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Bone marrow niche-inspired, multi-phase expansion of megakaryocytic progenitors with high polyploidization potential

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Abstract

Background—Megakaryopoiesis encompasses hematopoietic stem and progenitor cell (HSPC) commitment to the megakaryocytic cell (Mk) lineage, expansion of Mk progenitors and mature Mks, polyploidization, and platelet release. pH and pO₂ increase from the endosteum to sinuses, and different cytokines are important for various stages of differentiation. We hypothesized that mimicking the changing conditions during Mk differentiation in the bone marrow would facilitate expansion of progenitors that could generate many high-ploidy Mks.

Methods—CD34⁺ HSPCs were cultured at pH 7.2 and 5% O₂ with stem cell factor (SCF), thrombopoietin (Tpo), and all combinations of Interleukin (IL)-3, IL-6, IL-11, and Flt-3 ligand to promote Mk progenitor expansion. Cells cultured with selected cytokines were shifted to pH 7.4 and 20% O₂ to generate mature Mks, and treated with nicotinamide to enhance polyploidization.

Results—Using Tpo+SCF+IL-3+IL-11, we obtained 3.5 CD34⁺CD41⁺ Mk progenitors per input HSPC, while increasing purity from 1% to 17%. Cytokine cocktails with IL-3 yielded more progenitors and mature Mks, although the purities were lower. Mk production was much greater at higher pH and pO₂. Although fewer progenitors were present, shifting to 20% O₂/pH 7.4 at day 5 (versus days 7 or 9) yielded the greatest mature Mk production, 14 per input HSPC. Nicotinamide more than doubled the percentage of high-ploidy Mks to 40%.

Discussion—We obtained extensive Mk progenitor expansion, while ensuring that the progenitors could produce high-ploidy Mks. We anticipate that subsequent optimization of cytokines for mature Mk production and delayed nicotinamide addition will greatly increase high-ploidy Mk production.

Keywords

hematopoietic stem cells; megakaryocytic cells; nicotinamide; megakaryocyte progenitor cells

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Disclosures of Interest

The authors declare no competing financial interests.

Introduction

Platelets, which are necessary for clotting and hemostasis, are derived from hematopoietic stem cells through a multi-step process [1]. First, CD34⁺ stem cells commit to the megakaryocytic cell (Mk) lineage and co-express the surface marker CD41. Each CD34⁺CD41⁺ Mk progenitor can give rise to multiple mature Mks. Maturing Mks lose expression of CD34 and acquire expression of CD42b. Mature Mks subsequently undergo endomitosis, a variation of the cell cycle whereby Mks duplicate their DNA without completing cytokinesis, thereby creating cells that have DNA content of 4N, 8N, 16N, etc. [2]. Higher Mk ploidy, or DNA content, has been correlated with greater platelet production [3]. Ultimately, high-ploidy Mks form long, branched cytoplasmic extensions, called proplatelets, from which platelets are released.

Thrombocytopenia, a deficiency in blood platelet counts, can be caused by various hematologic malignancies, and is often onset as a side effect of chemotherapy [4]. Prophylactic platelet transfusions are often prescribed for leukemia patients who have a severe risk of bleeding or are scheduled to undergo surgical procedures [5]. Although widely used, there are problems associated with platelet collection and transfusions. Collecting enough platelets for a single transfusion requires either expensive apheresis equipment or the pooling of platelets isolated from platelet-rich-plasma or buffy coats from 4 to 8 different donors [6,7]. Platelets are damaged by the cold and are stored at 20–24°C, so that bacterial contamination during blood collection is a serious concern [6,8,9].

The production of platelets from hematopoietic stem and progenitor cells (HSPCs) in culture using Good Manufacturing Practices (GMP) has the potential to augment the platelet supply, while also decreasing the likelihood of contamination and adverse immune responses. Many investigators have produced Mks in culture using CD34⁺ HSPCs derived from umbilical cord blood (CB) or mobilized peripheral blood (mPB) [3,10–19]. However, conditions that result in the greatest Mk production often yield lower Mk purity and/or ploidy.

Because the process by which HSPCs differentiate into high-ploidy Mks consists of multiple, sequentially-dependent steps, we hypothesize that production of high-ploidy Mks could be greatly enhanced by dividing the process into three separate phases – with environmental conditions and growth factors separately optimized for Mk commitment and progenitor expansion, mature Mk production, and Mk polyploidization. The arrangement of diverse hematopoietic cell lineages within the bone marrow (BM) suggests that their development is differentially orchestrated by their microenvironment. As a result of metabolic activity within the intravascular space, substantial gradients in pH and oxygen partial pressure (pO₂) are established between the bone surface and the BM sinuses [20]. Hematopoietic stem cells are widely accepted to reside in niches at the bone surface, far from the sinuses [21,22]. Granulocytic (G) precursors remain deep within the BM until they reach the metamyelocyte stage and become motile [23,24], and this is consistent with greater mature G cell production in cultures carried out at pH 7.1–7.2 under an atmosphere containing 5% O₂ [25]. In contrast, production of mature erythroid (E) cells and Mks occurs adjacent to the sinus wall [23,24]. Consistent with this observation, the production of mature E cells and Mks is increased under 20% O₂ [10,26,27] and the differentiation of Mks and E cells is more rapid at higher pH [28,29].

Our objective in the present study was to focus on the first phase of Mk commitment and progenitor expansion, but we also ensured that the expanded progenitors retained the potential to produce large numbers of mature Mks and to yield high-ploidy Mks. Progenitors produced under the conditions identified in this study will be used as input cells in subsequent studies for the optimization of mature Mk production and polyploidization.

Thrombopoietin (Tpo) and stem cell factor (SCF) were included at a saturating concentration of 100 ng/mL because they have been used extensively for Mk progenitor expansion [10–16,19,29–34]. We evaluated all combinations of interleukin (IL)-3, IL-6, IL-11, and Flt3-ligand (FL) at 10 ng/mL using a 2⁴ factorial design that facilitates analysis of the effects of individual cytokines, as well as cytokine-cytokine interactions. IL-3, IL-6, IL-11, and/or FL are often used with Tpo and/or SCF in Mk cultures, and are typically used at 10 ng/mL [12–16,32,33]. IL-3 has consistently been shown to increase HSPC expansion, but there are conflicting reports regarding its effectiveness for increasing CD41 expression and Mk colony forming unit (CFU-Mk) potential [12–15,31,32]. IL-6 and IL-11 increase Mk progenitor expansion in CD34⁺ cell cultures [12,15,16,33,34]. FL increases cell expansion in Mk cultures, but also decreases the fraction of CD41⁺ cells [12,33]. Because we previously showed that subvascular pH and pO₂ promote Mk progenitor expansion and maintenance [10,27,29], the cultures were carried out at pH 7.2 and 5% O₂.

Based on the factorial design analysis, we selected two cytokine cocktails that yielded high levels of Mk progenitor production and purity for further optimization via modulation of the IL-3 concentration. We also evaluated the effect of heparin, which has been shown to synergize with Tpo and IL-11 to increase CFU-Mk production [35]. Mk progenitors were removed from these cultures after 5, 7, and 9 days to evaluate their ability to form mature Mks that undergo polyploidization. These second phase cultures were carried out at 20% O₂ and pH 7.4 to enhance Mk production and ploidy [10,29]. Replicate cultures were also supplemented with nicotinamide (NIC), which our group has previously shown to greatly enhance Mk ploidy under a wide range of Mk-promoting conditions [36,37]. Using this approach, we obtained extensive progenitor expansion, while retaining the potential for high ploidy. Our results provide the basis for further optimization via modulation of the conditions used for Mk production and polyploidization.

Materials and Methods

Unless otherwise noted, all chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) and all antibodies were obtained from BD Biosciences (San Jose, CA, USA).

Cell Culture for Primary (1°) Cytokine Evaluation

Cultures were initiated in T-flasks with previously frozen mPB CD34-selected cells (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) seeded at 50,000 cells/mL in serum-free X-VIVO 20 media (Lonza, Walkersville, MD, USA) adjusted to pH 7.2 by addition of less than 0.05% of 12.1 M hydrochloric acid. Cultures were maintained at 100,000–350,000 cells/mL via dilution feeding in a fully-humidified chamber at 37°C, 5% CO₂, and 5% O₂ for 11 days. Care was taken to minimize the amount of time the cultures were outside the chamber for sampling. At day 0, cells were cultured with one of 16 cytokine cocktails comprising all combinations of IL-3, IL-6, IL-11, and FL (all from Peprotech, Rocky Hill, NJ, USA) at 0 or 10 ng/mL, as outlined in Table 1. All cocktails contained 100 ng/mL Tpo (Peprotech) and 100 ng/mL SCF (donated by Amgen, Thousand Oaks, CA, USA).

Cell Culture for Secondary (2°) Cytokine Evaluation

Cultures were initiated and maintained as outlined above for the 1° cytokine evaluation. At day 0, cells were cultured with one of six cytokine combinations, based on conditions 6 and 10 (Table 1) from the 1° cytokine evaluation. The conditions evaluated all included 100 ng/mL Tpo, 100 ng/mL SCF, and 10 ng/mL IL-11 in addition to: 10 ng/mL IL-3 (6-I), 2.5 ng/mL IL-3 (6-II), 25 U/mL heparin (Pfizer, New York, NY, USA) + 10 ng/mL IL-3 (6-III; data not shown), 10 ng/mL IL-6 (10-I), 10 ng/mL IL-6 + 2.5 ng/mL IL-3 (10-II), or 25 U/

mL heparin + 10 ng/mL IL-6 (10-III; data not shown). At days 5, 7, or 9, cells were removed from each of the conditions, centrifuged to remove spent media, washed, and resuspended at 100,000 cells/mL in X-VIVO 20 media containing the same cytokines, but adjusted to pH 7.4 by addition of less than 0.05% of 5 M sodium hydroxide. The resuspended cells were seeded into fresh T-flasks and maintained in a fully-humidified chamber at 37°C, 5% CO₂, and 20% O₂ for an additional eight days if shifted at days 5 or 7 or an additional six days if shifted at day 9 (day 9 shift data not shown). When cells were shifted to pH 7.4 and 20% O₂, half of the cells in each of the conditions were also treated with 6.25 mM NIC.

Flow Cytometry during 1° Cytokine Evaluation

Twenty-five thousand cells were removed from each condition, washed with phosphate-buffered saline containing 2 mM EDTA and 0.5% bovine serum albumin (PEB) twice, incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD41 (Beckman Coulter, Fullerton, CA, USA), phycoerythrin (PE)Cy5-conjugated anti-CD34, PE-conjugated anti-CD14, and allophycocyanin (APC)-conjugated anti-GlyA for 30 min at 4°C, washed twice with PEB, and incubated with 5 µg/mL 4',6-diamidino-2-phenylindole (DAPI) for 15 min at room temperature. Samples were acquired using an LSRII flow cytometer (BD Biosciences) and analyzed with FACSDiva software (BD Biosciences).

Flow Cytometry during 2° Cytokine Evaluation

Cells were removed from each of the conditions maintained at 5% O₂/pH 7.2 and stained for surface markers as described above. From day 5 until the end of the culture period, 25,000 cells were removed from each of the conditions to assay for Mk surface markers. These samples were washed twice with PEB, incubated with FITC-conjugated anti-CD41, PE-Cy5-conjugated anti-CD34, and PE-conjugated anti-CD42b for 30 min at 4°C, washed twice with PEB, and incubated with 5 µg/mL DAPI for 15 min at room temperature. An additional 50,000 cells were removed from each condition and assayed for Mk ploidy as described [36].

Colony Assays

CFU-Mks were detected by seeding cells from 5% O₂ cultures at days 5 (5000 cells/mL), 7 (7000 cells/mL), and 9 (9000 cells/mL) into semi-solid MegaCult medium (StemCell Technologies, Vancouver, BC, Canada) with 10 ng/mL IL-3 and 50 ng/mL Tpo. After being maintained for 12 days at 37°C, 5% CO₂, and 5% O₂, colonies were fixed and stained with a streptavidin-horseradish peroxidase system (Biomedex, Foster City, CA, USA) [10] according to the manufacturer's protocol. CFU-Mk colonies were also scored for size and characterized as either small (containing 3–20 cells), medium (21–49 cells), or large (≥ 50 cells).

Formation of E, G, monocytic (M), and GM colony forming units (CFU-Mix) was performed by seeding cells from 5% O₂ cultures at days 5 (1500 cells/mL), 7 (2000 cells/mL), and 9 (2500 cells/mL) into semi-solid MethoCult medium (StemCell Technologies) with 20 ng/mL GM-colony-stimulating factor (GM-CSF; Peprotech), 20 ng/mL G-CSF (Amgen), 20 ng/mL IL-6, 3 U/mL erythropoietin (Epo; Amgen), 50 ng/mL SCF, and 20 ng/mL IL-3. After being maintained for 12 days at 37°C, 5% CO₂, and 5% O₂, colonies were scored for CFU-Mix formation according to the manufacturer's protocol.

Statistical Analysis

Results are expressed as mean ± standard error (SEM). Statistical significance was determined using either a paired two-tailed t-test for a particular day in culture or a Kruskal-Wallis test for particular time intervals or for groups of conditions at a particular day; use of

the Kruskal-Wallis test is indicated in the text. p-values for significant effects are noted. Minitab 15 software (State College, PA, USA) was used for ANOVA to calculate coefficients of correlation and identify which individual cytokines or combination of cytokines yielded statistically significant ($p < 0.05$) effects for each parameter in the factorial design.

Results

Mk Progenitor Production using 1° Cytokine Cocktails

The effects of IL-3, IL-6, IL-11, and FL on CD34⁺CD41⁺ cell production in cultures containing SCF and Tpo were evaluated using a 2⁴ full factorial design (Table 1). A significance analysis was performed on the expansion and surface marker expression data from three biological replicates (Figure 1). IL-3 had, by far, the most profound positive effect on total cell expansion (Figure 1A). Adding IL-3 alone to cultures with SCF and Tpo (condition 8) gave equivalent expansion as that for adding IL-6, IL-11, and FL (condition 9) (supplementary Figure S1A). IL-6 and FL also significantly increased cell expansion, but the effect was much smaller (Figure 1A). IL-3, and to a lesser extent IL-6, significantly accelerated the loss of CD34 expression (Figure 1B and data not shown). IL-3 and FL main effects decreased Mk commitment (Figure 1C) and Mk progenitor purity (Figure 1D) to a similar extent. The negative effects of IL-3 (black vs. gray) and FL (open vs. closed symbols) on Mk progenitor purity are also evident in Figure 2A. IL-6 also significantly decreased the percentages of CD41⁺ and CD34⁺CD41⁺ cells, but to a much smaller extent (Figure 1C–D). Conditions that maintained CD34 expression also tended to promote CD41 expression (supplementary Figure S1B–C and data not shown). The beneficial effect of IL-3 on total cell fold expansion more than offset the negative effects on CD41 and CD34 expression, such that IL-3 greatly increased the number of CD34⁺CD41⁺ cells produced per HSPC seeded at day 0 (Figures 1E, 2B). In contrast, FL tended to decrease the number of CD34⁺CD41⁺ cells produced per input HSPC (Figures 1E, 2B). Only the IL-3-IL-6 interaction for CD34 expression reached statistical significance (Figure 1B) among the parameters evaluated in cultures with SCF and Tpo, and none of the cytokines significantly affected viability (Figure 1F).

Although co-expression of CD34 and CD41 is a marker of Mk progenitors, it does not provide any information on their clonogenic potential. Therefore, we seeded cells from all 16 conditions into CFU-Mk assays to determine the capacity of the progenitors in different conditions to give rise to mature Mks. On day 5, cultures without IL-3 (conditions 9–16) tended to contain a greater frequency of Mk progenitors, as well as a greater proportion of progenitors with the capacity to produce large colonies (Figure 3A). A representative large colony formed by cells from condition 10 is shown in Figure 3B. Condition 10 tended to yield the greatest frequency of large-colony CFU-Mks (Figure 3A). Progenitors removed from liquid culture at day 5 gave rise to a far greater proportion of large-colony CFU-Mks than progenitors removed and seeded at days 7 or 9 (data not shown), indicating a rapid decline in clonogenic capacity with time in culture. Cultures were also evaluated for myeloid progenitors. Flow cytometric detection of G, M, and E surface markers was minimal at all time points and the cells exhibited poor clonogenic potential in CFU-Mix assays (data not shown).

Based on the 1° cytokine evaluation, there appears to be a trade-off between Mk progenitor production and purity (Figure 2). This is especially evident regarding the effects of IL-3. We selected conditions 6 (Tpo + SCF + IL-3 + IL-11) and 10 (Tpo + SCF + IL-6 + IL-11) as the basis for further cytokine optimization. Condition 6 tended to yield the greatest CD34⁺CD41⁺ cell production (Figure 2B) and the highest CD34⁺CD41⁺ Mk progenitor purity among the conditions with IL-3 (Figure 2A). Although conditions 14 and 16 tended to

yield higher CD34⁺CD41⁺ cell purity (Figure 2A), condition 10 tended to produce a greater number of CD34⁺CD41⁺ cells per HSPC (Figure 2B), as well as the largest fraction of large-colony CFU-Mks and the greatest total number of CFU-Mks (Figure 3A).

Mk Progenitor Production with 2° Cytokine Cocktails

For the 2° cytokine evaluation, we carried forward conditions 6 (termed 6-I) and 10 (10-I) from the 1° cytokine evaluation. We also investigated the effects of using an intermediate level of IL-3 (2.5 ng/mL; 6-II and 10-II) in an effort to obtain high Mk progenitor production per input HSPC, while maintaining relatively high purity and clonogenic capacity. Finally, we added heparin (25 U/mL) to conditions 6-I (6-III) and 10-I (10-III) to evaluate its impact on Mk progenitor production. Others have used heparin, often together with Tpo and IL-11, to increase CFU-Mk and mature Mk production [35,38].

An intermediate dose of IL-3 yielded an intermediate response in terms of total cell expansion; the percentages of CD34⁺, CD41⁺, and CD34⁺CD41⁺ cells; and the number of CD34⁺CD41⁺ cells produced per HSPC (Figure 4). Reducing the concentration of IL-3 from 10 ng/mL (condition 6-I) to 2.5 ng/mL (6-II) resulted in a 40% decrease in total cell fold expansion by day 11 ($p < 0.01$), while addition of 2.5 ng/mL IL-3 to condition 10-I (10-II) increased total cell expansion by 95% ($p < 0.01$; Figure 4A). Cultures with 10 ng/mL IL-6 and 2.5 ng/mL IL-3 gave intermediate expansion compared to cultures containing 2.5 and 10 ng/mL IL-3 without IL-6, which is consistent with the much smaller effect of IL-6 than IL-3 for cell expansion (Figure 1A). Adding 25 U/mL heparin slightly reduced expansion in cultures without IL-3 (data not shown). At day 9, viability was between 70–90% for all conditions, and tended to be higher in the conditions containing heparin (supplementary Figure S2A and data not shown). By day 11, CD34 expression decreased more rapidly in cultures with IL-3 in a dose-dependent manner ($p < 0.02$; Figure 4B). Similar to what was observed in the 1° cytokine evaluation, conditions that resulted in greater total cell expansion tended to yield lower percentages of CD41⁺ cells (Figure 4C) and CD34⁺CD41⁺ cells (Figure 4D). Heparin tended to decrease CD41 expression in cultures without IL-3 (data not shown). The ranking of conditions for CD34⁺CD41⁺ cell production per input HSPC (Figure 4E) tended to be the same as that for total cell production (Figure 4A). Condition 6-I tended to produce the greatest number of CD34⁺CD41⁺ cells at day 11 with about 3.5 cells per HSPC seeded (Figure 4E). This represents a 175-fold increase in the number of Mk progenitors compared to the CD34⁺CD41⁺ cells present in the HSPC population at day 0.

The cultures contained less than 10% of M plus E progenitors at all time points (supplementary Figure S2B–C), which is consistent with the formation of few M or E colonies in CFU-Mix assays (data not shown). Conditions with IL-6 tended to contain more M (supplementary Figure S2B) and fewer E progenitors (supplementary Figure S2C). Analysis of the 1° cytokine evaluation cultures also showed very low levels of CD34⁺CD15⁺ G progenitors (data not shown).

We evaluated the clonogenic capacity of Mk progenitors from the six 2° cytokine cocktails using CFU-Mk assays. Consistent with the increase in CD34⁺CD41⁺ cells during culture (Figure 4E), the total number of CFU-Mks per input HSPC increased significantly between days 5 and 9 (Figure 5A–C) for all conditions shown ($p < 0.001$; Kruskal-Wallis). However, the increase in CFU-Mks was much less than for CD34⁺CD41⁺ cells and the proportion of large colonies decreased dramatically ($p < 10^{-5}$; Kruskal-Wallis). The cloning efficiency of small-, medium-, and large-colony CFU-Mks decreased between days 5 and 7 ($p < 0.005$; Kruskal-Wallis; Figure 5D–E). Although the overall cloning efficiency was similar at days 7 and 9, the percentages of medium- and large-colony CFU-Mks tended to be much smaller at day 9 (Figure 5E–F). This suggests that the potential of CD34⁺CD41⁺ cells to produce

mature Mks would also decline after day 5 in culture. Therefore, we removed cells at days 5, 7, and 9 to evaluate their potential to form mature Mks and undergo polyploidization.

Mk Production from Progenitors using 2° Cytokine Cocktails

Since Mk colony size and CD34⁺CD41⁺ cell cloning efficiency decreased after day 5 (Figure 5D–F), while CD34⁺CD41⁺ cell numbers continued to increase, we investigated which time point would be best for shifting the cells to higher pH and pO₂ to promote mature Mk production from the progenitors. At days 5, 7, and 9, we removed a portion of the cells from each of the six 2° cytokine evaluation cocktails and reseeded them at 20% O₂/pH 7.4. The remaining cells for all six conditions were maintained at pH 7.2/5% O₂ for the entire culture period as a control.

For all conditions containing IL-3, the shift to higher pO₂ and pH tended to increase total cell fold expansion, regardless of whether the shift occurred at day 5 or 7 (supplementary Figure S3A–B). Total cell production was approximately the same for a given condition no matter which day the shift occurred. Viability tended to decrease more rapidly at 20% O₂ for cultures with IL-3 (supplementary Figure S3C–D). There was little, if any, effect of the shift in pO₂ and pH on the loss of CD34 expression (data not shown). After being switched to 20% O₂/pH 7.4 at day 5, cells in all conditions tended to exhibit a large increase in CD41 expression compared to that in cultures with the same cytokine cocktails maintained at 5% O₂/pH 7.2, and this increase was greatest in cultures without IL-3 ($p < 0.03$ for 10-I; Figure 6A). Cells in all conditions also tended to increase CD41 expression after being switched to 20% O₂/pH 7.4 at day 7 (Figure 6B), but the differences were not as great as when the shift was performed at day 5. Switching to 20% O₂/pH 7.4 at day 9 had little effect on CD41 expression (data not shown). Expression of CD42b was delayed compared to CD41, but switching to 20% O₂/pH 7.4 tended to increase CD42b expression in a similar manner (supplementary Figure S3E–F).

The greatest production of CD41⁺ Mks occurred on day 13 in cultures containing IL-3 that were shifted to higher pO₂ and pH on day 5 ($p < 0.06$; Kruskal-Wallis; Figure 6C). This suggests that greater clonogenic capacity more than offset the smaller number of CD34⁺CD41⁺ cells produced earlier in the culture. When shifted to 20% O₂/pH 7.4 at day 5, conditions 6-I and 10-II gave rise to 13.1 ± 2.1 and 12.6 ± 4.5 CD41⁺ cells, respectively, per input HSPC (Figure 6C), and condition 6-I treated with heparin (6-III) gave rise to 13.7 ± 4.2 CD41⁺ cells per input HSPC (data not shown). Shifting to 20% O₂/pH 7.4 at day 7 resulted in a lower maximum Mk production of 10.7 ± 2.0 CD41⁺ cells (at day 15) per input HSPC for condition 6-I (Figure 6D). All cocktails gave rise to a significantly greater number of Mks upon shifting to higher pH and pO₂ at day 5 ($p < 0.01$; Kruskal-Wallis; Figure 6C). When pO₂ and pH were increased at day 9, fewer than 10 CD41⁺ cells were produced per input HSPC and the benefit of shifting to 20% O₂/pH 7.4 was lost (data not shown).

Mk Polyploidization using 2° Cytokines and NIC

To ensure that the Mks produced retained high-polyploidization potential when they were shifted to 20% O₂/pH 7.4, cells from all 2° cytokine cocktails were also treated with 6.25 mM NIC. NIC greatly reduced total cell fold expansion for all conditions ($p < 0.0006$ for shift at day 5 and $p < 0.007$ for shift at day 7; Kruskal-Wallis; supplementary Figure S4A–B). Consistent with our previous findings [36], the inhibitory effect of NIC on expansion was less extensive for later NIC addition. There was a transient decrease in viability when cells were treated with NIC at day 5 ($p < 0.05$; Kruskal-Wallis), but NIC did not affect viability when it was added at later time points (supplementary Figure S4C–D). NIC tended to increase CD41 (supplementary Figure S4E–F) and CD42b (data not shown) expression late in cultures without IL-3, especially when applied at day 5, but had little effect on CD41

or CD42b expression in cultures containing IL-3. Due to the large decrease in total cell fold expansion with NIC, the number of Mks produced per HSPC in cultures with NIC was much lower than that for cells cultured at 20% O₂ without NIC ($p < 10^{-7}$ for shift at day 5 and $p < 10^{-5}$ for shift at day 7; Kruskal-Wallis; Figure 7A–B). In contrast to lower CD41⁺ cell production for later switch times in cultures without NIC, the maximum CD41⁺ cell production in cultures with NIC was similar for cells switched to 20% O₂/pH 7.4 on all three days (Figure 7A–B and data not shown). NIC greatly increased the percentage of high-ploidy Mks for all conditions, even when added at day 9 ($p < 10^{-12}$; Kruskal-Wallis; Figure 7C–D and data not shown). Representative day 11 ploidy distributions are shown in Figure 8 for conditions 6-II (A–C) and 10-II (D–F). Shifting to 20% O₂/pH 7.4 without NIC moderately increased the percentage of high-ploidy Mks and the maximal ploidy level (Figure 8A–B,D–E). In contrast, NIC greatly increased the percentage of high-ploidy Mks and increased the maximal ploidy to 32N (Figure 8C,F). Also, the ploidy continued to increase until day 13 in cultures with NIC, but decreased after day 11 in cultures without NIC. The maximum production of high-ploidy Mks was similar for cells shifted to higher pO₂ and pH on all three days, but tended to be greatest for the shift at day 7 (Figure 7E–F and data not shown). The condition that gave rise to the largest number of high-ploidy Mks varied with the shift day, but condition 6-I + NIC was consistently among the best. The maximum number of high-ploidy Mks produced was similar for cells treated with or without NIC, but the maximum value tended to be sustained for a longer time with NIC.

Discussion

A full 2⁴ factorial design in the 1^o cytokine evaluation step allowed us to examine the impact of all possible combinations of IL-3, IL-6, IL-11, and FL (together with Tpo and SCF) and determine which individual cytokines or cytokine-cytokine interactions had statistically significant effects on particular Mk parameters. We observed wide ranges of cell expansion and Mk progenitor purity (supplementary Figure S1A, Figure 2). IL-3 greatly increased total cell fold expansion and profoundly reduced CD34 and CD41 expression. The beneficial effect on expansion outweighed the negative effect on CD34 and CD41 expression, such that IL-3 had a large positive effect on CD34⁺CD41⁺ cell production per input HSPC (Figures 1, 2, 4). Similar to our findings, Case *et al.* found, using a fractional factorial design, that IL-3 had the greatest positive effect on increasing CD41⁺ cell production from mPB HSPCs [12]. Drayer *et al.* found that IL-3 increased by 5-fold the number of Mk progenitors produced per mPB HSPC when added to cultures containing Tpo and SCF [15]. Adding IL-6, IL-11, and FL in addition to Tpo + SCF + IL-3 did not substantially increase Mk progenitor production [15], which is consistent with our results (Figure 2B; condition 1 vs. condition 8). FL moderately increased total cell expansion, but decreased CD41 expression to a greater extent (Figures 1A, C and 2, supplementary Figure S1A), so that FL had a net negative effect on Mk progenitor production. Other investigators have also shown that FL increases cell expansion in Mk cultures, but decreases the fraction of CD41⁺ cells [12, 33]. However, the reverse was observed in another study [14]. Neither IL-6 nor IL-11 had a significant positive effect on CD34⁺CD41⁺ cell production or purity (Figure 1D–E). However, IL-11 was present in the cocktails that tended to yield the greatest CD34⁺CD41⁺ cell production (Figure 2B; conditions 6 and 2) and both IL-6 and IL-11 were present in the cocktail that tended to yield the greatest fraction of large-colony CFU-Mks (Figure 3A; condition 10). We previously demonstrated that adding either IL-6 or Tpo to mPB CD34⁺ cell cultures with SCF and IL-3 doubled CFU-Mk production, and that adding both IL-6 and Tpo resulted in a 4-fold increase [34]. IL-11 has been shown to support burst-forming unit (BFU)-Mk and CFU-Mk formation [35, 39], and this is consistent with our findings regarding condition 10 in the 1^o cytokine optimization step. Using an intermediate dose of IL-3 in the 2^o cytokine evaluation gave intermediate responses in terms of expansion and CD34 and CD41 expression (Figure 4), which is consistent with the differences between

conditions with and without IL-3 in the 1° cytokine evaluation (Figure 2, supplementary Figure S1A). Heparin was included in the 2° cytokine evaluation because it was shown to increase large-colony CFU-Mk formation 1.5-fold and CD34⁺CD41⁺ cell production 3-fold in conjunction with Tpo and IL-11 [35]. However, this effect was not observed in our study (data not shown). Since the trends observed in the 1° cytokine evaluation were also present in the 2° cytokine evaluation, there was no change in the best cocktails for CD34⁺CD41⁺ cell purity or production per HSPC.

Using the most effective cocktail from the 2° cytokine evaluation (100 ng/mL Tpo + 100 ng/mL SCF + 10 ng/mL IL-3 + 10 ng/mL IL-11), we produced 3.2 ± 0.3 CD34⁺CD41⁺ Mk progenitors per HSPC by day 9 of culture and 3.5 ± 0.5 by day 11 (Figure 4E). This value is comparable to the best Mk progenitor yields from mPB CD34⁺ cells in serum-free cultures reported by other investigators. Fukushima-Shintani *et al.* produced 3.8 CD34⁺CD41⁺ cells per input CD34⁺ cell in cultures with Tpo plus AKR-501, a Tpo receptor agonist, but only 1.6 CD34⁺CD41⁺ cells with Tpo alone [40]. Lefebvre *et al.* produced 5.2 Mk progenitors per input HPSC in cultures with 100 ng/mL Tpo and 100 ng/mL SCF [11]. Tijssen *et al.* produced on average circa (ca.) 3 Mk progenitors per input HPSC in cultures with 100 ng/mL Tpo and 10 ng/mL IL-1β [41]. However, many groups have reported fewer than 2.5 CD34⁺CD41⁺ cells per input CD34⁺ cell [13, 17, 42–45]. For example, De Bruyn *et al.* reported 1.8 Mk progenitors per input HPSC in cultures with 100 ng/mL Tpo, 100 ng/mL FL, 10 ng/mL IL-6, and 10 ng/mL IL-11 [13]. Consistent with reports by other investigators [11, 16, 33], we saw a substantial decrease in large-colony CFU-Mk potential after a particular point in culture (Figure 5).

Because colony size and cloning efficiency were decreasing, while the number of CD34⁺CD41⁺ cells produced was increasing, we removed cells at days 5, 7, and 9 and seeded them into culture at pH 7.4 and 20% O₂ to see which day would yield the largest number of Mks per input CD34⁺ cell. We found that, while more Mk progenitors were produced in culture by day 9 than day 5, the progenitors at day 5 had a much greater capacity to give rise to mature Mks. Although there was similar total cell production among the three shift days, the percentage of CD41⁺ cells and total Mk production tended to be much greater when the cells were shifted at day 5 (Figure 6). Consistent with our past findings [10,27,29] and with Mk maturation near the BM sinuses *in vivo* [23,24], shifting the cells to 20% O₂ and pH 7.4 on day 5 yielded about twice as many mature Mks as maintaining cells at 5% O₂ and pH 7.2.

We identified a cytokine combination (100 ng/mL Tpo + 100 ng/mL SCF + 10 ng/mL IL-3 + 10 ng/mL IL-11) at 20% O₂/pH 7.4 that generated ca. 14 Mks and 2 high-ploidy Mks per HSPC (Figure 7). Our maximum average yield of 14 Mks per input HPSC compares well to what others have reported using mPB CD34⁺ cells. Lefebvre *et al.* produced 48 Mks per input CD34⁺ cell [11] using mPB CD34⁺ cells from breast cancer patients with daily dilution-feeding and Fukushima-Shintani *et al.* [40] obtained 36 CD41⁺ cells per CD34⁺ cell. This level of Mk production from HSPCs derived from mPB donors is rare, although one donor sample from the present study produced 30 Mks per input HSPC. Most groups report fewer than 7 Mks per mPB CD34⁺ cell [13,17,32,41,42,45–48]. Further, relatively few groups have reported whether the expanded Mks attained high-ploidy. Lefebvre *et al.* reported 3.8 high-ploidy Mks per input CD34⁺ cell in cultures with Tpo and SCF, but these cells represented only 8% of the Mk population [11]. Bertolini *et al.* produced ca. 9 mature Mks per input CD34⁺ cell of which 18% were high-ploidy [44]. Many groups have reported fewer than 1.5 high-ploidy Mks per CD34⁺ cell [13,18,49]. While different cytokine combinations and the day at which cells were shifted from 5% O₂/pH 7.2 to 20% O₂/pH 7.4 had profound effects on total cell fold expansion and CD41 expression (Figure 6 and supplementary Figure S3A–B), NIC greatly decreased expansion and increased ploidy in a

similar manner across all conditions (Figure 7 and supplementary Figure S4A–B). In contrast to previous studies reporting an inhibitory effect of SCF on Mk polyploidization [19,50] and consistent with our recent report [37], Mks in conditions containing both NIC and a high concentration of SCF reached ca. 45% high-ploidy.

Relatively few investigators have divided the process of Mk production from HSPCs into phases using growth factors that optimize distinct aspects of Mk differentiation. The current study is unique in that we have also incorporated the changes in pH and oxygen tension that occur during Mk development in the bone marrow niche. The cytokines used in this study were chosen specifically to promote Mk progenitor expansion and not overall Mk production. Further increases in Mk production will likely be obtained by evaluating different combinations of cytokines for use when cells are shifted to 20% O₂ and pH 7.4 on day 5. Two-phase cultures with different cytokine combinations for Mk progenitor and mature Mk production have been used successfully for CB CD34⁺ cells [19,51]. While NIC is typically most effective when added at day 5 in Tpo-only cultures [36], cells treated at day 9 were still responsive to NIC and became high-ploidy (data not shown). Delayed addition of NIC, relative to the shift in pH/pO₂ and cytokines, is likely to substantially increase the production of high-ploidy Mks, thereby creating a three-phase process to maximize high-ploidy Mk production from mPB CD34⁺ cells. During the third phase of Mk differentiation, Mks could also be cultured at pH 7.6 to increase ploidy [29]. We and other groups have shown that CD34⁺ cells can be effectively cultured in stirred bioreactors with pH and pO₂ control [52–56]. Thus, it is likely that the culture environment in large-scale processes would be regulated more efficiently using probes in bioreactors to constantly monitor and adjust pH and pO₂. Lastly, it will be important to evaluate the proplatelet-forming ability of Mks produced using our multi-phase culture process (e.g., by plating Mks on fibrinogen or von Willebrand factor, which have been shown to promote Mk proplatelet formation [57]) and ultimately to determine whether platelets produced from such a process are functional and provide a viable alternative to standard platelet transfusions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

APC	allophycocyanin
BFU	burst forming unit
BM	bone marrow
CB	umbilical cord blood
CFU	colony forming unit
DAPI	4',6-diamidino-2-phenylindole
E	erythroid
FITC	fluorescein isothiocyanate

FL	Flt-3 ligand
G	granulocytic
GMP	good manufacturing practices
HSPC	hematopoietic stem and progenitor cell
IL	interleukin
M	monocytic
Mk	megakaryocytic cell
mPB	mobilized peripheral blood
NIC	nicotinamide
PE	phycoerythrin
PEB	phosphate-buffered saline containing 2 mM EDTA and 0.5% bovine serum albumin
pO₂	oxygen partial pressure
SCF	stem cell factor
Tpo	thrombopoietin

References

1. Chang Y, Bluteau D, Debili N, Vainchenker W. From hematopoietic stem cells to platelets. *J Thromb Haemost*. 2007 Jul; 5(Suppl 1):318–27. [PubMed: 17635743]
2. Vitrat N, Cohen-Solal K, Pique C, Le Couedic JP, Norol F, Larsen AK, et al. Endomitosis of human megakaryocytes are due to abortive mitosis. *Blood*. 1998 May 15; 91(10):3711–23. [PubMed: 9573008]
3. Mattia G, Vulcano F, Milazzo L, Barca A, Macioce G, Giampaolo A, et al. Different ploidy levels of megakaryocytes generated from peripheral or cord blood CD34+ cells are correlated with different levels of platelet release. *Blood*. 2002 Feb 1; 99(3):888–97. [PubMed: 11806991]
4. Vadhan-Raj S. Management of chemotherapy-induced thrombocytopenia: current status of thrombopoietic agents. *Seminars in hematology*. 2009 Jan; 46(1 Suppl 2):S26–32. [PubMed: 19245931]
5. Stanworth SJ, Hyde C, Heddle N, Rebulla P, Brunskill S, Murphy MF. Prophylactic platelet transfusion for haemorrhage after chemotherapy and stem cell transplantation. *Cochrane database of systematic reviews (Online)*. 2004; (4):CD004269. [PubMed: 15495093]
6. Stroncek DF, Rebulla P. Platelet transfusions. *Lancet*. 2007 Aug 4; 370(9585):427–38. [PubMed: 17679020]
7. Vassallo RR, Murphy S. A critical comparison of platelet preparation methods. *Curr Opin Hematol*. 2006 Sep; 13(5):323–30. [PubMed: 16888436]
8. Pereira A, Sanz C. Effect of extending the platelet storage time on platelet utilization: predictions from a mathematical model of prophylactic platelet support. *Transfus Med*. 2007 Apr; 17(2):119–27. [PubMed: 17430468]
9. Dutcher, JP. Transfusion supportive care. In: Wiernik, PH., editor. *Adult leukemias*. Hamilton, ON: BC Decker; 2001. p. 307-15.
10. Mostafa SS, Miller WM, Papoutsakis ET. Oxygen tension influences the differentiation, maturation and apoptosis of human megakaryocytes. *Br J Haematol*. 2000 Dec; 111(3):879–89. [PubMed: 11122151]
11. Lefebvre P, Winter JN, Meng Y, Cohen I. Ex vivo expansion of early and late megakaryocyte progenitors. *J Hematother Stem Cell Res*. 2000 Dec; 9(6):913–21. [PubMed: 11177605]

12. Case J, Hicks C, Trickett A, Kwan YL, Manoharan A. The expansion of megakaryocyte progenitors from CD34+-enriched mobilized peripheral blood stem cells is inhibited by Flt3-L. *J Interferon Cytokine Res.* 2006 Feb; 26(2):76–82. [PubMed: 16487027]
13. De Bruyn C, Delforge A, Martiat P, Bron D. Ex vivo expansion of megakaryocyte progenitor cells: cord blood versus mobilized peripheral blood. *Stem Cells Dev.* 2005 Aug; 14(4):415–24. [PubMed: 16137231]
14. Balducci E, Azzarello G, Valenti MT, Capuzzo GM, Pappagallo GL, Pilotti I, et al. The impact of progenitor enrichment, serum, and cytokines on the ex vivo expansion of mobilized peripheral blood stem cells: a controlled trial. *Stem Cells.* 2003; 21(1):33–40. [PubMed: 12529549]
15. Drayer AL, Sibinga CT, Blom NR, De Wolf JT, Vellenga E. The in vitro effects of cytokines on expansion and migration of megakaryocyte progenitors. *Br J Haematol.* 2000 Jun; 109(4):776–84. [PubMed: 10929029]
16. Halle P, Rouzier C, Kanold J, Boiret N, Rapatel C, Mareynat G, et al. Ex vivo expansion of CD34+/CD41+ late progenitors from enriched peripheral blood CD34+ cells. *Ann Hematol.* 2000 Jan; 79(1):13–9. [PubMed: 10663616]
17. Schipper LF, Brand A, Reniers N, Melief CJ, Willemze R, Fibbe WE. Differential maturation of megakaryocyte progenitor cells from cord blood and mobilized peripheral blood. *Exp Hematol.* 2003 Apr; 31(4):324–30. [PubMed: 12691920]
18. van den Oudenrijn S, von dem Borne AE, de Haas M. Differences in megakaryocyte expansion potential between CD34(+) stem cells derived from cord blood, peripheral blood, and bone marrow from adults and children. *Exp Hematol.* 2000 Sep; 28(9):1054–61. [PubMed: 11008018]
19. Cortin V, Garnier A, Pineault N, Lemieux R, Boyer L, Proulx C. Efficient in vitro megakaryocyte maturation using cytokine cocktails optimized by statistical experimental design. *Exp Hematol.* 2005 Oct; 33(10):1182–91. [PubMed: 16219540]
20. Chow DC, Wenning LA, Miller WM, Papoutsakis ET. Modeling pO₂ distributions in the bone marrow hematopoietic compartment. II. Modified Kroghian models. *Biophys J.* 2001 Aug; 81(2):685–96. [PubMed: 11463617]
21. Taichman RS. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. *Blood.* 2005 Apr 1; 105(7):2631–9. [PubMed: 15585658]
22. Wilson A, Trumpp A. Bone-marrow haematopoietic-stem-cell niches. *Nat Rev Immunol.* 2006 Feb; 6(2):93–106. [PubMed: 16491134]
23. Weiss, LP. Functional organization of the hematopoietic tissues. In: Hoffman, R.; Benz, EJJ.; Shattil, SJ.; Furie, B.; Cohen, HJ., editors. *Hematology: basic principles and practice.* New York: Churchill Livingstone; 1991. p. 82
24. Jandl, JH. *Blood: textbook of hematology.* 2. Boston: Little, Brown; 1996.
25. Hevehan DL, Papoutsakis ET, Miller WM. Physiologically significant effects of pH and oxygen tension on granulopoiesis. *Exp Hematol.* 2000 Mar; 28(3):267–75. [PubMed: 10720691]
26. Cipolleschi MG, D'Ippolito G, Bernabei PA, Caporale R, Nannini R, Mariani M, et al. Severe hypoxia enhances the formation of erythroid bursts from human cord blood cells and the maintenance of BFU-E in vitro. *Exp Hematol.* 1997 Oct; 25(11):1187–94. [PubMed: 9328456]
27. LaIuppa JA, Papoutsakis ET, Miller WM. Oxygen tension alters the effects of cytokines on the megakaryocyte, erythrocyte, and granulocyte lineages. *Exp Hematol.* 1998 Aug; 26(9):835–43. [PubMed: 9694504]
28. McAdams TA, Miller WM, Papoutsakis ET. pH is a potent modulator of erythroid differentiation. *Br J Haematol.* 1998 Nov; 103(2):317–25. [PubMed: 9827900]
29. Yang H, Miller WM, Papoutsakis ET. Higher pH promotes megakaryocytic maturation and apoptosis. *Stem Cells.* 2002; 20(4):320–8. [PubMed: 12110701]
30. Debili N, Coulombel L, Croisille L, Katz A, Guichard J, Breton-Gorius J, et al. Characterization of a bipotent erythro-megakaryocytic progenitor in human bone marrow. *Blood.* August 15; 1996 88(4):1284–96. [PubMed: 8695846]
31. Majka M, Baj-Krzyworzeka M, Kijowski J, Reca R, Ratajczak J, Ratajczak MZ. In vitro expansion of human megakaryocytes as a tool for studying megakaryocytic development and function. *Platelets.* 2001 Sep; 12(6):325–32. [PubMed: 11672471]

32. Birkmann J, Oez S, Smetak M, Kaiser G, Kappauf H, Gallmeier WM. Effects of recombinant human thrombopoietin alone and in combination with erythropoietin and early-acting cytokines on human mobilized purified CD34+ progenitor cells cultured in serum-depleted medium. *Stem Cells*. 1997; 15(1):18–32. [PubMed: 9007219]
33. Williams JL, Pipia GG, Datta NS, Long MW. Thrombopoietin requires additional megakaryocyte-active cytokines for optimal ex vivo expansion of megakaryocyte precursor cells. *Blood*. 1998 Jun 1; 91(11):4118–26. [PubMed: 9596657]
34. LaFuppa JA, Papoutsakis ET, Miller WM. Evaluation of cytokines for expansion of the megakaryocyte and granulocyte lineages. *Stem Cells*. 1997; 15(3):198–206. [PubMed: 9170211]
35. Feng Y, Zhang L, Xiao ZJ, Li B, Liu B, Fan CG, et al. An effective and simple expansion system for megakaryocyte progenitor cells using a combination of heparin with thrombopoietin and interleukin-11. *Exp Hematol*. 2005 Dec; 33(12):1537–43. [PubMed: 16338497]
36. Giammona LM, Fuhrken PG, Papoutsakis ET, Miller WM. Nicotinamide (vitamin B3) increases the polyploidisation and proplatelet formation of cultured primary human megakaryocytes. *Br J Haematol*. 2006 Nov; 135(4):554–66. [PubMed: 17054670]
37. Giammona LM, Panuganti S, Kemper JM, Apostolidis PA, Lindsey S, Papoutsakis ET, et al. Mechanistic studies on the effects of nicotinamide on megakaryocytic polyploidization and the roles of NAD+ levels and SIRT inhibition. *Experimental hematology*. 2009 Nov; 37(11):1340–52. e3. [PubMed: 19715739]
38. Takayama N, Nishikii H, Usui J, Tsukui H, Sawaguchi A, Hiroyama T, et al. Generation of functional platelets from human embryonic stem cells in vitro via ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors. *Blood*. 2008 Jun 1; 111(11):5298–306. [PubMed: 18388179]
39. Ciurea SO, Hoffman R. Cytokines for the treatment of thrombocytopenia. *Semin Hematol*. 2007 Jul; 44(3):166–82. [PubMed: 17631181]
40. Fukushima-Shintani M, Suzuki K, Iwatsuki Y, Abe M, Sugawara K, Hirayama F, et al. AKR-501 (YM477) in combination with thrombopoietin enhances human megakaryocytopoiesis. *Exp Hematol*. 2008 Oct; 36(10):1337–42. [PubMed: 18619724]
41. Tijssen MR, van Hennik PB, di Summa F, Zwaginga JJ, van der Schoot CE, Voermans C. Transplantation of human peripheral blood CD34-positive cells in combination with ex vivo generated megakaryocytes results in fast platelet formation in NOD/SCID mice. *Leukemia*. 2008 Jan; 22(1):203–8. [PubMed: 17943170]
42. van den Oudenrijn S, von dem Borne AE, de Haas M. Influence of medium components on ex vivo megakaryocyte expansion. *J Hematother Stem Cell Res*. 2001 Feb; 10(1):193–200. [PubMed: 11276373]
43. Rossi B, Zanolin E, Vincenzi C, Diani F, Pizzolo G, de Wynter E, et al. Effect of addition of FLT-3 ligand and megakaryocyte growth and development factor on hemopoietic cells in serum-free conditions. *Stem Cells Dev*. 2004 Aug; 13(4):362–71. [PubMed: 15345130]
44. Bertolini F, Battaglia M, Pedrazzoli P, Da Prada GA, Lanza A, Soligo D, et al. Megakaryocytic progenitors can be generated ex vivo and safely administered to autologous peripheral blood progenitor cell transplant recipients. *Blood*. 1997 Apr 15; 89(8):2679–88. [PubMed: 9108385]
45. Perez LE, Rinder HM, Wang C, Tracey JB, Maun N, Krause DS. Xenotransplantation of immunodeficient mice with mobilized human blood CD34+ cells provides an in vivo model for human megakaryocytopoiesis and platelet production. *Blood*. 2001 Mar 15; 97(6):1635–43. [PubMed: 11238102]
46. Decaudin D, Vantelon JM, Bourhis JH, Farace F, Bonnet ML, Guillier M, et al. Ex vivo expansion of megakaryocyte precursor cells in autologous stem cell transplantation for relapsed malignant lymphoma. *Bone Marrow Transplant*. 2004 Dec; 34(12):1089–93. [PubMed: 15489877]
47. Scheduling S, Bergmann M, Rathke G, Vogel W, Brugger W, Kanz L. Additional transplantation of ex vivo generated megakaryocytic cells after high-dose chemotherapy. *Haematologica*. 2004 May; 89(5):630–1. [PubMed: 15136238]
48. Prince HM, Simmons PJ, Whitty G, Wall DP, Barber L, Toner GC, et al. Improved haematopoietic recovery following transplantation with ex vivo-expanded mobilized blood cells. *Br J Haematol*. 2004 Aug; 126(4):536–45. [PubMed: 15287947]

49. Kratz-Albers K, Scheduling S, Mohle R, Buhring HJ, Baum CM, Mc Kearn JP, et al. Effective ex vivo generation of megakaryocytic cells from mobilized peripheral blood CD34(+) cells with stem cell factor and promegapoeitin. *Exp Hematol*. 2000 Mar; 28(3):335–46. [PubMed: 10720698]
50. Proulx C, Boyer L, Hurnanen DR, Lemieux R. Preferential ex vivo expansion of megakaryocytes from human cord blood CD34+-enriched cells in the presence of thrombopoietin and limiting amounts of stem cell factor and Flt-3 ligand. *J Hematother Stem Cell Res*. 2003 Apr; 12(2):179–88. [PubMed: 12804177]
51. Boyer L, Robert A, Proulx C, Pineault N. Increased production of megakaryocytes near purity from cord blood CD34+ cells using a short two-phase culture system. *Journal of immunological methods*. 2008 Mar 20; 332(1–2):82–91. [PubMed: 18234208]
52. Kogler G, Callejas J, Sorg RV, Wernet P. An eight-fold ex vivo expansion of long-term culture-initiating cells from umbilical cord blood in stirred suspension cultures. *Bone Marrow Transplant*. 1998 Jun; 21(Suppl 3):S48–53. [PubMed: 9712494]
53. Collins PC, Miller WM, Papoutsakis ET. Stirred culture of peripheral and cord blood hematopoietic cells offers advantages over traditional static systems for clinically relevant applications. *Biotechnol Bioeng*. 1998 Sep 5; 59(5):534–43. [PubMed: 10099369]
54. Zandstra PW, Eaves CJ, Piret JM. Expansion of hematopoietic progenitor cell populations in stirred suspension bioreactors of normal human bone marrow cells. *Bio/technology (Nature Publishing Company)*. 1994 Sep; 12(9):909–14.
55. De Leon A, Mayani H, Ramirez OT. Design, characterization and application of a minibioreactor for the culture of human hematopoietic cells under controlled conditions. *Cytotechnology*. 1998 Nov; 28(1–3):127–38. [PubMed: 19003414]
56. Collins PC, Nielsen LK, Patel SD, Papoutsakis ET, Miller WM. Characterization of hematopoietic cell expansion, oxygen uptake, and glycolysis in a controlled, stirred-tank bioreactor system. *Biotechnology progress*. 1998 May-Jun; 14(3):466–72. [PubMed: 9622528]
57. Balduini A, Pallotta I, Malara A, Lova P, Pecci A, Viarengo G, et al. Adhesive receptors, extracellular proteins and myosin IIA orchestrate proplatelet formation by human megakaryocytes. *J Thromb Haemost*. 2008 Nov; 6(11):1900–7. [PubMed: 18752571]

Figure 1.

Statistical significance analysis of 2^4 factorial design for 1° cytokine evaluation. Main and interaction effects for each cytokine, either individually or in combination, were calculated at day 7 for (A) total cell fold expansion; the percentages of (B) CD34⁺, (C) CD41⁺, and (D) CD34⁺CD41⁺ cells; (E) the number of CD34⁺CD41⁺ cells produced per input HSPC; and (F) total cell viability. Data represent $n = 3$ donors. An asterisk (*) indicates that a main or interaction effect fell outside the 95% confidence interval and was considered to have a significant effect on influencing that parameter on day 7. Day 7 was chosen for comparison because, after that time point, CD34⁺CD41⁺ cell purity had peaked and begun to decrease for some donors/conditions. Coefficients of correlation were (A) 0.94, (B) 0.98, (C) 0.94, (D) 0.95, (E) 0.81, and (F) 0.57.

Figure 2.

Mk progenitor production using 1° cytokine cocktails. (A) Mk progenitor purity and (B) the number of CD34⁺CD41⁺ Mk progenitors produced per input CD34⁺ HSPC are shown for the 11-day culture for all 16 cytokine combinations evaluated. Data points represent the average $\pm$ SEM for $n = 3$ donors. Symbols correspond to conditions as indicated in Table 1.

Figure 3.

Mk colony production by progenitors from cultures with 1° cytokine cocktails. (A) Cells were removed from each of the sixteen conditions (see Table 1) at day 5 and seeded into CFU-Mk assays. Colonies of CD41⁺ cells were enumerated and scored for size. Data represent the average $\pm$ SEM for $n = 3$ donors. (B) A representative large colony from condition 10 is shown. CD41 expression is indicated by a red stain.

Figure 4.

Mk progenitor production at 5% O₂ and pH 7.2 using 2° cytokine cocktails. 100 ng/mL Tpo and 100 ng/mL SCF were present in all conditions. IL-11 and IL-6 were used at 10 ng/mL. Total cell fold expansion (A); the percentages of (B) CD34⁺, (C) CD41⁺, and (D) CD34⁺CD41⁺ cells in the viable population; and the number of CD34⁺CD41⁺ Mk progenitors produced per input CD34⁺ HSPC (E) are shown for the entire culture period. Data represent the average ± SEM for n = 4 donors.

Figure 5.

Mk colony production by progenitors from cultures with 2° cytokine cocktails at pH 7.2 and 5% O₂. Cells were removed from each of the six conditions at days 5 (A,D), 7 (B,E), and 9 (C,F) and seeded into CFU-Mk assays. Mk colonies formed were enumerated and scored for size. Data were normalized to account for the number of colonies produced per 1000 input cells on day 0 (A-C) or the number of colonies produced per 1000 cells seeded on either day 5, 7, or 9 (D-F). Data represent the average ± SEM for n = 3 donors.

Figure 6.

Total Mk production in cultures with 2° cytokines after shifting from 5% O₂/pH 7.2 to 20% O₂/pH 7.4. Cells from each of the conditions at 5% O₂/pH 7.2 were removed at days 5 (A,C) and 7 (B,D) and seeded into fresh media at 20% O₂/pH 7.4 (open symbols). (A-B) The percentage of CD41⁺ cells in the viable population and (C-D) the number of CD41⁺ Mks produced per input CD34⁺ HSPC are shown for the entire culture period. Control cultures maintained at 5% O₂ are represented by solid symbols. Data represent the average ± SEM for n = 3 donors.

Figure 7.

Effect of NIC on Mk polyplodization in cultures with 2^o cytokines after shifting to 20% O₂/pH 7.4. Cells from each of the conditions were removed from 5% O₂/pH 7.2 at days 5 (A,C,E) and 7 (B,D,F) and seeded into fresh media at 20% O₂/pH 7.4 with (dashed lines) or without NIC (solid lines). (A-B) The number of CD41⁺ Mk produced per input CD34⁺ HSPC, (C-D) the percentage of high-ploidy Mk in culture, and (E-F) the number of high-ploidy Mk produced per input CD34⁺ HSPC are shown for the entire culture period. Data represent the average $\pm$ SEM for n = 3 donors.

Figure 8.

Ploidy distribution of Mks in cultures with selected 2° cytokines. Histograms show DNA staining with propidium iodide (PI) in permeabilized CD41⁺ Mks from conditions 6-II (A-C) and 10-II (D-F). Representative histograms for day 11 are shown for cells maintained at 5% O₂/pH 7.2 (A,D) or shifted to 20% O₂/pH 7.4 at day 7 without (B,E) or with NIC (C,F).

Total cell fold expansion and Mk progenitor production using 1° cytokine cocktails. Total cell fold expansion, Mk progenitor purity and the number of CD34⁺CD41⁺ Mk progenitors produced per input CD34⁺ HSPC are shown for all 16 cytokine combinations evaluated at days 7, 9, and 11.

Table 1

	IL-3	IL-6	IL-11	FL	expansion (day 7)	expansion (day 9)	expansion (day 11)	%CD34+CD41+ (day 7)	%CD34+CD41+ (day 9)	%CD34+CD41+ (day 11)	CD34+CD41+ yield (day 7)	CD34+CD41+ yield (day 9)	CD34+CD41+ yield (day 11)
1	+	+	+	+	13.4 ± 0.8	22.3 ± 0.2	30.1 ± 0.8	16.0 ± 4.4	10.2 ± 1.3	6.7 ± 4.5	2.1 ± 0.4	2.3 ± 0.3	2.0 ± 1.3
2	+	+	+	-	10.4 ± 1.3	17.0 ± 1.7	23.0 ± 3.1	21.8 ± 3.6	16.6 ± 4.6	11.8 ± 6.1	2.2 ± 0.1	2.7 ± 0.5	2.5 ± 1.0
3	+	+	-	+	12.2 ± 2.0	24.1 ± 6.1	33.3 ± 10.2	14.9 ± 2.7	11.5 ± 3.1	8.9 ± 6.8	1.7 ± 0.1	2.6 ± 0.0	2.3 ± 1.3
4	+	+	-	-	10.3 ± 1.5	17.3 ± 2.2	22.6 ± 3.0	21.3 ± 3.4	16.1 ± 1.2	9.6 ± 5.0	2.1 ± 0.1	2.8 ± 0.2	2.0 ± 0.8
5	+	-	+	+	11.8 ± 2.7	23.5 ± 6.6	30.5 ± 8.8	18.6 ± 4.3	11.2 ± 3.3	9.1 ± 6.4	2.1 ± 0.3	2.4 ± 0.0	2.2 ± 1.1
6	+	-	+	-	9.2 ± 0.7	16.1 ± 1.7	20.1 ± 0.9	24.4 ± 3.4	18.9 ± 2.6	12.2 ± 7.4	2.2 ± 0.2	3.0 ± 0.1	2.4 ± 1.4
7	+	-	-	+	10.6 ± 0.6	17.3 ± 0.8	21.8 ± 1.6	17.4 ± 3.2	13.4 ± 3.0	8.9 ± 6.9	1.8 ± 0.2	2.3 ± 0.6	2.1 ± 1.6
8	+	-	-	-	8.0 ± 1.2	12.4 ± 1.9	14.7 ± 1.1	24.3 ± 2.9	18.3 ± 2.5	11.2 ± 7.5	1.9 ± 0.1	2.2 ± 0.0	1.6 ± 1.0
9	-	+	+	+	7.4 ± 1.7	10.9 ± 2.9	14.7 ± 3.2	20.0 ± 5.4	19.0 ± 8.1	12.4 ± 9.8	1.3 ± 0.1	1.8 ± 0.3	1.5 ± 1.0
10	-	+	+	-	5.0 ± 1.3	6.9 ± 2.6	8.4 ± 2.6	31.6 ± 6.9	35.1 ± 10.7	21.8 ± 11.5	1.4 ± 0.2	2.1 ± 0.2	1.5 ± 0.4
11	-	+	-	+	3.9 ± 1.8	4.0 ± 3.1	5.0 ± 4.3	26.0 ± 4.7	29.0 ± 1.3	18.7 ± 4.4	0.9 ± 0.4	1.1 ± 0.8	1.1 ± 1.0
12	-	+	-	-	5.2 ± 1.1	7.8 ± 2.8	10.3 ± 3.9	32.2 ± 6.6	32.3 ± 10.0	22.8 ± 13.6	1.5 ± 0.1	2.2 ± 0.1	1.8 ± 0.5

	IL-3	IL-6	IL-11	FL	expansion (day 7)	expansion (day 9)	expansion (day 11)	%CD34+CD41+ (day 7)	%CD34+CD41+ (day 9)	%CD34+CD41+ (day 11)	CD34+CD41+ yield (day 7)	CD34+CD41+ yield (day 9)	CD34+CD41+ yield (day 11)
13	–	–	+	+	4.4 ± 1.0	4.3 ± 0.3	4.5 ± 1.9	25.3 ± 8.6	25.2 ± 13.0	15.9 ± 13.7	0.9 ± 0.1	1.1 ± 0.6	1.0 ± 0.9
14	–	–	+	–	3.8 ± 1.4	5.2 ± 3.1	4.9 ± 1.7	37.0 ± 7.7	39.4 ± 12.0	27.6 ± 16.8	1.2 ± 0.3	1.7 ± 0.6	1.1 ± 0.4
15	–	–	–	+	5.4 ± 1.6	7.9 ± 3.2	8.2 ± 1.6	26.5 ± 5.9	23.8 ± 10.6	16.8 ± 11.7	1.3 ± 0.1	1.5 ± 0.1	1.2 ± 0.7
16	–	–	–	–	3.8 ± 1.5	6.3 ± 4.1	6.0 ± 2.9	37.4 ± 6.1	36.8 ± 13.1	27.4 ± 12.3	1.2 ± 0.3	1.8 ± 0.7	1.3 ± 0.1
	K				7.8	12.7	16.1	24.7	22.3	15.1	1.6	2.1	1.7

All cocktails shown also included 100 ng/mL Tpo and 100 ng/mL SCF. The “+” and “–” symbols represent addition of 10 ng/mL or 0 ng/mL, respectively, of the indicated cytokine to the culture media. K is the mean of all conditions. Data shown represents the average ± SEM for n = 3 donors. Symbols on the left-hand side of the table correspond to the symbols used in Figure 2 and supplementary Figure S1.