Identification of growth factor conditions that reduce *ex vivo* cord blood progenitor expansion but do not alter human repopulating cell function *in vivo*

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Background and Objectives. *Ex vivo* expansion of primitive hematopoietic cells for transplantation is an important step to realizing the optimal clinical potential of human cord blood (CB). We aimed to characterize minimal growth factor (GF) conditions that allow *ex vivo* expansion of primitive cells, including candidate hematopoietic stem cells.

Design and Methods. Here, we directly investigated the effect of thrombopoietin (TPO) on progenitors and repopulating cells using serum-free culture of CB $Lin^-CD34^+CD38^-$ cells in two different minimal GF conditions: stem cell factor (SCF)+FLT-3-L (termed S/F) and SCF+FLT-3-L+TPO (termed S/F/T).

Results. While S/F media supported only low levels of total cell and CFU (colony-forming unit) expansion, the addition of TPO (S/F/T) partially restored cell proliferation, and completely restored CFU expansion to levels observed using full GF conditions (SCF+FLT-3-L+interleukin (IL)-3 (IL-3)+IL-6+ granulocyte colony-stimulating factor (G-CSF). Intravenous transplantation of either S/F- or S/F/T-expanded cells into NOD/SCID mice resulted in similar frequencies and levels of multilineage reconstitution.

Interpretation and Conclusions. The use of minimal cytokine stimulation and simultaneous assessment of CFU and SCID-repopulating cells (SRC) indicate that hematopoietic progenitors and *in vivo*-detected repopulating cells are differentially responsive to TPO; CFU expand in response to TPO but SRC do not. In addition, our study suggests that TPO can functionally replace IL-3+IL-6+G-CSF for CFU expansion of *ex vivo* cultured CB $Lin^-CD34^+CD38^-$ hematopoietic stem cells.

Key words: cord blood, *ex vivo* expansion, hematopoietic growth factors, progenitors, SCID-repopulating cells.

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Primitive hematopoietic cells include two different cell types: short-lived lineage-restricted progenitors, and long-lived hematopoietic stem cells (HSC) that sustain multilineage hematopoiesis.[1] Neither cell type can be prospectively purified in the human, since there is no combination of known cell surface markers that identifies a homogenous population of progenitors or HSC. Therefore, human cells with progenitor or HSC function are identified retrospectively, by their unique behavior in different assays. Myeloid progenitors (colony-forming units, CFU) can be identified *in vitro* because they form discrete colonies of mature cells in response to hematopoietic cytokines in semi-solid medium. In contrast, human HSC function can be assayed only *in vivo*, most commonly using a xenotransplantation assay that retrospectively identifies the rare cells that support long-term multilineage hematopoiesis in immune-deficient NOD/SCID mice (termed SCID-repopulating cells, SRC).[2]

For clinical transplantation, both progenitors and HSC are important since progenitors provide cells that transiently sustain hematopoietic function until a permanent HSC-derived graft is established. Cord blood (CB) is an attractive alternative to bone marrow or mobilized peripheral blood for transplantation since it is readily available and is enriched in primitive hematopoietic cells.[3] However, the low yield of transplantable cells and delayed engraftment observed in adult recipients may limit the direct application of single CB units to patients without a suitable bone marrow donor;[4,5] *ex vivo* amplification of primitive CB cells would broaden the therapeutic potential of this cell source. While many groups have reported expan-

sion of CB-derived CFU, reconstituting cells (SRC) are usually lost or only modestly expanded, suggesting that progenitors and HSC respond differently to a given culture condition.[6-9] This is not surprising, since their distinct functional properties predict distinct regulatory mechanisms.

For serum-free *ex vivo* expansion of CB Lin$^-$CD34$^+$ CD38$^-$ cells, our laboratory and others developed a growth factor (GF) cocktail containing stem cell factor (SCF), fetal liver tyrosine kinase-3-ligand (FLT-3-L), interleukin-3 (IL-3), interleukin-6 (IL-6) and granulocyte colony-stimulating factor (G-CSF), which has been shown to expand CFU and maintain or modestly expand SRC.[6,8,10] In this study, we aimed to refine these culture conditions and reasoned that reducing the number of GF in the culture might directly identify those GF that regulate both CFU and SRC and highlight those that are specific for CFU and/or SRC expansion. IL-3 has been reported to play a negative role in primitive cell expansion,[9,11-13] and IL-6 has generally been reported to have a neutral,[14,15] or very modest positive effect[12,13,16] on expansion of primitive human cells, so both IL-3 and IL-6 were excluded from our minimal GF conditions. Despite its clinical utility,[17] there is little evidence that G-CSF promotes self-renewal of primitive multipotent hematopoietic cells, so this cytokine was not added. In contrast, SCF and FLT-3-L have been shown to have non-redundant, synergistic effects on primitive hematopoietic cells,[18,19] thereby defining SCF+FLT-3-L as the minimal growth factor combination evaluated. There is good evidence in the mouse that thrombopoietin (TPO), originally characterized as a key regulator of megakaryocyte development,[20] has a non-redundant role in regulating primitive hematopoietic cells.[21,22] Based on these studies, the effect of adding TPO to SCF+FLT-3-L was also evaluated.

Here, we report for the first time on the effect of the particular cytokine combinations of SCF+FLT-3-L and SCF+FLT-3-L+TPO on highly purified CB Lin$^-$CD34$^+$ CD38$^-$ cells using simultaneous assessment of *in vitro* progenitors (CFU) and *in vivo* repopulating cells (SRC) after culture.

Design and Methods

Cell purification

Human CB mononuclear cells were isolated using Ficoll-Hypaque (Amersham Biosciences, Baie d'Urfé, Quebec, Canada), and primitive, lineage-depleted (Lin$^-$) cells were purified by negative selection using a StemSep™ immunomagnetic column (StemCell Technologies, Vancouver, British Columbia, Canada). Lin$^-$ cells were stained with anti-CD34-APC, anti-CD38-PE (both from Becton-Dickinson, Mississauga, Ontario, Canada), and 7-AAD (Immunotech, Beckman Coulter, Mississauga, Ontario, Canada), and live Lin$^-$CD34$^+$CD38$^-$ cells were isolated by flow cytometry using a FACSVantage™ (Becton-Dickinson).

Ex vivo culture

Purified Lin$^-$CD34$^+$ CD38$^-$ cells were seeded in human fibronectin-coated 96-well plates (Biocoat® Cellware, Becton-Dickinson) at a density of 2,000 or 6,000 cells per well. The basal medium for all GF conditions was 1X BIT 9500 medium (StemCell Technologies), which contains 1% bovine serum albumin, 10 μg/mL bovine insulin, and 0.2 mg/mL human transferrin in IMDM. Growth factor (GF) concentrations were as follows: 300 ng/mL SCF (donated by Amgen), 300 ng/mL FLT-3-L (R&D Systems, Minneapolis, MN, USA), 10 ng/mL IL-3 (donated by Novartis), 10 ng/mL IL-6 (R&D Systems), 50 ng/mL G-CSF (Neupogen®, donated by Amgen), and 20 ng/mL TPO (donated by Amgen). GF combinations were as indicated in the text. Media were changed every 2 days, and cells were expanded into new wells as needed. On day 9 of culture, all cells were harvested using an enzyme-free cell dissociation buffer (Gibco Invitrogen Corp., Burlington, Ontario, Canada).

Clonogenic progenitor assays and flow cytometry

On day 9 of culture, harvested cells were counted with a hemacytometer, and appropriate aliquots were plated in a 1% methylcellulose-based medium (MethoCult™ H4230, StemCell Technologies) for identification of clonogenic progenitors (CFU). Added cytokines were 50 ng/mL SCF, 10 ng/mL IL-3, 10 ng/mL GM-CSF (donated by Novartis), and 3 U/mL erythropoietin (donated by Amgen). CFU-granulocyte, CFU-granulocyte/macrophage, CFU-macrophage, and CFU-mixed (granulocyte, macrophage, erythroid) were enumerated after 10-14 days of culture using standard scoring criteria.

NOD/SCID mouse transplantation and analysis

NOD/SCID mice (NOD/LtSz-scid/scid, originally from Jackson Laboratory, Bar Harbor, ME, USA) were housed in an exclusion barrier facility at the Robarts Research Institute, and handled under sterile conditions. Mice at 8-12 weeks of age were transplanted by tail vein injection according to standard protocols.[8] Six weeks post-transplant, the mice were killed and bone marrow was flushed from the femora, tibiae, and iliac crests. A small aliquot of cells from each mouse was stained with anti-CD45-FITC and anti-CD38-PE (both from Becton-Dickinson) to analyze human cell engraftment by flow cytometry. For mice with greater than 1% engraftment, multilineage analysis was performed using aliquots for primitive (anti-CD34-FITC, anti-CD38-PE), myeloid (anti-CD33-FITC, anti-CD15-

PE), and B-lymphoid (anti-CD20-FITC, anti-CD19-PE) cells. Anti-CD45-APC was used to gate human cells for multilineage analysis. Appropriate isotype-matched control antibodies were used for all flow cytometric analyses. CD38-PE, CD33-FITC and CD45-APC antibodies were from Becton-Dickinson, CD34-FITC was from BD Pharmingen and CD15-PE, CD19-PE, and CD20-FITC were from Immunotech Beckman Coulter.

Statistical analyses

Data were analyzed, with either unpaired t-tests or one-way ANOVA as appropriate, using GraphPad Prism Software. Results were considered statistically significant at $p \leq 0.05$.

Results

Different minimal GF conditions have different effects on CB Lin$^-$CD34$^+$CD38$^-$ cell expansion and primitive phenotype

We aimed to define the minimal GF required for *ex vivo* expansion of CB-derived CFU and SRC, by modifying our previously established GF cocktail of SCF$^+$FLT-3-L+IL-3+IL-6+G-CSF. CB Lin$^-$CD34$^+$CD38$^-$ cells were cultured for 9 days in full GF medium (SCF+FLT-3-L+IL-3+IL-6+G-CSF), S/F medium (SCF+FLT-3-L), or S/F/T medium (SCF+FLT-3-L+TPO). Compared to cells cultured in full GF, which achieved an average cell expansion of 447-fold (447.5 ± 84.9, range: 189- to 893-fold), cells cultured in S/F alone expanded only 14-fold (14.0±2.8, range: 2.7- to 24.2-fold) (Figure 1A). Strikingly, the addition of TPO to S/F largely restored the level of proliferation seen with the full GF condition, with an average 241-fold cell expansion (241.3±121.8, range: 55.7- to 395-fold) (Figure 1A). As a first measure of the quality of the expanded cells, we assessed their primitive phenotype by flow cytometry for the primitive marker CD34 and the differentiation marker CD38. As shown in representative dot plots, the majority of expanded cells in all GF conditions that retained CD34 expression were also negative for CD38 (Figure 1B). After 9 days of culture, 22.5±2.6% of cells grown in full GF conditions retained CD34 expression, similar to the 29.7±4.6% CD34$^+$ cells in S/F/T-expanded cultures (Figure 1C). For cells expanded in S/F, half (48.8±1.2%) retained CD34$^+$ cells (Figure 1C), consistent with the low level of proliferation (Figure 1A). From 2,000 initial CD34$^+$ CD38$^-$ cells, full GF conditions produced an absolute yield of 22.6×10^4 CD34$^+$ cells (SEM: 5.4×10^4, range: 4.6×10^4 to 45.0×10^4), similar to that achieved with S/F/T: 17.1×10^4 CD34$^+$ cells (SEM: 4.1×10^4, range: 3.6×10^4 to 31.1×10^4) (Figure 1D). As predicted from the low level of cell expansion, S/F culture conditions generated only 1.4×10^4 CD34$^+$ cells (SEM: 0.4×10^4, range: 0.5×10^4 to 2.4×10^4). Together, these observations indicate that all GF conditions tested permit expansion of cells with a primitive phenotype, albeit with quantitative differences.

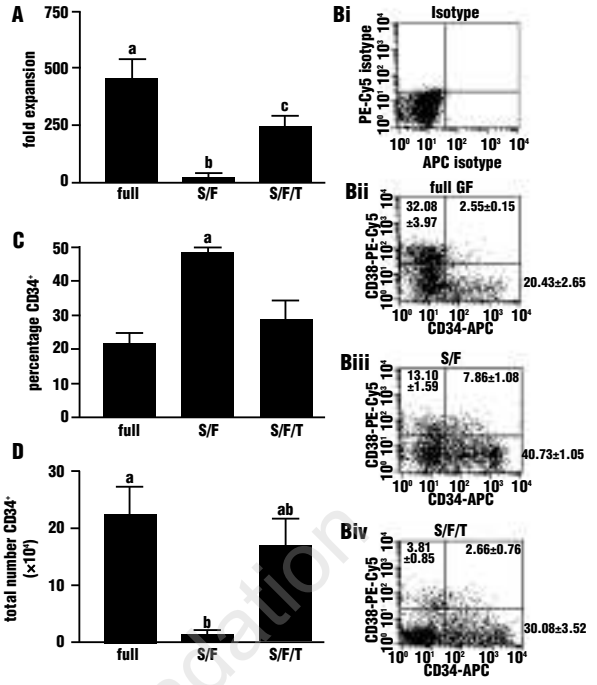

Figure 1. Cell expansion and phenotypic analysis of CB Lin$^-$CD34$^+$38$^-$ cells grown in different growth factor conditions. Cells were seeded typically at 2,000 cells per well of a 96-well plate, and cultured for 9 days in full GF, S/F, or S/F/T, as detailed in the Methods. (A). Fold increase in cell number, calculated as the number of cells on day 9 divided by the number of cells originally seeded. (B). Representative dot plots for full GF (ii), S/F (iii), and S/F/T (iv) expanded cells after 9 days showing CD34 and CD38 phenotype. Isotype control staining is shown in (i). The numbers in each quadrant are the averages for all experiments. (C). Percentage of cells retaining CD34 expression on day 9, as determined by flow cytometry. (D). Yield of cells retaining CD34 expression, calculated using the total cell count and the frequency of CD34$^+$ cells on day 9 of culture. All values shown are mean±SEM, (n=9 independent CB for fold expansion and n=6-8 independent CB for phenotype). Statistically significant differences, by one-way ANOVA, are indicated above the bars, $p \leq 0.05$.

CFU expansion is responsive to different GF conditions

After examining the primitive phenotype, we assessed the primitive function of the expanded cells using clonogenic progenitor assays. The frequency of progenitors within the expanded cultures (plating efficiency) was highest for cells expanded in S/F, and lowest for cells expanded in full GF conditions, at 78±21 and 36±9 colonies per 1,000 cells, respectively. Cells grown in S/F/T showed an intermediate plating efficiency, at 50±14 colonies per 1,000 cells (Figure 2A, left axis). None of these absolute differences reached statistical significance, although when the results for the minimal GF combinations were expressed relative

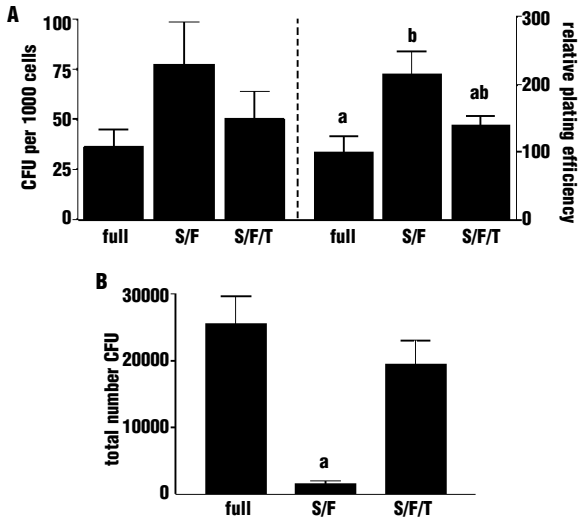

Figure 2. Functional analysis of CB Lin$^-$CD34$^+$38$^-$ expanded for 9 days in different GF conditions. Suitable aliquots of each expanded cell population were plated in methylcellulose media and scored after 12-14 days for myeloid CFU. (A) Frequency of CFU in the expanded cell population. The left axis shows plating efficiency as the number of CFU per 1,000 expanded cells, and the right axis shows the same data after the plating efficiency for S/F and S/F/T was expressed relative to that of full GF (100%) for each individual CB. (B) Total yield of CFU. The total number of CFU was calculated using the total cell count and the plating efficiency on day 9 of culture. All values shown are mean±SEM, (n=9 independent CB). Statistically significant differences were determined by one-way ANOVA, $p \leq 0.05$.

to full GF conditions for each cord blood, the difference between full GF conditions and S/F was statistically significant ($p<0.05$), and the plating efficiency for S/F/T was intermediate (Figure 2A right axis). When the total yield of CFU was calculated, cells expanded in full GF and S/F/T generated similar numbers of CFU, with averages of 25,588 ± 1,356 (range: 12145 to 43,650) and 19,540±3,520 (range: 3767 to 35,806), respectively (Figure 2B). While the plating efficiency of cells expanded in S/F was the highest (Figure 2A), the total yield of CFU was very low, at 1,593±372 (range: 233 to 3,934) (Figure 2B) due to the low level of cell expansion. All lineages of myeloid colonies (burst-forming unit-erythroid/BFU-E; CFU-macrophage/CFU-M; CFU-granulocyte/CFU-G; CFU-granulocyte/macrophage/CFU-GM; CFU-mixed/CFU-MIX (erythroid, granulocyte/macrophage, megakaryocyte) were contained within cell populations expanded using each of the different GF conditions. S/F and S/F/T conditions generated a slightly higher proportion of BFU-E, and correspondingly lower proportions of CFU-M, CFU-G, and CFU-GM compared to full GF conditions, which may have been due to the absence of IL-3+IL-6+G-CSF (Table 1). Together, these data demonstrate that in serum-free culture, the combination of

Table 1. CFU composition of primitive CB cells expanded in different GF conditions.

	%BFU-E	%CFU-M	%CFU-G	%CFU-GM	%CFU-MIX
Full GF	34.6±3.9[a]	28.5±3.0	22.7±3.0	11.4±2.2	2.5±1.1
S/F	53.0±5.0[b]	19.1±1.6	12.7±2.7	7.9±2.2	7.3±2.1
S/F/T	55.6±3.6[b]	17.7±2.6	11.7±1.3	7.9±1.3	6.4±1.4

Values shown are mean ± SEM, and 8 different CB samples were used for each GF condition. As indicated, the only statistically significant difference between GF conditions was in BFU-E percentage (values noted with [a]are significantly different from values noted with [b]within a given CFU lineage, $p \leq 0.05$ by ANOVA).

SCF+FLT-3-L is ineffective at promoting the expansion of CB-derived progenitors. The addition of a single GF, TPO, can increase total CFU expansion to a level that is identical to that produced by full GF-treated cultures, suggesting that TPO can replace IL-3+IL-6+G-CSF for progenitor expansion.

SRC activity is not affected by different GF conditions

Preliminary transplants of cells cultured in each of the three different GF conditions consistently resulted in multilineage hematopoietic repopulation in NOD/SCID mice, indicating that SRC were maintained in all GF conditions tested. Since we were most interested in the minimal growth factors and there is abundant historical data published from our laboratory and others regarding the SRC content of full GF-expanded cells,[6,8,10] we concentrated on transplantation of cells expanded in S/F and S/F/T. SRC are quantitatively and qualitatively assessed by NOD/SCID reconstitution frequency and level, respectively. Following transplantation of the 9-day expansion equivalent (all cells generated over 9 days of culture) of either 2,000 or 6,000 Lin$^-$CD34$^+$CD38$^-$ cells into NOD/SCID mice, S/F-treated and S/F/T-treated cells supported similar frequencies and levels of human hematopoietic engraftment (Figure 3Ai).

No differences in the B-lymphoid-dominant multilineage reconstitution profile for engrafted mice that received cells from either growth factor condition were observed (Figure 3B), nor did the reconstitution profile differ from that observed previously for mice transplanted with Lin$^-$CD34$^+$CD38$^-$ cells expanded in full GF conditions.[8] While mice transplanted with the S/F/T-treated expansion equivalent of 2,000 and 6,000 cells had levels of reconstitution that were respectively only 1.35- and 2.37-times higher than those off mice transplanted with S/F-treated cells (not significant), the S/F/T-expanded cells contained 12.27-times more CFU than their S/F-expanded counterparts

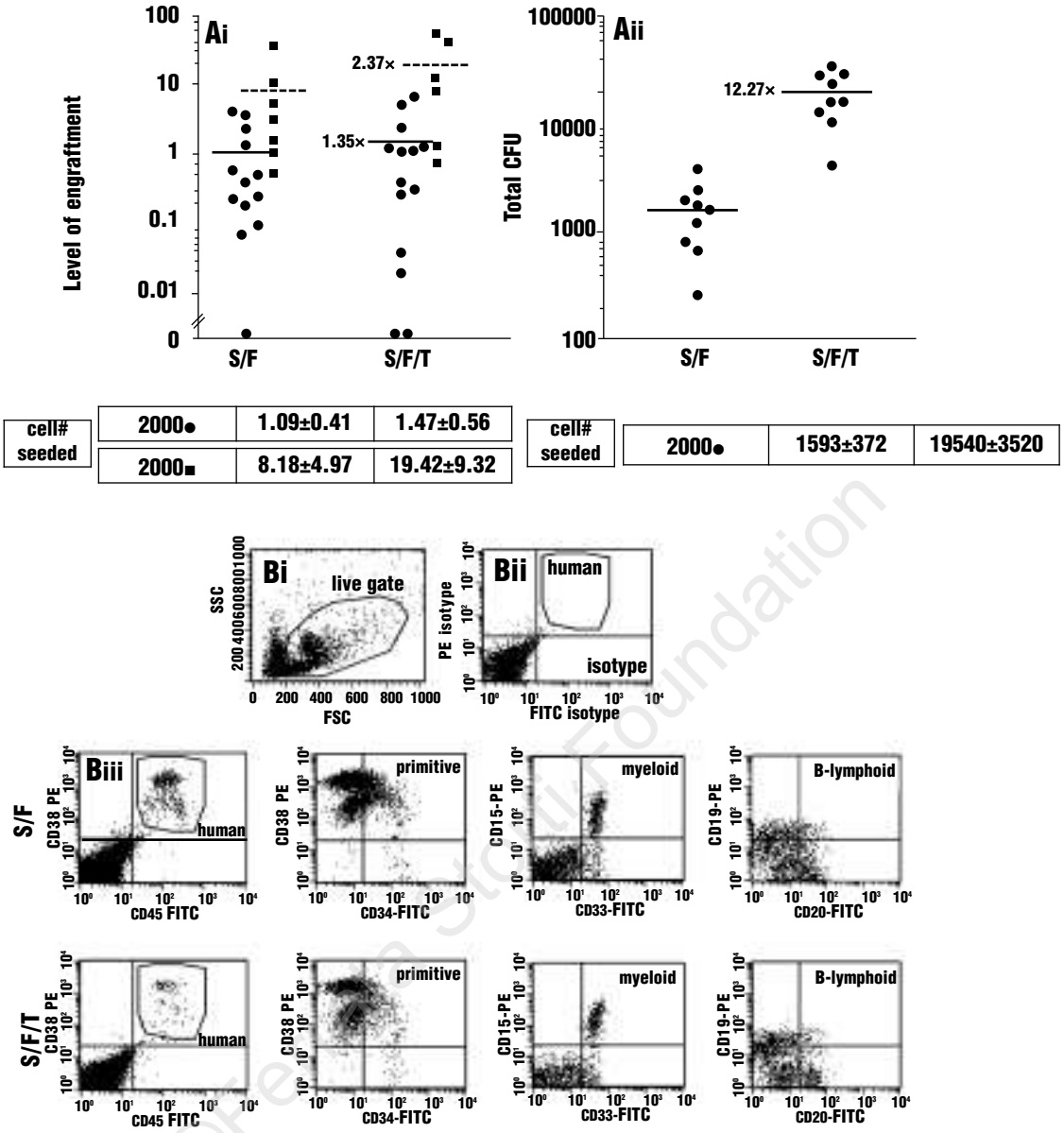

Figure 3. Analysis of the *in vivo* NOD/SCID reconstituting capacity of Lin⁻CD34⁺38⁻ cells expanded in S/F and S/F/T media. (A)i. The entire 9-day expansion equivalent of 2000 (●) (n=3 independent CB) or 6,000 (■) (n=4 independent CB) Lin⁻CD34⁺38⁻ cells was transplanted into sublethally irradiated NOD/SCID mice and human engraftment in the mouse bone marrow was analyzed by flow cytometry after 6 weeks. Each dot represents one mouse, and the mean level of engraftment for the expansion equivalent of 2,000 cells and 6,000 cells is indicated by a solid and dashed horizontal line, respectively, as well as in the chart below the graph. The difference in engraftment for S/F/T- versus S/F-expanded cells is also indicated (not significant; p=0.6 and p=0.3 for 2,000 and 6,000 cell expansion equivalents, respectively). (A)ii. For comparative purposes, the total number of CFU generated by each CB cultured in S/F versus S/F/T is shown. The mean number of CFU is indicated by a solid horizontal line, as well as in the chart below the graph; the mean fold increase in CFU yield is also indicated. (B) Representative multilineage reconstitution profiles for mice engrafted with S/F- and S/F/T-expanded cells. Mouse BM cells were gated on forward and side scatter to include all live cells (i), which were analyzed for human cells using CD45-FITC and CD38-PE (iii, as indicated). A second aliquot was used to determine the expression of lineage markers on the human cells after gating on CD45-APC-positive events. Representative dot plots are shown for primitive cells, myeloid and B-lymphoid lineages (iii, as indicated), and an isotype control (ii).

(p<0.05) (Figure 3Aii). Based on this comparative analysis of culture conditions using both *in vitro* progenitor and *in vivo* repopulation assays, we conclude that addition of TPO to SCF+FLT-3-L in serum-free culture promotes progenitor expansion but does not substantially affect *ex vivo* culture of SRC.

Discussion

The goal of our current study was to evaluate the effects of minimal GF conditions on the *ex vivo* expan-

sion of CB-derived progenitors and SRC, specifically to identify minimal culture conditions that preserve repopulating activity. We reasoned that for future experiments, the effects of a putative exogenous stem cell regulator would be more easily observed if the target cells were receiving minimal stimulation from other growth factors. We used SCF+FLT-3-L because of their non-redundant, synergistic effects on primitive hematopoietic cells.[18,19] TPO was added to the S/F combination because previous *in vitro* work suggested that TPO stimulates proliferation at the clonal level[23-25] and expands clonogenic progenitors.[12,16,24,26-28] Also, TPO is often present in long-term culture conditions that support the maintenance or expansion of SRC, but with one exception,[12] these studies have not directly investigated the specific effect of TPO by simultaneously assessing the repopulating ability of cells cultured in the absence of TPO.[13,15,16]

To the best of our knowledge, our study is the first to investigate the particular cytokine combinations of SCF+FLT-3-L and SCF+FLT-3-L+TPO on purified CB $Lin^-CD34^+CD38^-$ cells, and to simultaneously measure *in vitro* progenitors (CFU) and *in vivo* repopulating cells (SRC) after culture. We have shown that for progenitor expansion, the addition of TPO can functionally replace IL-3+IL-6+G-CSF when SCF and FLT-3-L are present in the culture medium. While expansion of CFU was very low using S/F culture conditions, the addition of TPO resulted in 12-fold higher CFU expansion, similar to the level observed with full GF conditions. Although CB-derived CFU had a large proliferative response to TPO, comparable to that of BM-derived CFU under similar culture conditions,[24] we found that SRC expansion was not similarly affected.

Indeed, both S/F and S/F/T-cultured cells supported similar frequencies and levels of hematopoietic repopulation in NOD/SCID mice. Previous work from our laboratory has shown that the maintenance of SRC activity in full GF *ex vivo* cultures of 9 days' duration is highly variable, and NOD/SCID reconstitution frequencies rarely reach the 85-90% observed in the current study using S/F and S/F/T,[6,8] suggesting that these minimal GF conditions are superior to full GF for consistent maintenance of SRC. Since the NOD/SCID assay is quantitative,[3,10] if the addition of TPO to S/F culture conditions had caused a 12-fold increase in SRC expansion, in parallel to its effect on CFU expansion, we would have observed a much greater difference in the levels of human chimerism. Thus, S/F/T appears to be no more effective at promoting CB-derived SRC survival and/or proliferation than is S/F alone. A different laboratory showed previously that addition of TPO to S/F improved the level, but not frequency of engraftment of *ex vivo* cultured $CD34^+$ CB cells.[12] The fact that we did not observe a similar improvement in engraftment level may be due to the different starting population of cells ($Lin^-CD34^+CD38^-$ vs. $CD34^+$) and/or the different immune-deficient recipient mouse strain (NOD/LtSz-scid vs. NOD/Shi-scid with anti-NK antibody treatment). Together, our observations indicate that progenitor and SRC expansion are independently regulated and have differential responsiveness to TPO.

Although there is no direct evidence that SRC are the same as HSC, the NOD/SCID mouse is an excellent surrogate host for evaluating the *in vivo* repopulating ability of human cells, and SRC activity is widely considered to be indicative of true HSC function.[29,30]

Therefore, our current observations have important clinical implications for *ex vivo* expansion of hematopoietic cells for transplantation. Although it is easy to assess primitive cell function using CFU assays, progenitor expansion is not necessarily a reliable indicator of HSC expansion. In addition, while phenotypic markers are indispensable for *de novo* isolation of primitive cell populations both in the laboratory and the clinic, *ex vivo* expansion causes a clear dissociation between phenotype and function, due at least in part to the culture-induced alteration of surface marker expression.[7] In our study, there was no positive correlation between CFU plating efficiency and the frequency of any primitive phenotype, including $CD34^+$, $CD38^-$, $CD34^+CD38^-$, and $CD34^+CD38^+$ (*data not shown*), supporting the conclusion that for a given CB sample, primitive phenotype after culture is not predictive of primitive function. Our observations, like those of previous studies, support the use of SCF+FLT-3-L+TPO for clinical *ex vivo* expansion protocols of CB-derived primitive cells,[31] since repopulating activity is not compromised and progenitors are greatly expanded.

In summary, our work uses minimal cytokine stimulation to demonstrate that human CFU and SRC are differentially responsive to TPO. Our paper is the first to suggest that TPO does not effectively expand SRC *ex vivo*, since CB $Lin^-CD34^+CD38^-$ cells grown in minimal culture conditions with or without TPO possess equivalent repopulating ability in NOD/SCID mice. In contrast, our study clearly identifies TPO as an effective cytokine for expanding progenitors since cells cultured in SCF+FLT-3-L+TPO contained 12 times more CFU than cells cultured in SCF+FLT-3-L. It is striking that TPO can functionally replace IL-3+IL-6+G-CSF for *ex vivo* expansion of CB-derived primitive hematopoietic cells.

KL was the principal author responsible for experiments, data collection, analysis, and interpretation, and writing the manuscript. FK assisted with experiments, and provided intellectual contributions to manucript revision. MB contributed with experimental

design, data interpretation, and writing the manuscript.We gratefully acknowledge the labor and delivery nurses at St. Joseph's Health Care and the London Health Sciences Centre Westminster Campus for collection of cord blood samples.

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