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Different growth factor requirements for the *ex vivo* amplification of transplantable human cord blood cells in a NOD/SCID mouse model

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ABSTRACT: The growth factor combination containing early acting cytokines FLT-3 ligand (FL), Stem Cell Factor (SCF) and thrombopoietin (TPO) is able to maintain, for an extended culture period, early stem cells, defined as long-term repopulating NOD/SCID mice (Scid Repopulating Cell-SRC) contained in cord blood (CB). In this culture system, the role of IL-6 and IL-3 has not been clearly established.

Using a combination of FL+TPO+SCF with or without IL-6, we were able to form CB CD34$^+$ cells for 30 weeks. The CB CD34$^+$ cells cultured in this system engrafted NOD/SCID mice after 6 weeks of culture; the cells from primary recipients were also able to engraft secondary NOD/SCID mice.

When CB CD34$^+$ cells were cultured in the presence of IL-3 in the place of IL-6 we observed an even better expansion of cells and a similar clonogenic progenitor output in the first 8 weeks of culture. However, more primitive LTC-IC output increased up to week 6 with the growth factor combination containing IL-3 and then decreased and disappeared, while with the growth factor combination with or without IL-6 increased up to week 23. Cells cultured for 4 weeks with the 4-factor combination containing IL-3 engrafted NOD/SCID mice less efficiently. Repopulation of NOD/SCID mice was no longer observed when *ex vivo* expansion was performed for 6 weeks.

This study provides some evidence that no differences could be detected in long-term maintenance and even expansion of human primitive cord blood cells cultured with FL+TPO+SCF in the presence or absence of IL-6. Under the culture conditions employed in this study, the presence of IL-3 reduced the repopulating potential of expanded CB CD34$^+$ cells. (J Biol Regul Homeost Agents 2001; 15: 38-48)

KEY WORDS: *Cord blood, Long-term culture, Expansion, Cytokines, NOD/SCID mice*

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INTRODUCTION

High-dose chemotherapy protocols are increasingly used to treat a number of malignant diseases, solid tumors and hematological diseases. The most prominent effect of the treatment is hematological toxicity, which requires stem cell rescue. For this reason transplants of hematopoietic stem cells (HSC) play an important role in the treatment of many malignancies (1-4).

The use of bone marrow as a source of transplantable hematopoietic progenitors has long been established. Also mobilized peripheral blood cells, because of easier collection and shorter duration of cytopenia following cell reinfusion, have been used to transplant myeloablated patients (5-7).

Human umbilical cord blood has long been recognized as a rich source of primitive and committed hematopoietic progenitors. In addition, the general availability and the ease of procurement make cord blood a very attractive alternative source of transplantable hematopoietic tissue (8). However, the major limitation to a widespread use of cord blood for transplantation lies is its physiological small volume. Until now, cord blood transplant has been mainly restricted to children and small size adults (weight < 40 kg). Therefore its use as graft for adult patients might require *ex vivo* manipulation.

Thus, an important goal is the identification of mechanisms and conditions that support the expansion of transplantable hematopoietic stem cells. Indeed the *ex vivo* expansion can only be considered successful when progeny cells retain both the pluripotent differentiation and self-renewal capacities of the original stem cells (9, 10).

Many studies unequivocally demonstrated that combinations of early-acting cytokines efficiently promote long-term repopulating hematopoietic stem cells to undergo self-renewing divisions (11). It has been demonstrated that early acting cytokines like Stem Cell Factor (SCF), FLT3-ligand (FL) and thrombopoietin (TPO) can be used for more primitive hematopoietic stem cell expansion (12). In the last decade some

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works demonstrated that Interleukin-6 (IL-6) is an efficient cofactor for survival and proliferation of primitive multilineage progenitors cells (13, 14). Controversial instead is the role of Interleukin-3 (IL-3) on the expansion of primitive hemopoietic stem cells. Some studies showed that IL-3 supports expansion of long-term multilineage repopulating activity (15). By contrast several papers demonstrate the negative influence of IL-3 on the expansion of primitive hematopoietic stem cells, suggesting that it stimulates the expansion of relatively mature hematopoietic cells (16-18).

The development of assays suitable for the detection of human stem cells, their distinction from other hematopoietic stem cells with more restricted proliferative potentials, and their selective expansion are important tasks. Over the past decade various *in vitro* and *in vivo* assays have been developed to estimate human stem cell frequencies. The Colony-Forming Cells (CFC) (20, 21) and Long-Term Culture -Initiating Cell (LTC-IC) (22) assays have been proposed to determine and enumerate primitive progenitor cells with long-term repopulating ability *in vitro*. A quantitative *in vivo* assay for human hematopoietic stem cells has been established by the recent development of the Nonobese Diabetic/Severe Combine Immune Deficiency (NOD/SCID) mouse model (23).

In this work we examined the expansion of human long-term repopulating stem cells cultured for several weeks on a stoma free system, in the presence of a three growth factor combination. We compared the CFC and LTC-IC output and *in vivo* repopulating capacity of human hematopoietic stem cells expanded using FL+TPO+SCF±IL-6 and FL+TPO+SCF±IL-3. The presence of IL-3 was found to be negative, in that it inhibited both the extent of LTC-IC expansion and the long-term generation of CFC. This negative effect was also visible in the NOD/SCID mouse transplantation model. By contrast, the addition of IL-6 did not appear to substantially modify the extent of LTC-IC expansion, but however appeared capable of enhancing the proliferative potential of at least a subpopulation of daughter stem cells.

Materials and methods

Recombinant human cytokines

The following recombinant purified human cytokines were used in these studies: recombinant human (rh) Thrombopoietin (rhTPO) and rh Stem Cell Factor (rhSCF) were generous gifts from Kirin (Kirin Brewery, Tokyo, Japan); Granulocyte Colony-Stimulating Factor (rhG-CSF) was from Genzyme (Cambridge, MA); rh Granulocyte-Macrophage Colony-Stimulating Factor (rhGM-CSF), rh Interleukin-6 (rhIL-6) (Peprothec Inc (NJ USA) and rh Interleukin-3 (rhIL-3) were from Sandoz (Basel, Switzerland); rh Erythropoietin (rhEPO; EPREX) was from Cilag (Milan, Italy); rh FLT-3 Ligand was kindly provided by SD Lyman (Immunex Corp, Seattle, WA).

Human cells

Umbilical cord blood (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 g/cm^3; Nyegaard, Oslo, Norway) density centrifugation. Cells were subjected to two cycles of plastic adherence (60 minutes each); and then washed with Hanks' Balanced Salt Solution (HBBS, GIBCO BRL, Grand Island, NY). The $CD34^+$ fraction was isolated with superparamagnetic microbead selection using high-gradient magnetic field and miniMACS column (Miltenyi Biotech, Gladbach, Germany). The efficiency of the purification was verified by flow cytometry counter staining with an anti-CD34-phycoerythrin (PE; HPCA-2; Becton Dickinson, San Jose, CA) and anti-CD38-fluorescein-isothiocyanate (FITC, Dako A/S Denmark) antibodies. In the cell fraction containing purified cells, the percentage of $CD34^+$ cells ranged from 90 to 98%.

Stroma-free liquid cultures

Stroma-free expansion cultures were performed as follows:

I. A total of twenty thousand $CD34^+$ cord blood cells were cultured in quadruplicate flat-bottomed 24-well plates in 1 ml of Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% Fetal Calf Serum (FCS) in the presence of the following growth factor combinations: FL (50 ng/ml) + SCF (50 ng/ml) +TPO (20 ng/ml) ±IL-3 (20 ng/ml) or ±IL-6 (10 ng/ml), which were added twice a week. The wells were incubated at 37° C and 5% of CO_2. At initiation of the cultures the number of CFC and CFU-Mk present in 1 ml of a single well was determined by triplicate Plasma Clot assays. Every week all of the wells were demidepopulated by removal of one half the culture volume (and cells), which was replaced with fresh medium and growth factors. Cells of the harvested media were counted and suitable aliquots of the cell suspensions were assayed for CFC and CFU-Mk content, for immunophenotype analysis ($CD34^+$, $CD34^+$ $CD38^-$) and for LTC-IC determination every 2 to 3 weeks by Limiting Dilution Assays (LDA). The total number of CFC or for LTC-IC generated CFC was calculated as previously reported (23).

II. Then, in a series of experiments 5 x 10^4 $CD34^+$ cells/ml were deposited on the bottom of tissue culture

T75 flask in quadruplicate. Every week the culture volume was doubled. Cell counts were performed every week. At weeks 2 and 4 the immunophenotype of the cells harvested from the different sets of expansion was performed, and the CFC content of each expansion set was determined by seeding suitable aliquots of the pooled flasks in triplicate Plasma Clot cultures. For LTC-IC assay, limiting dilutions of the cell suspensions deriving from each series of expansion flasks were seeded onto pre-irradiated stroma layers in 24-well-platets for 6 weeks and then the number of CFC generated by Plasma Clot cultures was enumerated as described elsewhere (23).

Clonogenic assays

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 follows. For CFU-GM, 1 x 10^3 $CD34^+$ CB cells of the initial cell suspension or suitable aliquots of the stroma-free long-term cultures were cultured at 4 platelets per point in 3% Agar, 15% FCS (Hyclone, Logan, UT) in IMDM. For CFU-Mk, the same number of cells was cultured in 1.3% MTC and IMDM containing 30% FCS at 37° C in a humidified atmosphere at 5% CO_2 in air. Colony scoring was performed on day 12 for CFU-Mk (at the immunophosfluorescent microscope after staining with an FITC-conjugated MoAb recognizing Human GPIIbIIIa) and on day 14 for CFU-GM, BFU-E and CFU-GEMM (24, 25). Several growth factors were added at optimum concentrations to sustain the formation of BFU-E and CFU-GEMM: rhIL-3 (20ng/ml), rh SCF (50ng/ml), rh GM-CSF (10ng/ml, rh Epo (3U/ml). For CFU-Gm, rh GM-CSF (20ng/ml,) rh IL-3 (20ng/ml), rh SCF (50 ng/ml) were added. For CFU-Mk, rh IL-3 (5ng/ml) was used as a single growth factor.

When transplanted NOD/SCID mouse BM cells were to be evaluated for their human hemopoietic progenitor content, the 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 rhIL-3), but for CFU-GM, BFU-E and CFU-GEMM as well (with the addition of rhIL-3, rhGM-CSF, rhSCF and rhEPO). 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 (8, 13). In addition, colonies grown in plasma-clot and colonies plucked from methylcellulose cultures were stained with FITC-conjugated anti-human GPIIbIIIa, CD45, CD13 and GpA and scored at the immunofluorescence microscope. The presence of fluorescent colonies was an index of their human origin. As a control, bone marrow 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.

The LTC-IC content of cell suspension was determined by limiting dilution assays as previously described (24, 25).

Injection of cells in NOD/SCID mice

NOD/LtSz scid/scid (NOD/SCID) mice bred at Jackson Laboratories (MI, USA) were obtained from Charles River Italia (Calco, Italy), and maintained in the animal facilities at C.I.O.S. (Torino, Italy).

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 hours given a single intravenous injection.

Mice were sacrificed 6-8 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 bone marrow

Bone marrow cells were flushed from the femurs and tibias to be assessed using a syringe and 26-gauge needle. The cells (from bone marrow and peripheral blood) were resuspended at 1 to $2x10^6$ cells/ml and incubated with mouse IgG (Fluka Chemika Biochemika, Buchs, Switzerland), to block non-specific binding to Fc receptor. Cells were then incubated with MoAb specific for human CD45, CD71 and glycophorin-A (GpA), directly labeled with FITC or PE for 30 minutes at 4° C to assess population of human hematopoietic cells. Additional aliquots of cells were stained with antihuman CD33-PE (DAKO A/S Denmark), CD19-PE (DAKO), CD41-PE (DAKO), CD34-PE (Becton Dickinson) in combination with anti-human CD45-FITC (CALTAG Laboratory) to allow discrimination of subpopulation with the CD45 gate. Some cells from each suspension were similarly incubated with irrelevant (control) MoAbs labeled with FITC and PE. After staining all cells were washed once in PBS with 0.1% BSA and 0.01% sodium azide. Then, contaminating red blood cells were lysed with EDTA 10-4 mol/L, $KHCO_3$ 10^{-3} mol/L, NH_4CL 0.17 mol/L. Flow cytometric analysis was performed using a FACSCalibur cytometer (Becton Dickinson). At least 10,000 events were acquired for each analysis. Analysis was performed with CellQuest software (Becton Dickinson).

RESULTS

In vitro *effects of different hematopoietic growth factor combinations*

In an effort to induce long-term proliferation and amplification of primitive hematopoietic progenitors contained in CD34$^+$ CB cells, we set up stroma-free suspension cultures in which a number of cytokines known to be active on more immature hematopoietic progenitors (IL-3 and IL-6) in combination with early acting cytokines SCF, TPO and FL were used.

Three different combinations of growth factors have been used (FL+TPO+SCF; FL+TPO+SCF+IL-3; FL+TPO+SCF+IL-6) in order to identify the specific growth factor combination that could stimulate an optimum expansion of LTC-IC in stroma-free liquid cultures initiated with CB CD34$^+$ cells and to determine how these growth factor requirements might compare to those needed to maximize CFC production from the same starting population.

Purified CB CD34$^+$ cells were grown in 24 well plates or in tissue culture T75 flasks (for small and large scale expansions respectively). In an attempt to carefully investigate the proliferative events that take place during the long-term culture period, different stages of hematopoiesis were monitored:

CD34$^+$ subpopulation: The CD34 expression by hematopoietic cells was maintained throughout the course of LTC. The CD34$^+$ population underwent continuous expansion in the presence of FL+TPO+SCF and FL+TPO+SCF+IL-6 (more than 190,000-fold and 170,000-fold the initial number after 20 weeks of liquid culture respectively) (Fig. 1). Expansion of CD34$^+$ cells reached a peak at week 8 in the presence of FL+TPO+SCF+IL-3 and then decreased, so that the cultures lasted only 12 to 16 weeks (Fig. 1).

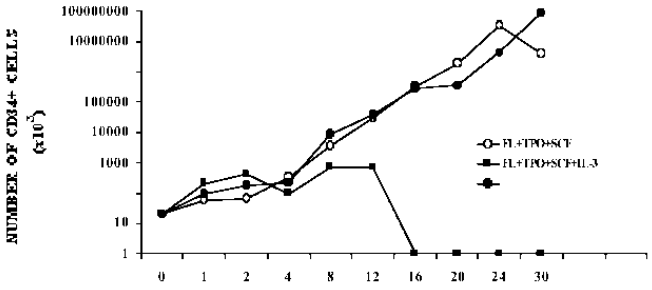

Fig. 1 - Long-term expansion of CD34$^+$ CB cells in stroma-free LTC. The results represent the mean fold increase of four representative experiments performed in quadruplicate.

CFC output (i.e. CFU-GM+BFU-E+CFU-GEMM+CFU-Mk number): During the long-term suspension cultures, the granulocyte-macrophage, megakaryocyte, erythroid and mixed progenitors (CFC) were generated and amplified (Tab. I). As shown in Figure 1, in the presence of FL+TPO+SCF, the CFC output reached 109 million-fold the initial number and in the presence of FL+TPO+SCF+IL-6 the CFC levels were ~ 48 million-fold the number of CFC at start of the suspension cultures. A decline of the CFC production was initially seen between week 28 and 30 and after week 33 hemopoietic colonies could no longer be detected (Fig. 2). The presence of IL-3 in addition to FL+TPO+SCF improved neither the extent nor the persistence of CFC output and induced a ~ 6,000-fold increase of CFC output by week 12 and no colonies were detectable by week 16 (Fig. 2).

LTC-IC output (i.e. number of CFU-GM+BFU-E+CFU-GEMM generated in 14 to 21 day methylcellulose cultures generated by trypsinized+nonadherent cells obtained after 6 weeks of stroma co-cultures): The ability of the three different growth factor combinations to

TABLE I - EFFECT OF DIFFERENT GROWTH FACTOR COMBINATIONS ON CFCS EXPANSION

	WEEKS OF CULTURE																	
	week 2			week 6			week 12			week 16			week 20			week 24		
Growth factors	-GM	-Mk	-E	-GM	-Mk	-E	-GM	-Mk	-E	-GM	-Mk	-E	-GM	-Mk	-E	-GM	-Mk	-E
FL+TPO+SCF	69 ±7	4.5 ±2	1.2 ±0.8	77 ±11	32 ±8	0 ±0	12,453 ±998	70 ±15	404 ±91	168,292 ±1,195	3,273 ±119	421 ±93	545,569 ±5349	10,278 ±3135	936 ±129	17,373,935 ±17,229	146,526 ±27,653	586,104 ±94,675
FL+TPO+SCF+IL-6	50 ±8	5 ±1	1.4 ±0.2	386 ±97	39 ±12	0 ±0	13,639 ±895	350 ±28	280 ±27	117,497 ±618	3,152 ±426	2,656 ±258	875,577 ±65,768	47,018 ±4,305	29,209 ±2,441	38,035,423 ±26,925	190,650 ±34,732	266,684 ±29,222
FL+TPO+SCF+IL-3	11 ±2	6.4 ±4	0.9 ±0.2	100 ±22	15 ±4	0 ±0	744 ±71	90 ±80	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0

20-50 x 10^3 CD34$^+$ cord blood cells were grown in stroma-free cultures with indicated growth factors for 24 weeks. At the indicated time intervals suitable aliquots of each culture were seeded in agar, plasma clot and methylcellulose assays.
Values shown represent the mean±s.e.m of the fold increase, relative to the input number obtained from four separate representative experiments.

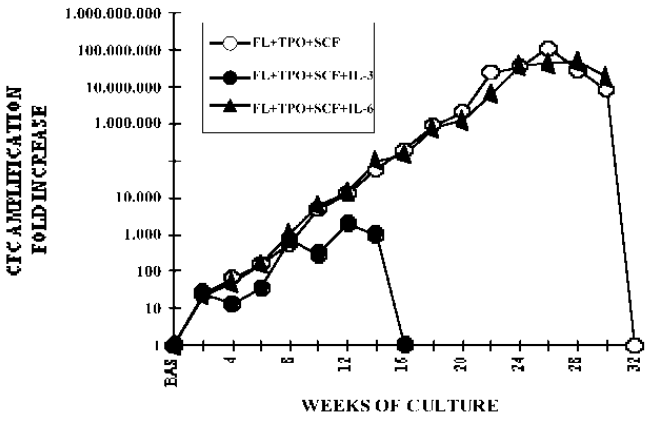

Fig. 2 - Long-term expansion of CFCs from CD34⁺ CB cells in stroma-free LTC. The results represent the mean fold increase of four representative experiments performed in quadruplicate.

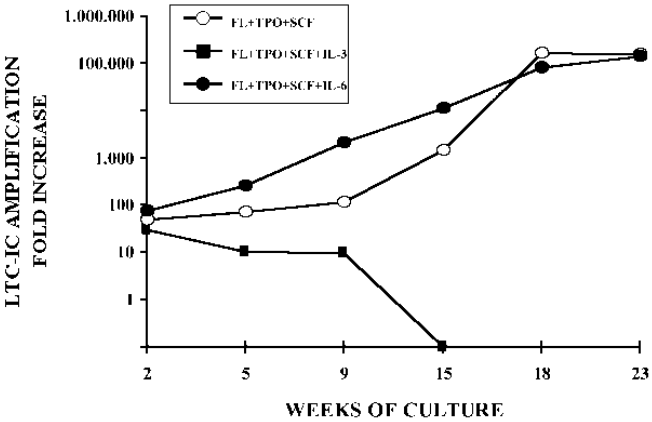

Fig. 3 - Kinetics of CB LTC-IC expansion over time. Generation and expansion of primitive stem cells (identified as LTC-IC). The results represent the mean fold increase of four representative experiments performed in quadruplicate.

TABLE II - COMPARISON BETWEEN TWO DIFFERENT EXPANSION PROTOCOLS AND THREE DIFFERENT GROWTH FACTOR COMBINATIONS

		Input		Week 4		Week 6		Week 8	
Growth factors				Demi-depopulation well	Flasks	Demi depopulation well	Flasks	Demi depopulation well	Flasks
FL+TPO+SCF	Cells	x10⁶	0.02	9.09±3.2	10.3±1.9	31.28±8	27.9 ±6.9	103.7±32.9	106±40.6
	CD34⁺	%	91	3.28 ±0.29	3.0 2±0.7	1.67±0.9	1.7 ±0.9	2.97±0.7	2.06±0.85
	CFC	x10³	2.8	194.2 ±60.2	255.3 ±41	314.7±99.1	298.7±106.2	1,252.6±508.1	1,066.5±737.4
	LTC-IC	x10³	3.6	48.2±24.8	57.4±22	172.8±122.4	204.6±79	1,012.9±795.6	1,003±604
FL+TPO+SCF+IL-6	Cells	x10⁶	0.02	10.01±3.2	6.96±2.91	55.6±21.7	57.1±23.6	113.4±49.8	249±73.1
	CD34⁺	%	91	4.32±0.95	3.73±0.7	2.47±0.8	2.55±0.8	2.87±0.85	2.03±0.9
	CFC	x10³	2.8	180.6±46.8	223.2±58.8	473±16	389.9±76.1	1,898.6±1,785	2,696±863.4
	LTC-IC	x10³	3.6	52.2±21.9	55.2±41	290.6±78.5	256.4±91.3	1,100.5±665	1,264±592
FL+TPO+SCF+IL-3	Cells	x10⁶	0.02	19.8±4.6	17.4±5.1	78.6±12.8	69.4±8.3	295.6 ±64.9	225.6±78.2
	CD34⁺	%	91	2.98±0.7	2.8 ±1.8	2.1±1.7	1.9±1.1	0.4±0.4	0.21 ±0.13
	CFC	x10³	2.8	28.2±6.4	19.4±4.8	84.7±12.4	79.8 ±17.2	1,134.3±175.5	996.9±81.7
	LTC-IC	x10³	3.6	4.2±0.7	3.9 ±1.1	3.8 ±1.2	3.55 ±1.8	0.56 ±0.2	0.23 ±0.13

20-50 x 10³ CD34⁺ cord blood cells were grown in stroma-free cultures with indicated growth factors for 8 weeks. At the indicated time points, the cells were harvested, counted, and aliquots of each culture were analyzed for CD34 antigen expression. Suitable aliquots were assayed in semisolid assays for CFC and on stroma co-cultures for LTC-IC enumeration.
Values shown represent the mean ± s.e.m of the fold increase, relative to the input number obtained from four separate representative experiments.

support an amplification of the LTC-IC compartment was evaluated after increasingly longer periods of liquid cultures. The results generated by these experiments are summarized in Figure 3. The association of FL+TPO+SCF and FL+TPO+SCF+IL-6 resulted in a massive stimulation of LTC-IC expansion over time, which reached respectively a 155,000-fold increase and a 139,000-fold increase after 23 weeks of culture.

On the other hand, when IL-3 was added as a supplement to the FL+TPO+SCF combination, not only was it incapable of further enhancing the already stimulated LTC-IC expansion, but it also produced a negative effect, resulting in a decrease of the LTC-IC number. This was not so in the case of IL-6, which appeared to be neutral, as no modifying effect on the LTC-IC output was observed. Evident from these experiments are the findings that only marginal differences could be detected in the qualitative and quantitative content of the two different (small and large scale systems) expansion procedures (Tab. II).

Long-term repopulating ability of the cells cultured with 3-early acting cytokine combination ± IL-6

We initially examined NOD/SCID mice repopulating capacity of variable doses of fresh CB CD34⁺ cells. A

TABLE III - ABILITY OF FRESH CB CD34$^+$ CELLS TO ENGRAFT THE BONE MARROW OF NOD/SCID MICE

CB CD34$^+$	No. of positive mice	Level of engraftment*
$2x10^5$	3/3	12.5; 21; 17
$1x10^5$	4/4	1.3; 5; 12; 25
$5x10^4$	4/5	1.5; 9; 6; 7.1; 0
$2x10^4$	1/4	0.12
$1x10^4$	0/4	0

CD34+ cells deriving from different CB samples were separated as described, pooled and then injected in the mice. BM of NOD/SCID recipients was recovered from both tibias and femurs at weeks 6-8 post transplant.
* Positive mouse: >0.1% of human CD45$^+$, CD71$^+$ and GpA$^+$ cells in the whole BM of FACS analysis.

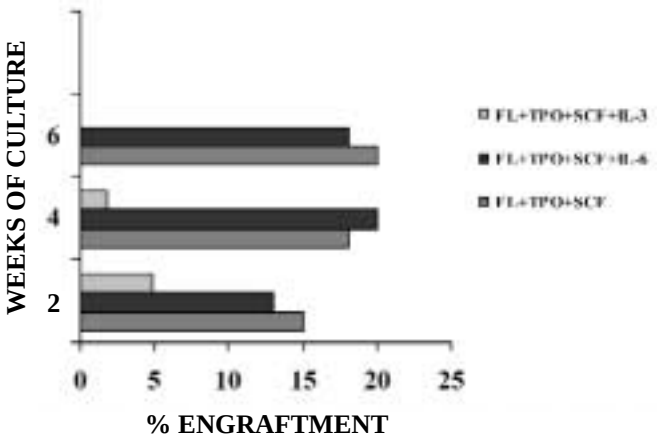

Fig. 4 - Long-term repopulating capacity of CD34$^+$ cells cultured in presence of FL+SCF+TPO ±IL-6 ±IL-3 at different time points (2-4-6 weeks of culture).

group of sublethally irradiated NOD/SCID mice were injected with CD34$^+$ cells, which have been separated from several cord blood samples and then pooled. Six-eight weeks later, BM cells of the sacrificed animals were harvested from both femurs and both tibias and assessed for the presence of human hematopoietic cells. As shown in Table III, $2x10^5$, $1x10^5$, $5x10^4$ CB CD34$^+$ cells engrafted the near totality of mice. The level of engraftment was variable and dependent on the number of injected CB CD34$^+$ cells. Human CD45$^+$, CD71$^+$ and GpA$^+$ cells ranged from 1.4 to 25%. None of 4 NOD/SCID mice transplanted with less than $2x10^4$ cells revealed successful engraftment. BM cells of engrafted mice were analyzed for evidence of multilineage development from input CD34$^+$ cells. Cells were quantified for myeloid, lymphoid, erytroid, megakaryocyte surface markers as well as for the expression of the CD34 antigen. Flow cytometric analysis showed that the human cells belonged to all hematopoietic lineages. As an additional proof of BM, cells of sacrificed mice were cultured in semisolid as-

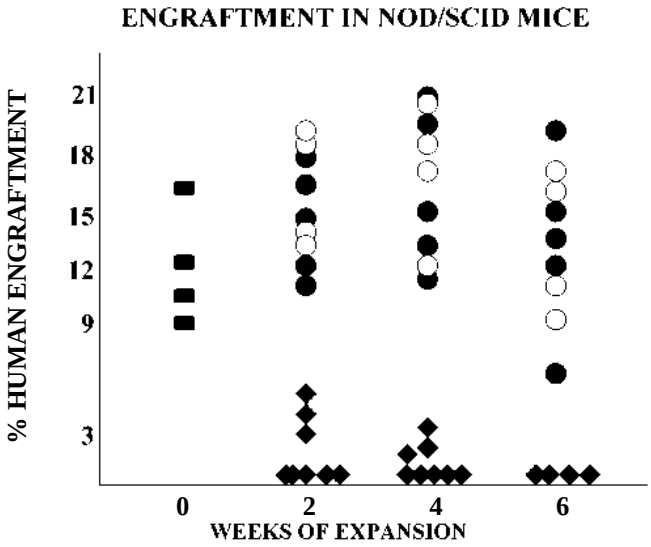

Fig. 5 - Summary of the level of human cell engraftment in the BM of mice transplanted with 100,000 fresh CB CD34$^+$ (■), and with cells harvested at different time points from *ex vivo* expansion cultures in the presence of FL+SCF+TPO (○), FL+SCF+TPO+IL-6 (●) or FL+SCF+TPO+IL-3 (♦).
Individual NOD/SCID mice (each symbol represents a mouse) were injected 8 weeks previously with expanded cells, corresponding to $1x10^5$ initial CD34$^+$ cells. The level of human engraftment in the mouse BM was evaluated by flow cytometry (as % of human CD45, CD71 and GpA positive cells).

say (plasma-clot) in culture conditions that have been reported to allow human and not mouse colony growth (8, 26, 27). In all engrafted animals human colonies was found.

In order to assess whether the different sets of expanded cells retained their *in vivo* repopulating capacity, NOD/SCID mice were irradiated with 350 cGy from a ^{137}Cs source and then injected with the corresponding progeny of 100,000 initial numbers of CD34$^+$ that were grown 2, 4, and 6 weeks with FL+TPO+SCF±IL-6 (Fig. 4). Progeny of 100,000 initial CD34$^+$ cells could engraft NOD/SCID mice at very good levels after 6 weeks of liquid cultures in the presence of FL+TPO+SCF±IL-6. In both cases (with or without IL-6) engraftment of human CD45$^+$, CD71$^+$ and GpA$^+$ cells ranged from 5 to 18% (Fig. 5). Flow cytometric analysis showed that the human cells in the BM of the engrafted mice belonged to all hematopoietic lineages. In addition, the cells from BM of mice transplanted with expanded cells are able to generate human colonies. Also, human CD45$^+$ cells were again separated from the BM of the primary mice and injected into sublethally irradiated secondary NOD/SCID mice. Human engraftment was determined by FACS analysis and by semisolid culture assays. Human cells were present in all evaluated cases, their percentage ranging from 4.8 to 11% (Fig. 6).

These date don't highlight the considerable and important differences in NOD/SCID repopulating capacity between cells cultured with FL+TPO+SCF and cell growth with this cytokine combination +IL-6.

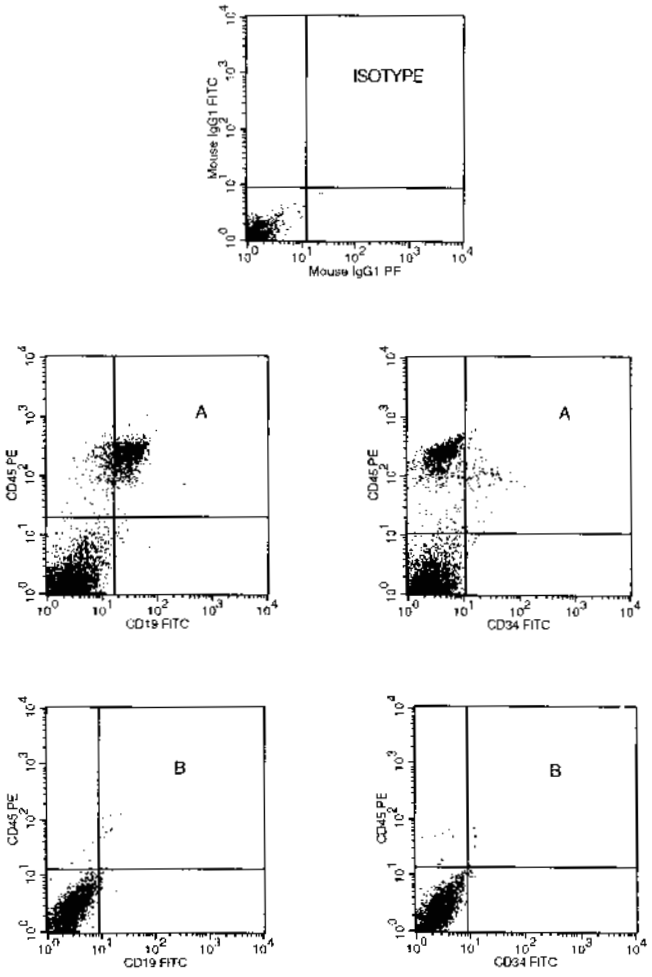

Fig - 6. A) Engraftment in the BM of a representative primary NOD/SCID mouse transplanted with 100,000 CB CD34$^+$ expanded for 4 weeks with FL+TPO+SCF+IL-6.
B) Representative FACS profile of marrow cells from individual secondary NOD/SCID mouse repopulating with cells derived from primary mouse (A) injected with expanded cells.

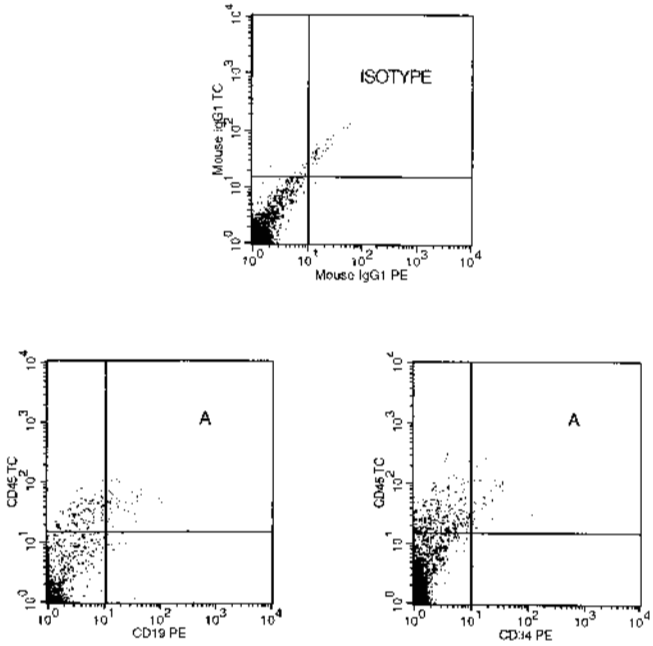

Fig. 7 - A) Representative FACS profile of marrow cells from individual primary NOD/SCID mouse transplanted 6 weeks previously with 100,000 CB CD34$^+$ expanded for 4 weeks with FL+TPO+SCF+IL-3.

Inhibitory effect of IL-3 on the NOD/SCID repopulating capacity

In order to assess whether the CB CD34$^+$ cells cultured with 3-early acting growth factor combination +IL-3 retained their *in vivo* repopulating capacity, NOD/SCID mice were irradiated with 350 cGy from a ^{137}Cs source and then injected with the corresponding progeny of 100,000 initial numbers of CD34$^+$ that were grown 2, 4, 6 weeks with FL+TPO+SCF±IL-3. Progeny of 100,000 initial CD34$^+$ cells, cultured with FL+TPO+SCF+IL-3 for 2 and 4 weeks, were capable of engrafting only a proportion (3 out of 8 and 4 out 10, respectively) of the injected mice. In the transplantation of the cells cultured with IL-3, the percentage of human CD45$^+$, CD71$^+$ and GpA$^+$ cells in BM was lower (4.8% and 1.8% for 2 and 4 weeks-expanded cells, respectively) than that of the initial unmanipulated 100,000 CD34$^+$ cells (Fig. 7). No mice injected with the cells cultured for 6 weeks in the presence of IL-3, were successfully engrafted. The BM of engrafted primary mouse was utilized for secondary injection; but, in this case, human cells in secondary recipients were not found.

These results indicate that IL-3 inhibits the expansion of the cells able to completely repopulate the BM of NOD/SCID mice.

Discussion

Several cytokine combinations have been tested for maintenance and expansion of human hematopoietic stem/progenitor cells (10, 24, 25, 28-34). 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, long-term culture-initiating cells (LTC-IC) to optimize the culture conditions, but these surrogate assays have been shown not to reflect stem cell activity correctly (3, 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 (3, 22).

The early acting cytokines SCF, FL, TPO and IL-6 have been used as key factors for HSC expansion (12). SCF and FL tyrosine kinase receptors were shown to transduce signals crucial for HSC development (37, 38); TPO has been shown to stimulate the expansion of primitive hematopoietic stem cells (12, 28); IL-6 is a potent cofactor for survival and prolifera-

tion of primitive multilineage progenitor cells (13, 14).

IL-3 supports the development of multiple hematopoietic lineages by interacting with multipotential and lineage-committed progenitors in culture (39, 40). Previous reports on the effect of IL-3 on the expansion of primitive hematopoietic cells were controversial even though they found it stimulates the expansion of relatively mature hemopoietic progenitor cells. IL-3, as a single factor, supports proliferation of the progenitors after they exit from the cell-cycle dormant state (G0) (39, 41). IL-3 also synergizes with IL-6 (42), IL-11 (43, 44), G-CSF (45), leukemia inhibitory factor (46), TPO (47, 48), and SCF (49, 50) in triggering cell divisions of the multipotential progenitors in G0. Recently, Bryder and colleagues reported that IL-3 is able to support FL+TPO+SCF-induced expansion of multilineage, long-term reconstituting activity in primary and secondary recipients (15).

Matsunaga et al noted negative effects of IL-3 on the ability of cultured cells to engraft the marrow of recipient mice (51). This observation is in agreement with the report from Peters et al (52) which demonstrated that suspension culture of murine marrow cells in the presence of IL-3, IL-6, IL-11 and SCF results in impairment of the engrafting capability of the cultured cells. These observations raised the possibility that IL-3 may be a stage-specific negative regulator and that it may suppress the earliest process of hematopoiesis, e.g., self-renewal of the stem cells. The negative effects of IL-3 observed in murine models may be relevant to *in vitro* manipulation of human stem cells.

IL-6 enhanced CXCR4 expression on $CD34^+$ cells (53), and the 70-fold expansion of NOD/SCID repopulating cells reported after 9-10 weeks of culture of CB with IL-6 in combination with FL,TPO and SCF, may in part be due to upregulation of CXCR4 (23). Defective hematopoiesis in IL-6-deficient mice indicates that IL-6 plays such a role *in vivo* (54). IL-6 acts on cells through a receptor system comprising two proteins, IL-6 receptor (IL-6R, gp80) and gp130 (55, 56). Signal transduction is solely due to dimerization of gp130 (57) upon formation of a hexameric complex of 2 gp130, 2 IL-6R, and 2 IL-6 ligands (58, 59). Soluble forms of IL-6R (sIL-6R) are produced by cells and are found in blood and urine (60, 61). These sIL-6R act as potent agonists of IL-6 on many cell types, because they retain the ability to induce IL-6 dependent gp130 dimerization (62, 55).

In order to address this issue in the human setting, in this study we compared the effect of three early acting cytokine combinations containing FL+TPO+SCF, in the presence or in the absence IL-6 or IL-3, 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 30 weeks in stroma-free cultures in the presence of FL+TPO+SCF±IL-6. When assayed in the NOD/SCID model expanded $CD34^+$ retained their capacity to completely engraft the bone marrow of sublethally irradiated NOD/SCID recipients. The experiments reported here show that CB $CD34^+$ cells during a six-week expansion in stroma-free liquid cultures containing FL+TPO+SCF±IL-6 retained their capacity to completely engraft the bone marrow of sublethally irradiated NOD/SCID recipients. The level of engraftment, similar or greater than that usually observed when the same number of uncultured cells was injected in the same recipients, suggested that SCID Repopulating Cells (SRC) were not only maintained, but also, probably, expanded. By contrast, when the same CB $CD34^+$ cells were cultured with FL+TPO+SCF+IL-3, their content in SRC was maintained, but only at very low degrees for no longer than four 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* (2, 25, 29, 63).

Bhatia et al (64) and Conneally et al (65) reported about the expansion of *in vivo* repopulating cells in liquid cultures containing also IL-3, but the role of IL-3 in that context was not further elucidated.

The reports on the effect of IL-3 on HSC expansion have differed for mice and humans. Culture of murine HSC with IL-3 was detrimental to the maintenance of stem cell activity, as was shown by the repopulating ability in lethally irradiated mice (16, 17). By contrast, it was reported that IL-3 did not negatively affect the long-term repopulating ability of human HSCs (18). Here, we demonstrated that IL-3 exhibits an inhibitory effect on the expansion of human LTR-HSCs. Because IL-3 could stimulate the increase of mature blood cells and HPCs, IL-3 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.

Ueda et al reported that a combination of SCF, FL, TPO, IL-6, and a soluble form of the IL-6 receptor (sIL-6R) was superior to other formulations for expanding CB cells with NOD/SCID engraftment potential. The proportion of human CD45 cells engrafted in the mouse marrow at 10-12 weeks was 10-fold higher with 7-day-cultured CB cells than with fresh cells, and repopulating cells were expanded 4.2-fold by limiting dilution assay. These same investigators had earlier reported potent synergism between SCF and a complex of IL-6 and sIL-6R for cord blood progenitor expansion, and their current study extends the observed synergism to expansion of cells with *in vivo* stem cell potential. The rationale for the use of sIL-6R was based on the earlier observation that human $CD34^+$ LTC-ICs expressed the signaling component of the IL-6 receptor pathway but not the ligand-binding (-chain of the IL-6R (66). Enhanced signaling through gp 130 on stem cells may be advantageous in two ways: favoring self-renewal and upregulating CXCR4, which

favors efficient homing to the marrow (67).

The present paper shows that CB SRC can be maintained for 12 weeks of *ex vivo* cultures in the presence of FL+TPO+SCF±IL-6. The difference between FL+TPO+SCF and FL+TPO+SCF+IL-6 in term of *in vitro* $CD34^+$ cells, CFC, and LTC-IC amplification and of *in vivo* repopulating capacity is not significant, probably because only IL-6 could be not sufficient to improve the expansion performances with respect to FL+TPO+SCF but the soluble form of IL-6R could be necessary. The degree of expansion has not been fully calculated, because of the low numbers 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 very large numbers of cells and progenitors belonging to the more mature hemopoietic compartments and, in 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 IL-3, which in the early phases of expansion seems to induce an even greater or equal expansion of cells, CFCs and also LTC-ICs and SRC, and 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 data could prove essential to design and test conditions for *ex vivo* activation and expansion of immature hematopoietic cells and for various experimental purposes, required for the development of efficient gene transfer protocols into hematopoietic cells with retention of repopulating ability.

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