at 24 hours post injection. BM samples were obtained by flushing their femurs and tibias with PBS at 4°C. Homing of human cells was detected by flow cytometry. The bright fluorescence of BCECF/AM was sufficient to separate labeled human cells from unlabeled murine cells by at least 1 log. To quantify homing of human progenitor cells, BM cells were stained with allophycocyanin-conjugated anti-human CD34 monoclonal antibodies (Becton Dickinson, Belgium) and $BCECF/AM^+CD34^+$ cells were enumerated. For each sample, 100,000 events were acquired and analyzed. The Animal Care Committee of Hadassah, Hebrew University Medical Center, Jerusalem, Israel approved these experiments. To evaluate BM homing after cotransplantation, noncultured cells were stained with BCECF/AM (fluorescein isothiocyanate) and cultured cells stained with PKH (PE), as described.

Transplantation of human $CD34^+$ cells into NOD/SCID mice and quantification of SCID repopulating cells (SRC)

NOD/SCID mice were bred and maintained at the Weizmann Institute, Rehovot, Israel in sterile intraventilated cages (Techniplast, Bugugiatte, Italy) or at Harlan Biotech. The experiments were approved by the Animal Care Committee of the Weizmann Institute and of Harlan Biotech. Eight-week-old mice were sublethally irradiated as described and transplanted with human CB-derived cells. Mice were sacrificed on week 6, and the BM cells were immunophenotyped as described here [31,33]. To compare engraftment before and after culture, each CB unit was frozen into two portions. CD34 cells purified from one portion were cultured for 3 weeks as described. The second portion was kept frozen. On the day of transplantation, this portion was thawed and TNC or purified $CD34^+$ cells were transplanted. In some experiments, to avoid donor variability, this procedure was carried out with $CD34^+$ cells pooled from several CB units. Single units were used in experiments where cultured and noncultured TNC were cotransplanted in the same mouse. In these experiments, to avoid clamping of noncultured cells, the two cell populations were mixed just before cell injection. The frequencies of SRC were quantified by a limiting dilution analysis and applying Poisson statistics to the single-hit model as described previously [34]. Mice were scored as positively engrafted if at least 0.5% of their marrow cells expressed human CD45. Frequencies of SRC and statistical comparison between individual populations were calculated by maximum likelihood estimator using L-Calc software (StemCell Technologies) [34,35].

Western blotting

$CD34^+$ cells were cultured with cytokines, with and without 5 mM NAM, 18 hours before the addition of lysis Tris/saline/azide (1 μM). For the immunoprecipitation, cells were lysed in lysis buffer containing (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA , 1% NP-40, and a cocktail of protease inhibitor). HDAC inhibitors were supplemented in the lysis buffer at the following final concentrations: 5 mM NAM and 1 μM Tris/saline/azide. Cellular lysates were incubated with agarose-conjugated anti-acetyllysine antibody (-Ac-K) (ImmuneChem Pharmaceuticals Inc., Burnaby, BC, Canada) overnight at 4°C on a rotation wheel. Immunocomplexes were washed four times with lysis buffer, boiled, and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Western blotting analysis of preimmunoprecipitation (input) and immunoprecipitated samples (-Ac-K) were performed with an anti-Ku70 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Statistics

The nonparametric Wilcoxon rank test was applied for testing differences between the study groups. All the tests applied were two-tailed, and a p value of ≤ 0.05 was considered statistically significant. Data were analyzed using SAS software (SAS Institute, Cary, NC, USA).

Results

Characterization of NAM effect on ex vivo $CD34^+$ cell cultures

The effect of NAM was determined in $CD34^+$ cells derived from UCB during 3 weeks in cultures supplemented with cytokines (i.e., fms-like tyrosine kinase–3, IL-6, thrombopoietin, and stem cell factor). Analysis included the number of TNCs, CFUc, and phenotypic characterization of hematopoietic progenitors, $CD34^+$ and $CD34^+CD38^-$ cells (Fig. 1A–L). As early as 1 week post seeding, the number of TNCs (Fig. 1A) and $CD34^+$ cells (Fig. 1B) was substantially lower, while percentages (Fig. 1C, D) and absolute number (Fig. 1E) of $CD34^+CD38^-$ cells were significantly higher in cultures treated with 2.5 and 5 mM NAM as compared with control cultures treated with cytokines only. The divisional history of seeded $CD34^+$ cells stained with PKH indicated that during the first week in culture, the vast majority of $CD34^+$ cells underwent several cycles under both culture conditions (Fig. 1F, G), with consistent lesser divisions (higher fluorescence intensity) of cells cultured with NAM (Fig. 1H). Slower cycling was particularly prominent in the $CD34^+CD38^-$ subset (Fig. 1G), which, nevertheless, increased within the expanded cell

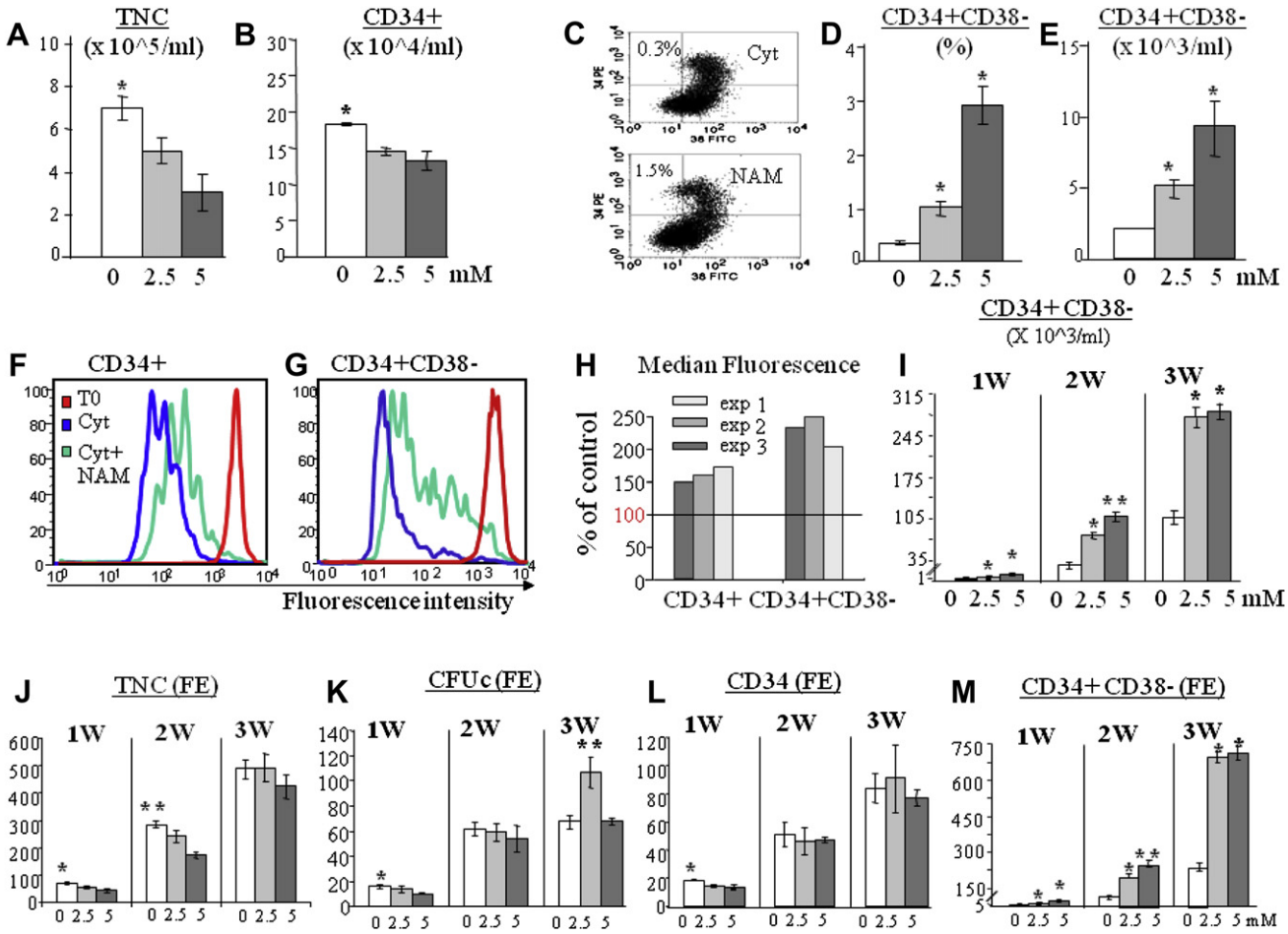

Figure 1. Effect of NAM on CD34$^+$ cell cultures. CB-derived purified CD34$^+$ cells were cultured for 3 weeks with cytokines or with cytokines and NAM at 2.5 and 5 mM. The culture, analysis procedures and calculations are described in Materials and Methods. Each bar represents the mean ± standard error of four independent experiments. (**A–E**) Analysis of cultured cells 1 week post seeding. (**A**) Number of TNCs (*$p < 0.01$ vs NAM, 2.5 and 5 mM). (**B**) Number of CD34$^+$ cells (*$p < 0.008$ vs NAM). (**C**) Representative flow cytometry analysis dot plots of cells double stained with CD34 PE and CD38 fluorescein isothiocyanate. (**D**) Percentages of CD34$^+$CD38$^-$ cells (*$p < 0.01$ vs NAM-nontreated cultures). (**E**) Numbers of CD34$^+$CD38$^-$ cells (*$p < 0.03$ vs NAM-nontreated cultures). (**F–H**) To track cell-division history, freshly purified CD34$^+$ cells were labeled with PKH2, cultured, and analyzed 7 days post seeding, as described in Material and Methods. Histograms of PKH fluorescence intensity of CD34$^+$ (**F**) and CD34$^+$CD38$^-$ (**G**) cells are shown. The histograms present the same number of cells for both control and NAM-treated cells in a representative experiment out of three experiments performed. (**H**) The median fluorescence intensities of NAM expanded cells on day 7 cultures of three separate experiments are shown as percentages of control cultures treated with cytokines alone. (**I**) Numbers of CD34$^+$CD38$^-$ cells, 1 week (*$p \leq 0.03$ vs NAM-nontreated), 2 weeks (*$p \leq 0.02$ and **$p \leq 0.02$ vs NAM-nontreated) and 3 weeks (*$p \leq 0.01$ vs NAM-nontreated) post seeding. (**J–M**) Fold expansion (FE) of TNC (*$p < 0.01$ vs NAM; **$p < 0.03$ vs NAM 5 mM) (**J**), CFUc (*$p < 0.03$ vs NAM; **$p < 0.02$ (**K**), CD34$^+$ cells (*$p \leq 0.01$ vs NAM) (**L**), and CD34$^+$38$^-$ cells 1 week (*$p \leq 0.04$ vs NAM-nontreated), 2 weeks (*$p \leq 0.03$ and **$p \leq 0.02$ vs NAM-nontreated), and 3 weeks (*$p \leq 0.01$ vs NAM-nontreated) post seeding (**M**), are shown.

population from week 1 through week –3 (Fig. 1I). After 3 weeks in culture, fold-expansion of TNCs (Fig. 1J), CFUc (Fig. 1K), and CD34$^+$ cells (Fig. 1L) in NAM-treated cultures reached the values in NAM-nontreated cultures, while fold-expansion of CD34$^+$CD38$^-$ cells was superior in cultures treated with NAM throughout the 3-week culture duration (Fig. 1M). Higher concentrations of NAM (10 mM) deteriorated TNC, CD34, and CFU proliferation throughout the culture duration, while a lower concentration of NAM (1 mM) had a slight effect on the expansion of CD34$^+$CD38$^-$ cells (Supplementary Figure E1; online only, available at www.exphem.org). Phenotype characterization of lineage differentiated cells in 3-week cultures revealed lessening of differentiation in cultures treated with NAM (2.5 and 5 mM) than in cultures treated with cytokines alone, as demonstrated by significantly lower percentages of CD14, CD11b, CD11c, and CD15$^+$ cells (Supplementary Figure E2; online only, available at www.exphem.org).

NAM attenuates differentiation and promotes long-term expansion of cultured CD34$^+$ cells

In order to test the long-term potential of short-term NAM-treated cultures, CD34$^+$ cells from 3 UCB units were cultured for 3 weeks with and without NAM, and

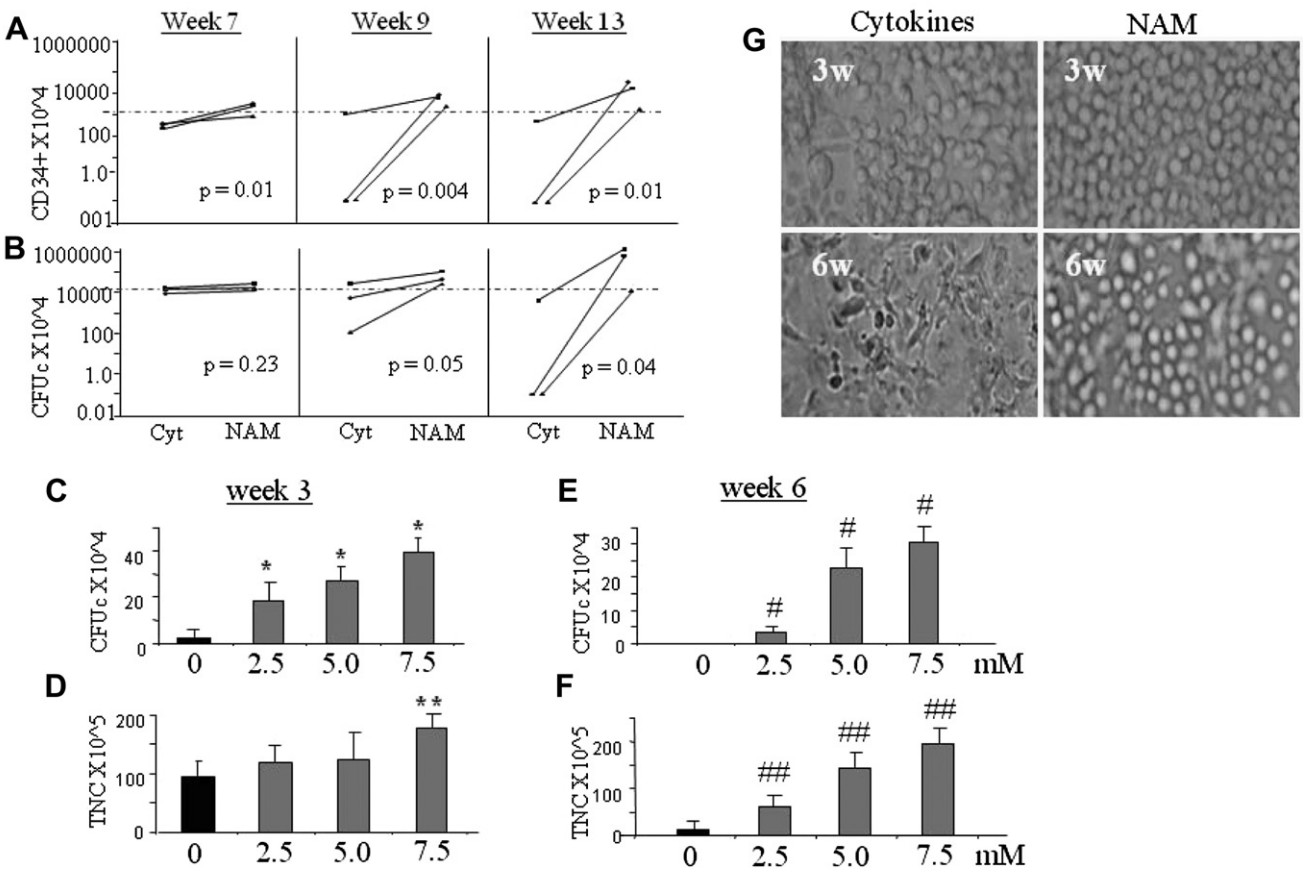

Figure 2. The long-term expansion potential of NAM-treated cells. (**A, B**) $CD34^+$ cells were cultured in medium supplemented with cytokines with or without NAM (5 mM). After 3 weeks, both cultures were supplemented with cytokines only. On the indicated weeks, $CD34^+$ cells (**A**) and CFUc (**B**) were determined. Each data point represents the mean count of two duplicates. The average number of three experiments initiated with cells derived from different donors ± standard error and the *p* values of the difference between cultures with and without NAM are indicated. (**C–F**) $CD34^+$ cells were cultured for 3 weeks with the four early-acting cytokines (fms-like tyrosine kinase–3 [FLT3], stem cell factor, thrombopoietin, and IL-6) and IL-3 at 20 ng/mL (Pepro Tech, Inc.), with or without NAM (2.5–7.5 mM). Number of 3-week CFUc (**C**) and TNC (**D**) are shown. After 3 weeks, all cultures were supplemented with four early-acting cytokines alone (without IL-3 and NAM) for an additional 3 weeks. Number of 6-week CFUc (**E**) and TNC (**F**) are shown. (**G**) Phase contrast images (×20) of 3- to 6-week cultures (n = 3, $^*p < 0.03$; $^{**}p < 0.04$; $^{\#}p < 0.001$, $^{\#\#}p < 0.002$ vs NAM-nontreated cultures).

subsequently monitored throughout an additional 10 weeks of culture in the absence of NAM. Regardless of NAM treatment, the number of CFUc in long-term expansion cultures are exceeding the number of $CD34^+$ cells, suggesting that the decline in $CD34^+$ cells precedes the decline in CFUc. However, after 9 and 13 weeks in culture, both the numbers of $CD34^+$ cells and their clonogenic activity (CFUc) were significantly increased by the initial treatment of the cultures for 3 weeks with NAM over control cultures not treated with NAM. Moreover, in two out of three cultures initially treated with NAM, numbers of $CD34^+$ cells and CFUc considerably increased throughout the culture duration (from week 7 to week 13) (Fig. 2A, B), suggesting that the 3-week treatment with NAM not only increased the number of cells displaying an early progenitor cell phenotype, but also preserved their potential for long-term expansion.

To further test the effect of NAM on in vitro differentiation, IL-3, a cytokine that hastens myeloid differentiation of ex vivo–expanded $CD34^+$ cells [36], was added to the medium of cultures supplemented with the four early-acting cytokines and increasing concentrations of NAM (2.5–7.5 mM). After 3 and 6 weeks, both clonogenic activity (Fig. 2C, E) and total cellularity (Fig. 2D, F) were increased, in a dose response, in cultures treated with NAM over control, NAM-nontreated cultures. Furthermore, phase-contrast images (Fig. 2G) visualize the differences in cell morphology between short and long-term cultures treated with and without NAM. Taken together, treatment with NAM delayed differentiation and promoted expansion of progenitors with enhanced self-renewal capacity.

Short-term SRC potential of NAM-treated cells

The short-term SCID reconstituting capacity of NAM-treated cells was evaluated in transplantation experiments using human anti-CD45 at a threshold level of 0.5% of

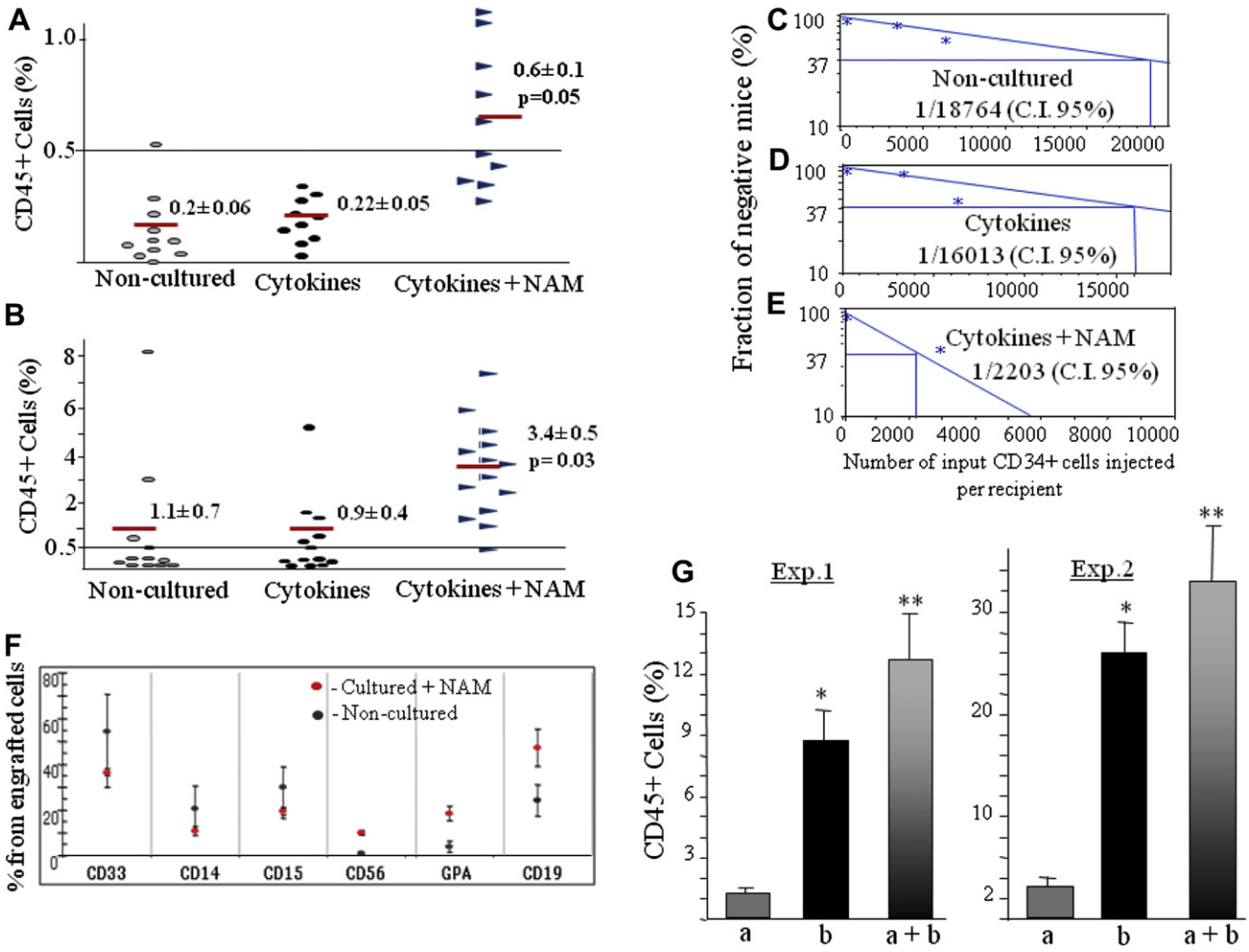

Figure 3. The short-term SCID-repopulating potential of NAM-treated cells. Noncultured $CD34^+$ cells from two independent experiments, 3×10^3 (**A**), 6×10^3 (**B**), or their entire progeny following 3-week culture with and without NAM, as described in Materials and Methods, were transplanted into NOD/SCID mice. Six weeks later, human CD45 cells in the mouse marrows were scored and presented as percentage (%) of the total nuclear cells. (**C–E**) SRCs were calculated by plotting the engraftment frequencies at each dose. The resultant curve indicates the estimated frequency of short-term SRC within noncultured $CD34^+$ cells (**C**) and the increase in short-term SRC number after culturing without (**D**) or with NAM (**E**) was determined. The number shown in each box indicates the calculated frequency of SRCs using the maximum likelihood estimator. (**F**) BM of grafted mice derived from the groups transplanted with 6×10^3 noncultured cells or their total progeny after culturing with NAM were dually stained with antibodies to human CD45 and with antibodies to human differentiation antigens as indicated. Percentages of dual-positive cells are shown. (**G**) NOD/SCID mice were inoculated with 2×10^6 noncultured cells (**a**) or 2×10^6 NAM-treated cultured cells (**b**) or with a mixture of cultured (2×10^6) and noncultured (2×10^6) cells (**a**+**b**) as described in Material and Methods. Each experiment was initiated with cells derived from a single CB unit. Percent (mean ± standard error) of engrafted human CD45 cells is shown (n = 10/experiment; $*p < 0.05$ and $**p < 0.05$ vs noncultured cells).

marrow cellularity. Sublethally irradiated NOD/SCID mice were grafted with 3×10^3 (Fig. 3A) or 6×10^3 (Fig. 3B) purified $CD34^+$ cells , or the entire progeny of the same number of cells was cultured for 3 weeks with or without NAM. Evidence of engraftment was found at 6 weeks post transplantation in 8% of mice grafted with 3×10^3 noncultured $CD34^+$ cells (Fig. 3A). During 3 weeks of culture, a total number of 1×10^6 cells was obtained, irrespective of the presence of NAM. However, while none of the mice grafted with cells cultured with cytokines showed engraftment, exposure to NAM resulted in engraftment in 50% of the mice (Fig. 3A). A donor inoculum of 6×10^3 cells and their entire progeny (2×10^6 cells) resulted in engraftment in 33%, 46%, and 93% of recipients of fresh $CD34^+$ cells, cytokine-cultured cells, and NAM-treated cells, respectively (Fig. 3B). These data suggested a significant advantage in engraftment of cells upon exposure to NAM, over both cultured and fresh $CD34^+$ cells. Short-term SRC frequency was 1 in 18,764 cells (95% confidence interval, 1/44,982–1/7828) and 1 in 16,013 cells (95% confidence interval, 1/38,347–1/6687) for noncultured and cytokine cultured cells, respectively (Fig. 3C, D). NAM-supplemented cultures yielded a short-term SRC frequency of 1 in 2203 cells (95% confidence interval,

1/3964–1/1224) (Fig. 3E). Applying the maximum likelihood estimator test [34,35], the short-term SRC frequency calculated within NAM-cultured cells was significantly higher than the short-term SRC frequency in either noncultured or cytokine-only cultured cells ($p = 0.05$ and $p = 0.045$, respectively). These data correspond to a net ninefold increase in short-term SRC activity of NAM-treated cells compared to fresh $CD34^+$ cells and a 7.6-fold increase over cells cultured without NAM. The in vivo differentiation potential to myeloid and lymphoid lineages after engraftment of noncultured and NAM-cultured cells is shown in Figure 3F.

Prior competitive assays reported that fresh (noncultured) $CD34^+$ cells had a significant engraftment advantage and therefore outcompeted engraftment of cytokine-cultured cells when cotransplanted in the same mouse [37–39]. We transplanted a similar number of cultured and noncultured cells (TNC) derived from the same CB unit (Fig. 3G), either separately or together (cotransplantation) in the same mouse. Mean engraftment (the percentage of human CD45 cells) of mice transplanted with 2×10^6 noncultured cells containing 0.5×10^4 $CD34^+$ cells (0.25% of 2×10^6 TNC) and that of 2×10^6 NAM cultured cells, the progeny of 0.5×10^4 culture seeded $CD34^+$ cells (representing a 400-fold TNC expansion throughout the 3-week culture) was 1.2 ± 0.3 and 8.7 ± 1.4, respectively (Fig. 3G, Experiment 1) and 3.4 ± 1.8 and 27 ± 2.6, respectively (Fig. 3G, Experiment 2). Based on these experiments, the net increase in the level of engraftment after a 3-week culture with NAM was 7.4- (Experiment 1) and 8.0- (Experiment 2) fold, respectively. Cotransplantation of equal numbers (2×10^6) of fresh and NAM-cultured cells resulted in levels of human cell engraftment of 12.2 ± 2.1 (Experiment 1) and 33.2 ± 4.1 (Experiment 2). The additive effect on engraftment suggests that these grafts did not compete for engraftment, but rather yielded distinct contribution to human short-term SRC function.

BM homing of NAM-treated cells

Earlier studies have attributed, in part, reduced engraftability of ex vivo expanded cells to defective homing to the BM caused by exposure to cytokines in culture [3]. In view of the superior engraftment of NAM-cultured cells (Fig. 3), we tested whether their homing ability was improved. NOD/SCID mice were grafted with 10×10^6 fresh (noncultured) UCB cells or with their total progeny after 3 weeks of culture, 10×10^6 cells (calculated as described here). Cells were labeled with BCECF/AM and homing to the mouse BM was assessed 24 hours after transplantation (Fig. 4A–C). Representative flow cytometry analysis of the BM of grafted mice is shown in Figure 4D–J, allowing the evaluation of BM-homed $CD45^+$ ($BCECF/AM^+$) (Fig. 4D–G) and $CD34^+$ ($BCECF/AM^+CD34^+$) cells (Fig. 4H–J) in the respective gates of labeled cells. Even though the same number of cells and $CD34^+$ cells were transplanted from both cultured groups (due to a similar fold expansion of TNC and $CD34^+$ cells following 3-week culture ± NAM, Figure 1J, L, respectively), the homing capacity of cells cultured with NAM increased compared to nontreated cultures (Fig. 4A). The number of $CD34^+$ cells that home to the BM after expansion with NAM was sixfold higher, while that of NAM-nontreated cultures was only twofold higher over the homing of culture input $CD34^+$ cells (Fig. 4B). In spite of the substantial advantage obtained following expansion with NAM, calculation of homing efficacy based on number of $CD34^+$ cells infused demonstrate higher efficacy of noncultured $CD34^+$ cells over the two cultured groups (Fig. 4C). The homing efficacy of NAM-cultured $CD34^+$ cells was significantly higher over the homing efficacy of $CD34^+$ cells cultured without NAM.

Therefore, although there is a significant improvement in homing, NAM treatment does not completely overcome homing defect resulting from in vitro culture. The increased numbers of $CD34^+$ cells being injected contribute to the substantial increase in the absolute number of $CD34^+$ cells that home to the BM after expansion with NAM over noncultured cells.

Next, we sought to determine the homing capacity of NAM-treated cultured cells when transplanted along or with noncultured competitor cells. To this end, 20×10^6 fresh cells (noncultured) labeled with BCECF/AM were cotransplanted with an equal number of NAM-treated cells labeled with PKH membrane linkers (Fig. 4K). The capacity of NAM-treated cells was not affected, indicating that NAM-cultured and fresh cells do not compete with each other during homing.

Dependency of migration and homing of NAM-treated cells on CXCR4

Next, we tested the migration capacity of $CD34^+$ cells toward SDF-1 gradient in vitro in a Transwell migration assay [40]. Background spontaneous migration of cells in a Transwell was very low in all groups of cells, in particular after 3 weeks of culture (Fig. 5A). Similar to their in vivo homing capacity, migration of NAM-treated cells in response to SDF-1 also increased compared to that of cytokine-cultured and freshly isolated cells (Fig. 5B). However, treatment of cells with NAM did not change the number of cells expressing CXCR4 or the intensity (mean fluorescence intensity) of CXCR4 cell surface expression. Number of cells expressing the cell adhesion molecules, very late antigen 4 and lymphocyte function–associated antigen 1, and their mean fluorescence intensity was increased in both cultured groups (Fig. 5C, D). Therefore, quantitative expression of these relevant cell surface molecules cannot explain the increased migration and homing potential of NAM-treated cultured cells.

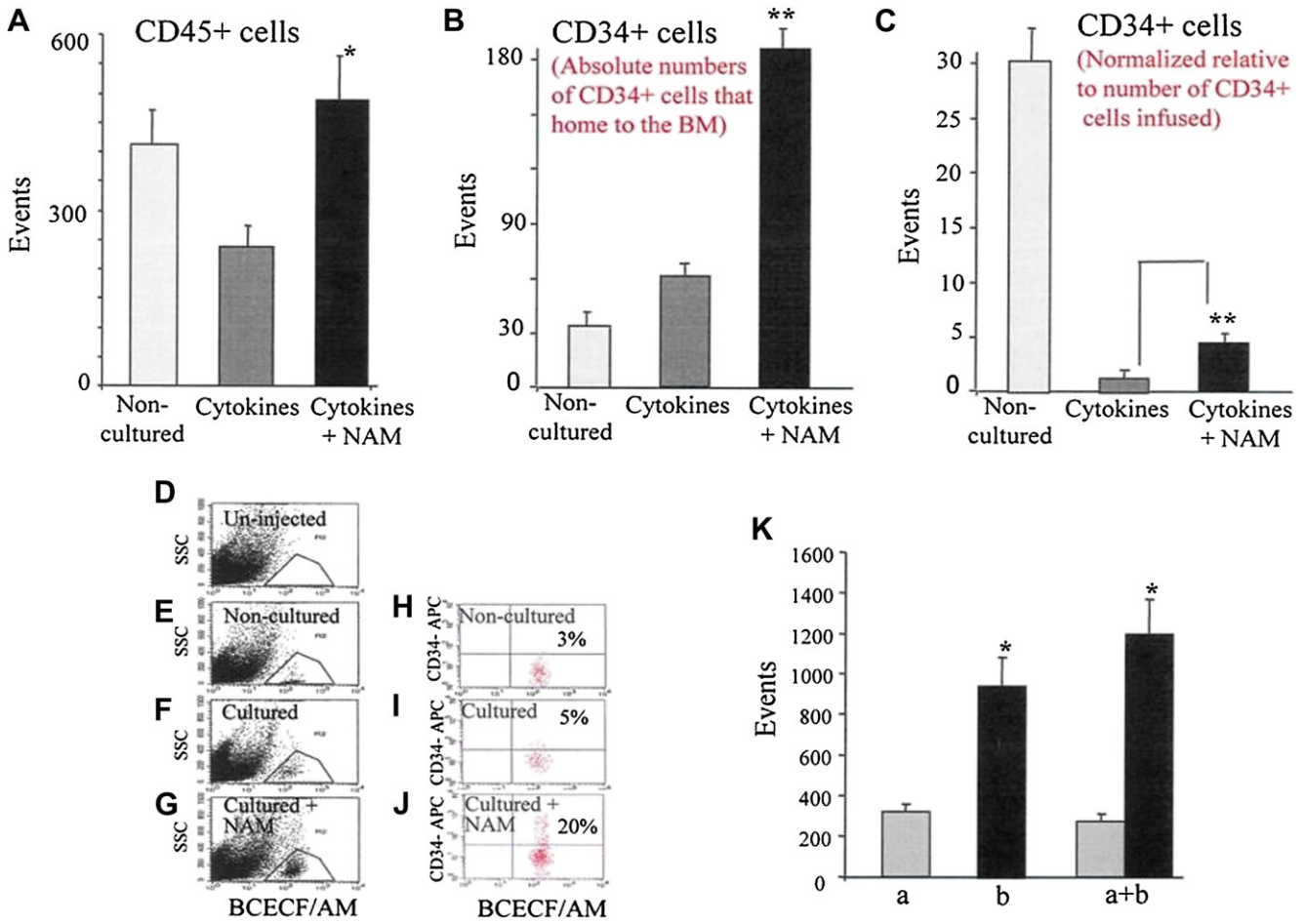

Figure 4. BM homing of cultured and noncultured cells. NOD/SCID mice were transplanted with either 10×10^6 noncultured TNC (containing 5×10^4 $CD34^+$ cells), or with the total progeny of their purified $CD34^+$ cells following 3-week culture with or without NAM (both containing 200×10^4 $CD34^+$ cells). Before transplantation, cells were labeled with BCECF/AM, and 24 hours later, BCECF/AM-labeled $CD45^+$ cells (**A**) and $CD34^+$ cells (**B**) in the BM were quantified by flow cytometry, as detailed in Materials and Methods. Homing efficacy of $CD34^+$ cells (number of events normalized to relative number of $CD34^+$ cells infused) is shown in (**C**). Homing of human cells is presented as the number of positive events per 100,000 marrow cells analyzed (each bar represents the mean ± standard error of three independent experiments, n = 8/experiment; $*p < 0.05$ vs cytokines only; $**p < 0.001$ vs cytokines only and noncultured cells). (**D–J**) Representative dot plots (side scatter vs BCECF/AM fluorescence) of BM cells from noninjected mice (**D**), mice injected with noncultured cells (**E**), mice injected with cells cultured without NAM (**F**), and mice injected with cells cultured with NAM (**G**). (**F–J**) BCECF/AM-positive cells were gated and analyzed for BCECF/AM (x axis) and CD34 (y axis). The upper and lower right quadrants represent homing of total human cells, and the upper right quadrant represents homing of human $CD34^+$ cells. (**K**) BCECF/AM-labeled noncultured cells (20×10^6) (**a**) and PKH-labeled NAM-cultured cells (20×10^6) (**b**) were transplanted separately or cotransplanted (**a+b**) in the same mouse (n = 10/experimental group). Twenty four hours later, BCECF/AM-positive cells (**a**), PKH-positive cells (**b**), BCECF/AM-positive and PKH-negative or PKH-positive and BCECF/AM-negative cells (**a+b**) were gated and quantified by flow cytometry as described ($*p < 0.04$ vs noncultured cells).

Cellular target of NAM on cultured $CD34^+$ cells

NAM was reported as a noncompetitive inhibitor of SIRTI [19], the class III HDAC. Inhibition of SIRT1 deacetylase activity by NAM in our cultures was tested by Western blotting with an antibody specific for acetylated Ku70, a deacetylation target of SIRT1 [41]. Figure 6A shows that Ku70 was highly acetylated in cells cultured with NAM, while the level of acetylation was substantially lower in cells cultured with cytokines only, indicating inhibition of SIRT1 deacetylase activity by NAM in our cultures.

To study the causal role of SIRT1, $CD34^+$ cultures were treated with EX-527, a selective inhibitor of SIRT1 that does not inhibit class I and II HDAC or other sirtuin deacetylase family members (IC_{50} values are 98; 19,600; 48,700; >100,000, and >100,000 nM for SIRT1, SIRT2, SIRT3, HDAC, and NADase, respectively) [22,23]. Similar to NAM, but at substantially lower concentrations, EX-527 increased the fraction of $CD34^+CD38^-$ cells and decreased the fraction of more differentiated, monocytic (CD14), dendritic (CD11c), and common myeloid (CD11b) cells (Fig. 6B, C). Both molecules also similarly attenuated the accelerated differentiation imposed by IL-3, as demonstrated by the increase in number of CFUc in 5-week cultures compared to cytokine-treated cultures (Fig. 6D).

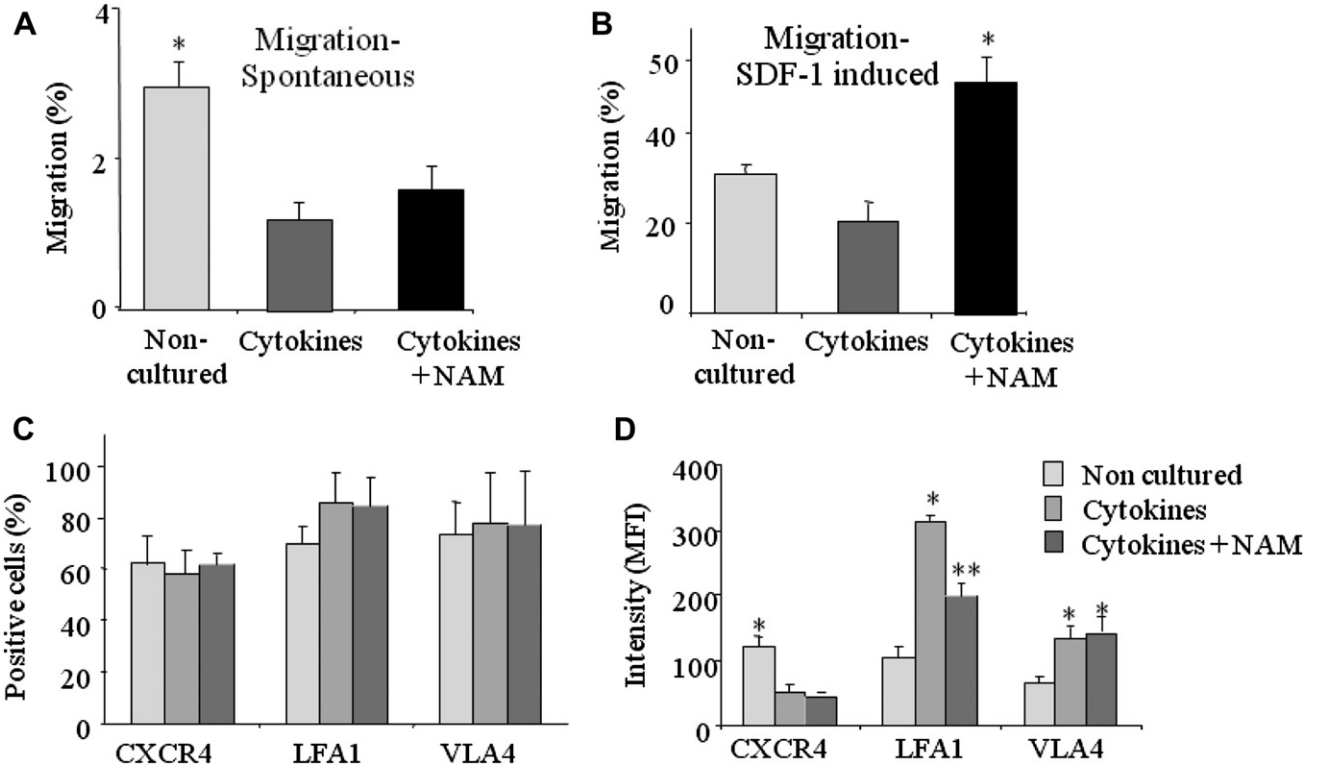

Figure 5. Dependency of migration and homing on CXCR4. Spontaneous (*$p < 0.03$ vs cultured cells) (**A**) and SDF-1 (100 ng/mL) induced Transwell migration (**B**) of $CD34^+$ cells either before or after 3-week culture are shown (*$p < 0.05$ vs cytokines and noncultured). $CD34^+$ cells, either before or after culture with or without NAM, were stained with antibodies to CXCR4, very late antigen 4 (VLA4), lymphocyte function–associated antigen 1 (LFA1), and flow cytometry analyzed. The percentages of positive cells (**C**) and the mean fluorescence intensity (MFI) (CXCR4: *$p < 0.02$ vs cultured cells, VLA4: *$p < 0.01$; **$p < 0.02$, LFA1: **$p < 0.02$ vs noncultured cells) (**D**) are shown. Each bar represents the mean ± standard error of four independent experiments.

To find out whether inhibition of ADP-ribosylation might also be mechanistically relevant to the effect of NAM, we used positional NAM isomers [26] and NAM-related molecules possessing [25] and lacking [24] the inhibitory activity over NAD(+)-dependent ADP-ribosyltransferase, NAM structurally unrelated inhibitors of ADP ribosylation including mono-ADP-ribosyltransferases [29] and poly-ADP-ribose polymerases [28] -ADP-ribosyltransferases and a CD38 antagonist [27] (Table 1). All of these molecules failed to enrich $CD34^+CD38^-$ and to decrease the fraction of differentiated $CD14^+$ cells, indicating that the effect of NAM on cultured $CD34^+$ cells was not mechanistically mediated by the inhibition of ADP-ribosylation. Taken together, these results imply that the NAM effect on CB-derived cultured $CD34^+$ cells is mediated by the inhibition of SIRT1.

Discussion

Here we show that NAM, a potent inhibitor of enzymes that require NAD^+ for their activity such as ribosylase enzymes [9–11] and class III HDAC [12], modulates in vitro expansion and differentiation and in vivo engraftability of ex vivo–cultured, CB-derived $CD34^+$ cells.

Treatment of $CD34^+$ cells with NAM together with cytokines resulted in about a 400-fold increase in total cellularity, a 80-fold increase in $CD34^+$ cells, and a 60-fold increase in clonogenic activity over input numbers that were not substantially different from cultures treated with cytokines only. However, in the cultures treated with NAM, the number of $CD34^+CD38^-$ cells was substantially increased compared to NAM-nontreated cultures. Furthermore, the number of differentiated cells was significantly reduced, the cycling of $CD34^+$ cells was delayed, and responsiveness to differentiation stimuli was attenuated. In vitro, the chemotactic migration toward SDF-1 was improved and, in parallel, the in vivo homing of NAM-treated cells to murine BM was increased by threefold compared to cultures without NAM. Importantly, the incidence of short-term SRC resulted in a 7.6-fold increase over cultures without NAM and 9-fold over culture input number of short-term SRC (noncultured cells). Finally, the advantage of NAM-cultured cells was evident when co-transplanted with noncultured competitor cells. Longer experiments will be required to demonstrate effects on long-term SRCs.

Phenotype analysis of cultures treated with NAM showed an increase in $CD34^+$ cell numbers during long-term cultures, which was primarily attributed to phenotypic stability of cycling $CD34^+CD38^-$ cells. In parallel, cells cultured with NAM displayed increased long-term

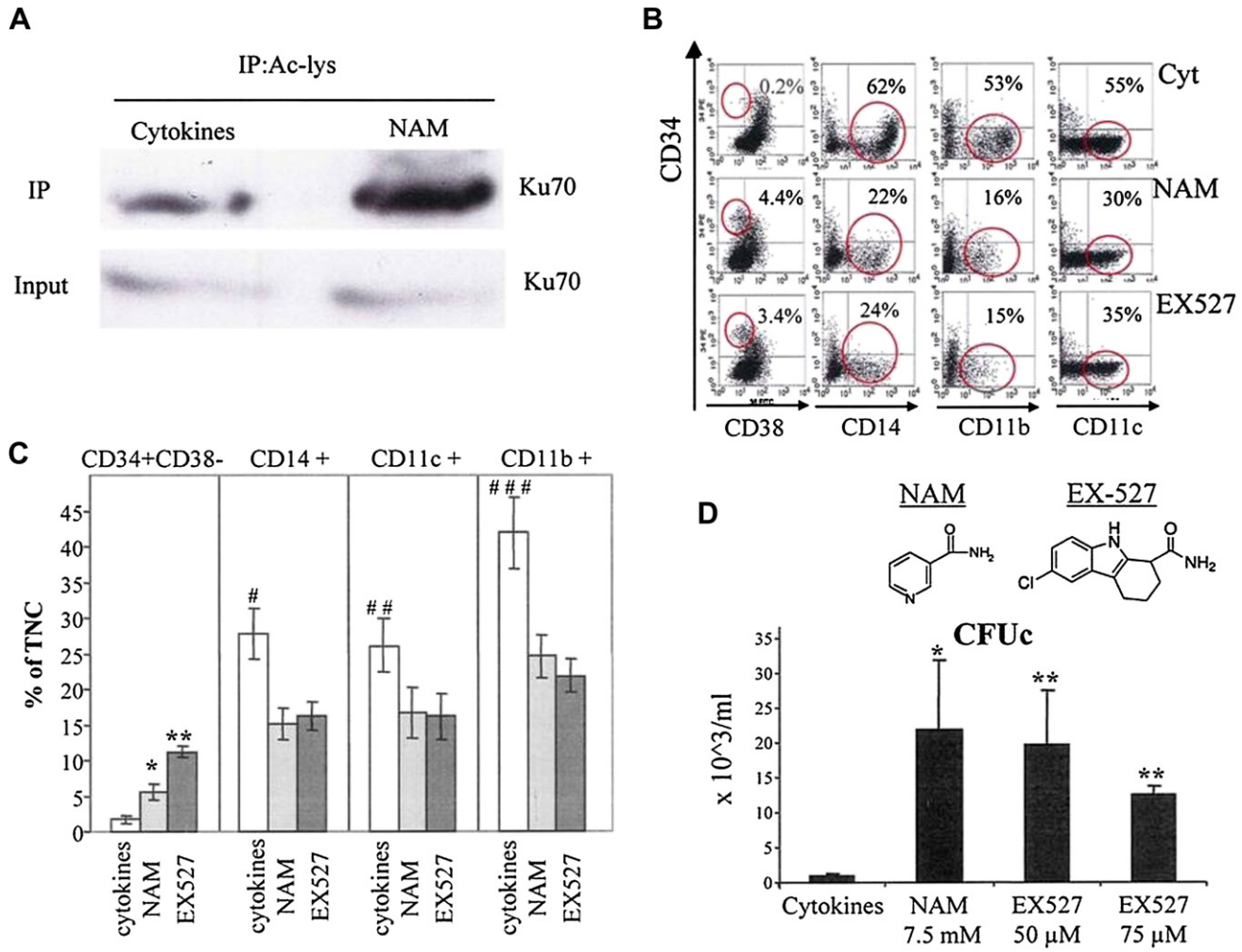

Figure 6. The cellular target of NAM in CD34$^+$ cell cultures. (**A**) CD34$^+$ cells were cultured for 3 weeks with NAM (5 mM) or without NAM. Western blotting analysis of preimmunoprecipitation (input) and immunoprecipitated (IP) samples (-Ac-K) were performed with an anti-Ku70 antibody, as described in Material and Methods. (**B, C**) CD34$^+$ cells were cultured with cytokines, cytokines + NAM (5 mM) or cytokines and EX527 (25–50 µM). After 3 weeks, cells were dually stained with antibodies to human CD34 and with antibodies to human differentiation antigens as indicated. Representative flow cytometry analysis dot plots (**B**) and percentages of CD34$^+$CD38$^-$ cells and of cells positive for differentiation Ags (**C**) (calculated from the upper and lower right quadrants) are shown (n = 3; *$p < 0.01$ NAM vs cytokines; **$p < 0.004$ EX527 vs cytokines and $^{\#}p < 0.02$; $^{\#\#}p < 0.03$; $^{\#\#\#}p < 0.01$ cytokines vs NAM and EX527). (**D**) CD34$^+$ cells were cultured with IL-3, as previously described, and treated without or with NAM and EX527 as indicated. Number of CFUc/mL culture, 4 weeks post seeding, are shown (n = 3; *$p < 0.05$; **$p < 0.05$ vs cytokines).

expansion potential of colony-forming cells. The relevance of these findings to the engraftability of NAM-cultured cells is difficult to interpret because it has already been shown that there is a dissociation between hematopoietic activity of expanded cells in vitro, such as long-term culture initiating cell, and their SCID-repopulating potential [2].

Furthermore, the CD34$^+$CD38$^-$ phenotype has been shown to be an unpredictable surrogate marker for culture expansion of in vivo repopulating cells [42], such as the phenotype is dissociated from their in vivo repopulating ability [43]. However, it was shown that SRCs following in vitro expansion reside exclusively within the CD34$^+$CD38$^-$ cell compartment, although their repopulating efficacy is significantly lower than that of noncultured cells displaying a similar phenotype [44]. The engraftment defect was attributed to cytokine-induced accelerated cycling of the cells in culture [5]. Hematopoietic progenitors usually cycle slower than their more differentiated progeny. Alteration of the rate of cell cycle transition, such as by deactivation of the G_1 check-point regulator and the cyclin-dependent kinase inhibitor p21, has been reported to accelerate HPC expansion and cause a defect in their engraftment [45]. We showed that CD34$^+$ cells cultured with NAM displayed an initial slower proliferation rate than their counterparts cultured without NAM. This was most pronounced in the putative HPC compartment defined as CD34$^+$CD38$^-$ cells, along with an increase in their numbers in the presence of NAM. Our results suggest that slower proliferation rates caused by NAM impacted positively on the preservation of the

Table 1. Screening for molecules with NAM-like activity on $CD34^+$ cell cultures

Tested compounds		Concentrations (mM)	ADPR inhibitory activity (published data)	NAM-like activity
NAM positional isomers	Isonicotinamide	1–10	+	–
	Picolinamide	1–10	+	–
NAM-related compounds	N-methylnicotinamide	1–10	+	–
	Nicotinic acid	1–10	–	–
NAM-nonrelated ADPR inhibitors	Meta-iodobenzylguanidine	2–1	+	–
	3-aminobenzamide	20–500	+	–
CD38 cADPR antagonist)	8- amino-cADPR	10–500	–	–
NAD		10–100	–	–

ADP ribosylation (ADPR), as indicated (N-methylnicotinamide was purchased from ABCR (Karlsruhe, Germany), 8-amino-cADPR from Invitrogen Corporation (Carlsbad, CA, USA), while the others were from Sigma Aldrich). Inhibition of differentiation (NAM-like activity) by the tested compounds was determined based on percentages of $CD34^+CD38^-$ and $CD14^+$ cells in 2- or 3-week cultures, relative to their percentages in cytokines only and NAM- treated cultures.

premature phenotype of cycling progenitor cells, as well as their functionality.

Engraftment is a multistep process involving directed migration of the inoculated cells, homing to the BM, retention within the BM niche, followed by self-renewal and differentiation [46]. One of the major causes of deficient engraftment of $CD34^+$ cells cultured with cytokines is ineffective homing to the murine BM as compared to fresh $CD34^+$ cells [3,6,47]. The homing deficiency acquired in culture explains, at least in part, the discrepancy between in vitro functional HPC assays and in vivo SRC activity. The superior engraftment obtained after culture of $CD34^+$ cells with NAM prompted us to evaluate whether these cells homed to the BM with higher efficacy than cells cultured with cytokine only. In our experiments, SCID mice were grafted with similar numbers of noncultured and cultured cells. Despite higher numbers of $CD34^+$ cells used for transplant after culture, homing of $CD34^+$ cells to the BM was increased by twofold in mice inoculated with cytokine-cultured cells and sixfold after treatment with NAM over homing of noncultured $CD34^+$ cells. Thus, NAM increases the homing potential of ex vivo–expanded $CD34^+$ cells. Interestingly, the effect of NAM on homing of cytokine-cultured cells required continuous exposure, as short-term treatment (1–48 hours) before transplantation was ineffective (data not shown). Hence, reversal of acquired deficiencies in cultured $CD34^+$ cells caused by NAM occurred through modulation of cytokine signaling in early stages of expansion.

The homing defect of cultured HPC was ascribed to a sustained increase in adhesion receptor expression along with a decrease in CXCR4 resulting in nonspecific binding of cultured cells to extramedullary endothelial surfaces [48]. Consistently, we found a substantial increase in the levels of the adhesion molecules expression, including very late antigen 4 and lymphocyte function–associated antigen 1, and a decrease in CXCR4 mean fluorescent intensity on cultured $CD34^+$ cells, irrespective of the presence of NAM. Despite that, $CD34^+$ cells cultured with NAM displayed increased migratory activity over cytokine-cultured cells, which is likely associated with superior homing and engraftment, suggesting modulation of CXCR4 downstream signaling by NAM.

NAM has been shown as a noncompetitive inhibitor of SIRT1 deacetylase [13]. In lower eukaryotes, Sir2 (the mammalian SIRT1 homolog) has been strongly implicated in the modulation of replicative life span and promotion of longevity in response to stress, while NAM strongly inhibits silencing and shortens replicative life span [13]. In contrast to its function in lower eukaryotes, SIRT1 function in higher eukaryotes is still debatable [16]. In higher eukaryotes, SIRT1 binds to and deacetylates a number of important transcription factors, such as nuclear factor–κB, Ku70, peroxisome proliferator-activated receptor–γ, peroxisome proliferator-activated receptor–α, peroxisome proliferator-activated receptor–γ coactivator 1 α, the FOXOs family of transcription factors, and others, thus linking SIRT1 activity to oxidative stress, cell survival, tumorigenesis, metabolism, and cell differentiation [49]. In neural progenitor cells, SIRT1 activator, resveratrol, was shown to suppress proliferation and directs their differentiation toward the astroglial lineage, thus mimicking the effect of oxidative conditions. This effect was blocked by small interfering RNAs against SIRT1 messenger RNA [49]. NAM, through inhibition of SIRT1, was shown to inhibit neural cells differentiation. In addition, NAM was shown to inhibit all-trans retinoic acid–induced differentiation of neuroblastoma cells, while class I and II HDAC inhibitors had no effect. Thus, SIRT1 was suggested as a novel regulator of neuronal differentiation [50]. In normal human keratinocytes, NAM was shown to inhibit the expression of keratinocyte differentiation markers, whereas the SIRT1 activator, resveratrol, to enhance the expression of differentiation markers. Similar results were obtained in keratinocytes manipulated to overexpress or underexpress SIRT1. It was concluded that SIRT1 functions in normal human keratinocytes to inhibit proliferation and to promote differentiation [51]. In contrast to SIRT1 effect on the differentiation of neural and keratinocyte progenitor cells, its overexpression in muscle and fat cells was shown

to inhibit differentiation whilst NAM greatly stimulated differentiation [52,53]. Therefore, the conflicting reports in higher eukaryotes demonstrating promotion or inhibition of differentiation depended on cell or tissue type [16].

Here we show that, similar to NAM, EX-527, a specific SIRT1 inhibitor [23], reduced significantly the expression of granulomonocytic (CD11b, CD11C, and CD14) markers, increased the fraction of $CD34^+CD38^-$ cells, and inhibited the differentiation forced by IL-3 on CB-derived, $CD34^+$ cell cultures. NAM positional isomers [26], NAM-related molecules [25], or NAM-nonrelated poly-ADP-ribose polymerases [28] and mono-ADP-ribosyltransferases [29] inhibitors did not inhibit differentiation, suggesting that the effect of NAM on cultured $CD34^+$ cells is probably through regulation of SIRT1 activity. Although Ex-527 has been reported to be a selective inhibitor of SIRT1, NAM inhibits most sirtuins. Our preliminary results suggest that although inhibition of SIRT2 by its specific inhibitor, AGK2, attenuates differentiation, its effect is less significant than that obtained with EX527 or NAM (data not shown). Current studies are designed to appraise the role of SIRT1 and to define the signaling pathways upstream and downstream of SIRT1 and characterize the role that each plays in the regulation of self-renewal and differentiation of CB-derived HPCs.

Several strategies have been reported to modulate the fate of HPCs cultured with different combinations of cytokines, including the immobilized form of the Notch ligand δ-1 [54], copper chelators [31,33,55], Wnt3a [56], prostaglandin E2 [57], enforced expression of the HOXB4 transcription factor [58], and coculture of HPCs with supportive stromal cells [59] . Even though all of these strategies promoted expansion in ex vivo cultures, each distinctly contributes and advances our understanding of the biology of HPCs and the pathways involved in the regulation of self-renewal and differentiation. However, the successful development of epigenetic strategies capable of inducing human HPC expansion ex vivo, while preserving their in vivo function after transplantation, remains a challenge. Several of these strategies are presently being evaluated in the clinic [54,55,59]; however, the optimal expansion conditions are still not known.

Conclusions

The results of the present study uncover SIRT1, class III NAD^+-dependent-histone-deacetylase, as a novel pathway involved in regulation of self-renewal and differentiation, as well as additional fundamental functions of CB-derived HPCs, such as directed migration, homing to the BM niche, and engraftment.

This novel expansion strategy based on downregulation of SIRT1 by NAM is currently being evaluated in a pilot study for CB transplantation in patients with hematological malignancies (ClinicalTrials.gov Identifier: NCT01221857).

Conflict of Interest Disclosure

The following authors may have a financial interest in the present work: Drs T. Peled, Shoham, Aschengrau, Yackoubov, Frei, and Rosenheimer G are employed by Gamida Cell Ltd., and Drs. T. Peled, Shoham, and Rosenheimer G have ownership interest. Drs. Nagler and A. Peled are consultants for Gamida Cell Ltd. For the remaining authors, no financial interest/relationships with financial interest relating to the topic of this article have been declared.

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Author contributions

Tony Peled: Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript; Hadas Shoham: Performed research, collection and/or assembly of data, data analysis and interpretation; Gabi Frei: Performed research, collection and/or assembly of data, data analysis and interpretation; Dorit Aschengrau: Performed research, collection and/or assembly of data, data analysis and interpretation; Dima Yackoubov: Performed research, collection and/or assembly of data, data analysis and interpretation; Noga Rosenheimer G: Performed research, data analysis and interpretation; Haim Y. Cohen: Data analysis and interpretation; Batya Lerrer: Performed research, Data analysis and interpretation; Arnon Nagler: Data analysis and interpretation; Eitan Fibach: Data analysis and interpretation, manuscript writing; Amnon Peled: Data analysis and interpretation, manuscript writing.

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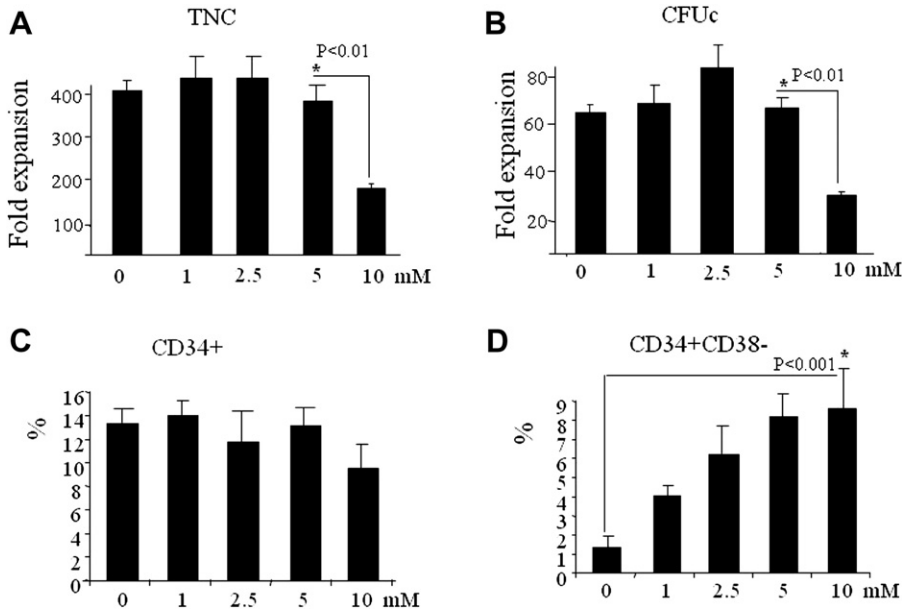

Supplementary Figure E1. The effect of increasing concentrations of NAM on cultured $CD34^+$ cells. CB–derived purified $CD34^+$ cells were cultured for 3 weeks with cytokines or with cytokines and increasing concentrations of NAM as indicated. The culture, analysis procedures and calculations are described in Materials and Methods. Each bar represents the mean ± standard error of three independent experiments. Results show the fold-expansion TNC (**A**) and of CFUc (**B**). Percentages in culture of $CD34^+$ and $CD34^+CD38^-$ cells are shown in (**C**) and (**D**), respectively.

	Percentage in culture		
	NAM (Mm)		
	0	2.5	5
CD14/CD45	20.71 ± 1.46	8.68 ± 1.46 $p = 0.035$	8.78 ± 1.99 $p = 0.028$
CD15/CD45	67.88 ± 3.33	65.06 ± 3.23	65.57 ± 3.67
CD15/CD34	7.81 ± 1.69	3.06 ± 1.01 $p = 0.048$	3.67 ± 1.06 $p = 0.044$
CD11b/CD45	46.2 ± 2.92	22.49 ± 2.46 $p = 0.001$	19.25 ± 0.94 $p = 0.001$
CD11c/CD45	20.17 ± 2.30	12.87 ± 1.68 $p = 0.033$	8.19 ± 1.17 $p = 0.002$
CD3/CD45	1<	1<	1<
CD19/CD45	1<	1<	1<
CD56/CD45	1<	1<	1<

Supplementary Figure E2. Phenotype characterization of lineage-differentiated cells in cultures treated with or without NAM. CB-derived purified $CD34^+$ cells were cultured with cytokines or with cytokines and 2.5 or 5 mM NAM. After 3 weeks in culture, cells were dually stained with antibodies to human CD45 or CD34 and with antibodies to human differentiation antigens as indicated. Percentages of dual-positive cells ± standard error are shown (n = 4).