Interleukin-3 and *ex vivo* maintenance of hematopoietic stem cells: facts and controversies

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ABSTRACT. Although the utilization of IL-3 in the *ex vivo* expansion of hematopoietic stem cells has been considered as an attractive possibility, its mode of action remains unclear and controversial. Some reports show that IL-3 maintains or even enhances primitive stem cell activity, whereas others show the opposite. The presence of serum in culture media enhances the pro-differentiating effect of IL-3 on stem cells. Conversely, addition of IL-3 to serum-free cultures improves the capacity of TPO, SCF and Flt3-ligand to promote the self-renewal of primitive stem cells. The presence or absence of serum or of some serum substitutes (in serum-free cultures), as well as other culture parameters are probably responsible for these contrasting effects of IL-3 on stem cells. However, none of the data presently evaluated bring a clear, definitive explanation to this apparent paradox. Those data that appear to be the most informative are presented and discussed in this "technical review".

Keywords: IL-3, *ex vivo* expansion, stem cells, progenitors, self-renewal, differentiation

The use of interleukin-3 (IL-3) in hematology and oncology has been previously reviewed [1, 2]. Although the utilization of this cytokine in the *ex vivo* expansion of hematopoietic stem cells has been considered as an attractive possibility, its mechanism of action remains complex and controversial. Here, some questions related to the factors conditioning the IL-3 effects on hematopoietic stem cell survival, self-renewal and differentiation in course of *ex vivo* expansion are discussed.

Initially, IL-3 was named "hemopoietic growth factor" [3] or "multi-colony stimulating factor (CSF)" [4], since its biological activity was related to survival, maintenance and stimulation of the proliferation of multi-lineage hematopoietic progenitors. Because of these properties, IL-3 has been used to develop *in vitro* tests for several categories of hematopoietic progenitors. In spite of a low homology between murine and human IL-3 molecules and consequent species-specificity of each of them, their biological actions on respective hematopoietic cells are practically identical. This is probably related to their similar, respective receptors [5].

The actions of IL-3 have been studied in both species (murine and human) using either established cell lines or hematopoietic cells recovered from murine bone marrow or spleen, as well as from human bone marrow, peripheral blood and cord blood. IL-3 that enhances the survival of hematopoietic progenitors and stimulates their proliferation and differentiation [6] also favors, under certain conditions, their self-renewal [7]. These apparently contradictory effects of IL-3 are influenced by various factors such as: the target cell population, its concentration, culture conditions, presence or absence of other cytokines, serum, etc.

This issue is complex not only because this cytokine exhibits various effects on the cells, but also because the stem cell compartment is heterogeneous, thus complicating the direct analysis of the stem cell response.

COMPLEXITY OF THE IL-3 TARGET STEM/PROGENITOR CELL COMPARTMENTS

Functional definition of stem cells

A major problem in distinguishing the actions of IL-3 is the heterogeneity of the stem/progenitor cell compartment. Stem cell subpopulations enriched by means of phenotypical analysis and selection, do not reach the level of a pure homogeneous population. This is even more evident for cultured cells, in which dissociation between phenotype and function [8-11] is frequent but neglected. Consequently, a direct molecular biology and biochemical approach is of limited interest due to the contamination of enriched stem cells by mature cells or progenitors of different classes. Since stem cells differ in their long-term and short-term repopulating ability [12, 13], the effect of IL-3 could be evaluated only by functional tests that make its analysis and interpretation indirect and delicate.

Heterogeneity of stem cells

IL-3 has been shown to stimulate the survival and proliferation of the multipotent stem cell line FDCP-Mix [14]. It

also has a growth promoting activity for CFU-Mix, BFU-E, CFU-GM [15, 16] as well as for CFU-Blast [7]. It is a potent stimulator of CFU-S *in vitro* and *in vivo* [17-20]. However, CFU-S is now considered to be a "pluripotent progenitor" with a limited self-renewal capacity originating from more primitive stem cells, known as "pre-CFU-S" or "pre-CFC". These latter cells are responsible for the phenomenon of "marrow repopulating ability" (MRA) *in vivo*, with some of them ensuring a long-term engraftment [21-24]. In fact, both the long-term and short-term repopulating cells seem to be responsible for the long-term maintenance of hematopoiesis since the latter should be activated sequentially assuring the succession of different hematopoietic clones [12, 13]. Overlapping of stem cell subpopulations [25] further increases the complexity of the system *(Figure 1)*. Thus, the system of heterogeneous hematopoietic stem cells is even more complex than two- or three- or several- compartment, linear and one-way systems. The population of CFU-S could expand from itself by "self-renewal" or derive from enhanced differentiation of more primitive stem cells (pre-CFU-S) sensitive to IL-3. These pre-CFU-S could either proliferate symmetrically (with accentuated differentiation or self-renewal), or asymmetrically (ensuring simultaneously both of these phenomena) [26, 27]. If the observation is limited only to pre-CFC and CFC compartments, there are several possibilities of response to IL-3, further increased by: concentration of IL-3, dose-delivery rate, reactive secretion of other cytokines and growth factors...

Expression of cytokine receptors and postreceptor signaling

Different hematopoietic cell populations are characterized by a specific expression of specific cytokine receptors, which determines their responsiveness. The current knowledge on IL-3 receptors comes mainly from the studies of IL-3 - dependent cell lines such as the FDCP-mix and IL-3 sensitive cells such as B6SUtA1 clone. The density of IL-3 receptors seems to be related to the proliferative response of the cells. Cells with high density of IL-3 receptors (approximately 100 000 receptors per cell i.e. ten times more than the low density cells), have a 30-fold higher, relative proliferative response than low IL-3-receptor density cells [28]. The receptor for IL-3 (a member of the cytokine receptor family) is composed of α and β subunits. The α subunit of IL-3 receptor binds its ligand with low affinity, and interacts with the β unit (βc) to form a functional, high affinity receptor complex [29]. Common to IL-3, GM-CSF and IL-5, β subunit lacks the ability to bind any of these cytokines independently. It is structurally related to gp 130 subunit of the IL-6 receptor family [30], which is implicated in the self-renewal of embryonic and adult stem cells [31].

Hematopoietic stem cells are characterized by low expression of IL-3 receptors. Autoradiographic analysis using radio-labeled^{125} I-rm-IL-3 [32] demonstrated that 65% of primitive stem cells, highly enriched for long-term repopulating ability (Rhodamine-123^{lo} lineage-Ly6A/E + c-kit +), express IL-3R with a density of 80 receptors per labeled cell. A similar percentage (69%) of stem cells, highly enriched for short-term repopulating ability and day 13 CFU-S (Rhodamine-123$^{med/hi}$ Lineage- Ly6A/E + c-kit +), express IL-3R (110 receptors per labeled cell). Nearly all *in vitro* colony-forming cells (Lineage -Ly6A/E- c-kit +) (93%) express the IL-3R (110 receptors/labeled cell). The expression of the β transducing subunit in murine bone marrow cells increases during the granulocytic and monocytic differentiation [33], while it remains low on the cells of megakaryocyte and erythroid lineage. A similar situation is observed for the α subunit of the IL-3 receptor, known as CD123: The majority of human CD34 + CD123^{bright} cells are myeloid and B-lymphoid progenitors, while erythroid progenitors are mainly in the CD34 + CD123^{negative} fraction. The CD34 + CD123$^{low/negative}$ population is heterogeneous as regards primitive progenitor cells. Their concentration is not further increased when CD34 + CD123$^{negative/low}$ cells are enriched in CD90 (c-kit) [34]. The CD33- 7B9- subpopulation of human CD34 + cells that is considered to be relatively more primitive than the overall CD34 + population, is characterized by a synergistic proliferative response to IL-3 and GM-CSF [35].

In our hands, addition of IL-3 to 72 h serum free cultures induced cycling of most human cord blood G0 CD34 + cells, demonstrating that even CD34 + cells, supposed to express very low numbers of IL-3 receptors, exhibit IL-3 responsiveness [36].

Binding of IL-3 to its receptor molecules results in their dimerisation that activates a ras-mediated (res-raf-1-MAP kinase) pathway [37, 38] and a ras-independent JAK (janus kinase)-STAT (signal transducer and activator of transcription) system [39, 40]. Murine IL-3 at high concentrations promotes the self-renewal of the FDCP-mix preventing their differentiation in the presence of other growth factors [41]. Activation of hIL-3R α, β promotes prolonged stimulation of proliferation and suppression of clonogenic extinction of FDCP-mix cells transfected with the human IL-3R α and hβc subunits, even in the presence of cytokines that promote granulocyte-macrophage differentiation [5]. These data, indicate an anti-differentiating effect of IL-3 on the cells expressing IL-3Rα,β. IL-3 also avoids inappropriate apoptotic cell death [42] by activating, through different signaling pathways, the transcriptional repressor DREAM, which in turn binds to a silencer sequence in the *hrk* gene (apoptotic protein Hrk) and blocks its transcription. In this way, IL-3 allows, at the same time, survival and maintenance of primitiveness of the FDCP-mix cells. Another fundamental impact of IL-3 on cell survival, is its influence on ATP generation via glucose transport via ATP [3, 43, 44]. IL-3 maintains the permanent activity of the glucose transporter by a mechanism that involves tyrosine kinases and protein kinase C, an effect independent of respiration or signal transduction to the nucleus [45]. In some cells, IL-3 maintains the intrinsic transport properties of glucose transporters without markedly affecting their expression or translocation [46], whereas in others, it increases the transporter expression and its glucose affinity, thus affecting the glucose uptake [47]. By distinct mechanisms, IL-3 and the transforming oncogenes ras and abl, alter the activation state of glucose transporters [48]. Since the function of glucose transporters is crucial in the hypoxia that regulates their expression [49], IL-3 might be very important for the maintenance of stem and progenitor cells at low oxygen (O_2) concentrations by increasing glucose uptake. Recently, we have shown that a low dose of IL-3 (0.5 ng/ml) synergizes with a low O_2 concentration (3%) in the pre-CFC maintenance [81]. Interestingly, increasing the concentration of IL-

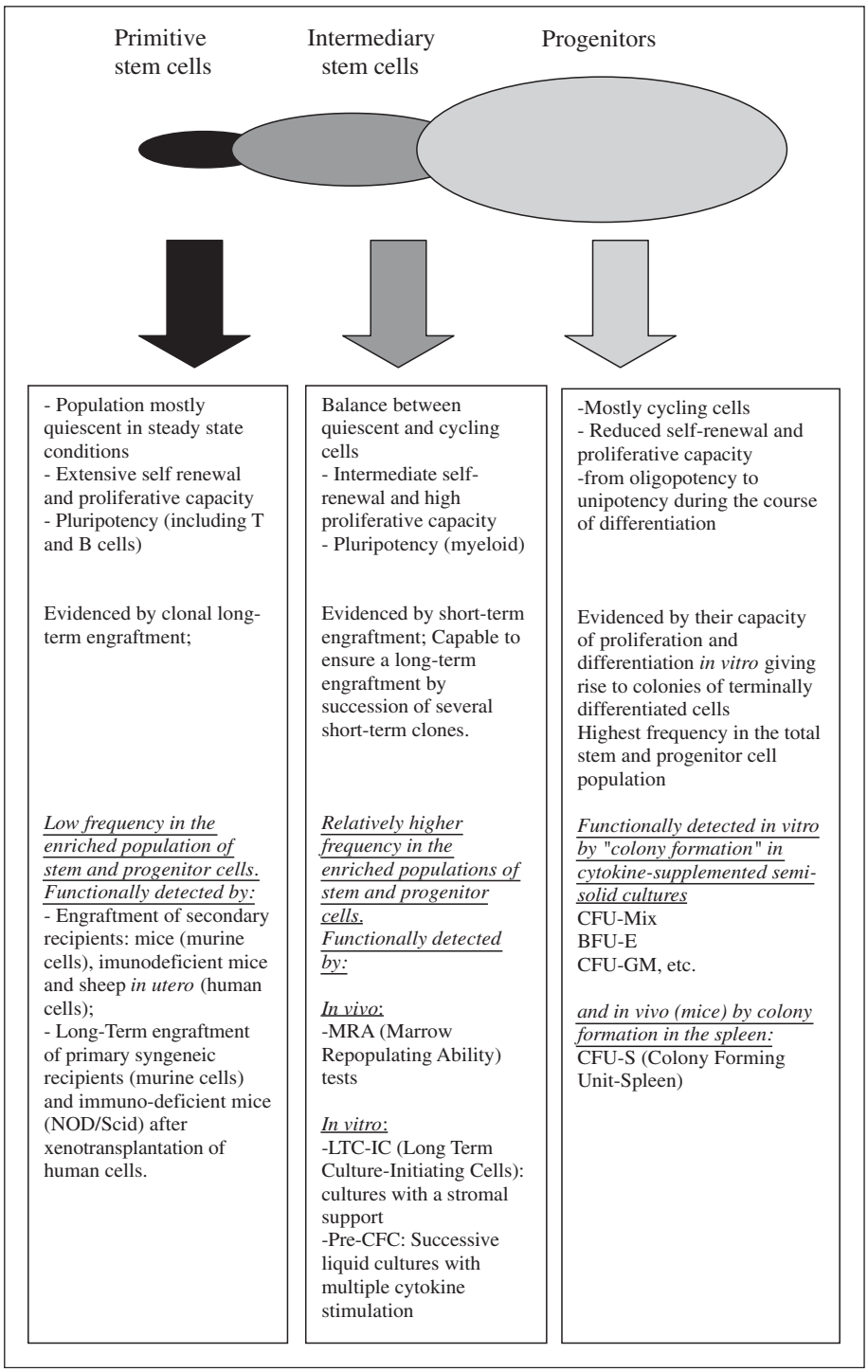

Figure 1
Schematic description of the compartments of hematopoietic stem and progenitor cells.

3 suppressed the synergistic effect, probably by enhancing the IL-3 induced differentiation of pre-CFC.

IL-3 AND *EX VIVO* EXPANSION OF HEMATOPOIETIC STEM AND PROGENITOR CELLS

Ex vivo expansion of hematopoietic stem cells with cytokines is an attractive approach that has already led to the shortening or abrogating of the period of post-transplantation neutropenias [51]. Amplification of small size explants, currently reserved almost exclusively for children, will also allow the engraftment of adults. The *ex vivo* maintenance of stem cells is necessary in course of the gene therapy procedures and the purging of malignant cells... The expansion product should contain primitive stem cells able to ensure a long-term engraftment [26], and induction of an early hematopoietic recovery [52, 53]. Therefore, the maintenance of primitive stem cells is an absolute requirement of *ex vivo* expansion, but it is also a very complex issue [54, 55] that depends on an optimal

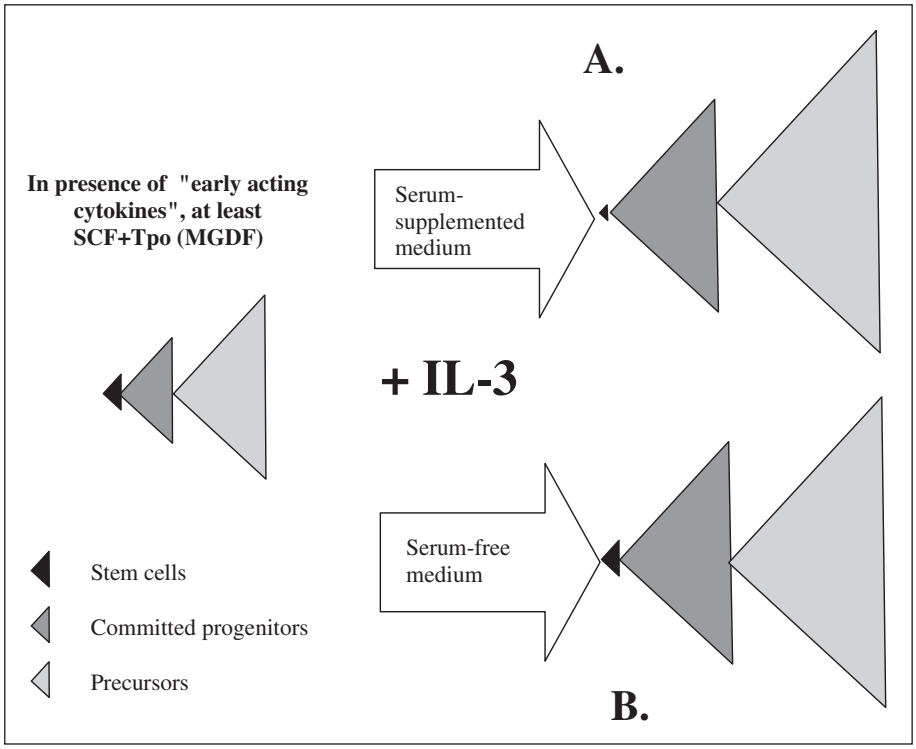

Figure 2
Effect of IL-3 on stem cell maintenance and amplification of progenitors in vitro in (A) serum supplemented culture and (B) in serum free medium. When compared to cultures performed without IL-3 in presence of early acting cytokines (SCF + TPO) addition of IL-3 in serum supplemented culture induces a reduction of stem cell functional activity.

combination of cytokines. IL-3 was present in numerous expansion protocols and most available data demonstrate that it increases the yield of CD34 + cells and committed progenitors. However, the action of IL-3 on more primitive stem cells, detected by *in vitro* (long-term culture- initiating cells -LTC-IC or pre-CFC) or *in vivo* assays (marrow repopulating ability- MRA, long-term repopulation activity - LTRA, serial transplantation of murine bone marrow cells, and engraftment of NOD/SCID mice with human cells), is still a contradictory issue.

The negative effect of IL-3 on primitive stem cells: serum-supplemented cultures

Some reports show a maintenance or even enhancement of primitive stem cells, while others show a negative effect of IL-3 on stem cells *(Figure 2A)* interpreted as exhaustion of their self-renewal, due to differentiation [56, 57]. Data supporting this theory come from the serum, supplemented, *in vitro* culture experiments. In these studies, IL-3 was used in combination with interleukin-11 (IL-11) and erythropoietin (Epo) on murine bone marrow Lin- Ly-6A/E +, c-Kit + cells) [57], in combination with stem cell factor (SCF) or SCF + Thrombopoietin (TPO) on human cord blood CD34 + cells [58, 59], in combination with Flt-3 ligand, SCF and TPO [60] or only with Flt-3 ligand and TPO [61]. The article of Nitsche *et al*. [62] provides well documented proof of an exhausting effect of IL-3 *in vivo* on primitive repopulating stem cells after the simultaneous engraftment of human cord blood CD34 + cells and a stably transfected rat fibroblast cell line that continuously produced IL-3. These mice had an increased number of human cells in bone marrow, spleen and in the peripheral blood when compared to control mice (simultaneously engrafted with the non-transfected rat fibroblasts). These results correspond well to those obtained in co-engraftment experiments using allogenic murine T-cell-depleted grafts together with the QXMSC stromal cell line engineered to secrete IL-3 [63]. However, when the femoral bone marrow cells from primary recipients were engrafted to secondary recipients, a significantly lower human-cell repopulating ability was found for mice initially co-engrafted with IL-3-producing cells [62]. Similarly, when cells from primary recipients were cultured for 2-3 weeks, there were fewer human cells in cultures of bone marrow cells from mice co-transplanted by IL-3-producing fibroblasts. Both serial transplantation and secondary cultures, which are considered as a stringent measure of true stem cell activity [64, 65] by enhancing proliferative and differentiation pressure on primitive stem cells, resulted in their exhaustion after a few passages, and in the loss of LTRC activity.

In serum-supplemented cultures, we have also demonstrated that in the presence of IL-3, the activity of murine bone marrow pre-CFC (closely related to MRA cells – [66]) decreases, while the number of CD34 + cells, CFU-GM, HPP-CFC3 and HPP-CFC2 increases [50]. These results confirm that IL-3 stimulates the proliferation of very primitive stem cells as well as progenitors. They also demonstrate that it induces the differentiation of dividing stem and progenitor cells, thus leading to an increased production of HPP-CFC2 (analogous to CFU-Sd14 - [67]) from pre-CFC, of HPP-CFC3 (analogous to CFU-S12 – [67]) from HPP-CFC2 and of CFU-GM from HPP-CFC3.

The positive effect of IL-3 on the maintenance of primitive stem cells: serum-free cultures

Recent studies performed in serum-free medium, demonstrate a positive effect of IL-3 on amplification of descendent progenitor populations, with no deleterious effect on primitive stem cells [68-72] *(Figure 2B)*. Furthermore, Bryder and Jacobson [72] even showed that IL-3 enhances the multilineage, long-term, reconstituting activity of murine bone marrow Lin- Sca-1 + c-kit + cells in serum-free cultures. Using SCF, Flt-3 ligand and MGDF -/ + IL-3, they confirmed earlier findings [68] that self-renewing divisions occur in serum-free cultures. Furthermore, even in this serum-free medium (with serum substitutes), a reduced LTRC activity was observed when fetal calf serum (FCS) was added [68]. This is in line with the data of Zanjani's group [73] showing in the human-to-sheep xenograft model that addition of serum to adult human bone marrow CD34 + cells cultured with IL-3, IL-6 and SCF, substantially decreased the *ex vivo* maintenance of repopulating stem cells. Similarly, Willems *et al*. demonstrated a monocyte differentiation-inducing effect of serum on primitive stem cells (pre-CFC) [74]. In conclusion, many studies agreed that cocktails of cytokines with IL-3 in a serum-free medium positively affected very primitive stem cells, ensuring their maintenance [70, 71] or even amplification [69, 72].

... But it is not so simple...

However, some studies also report negative effects of IL-3 on primitive stem cells in serum-free cultures [75-78]. Sekhar *et al*. [75] cultured murine bone marrow cells with IL-6, SCF and IL-3, but without TPO (MGDF) or Flt3-ligand, found to be essential for the maintenance of stem cell primitiveness [58, 79]. Since a culture condition without IL-3 is lacking in this study [75], IL-3 cannot be considered as the only factor responsible for abrogation of the repopulating ability of these cells. Mobest *et al*. [76] tested Flt3-ligand, SCF and IL-3 or, alternatively TPO, on expansion of mobilized CD34 + peripheral blood cells. TPO showed a better maintenance of repopulating cells in 7 days-old cultures than IL-3, but here again, the effect of IL-3 in the presence of TPO (condition FLT3-L, SCF, TPO + IL3) was not tested. Since the studies demonstrating a positive impact of IL-3 on repopulating cells have always been performed in the presence of SCF and TPO (MGDF) [68, 69, 72, 80], it should be considered that this combination of cytokines allows IL-3 to stimulate proliferation without enhancing, simultaneously, the differentiation of stem cells. Indeed, our results with SCF, G-CSF, MGDF -/ + IL-3 in serum-free medium demonstrated that cord blood pre-CFCs were better maintained with IL-3 (doses 0.5, 5 and 50 ng/ml). This effect is significantly enhanced at 3% O_2 concentration, with the lowest dose of IL-3 (0.5 ng/ml) [81] that also maintained 4 fold more SRC than at 20% O_2 without affecting CFC expansion [82].

In our experiments, increasing concentrations of IL-3 decreased the self-renewal of primitive stem cells and increased their differentiation, whereas Bryder and Jacobson [72] found a positive effect of IL-3 with much higher concentrations (20 ng/ml). It is difficult to explain why in the presence of SCF, TPO, and Flt3-ligand [78], IL-3 decreased LTC-IC and enhanced the telomerase activity. Abrogation of this negative effect of IL-3 by IL-6 [78], suggests that the action of IL-3 on stem cells in culture, varies with the presence and concentrations of other cytokines, as stressed by Ogawa group [83] as well as by Zandstra *et al*. [84]. Other factors such as quality and quantity of serum substitutes in medium and the culture atmosphere could influence stem cell survival, maintenance and differentiation. These variables should be analyzed more carefully to find out why the combination of IL-3 + IL-6 abrogates the NOD/SCID repopulating cells in CD34 + mobilized blood cell population in serum-free medium cultures, without any detectable effect on phenotypic or cycling features [77]. The ontogeny-associated changes in the cytokine responses of primitive hematopoietic cells should also be considered when comparing the results obtained with adult bone marrow and blood cells with those of neonatal placental blood [85].

Hematopoietic stem cell maintenance during ex vivo expansion is a delicate and still unresolved problem. It is of paramount interest to define conditions allowing in the same time the maintenance or better the increase of primitive stem cell activity together with progenitor cell amplification, but information is still lacking for this. As far as IL-3 is concerned, even if contradictory results cannot be ignored, it seems that a) it favors or at least does not influence maintenance/self renewal of stem cells in serum-free media when associated with "self renewing" cytokines (Figure 2B); b) its effects could be partially dependent on its concentration in the culture medium. From this point of view, to enable an easier and more rapid pre-clinical development, mini cultures should be performed with the media and cytokines certified for clinical cell therapy protocols.

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