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Negative Influence of IL3 on the Expansion of Human Cord Blood *In Vivo* Long-Term Repopulating Stem Cells

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ABSTRACT

Identification of culture conditions that support expansion or even long-term maintenance of *in vivo* repopulating human hematopoietic stem cells is still a major challenge. Using a combination of FLT3 ligand (FL), Stem Cell Factor (SCF), Thrombopoietin (TPO) and Interleukin 6 (IL6), we cultured cord blood (CB) $CD34^+$ cells for up to 12 weeks and transplanted their progeny into sublethally irradiated NOD/SCID mice. Bone marrow engraftment was considered successful when recipients contained measurable numbers of human $CD45^+$, $CD71^+$ and Glycophorin-A^+ (GpA) cells 8 weeks after transplantation. Twelve-week expanded cells with FL+SCF+TPO+IL6 successfully engrafted all of the recipients and human $CD45^+$+$CD71^+$+GpA^+ cells represented 4.3 to 22.4% of bone marrow. Substitution of IL6 with IL3 led to an even better expansion of cells and a similar clonogenic progenitor output in the first 8 weeks of culture; however, LTC-IC output increased up to week 6 and then decreased and disappeared. By contrast, with FL+SCF+TPO+IL6, LTC-IC kept increasing up to week 12. Four-week cultured cells with FL+SCF+TPO+IL3 less efficiently engrafted NOD/SCID mice, both as measured by frequency of positive recipients (4 out of 10) and percentage of engrafted human cells ($\leq$2%). Six-week expanded cells failed to engraft. This study provides evidence that many, but not all, of the so-called "early acting" cytokines, can sustain long-term maintenance and even expansion of human primitive *in vivo* repopulating stem cells. In particular, in the culture conditions used in this study, the presence of IL3 greatly reduces the repopulating potential of expanded $CD34^+$ CB cells.

INTRODUCTION

ALL OF THE DIFFERENT BLOOD CELL TYPES arise from pluripotent hematopoietic stem cells (HSC), which can support hematopoiesis throughout the life-span of an individual and can reconstitute and maintain hematopoiesis when transplanted into myeloablated recipients. Transplant experiments in mice using highly purified HSC indicate that complete repopulation of all hematopoietic lineages can be attained with one or very few cells (1–4).

Umbilical cord blood (CB) has been used as source of hematopoietic stem cells for transplant and is proving to be an acceptable alternative to bone marrow (5,6). The use of CB stem cells for transplants has many advantages, including the widespread availability. However, the major limitation resides in the limited number of cells available in each collection. For this reason, great interest has been paid to study aimed at the *ex vivo* expansion of primitive, repopulating hematopoietic stem cells. Because the development of HSC is thought to be regulated, at least in part, by interaction of cytokine receptor signals, many studies used various combinations of cytokines that have been shown to act on primitive hematopoietic cells to ob-

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tain the obtimal culture condition for HSC expansion. The early acting cytokines stem cell factor (SCF), FLT3-ligand (FL), c-mpl ligand or thrombopoietin (TPO) (7), and Interleukin 6 (IL6) have been used as key factors for HSC expansion.

SCF and FL tyrosine kinase receptors were shown to transduce signals crucial for HSC development (8,9); TPO has been shown to stimulate the expansion of primitive hematopoietic stem cells (7,10); IL6 is a potent cofactor for survival and proliferation of primitive multilineage progenitor cells (11,12). On the other hand, previous reports on the effect of Interleukin 3 (IL3) on the expansion of primitive hematopoietic cells were controversial, even though they found it stimulates the expansion of relatively mature HSC (13–15).

Of critical relevance for clinical application, is to establish whether *ex vivo* expanded HSC retain their long-term repopulating ability. In the majority of previous studies, this capacity could not be assessed (10,16–21).

The recent development of a functional in vivo assay for primitive human hematopoietic stem cells based on their ability to repopulate the bone marrow (BM) of sublethally irradiated Nonobese diabetic (NOD) severe combined immunodeficient (SCID) mice after intravenous transplantation enabled the stem cell activity of expanded hematopoietic cells to be evaluated and quantitated (22–27).

We compare here the expansion of human long-term *in vivo* repopulating stem cells cultured for several weeks in the presence of two growth factor combinations containing FL+SCF+TPO+IL6 and FL+SCF+TPO+IL3, respectively. We show that IL3 negatively affects the repopulating ability of cultured cells.

MATERIALS AND METHODS

Human cells

Human BM was obtained by aspiration from the posterior iliac crest of fully informed hematologically normal donors. Umbilical CB was obtained, with informed consent of the mother, at the end of full-term deliveries, after clamping and cutting of the cord, by drainage of blood into sterile collection tubes containing the anticoagulant citrate-phosphate dextrose.

$CD34^+$ cell purification

Mononuclear cells (MNC) were isolated from CB using Ficoll–Hypaque (density 1077; Nyegaard, Oslo, Norway) density centrifugation. Cells were subjected to two cycles of plastic adherence (60 min each) and then washed with Hanks' Balanced Salt Solution (HBBS; Gibco BRL, Grand Island, NY). The $CD34^+$ MNC fraction was isolated with superparamagnetic microbead selection using high-gradient magnetic field and mini-MACS column (Miltenyi Biotech, Gladbach, Germany). The efficiency of the purification was verified by flow cytometry counterstaining with anti-CD34-phycoerythrin (PE; HPCA-2; Becton Dickinson, San Jose, CA) and anti-CD38-fluorescein isothiocyanate (FITC) antibodies. In the cell fraction containing purified $CD34^+$ cells, the percentage of $CD34^+$ cells ranged from 84 to 95%.

Recombinant human cytokines

The following recombinant purified human cytokines were used in these studies: recombinant human (rh) stem cell factor (SCF), and rh TPO were a generous gift from Kirin (Kirin Brewery, Tokyo, Japan); rh granulocyte colony-stimulating factor (G-CSF) was from Genzyme (Cambridge, MA); rh granulocyte-macrophage colony-stimulating factor (GM-CSF); rh interleukin-6 (IL6) and rh interleukin-3 (IL3) were from Sandoz (Basel, Switzerland); rh Erythropoietin (EPO; EPREX) was from Cilag (Milan, Italy); rh FLT3-ligand (FL) was kindly provided by S.D. Lyman (Immunex Corp, Seattle, WA).

Stroma-free liquid cultures

Stroma-free expansion cultures were performed as follows:

Twenty to one-hundred thousand $CD34^+$ CB cells/ml in Iscove's Modified Dulbecco's medium (IMDM) supplemented with 10% pooled normal human serum in the presence of SCF (50 ng/ml) + FL (50 ng/ml) + TPO (20 ng/ml) + IL6 (10 ng/ml) or + IL3 (20 ng/ml) were deposited on the bottom of tissue culture T_{25} or T_{75} flasks in quadruplicate. Every week (or a few days, if the staring cell number was >50,000/ml) the culture volume was doubled (or more) according to cell counts. Cell counts were performed every week. At week 2, 4, 6, 8, 10, and 12 the immunophenotype of the cells harvested from the expansion cultures was performed and the colony-forming cells (CFC) content was determined by seeding suitable aliquots of the pooled flasks in triplicate plasma clot cultures. For long-term culture-initiating cell (LTC-IC) assay, limiting dilutions of the cell suspensions deriving from expansion flasks were seeded onto preirradiated stroma layers in 96-well plates for 5 weeks and then the number of CFC generated by methylcellulose cultures was enumerated as described belove.

Animals

NOD/LtSz scid/scid (NOD/SCID) mice were obtained from Charles River Italia, (Calco, Italy) and maintained in the animal facilities at the C.I.O.S. (Center of Immunogenetics and Experimental Oncology, C.N.R.,

Torino). All animals were handled under sterile conditions and maintained in cage microisolators. Mice to be transplanted were irradiated at 6 to 8 weeks of age with 350 cGy of total body irradiation from a ^{137}Cs source, and then within 24 h were given a single intravenous injection of: (1) human $CD34^+$ CB cells, which had previously separated from several CB samples, then pooled and cryopreserved (control cells). After thawing, 97 to 99% $CD34^+$ cells were viable by Trypan blue die exclusion; (2) cells harvested from expansion cultures as described. Also the latter cells were cryopreserved and injected at the same time as the control cells. When low numbers of unmanipulated $CD34^+$ cells were to be transplanted, at least 2×10^5 irradiated $CD34^-$ cells were coinjected as carrier cells. The same number of irradiated $CD34^-$ cells were also injected as a negative control, because no human engraftment could be detected. Mice were sacrificed 8 weeks post-transplant for assessment of the number and types of human cells detectable in both femurs and tibias.

Flow cytometric detection of human cells in murine BM

BM cells were flushed from the femurs and tibias of each mouse to be assessed using a syringe and a 26-gauge needle. To prepare cells for flow cytometry, contaminating red blood cells were lyzed with 8.3% ammonium chloride and the remaining cells were then washed in Hanks' Balanced Salt Solution (HBBS) with 0.1% bovine serum albumin (BSA; Sigma Chemical Co, Milan, Italy), 0.01% sodium azide. The cells were then resuspended at 1 to 2×10^6 cells/ml and processed as previously reported (28).

Hematopoietic cell cultures

Assays for granulopoietic, erythroid, megakaryocytic, and multilineage (granulocyte-erythroid-macrophage-megakaryocyte) colony-forming units (CFU-GM, BFU-E, CFU-Mk and CFU-GEMM, respectively) were usually performed as previously described (17,28). When transplanted NOD/SCID mouse BM cells were to be evaluated for their human hemopoietic progenitor content, the fetal calf serum (FCS) in the methylcellulose medium was replaced with an equivalent volume of a pretested pool of equivalently supportive normal human serum and bovine plasma in the plasma clot assay was replaced with an equivalent volume of human plasma. Plasma clot assays were adopted not only to detect CFU-Mk colonies (with the addition of IL3), but for CFU-GM, BFU-E, and CFU-GEMM as well (with the addition of IL3, GM-CSF, SCF, and EPO). G-CSF was omitted to minimize the stimulation of murine clonogenic cells. These culture conditions have been reported to be selective for colony formation by human progenitors and do not support coexisting murine progenitors (26,28). In addition, colonies grown in plasma-clot and colonies plucked from methylcellulose cultures were stained with FITC-conjugated anti-human GP IIbIIIa, CD45, CD13, and GpA and scored at the immunofluorescence microscope. The presence of fluorescent colonies was index of their human origin.

As a control, BM cells from untreated or irradiated carrier $CD34^-$-injected NOD/SCID mice were plated at identical cell concentrations in the same culture assays (plasma clot and methylcellulose) containing only human serum and/or human plasma and the above reported human-specific growth factors. Dishes were scored from day 12 up to day 21: in these culture conditions no colonies could be detected.

LTC-IC

The LTC-IC content of cell suspension was determined by limiting dilution assays as previously described (17,28).

RESULTS

Previous experiments have shown that CB $CD34^+$ cells, in fairly well-defined culture conditions, could be grown for extremely long periods of time and undergo a large expansion of more mature, and more primitive hematopoietic progenitors (17,18). In the experiments reported here, we cultured in the same stroma-free culture conditions $CD34^+$ cells in the presence of FL, SCF, TPO with the addition of either IL6 or IL3.

Figure 1 shows the proportion of different classes of hematopoietic progenitors at start of cultures and at different time points of expansion of $CD34^+$ CB cells. The four-factor combination containing IL3 (4F/3) appeared more powerful than the four-factor combination containing IL6 (4F/6), in that cell counts in the initial 8 weeks were nearly twice as much. Week 10 and week 12 cell counts were quite similar in both 4F/3 and 4F/6 (Fig. 1**A**). Expansion of $CD34^+$ cells and CFC output demonstrated different kinetics, in that it reached a peak at week 8 in the 4F/3 combination and then slowly decreased, while in the 4F/6 combination it kept increasing up to week 12 (Fig. 1**B** and 1**C**) and longer (not shown here). Accordingly, LTC-IC output appeared different for the two growth factor combinations: with the 4F/3 maximum LTC-IC number was obtained at week 4, but soon their number decreased and disappeared. By contrast with the 4F/6, LTC-IC number kept increasing with the passing of time (Fig. 1**D**).

To assess whether the two different sets of expanded cells retained their *in vivo* repopulating capacity,

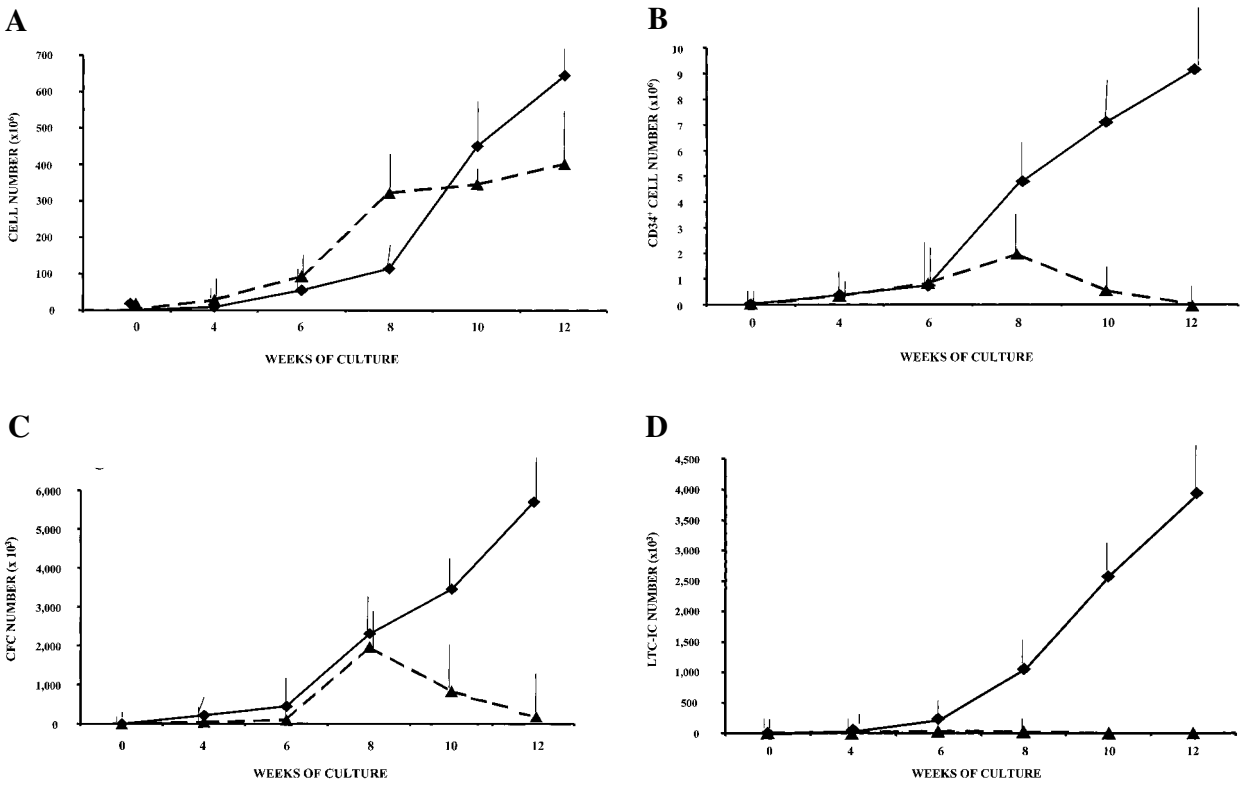

FIG. 1. Comparison between two growth factor combinations in stroma-free expansion cultures. (**A**) Total cell number counts; (**B**) Absolute $CD34^+$ cell counts; (**C**) Total CFC output numbers; (**D**) Total LTC-IC numbers. Twenty thousand $CD34^+$ CB cells/ml in IMDM containing 10% pooled AB serum were cultured in tissue culture flasks in the presence of FL+SCF+TPO+IL6 (—◆) and FL+SCF+TPO+IL3 (---▲) and cultured for up to 12 weeks. Every week the culture volume was doubled (or more, according to cell counts). At the indicated time points cells were harvested, counted, and aliquots analyzed for CD34 antigen expression. Suitable aliquots were plated in semisolid assays for CFC and layered onto preirradiated stroma layers for LTC-IC enumeration. Mean ± SEM of three separate experiments performed in quadruplicate.

NOD/SCID mice were transplanted with either unmanipulated $CD34^+$ either their progeny harvested at different points of expansion.

Groups of sublethally irradiated NOD/SCID mice were injected with $CD34^+$ CB cells, which had been separated from several CB samples and then pooled. Eight weeks after inoculation BM cells of the sacrificed animals were harvested from both femurs and both tibias and assessed for the presence of human hematopoietic cells. Table 1 presents a summary of these data.

TABLE 1. UNMANIPULATED CB $CD34^+$ CELLS ABILITY TO ENGRAFT THE BONE MARROW OF NOD/SCID RECIPIENTS

$CD34^+$[a]	*No. of positive mice*[b]	*Level of engraftment (%)*[c]
2×10^5	3/3	12.5; 21; 7
1×10^5	4/4	1.3; 3; 11; 25
5×10^4	4/5	1.4; 13; 6; 8; 0

[a]$CD34^+$ cells deriving from six different CB samples were separated as described, pooled, and then stored frozen. At the time of injection the samples were thawed, washed and resuspended in 300 μl of IMDM containing 10% FCS and then injected into three or five animals as described. BM of NOD/SCID recipients was recovered from both the femurs and the tibias at week 8 post-transplant and analyzed for the presence of human hematopoietic cells.

[b]Positive mouse: >0.1% of human CD45+, CD71+, and GpA+ cells.

[c]% of human (CD45, CD71, and GpA)$^+$ cells in the whole BM.

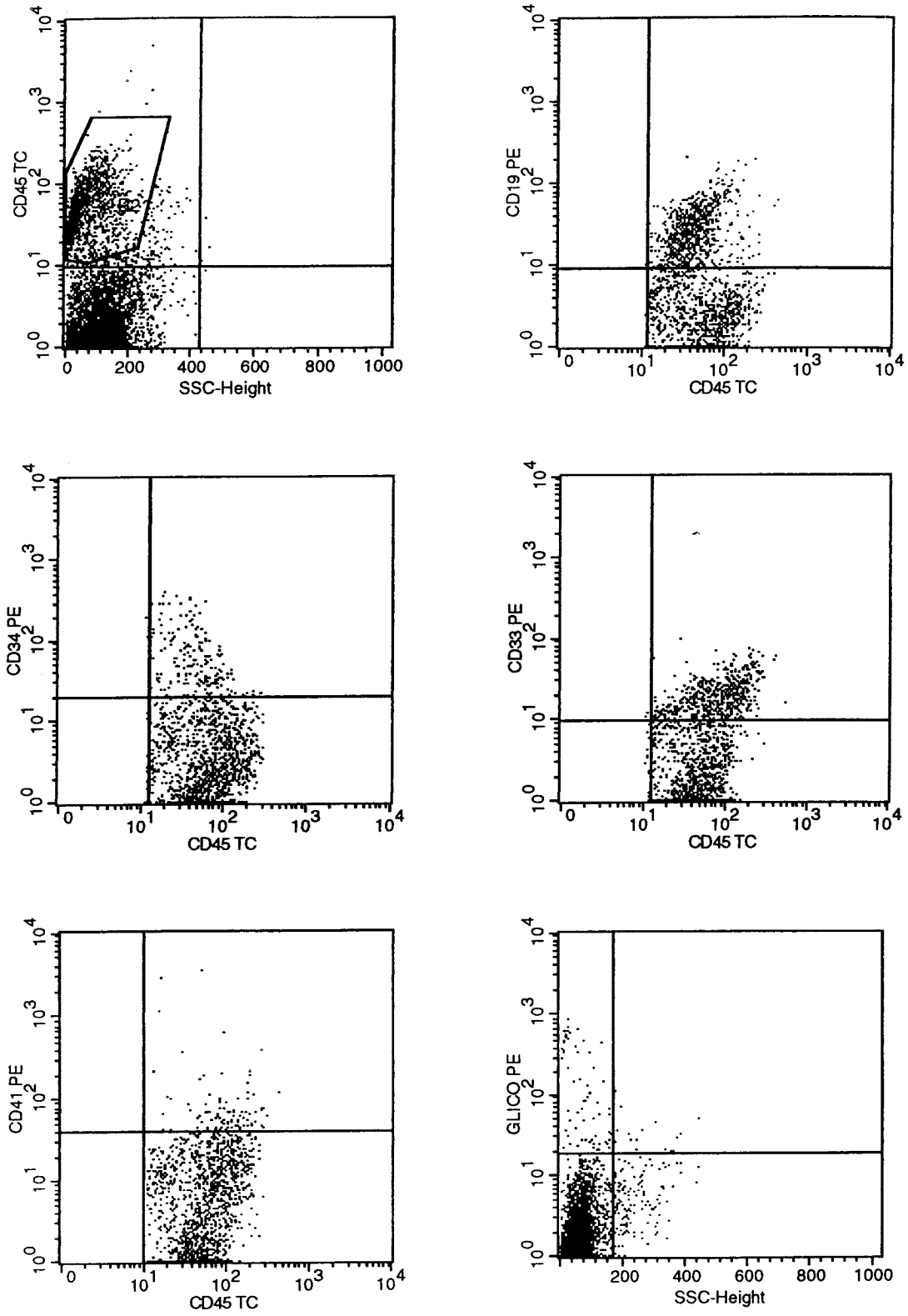

FIG. 2. Representative FACS profiles of marrow cells from a NOD/SCID mouse transplanted 8 weeks previously with 1×10^5 unmanipulated $CD34^+$ cells. Analysis of lineage markers was performed on cells comprised within the CD45 gate. Analysis of GpA/CD71-positive cells was performed on total BM cells.

2×10^5, 1×10^5, and 5×10^4 $CD34^+$ cells engrafted the near totality of mice. The level of engraftment was variable and dependent on the number of injected $CD34^+$ cells (human $CD45^+$, $CD71^+$ and GpA^+ cells ranged from 1.3 to 25%). BM cells of engrafted mice were further analyzed for evidence of multilineage development from input $CD34^+$ cells. Cells within the huCD45 gate or costained with CD45 were quantified for myeloid and lymphoid surface markers as well as for the expression of the CD34 antigen, erythroid, and megakaryocyte surface markers (Fig. 2). As additional proof of human myeloid engraftment, BM cells of sacrificed mice were cultured in semisolid assays in culture conditions that have been reported to allow human and not mouse colony growth (26,28). In addition, colonies were stained with monoclonal antibodies recognizing human total leukocytes, granulocyte-macrophages, erythroid cells and megakaryocytes, and only fluorescent colonies (consisting of human cells) were counted. BM of mice injected with 2×10^5 and 5×10^4 unmanipulated CB $CD34^+$ cells generated $55 \pm 36 \times 10^3$ and $30.5 \pm 14.76 \times 10^3$ human colonies (CFU-GM+BFU-E+CFU-GEMM+CFU-Mk), respectively.

To assess whether the expanded $CD34^+$ CB cells retained their ability of full hematopoietic reconstitution, NOD/SCID mice were irradiated with 350 from a ^{137}Cs source and injected with either 100,000 $CD34^+$ CB at start of cultures, either the corresponding progeny of similar initial number of $CD34^+$ cells that were grown for 4, 6, 8, 10, and 12 weeks with 4F/6 or 4F/3. Figures 3 and 4 summarize the data obtained.

In keeping with previously reported data, progeny of 1×10^5 initial $CD34^+$ cells could engraft at very good levels in NOD/SCID mice after 12 weeks of liquid cultures in the presence of FL, SCF, TPO, and IL6. Human $CD45^+$ cell range was 4.3 to 22.4%. Cells within the CD45 gate or co-stained with CD45 were also analyzed for evidence of multilineage engraftment (Fig. 5). The data obtained were quite similar to those found when unmanipulated $CD34^+$ cells were injected. In addition, the BM of mice transplanted with week 12-expanded cells generated $76 \pm 42.745 \times 10^3$ human colonies (mean $\pm$ SD) of the four experiments reported on Fig. 3). By contrast, the same $CD34^+$ cells, that had been cultured for 2 and 4 weeks in the presence of FL+SCF+TPO+IL3 were capable of engrafting only a proportion (3 out of 8 and 4 out of 10, respectively) of the injected animals. In addition, the level of engraftment was much lower (4.8 and 1.8% for 2 and 4 week-expanded cells, respectively) than that of the initial unmanipulated $CD34^+$ counterpart. Cells expanded for 6 weeks did not show any significant engraftment capacity.

The high levels of engraftment observed with FL+SCF+TPO+IL6-expanded cells and the excessively high cell counts reached after 12 weeks of expansion cultures prompted us to inoculate smaller and smaller fractions of the ex vivo generated cells. Table 2 summarizes the data obtained in two separate experiments.

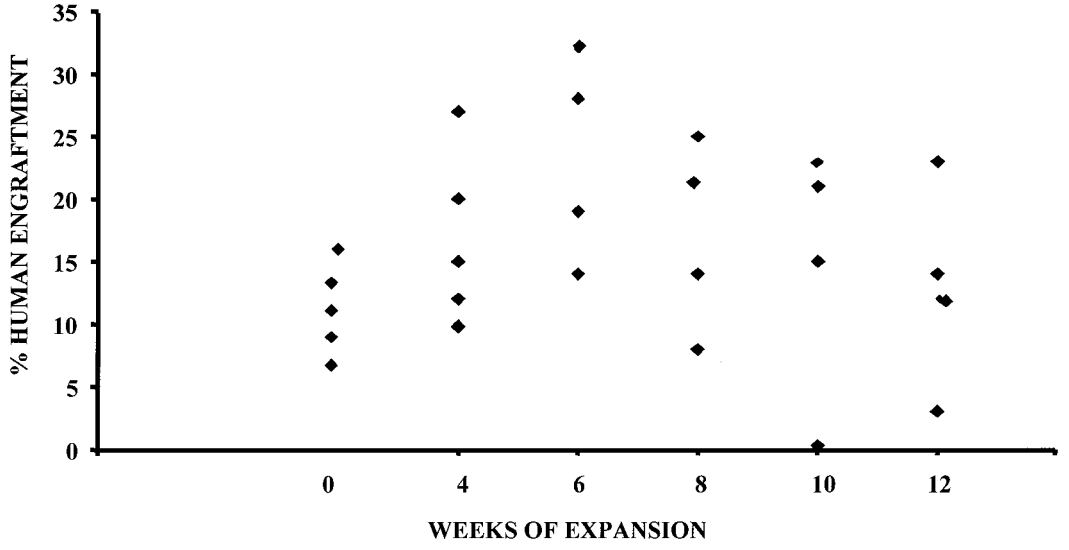

FIG. 3. Summary of the level of human cell engraftment in the BM of mice transplanted with cells harvested at different time points from *ex vivo* expansion cultures in the presence of FL, TPO, SCF, and IL6. Individual NOD/SCID mice (each symbol represents a mouse) were injected 8 weeks previously with fractions of the expanded cells, corresponding to 1×10^5 initial $CD34^+$ cells. The level of human engraftment in the mouse BM was evaluated by flow cytometry (as % of human CD45, GpA, and CD71 positive cells).

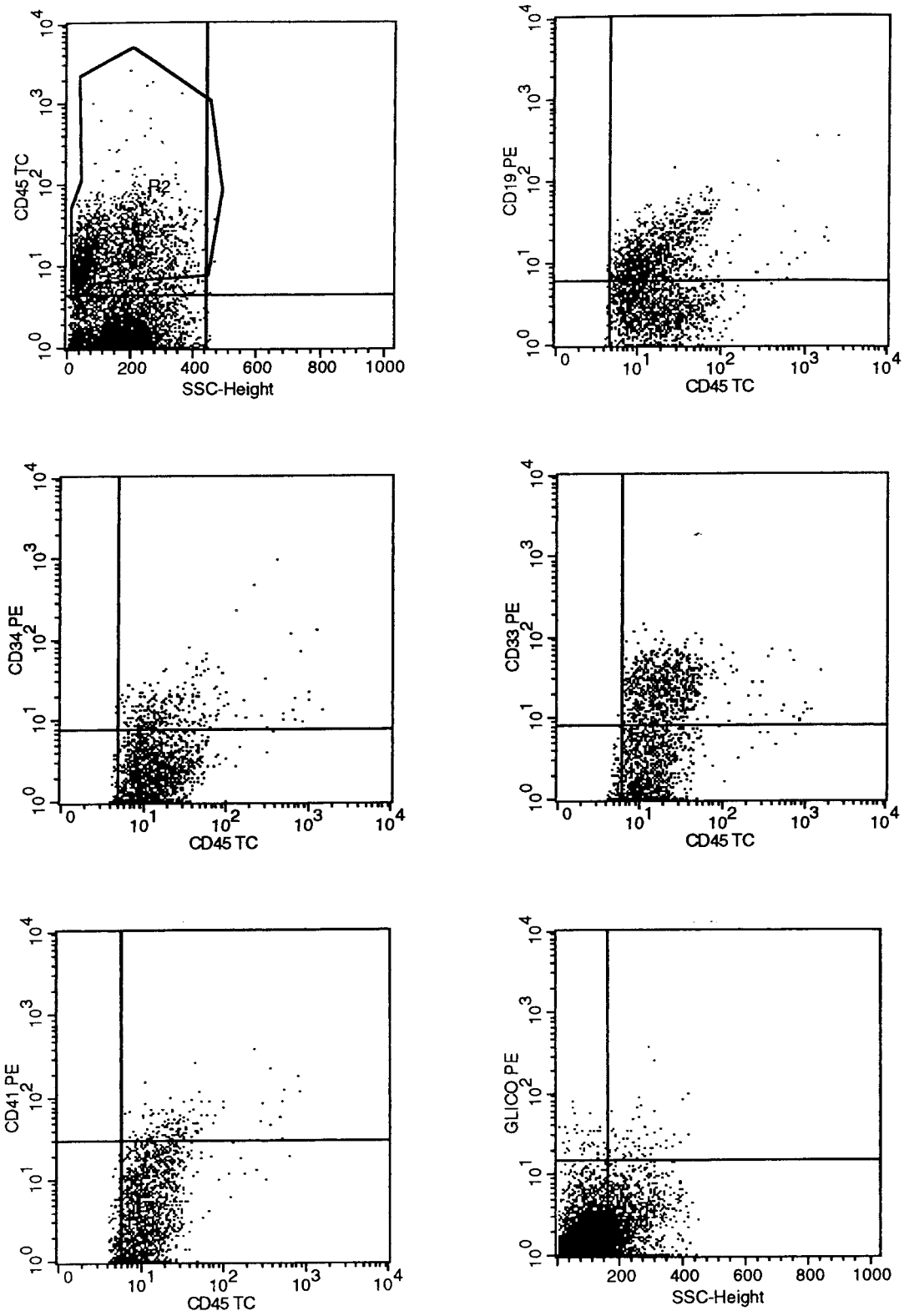

FIG. 4. Representative FACS profiles of marrow cells from a NOD/SCID mouse transplanted 8 weeks previously with the corresponding progeny generated by initial 1×10^5 $CD34^+$ cells expanded for 12 weeks in the presence of FL+SCF+TPO+IL6. Analysis of lineage markers was performed as explained in Fig. 2.

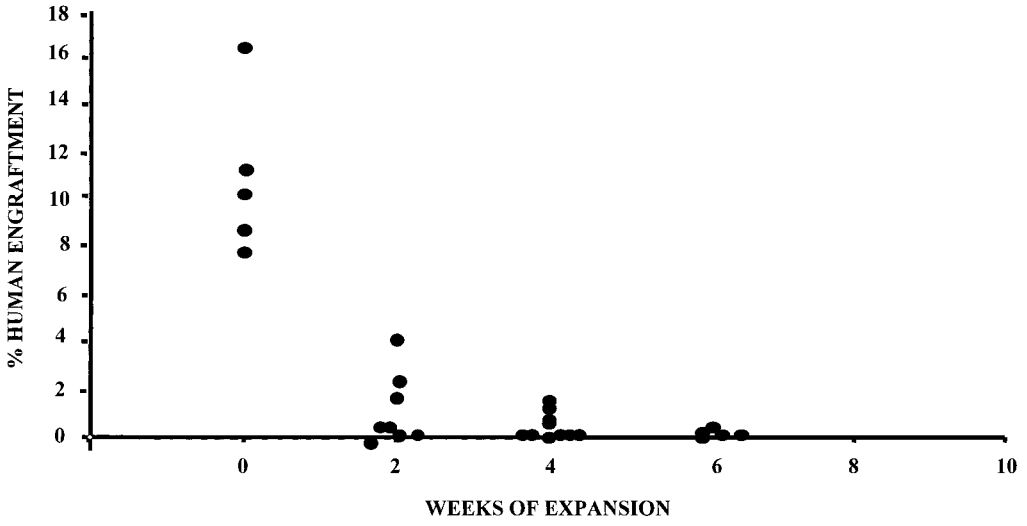

FIG. 5. Summary of the level of human cell engraftment in the BM of mice transplanted with cells harvested at different time points from ex-vivo expansion cultures in the presence of FL, TPO, SCF, and IL3. Individual NOD/SCID mice (each symbol represents a mouse) were injected 8 weeks previously with fractions of the expanded cells, corresponding to 1×10^5 initial $CD34^+$ cells. The level of human cell engraftment in mouse BM was evaluated by flow cytometry (as % of human CD45, GpA, and CD71 positive cells).

DISCUSSION

Several cytokine combinations have been tested for maintenance and expansion of human hematopoietic stem/progenitor cells (10,16–18,29–34). Until recently, however, most of the human HSC expansion studies aimed at clinical applications have used *in vitro* assay for $CD34^+$ cells, colony-forming cells in clonal culture, LTC-IC to optimize the culture conditions, but these surrogate assays have been shown not to reflect stem cell activity correctly (27,35,36). The establishment of the NOD/SCID mouse model has been instrumental in measuring the *in vivo* engraftment properties of human expanded hematopoietic stem cells (26,27).

Our previous studies show that a combination of FL, SCF, TPO, and IL6 was capable of supporting the expansion of hematopoietic progenitors and more primitive LTC-IC from CB $CD34^+$ cells. The same combination could also maintain and expand the *in vivo* repopulating ability of human stem cells (17,18,28).

IL3 supports the development of multiple hematopoietic lineages by interacting with multipotential and lineage-committed progenitors in culture (2,37,38). Previous reports on the effect of IL3 on the expansion of primitive hematopoietic cells were controversial even though they found it stimulates the expansion of relatively mature hemopoietic progenitor cells. IL3, as a single factor, supports proliferation of the progenitors after they exit from the cell-cycle dormant state (G0) (2,39). IL3 also synergizes with IL6 (40), IL11 (41,42), G-CSF (43), leukemia inhibitory factor (44), TPO (45,46), and SCF (47,48) in triggering cell divisions of the multipotential progenitors in G0.

Matsunaga and colleagues noted negative effects of IL3 on the ability of cultured cells to engraft the marrow of recipient mice (49). This observation is in agreement with the report from Peters and colleagues (50), demonstrated that suspension culture of murine marrow cells in the presence of IL3, IL6, IL11, and SCF results in impairment of the engrafting capability of the cultured cells. These observations raised the possibility that IL3 may be a stage-specific negative regulator and that it may suppress the earliest process of hematopoiesis, for example, self-renewal of the stem cells. The negative effects of IL3 observed in murine models may be relevant to *in vitro* manipulation of human stem cells.

To address this issue in the human setting, in this study we compared the effect of two early acting cytokine combinations containing FL+SCF+TPO with IL6 or IL3 on maintenance, proliferation, and *in vivo* repopulating capability of expanded CB $CD34^+$ cells. Our results show that CB $CD34^+$ cells can be grown and greatly expanded after 12 weeks in stroma-free cultures in the presence of FL, SCF, TPO, and IL6. When assayed in the NOD/SCID model, expanded $CD34^+$ cells retained their capacity to completely engraft the BM of sublethally irradiated NOD/SCID recipients. The level of engraftment, similar or greater than that usually observed when the same number of uncultured cells were injected in the same recipients, suggested that SCID repopulating cells (SRC) were not only maintained, but, probably, expanded. By contrast, when the same CB $CD34^+$ cells were cultured with

TABLE 2. CHARACTERISTICS OF WEEK 12-EXPANDED CELLS AND THEIR ENGRAFTMENT IN NOD/SCID MICE

Ex-vivo *expansion*					*Injection* in vivo			*Engraftment*	
Source of cells	*Growth factor added*	*Cells*[a] *(fold increase)*[b]	*CFC (fold increase)*	*LTC-IC (fold increase)*	*Cells injected*	*CFC injected*	*LTC-IC injected*	*% Human cells*[c]	
Exp. 1									
Start	no	50,000	3293	5,062	50,000	3,293	5,062	2.8	5.4
					10,000	866.4	1,332	0	0
					5,000	433	666	0	0
					2,500	216	333	0	0
					1,250	108	166	0	0
12 weeks	SCF + FL + TPO +	381×10^6	10,181,714	1,337,994	13.6×10^6	363,632	47,785	11	16
	IL6	(7,620)	(3,091.9)	(264)	6.8×10^6	181,816	23,892	8	6.8
					3.4×10^6	90,908	11,946	14	0.9
Exp. 2									
Start	no	100,000	9,442	15,254	100,000	9,442	15,254	14.3	9.8
					20,000	2,248	3,632	0	0
					5000	812	908	0	0
					2500	406	454	0	0
					1250	203	227	0	0
12 weeks	SCF + FL + TPO +	2059×10^6	8,493,056	2,843,334	88.75×10^6	366,080	122,580	ND	ND
	IL6	(20,590)	(899.5)	(186.4)	22.20×10^6	91,520	30,641	11	6.5
					11.10×10^6	45,760	15,322	0.6	3.9
					5.50×10^6	22,880	7,661	0.9	0.5

[a]$CD34^+$ cells were cultured at 5×10^4/ml or 1×10^5/ml in T-flasks as described. Cells at the start of cultures and cells harvested after 12 weeks of suspension cultures were cryopreserved and kept frozen until the time of injection in NOD/SCID mice.

The expansion cultures were performed in quadruplicate and then pooled. Two mice were injected each with 100,000, 50,000 (or less, as indicated) uncultured cells and two mice each with the content of a single expansion culture or a fraction of it (as indicated).

Two different experiments are represented.

[b]Between brackets: fold increase (compared to the input value).

[c]Each value represents the percentage of human $CD45^+$, $CD71^+$, and a-GPA^+ cells detected in the BM of each individual mouse.

FL, SCF, TPO and IL3, their content in SRC was maintained, but only at low degrees for no longer than 4 weeks.

Only a few studies with combinations of various cytokines have been reported so far on the *ex vivo* expansion of human hematopoietic stem cells, the long-term repopulating capacity of which has been proved *in vivo* (16,17,22,25,26).

Bhatia and colleagues (51) and Conneally and colleagues (52) reported about the expansion of *in vivo* repopulating cells in liquid cultures containing also IL3, but the role of IL3 in that context was not further elucidated.

The reports on the effect of IL3 on HSC expansion have differed for mice and humans. Culture of murine HSC with IL3 was detrimental to the maintenance of stem cell activity, as was shown by the repopulating ability in lethally irradiated mice (13,14). By contrast, it was reported that IL3 did not affect negatively the long-term repopulating ability of human HSCs (15). Here, we demonstrate that IL3 exhibits an inhibitory effect on the expansion of human LTR-HSCs. Because IL3 could stimulate the increase of mature blood cells and HPCs, IL3 might consume human HSCs by increasing their differentiation. The discrepancy between our observation and the previous reports may be caused by the difference in the culture conditions or target cells.

Our previous findings showed that $CD34^+$ CB cells could be grown in vitro for up to 9–10 weeks and still retain their ability to repopulate the bone marrow of sublethally irradiated NOD/SCID mice. By limiting dilution studies SRC expansion was calculated to be 70-fold (28).

The present paper shows that CB SRC can be maintained for 12 weeks of *ex vivo* cultures in the presence of FL, SCF, TPO, and IL6. The degree of expansion has not been fully calculated because of the low number of mice employed in this study.

The vast majority of prior studies aimed at developing clinical applications of expansion protocols have adopted culture conditions that resulted in a marked expansion of cell counts, $CD34^+$ cells, CFC, and even LTC-IC in a short period of time. However, the expansion was transient, soon followed by a rapid decline of cell number, of CFC output and disappearance of LTC-IC, which indicated the exhaustion of the stem cell pool. Our culture system shows that it is possible to obtain large numbers of cells and progenitors belonging to the more mature hemopoietic compartments and, at the same time, to maintain and even expand several-fold the primitive *in vivo* repopulating stem cells. However, in the same culture system, the presence of IL3, that in the early phases of expansion seems to induce an even greater or equal expansion of cells, CFCs and also LTC-ICs and SRC, in the long-run appears to be detrimental, probably because of the triggering of differentiation pathways, rather than self-renewal. Ongoing studies on secondary transplant will allow us to ascertain whether the cells expanded with the two growth factor combinations possess the same self-renewal potential.

These informations could prove essential to design and test conditions for *ex vivo* activation and expansion of immature hematopoietic cells and for various experimental purposes, such as required for the development of efficient gene transfer protocols into hematopoietic cells with retention of repopulating ability.

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