Cell cycle kinetic changes induced by interleukin-3 and interleukin-6 during *ex vivo* expansion of mobilized peripheral blood CD34 cells

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The long-term repopulating capacity of mobilized peripheral blood (MPB) CD34+ cells can be abrogated during *ex vivo* expansion due to cell-cycle related changes. In this study, we traced cell cycle kinetic and quiescent status of CD34+ cells during *ex vivo* expansion with exogenous cytokines. The addition of interleukin-3 (IL-3) and interleukin-6 (IL-6) to early acting cytokines resulted in a significantly lower percentage of cells remaining undivided or in quiescent G_0 phase when compared to the use of early acting cytokines alone. We conclude that *ex vivo* expansion of MPB CD34+ cells with a cocktail of five cytokines yields a highly cycling offspring, which could impair long-term repopulating capacity.

Key words: *ex vivo* expansion, peripheral blood CD34+ cells, PKH26 Cell tracking, quiescent cells.

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Ex vivo expansion of the primitive pluripotent hemotopoietic stem cell compartment from different sources has constituted an important challenge in recent years.[1-3] Unfortunately, up to now no clinical state has been identified in which transplantation with expanded cells is clearly advantageous and the procedure is costly and laborious.[4-5] However, *ex vivo* expansion would be the only alternative for a significant number of patients who are candidates for autologous transplantation but whose stem cell yield is poor as well as for adult patients who are candidates for allogeneic transplantation from a cord blood unit not containing sufficient cells.[1-3]

The ideal combination of cytokines to maintain long-term repopulating capacity still remains controversial.[6-7] In previous works, we have demonstrated that the addition of interleukin-3 (IL-3) and interleukin-6 (IL-6) to early acting cytokines such as thrombopoietin, stem cell factor and Flt-3 ligand, despite producing notably superior cellular expansion, abrogated the long-term repopulating ability of offspring cells. In addition, we have demonstrated that this deleterious effect is not related either to changes in surface adhesion molecules that allow the homing of CD34+ cells or to the loss of CXCR4 after culture.[9]

On the other hand, it seems clear that adult mobilized peripheral blood cells capable of engrafting non-obese diabetic/LtSz-severe combined immunodeficient (NOD/SCID) mice are predominantly found in the G_0 phase of the cell cycle and the frequency of SCID-repopulating cells (SRC) decreases in post-mitotic expanded CD34+ cells that have undergone more than three or four divisions.[10-15] *Ex vivo* expansion of mobilized peripheral blood with exogenous cytokines can induce G_0-G_1 progression that could eventually result in the abrogation of NOD/SICD repopulating capability. To test this hypothesis, we performed high-resolution cell division tracking using PKH26 labeling and assessment of quiescent G_0 cell cycle status using nuclear dyes, in mobilized peripheral blood CD34+ cells undergoing *ex vivo* cytokine-driven expansion.

Design and Methods

Cells

Mobilized peripheral blood samples were obtained from five healthy adult allogeneic transplant donors after they had given oral and written informed consent to the procedure. CD34+ cells were purified and stained with PKH26 as previously described.[8,9]

Suspension cultures

PKH26-labeled cells were cultured as described elsewhere[8-9] with the following cytokines or cytokine combinations: thrombopoietin (20 ng/mL; Genzyme, Cambridge, MA, USA), stem cell factor (100 ng/mL; R&D Systems, Minneapolis, MN, USA), and Flt3-ligand (100 ng/mL; R&D Systems). This was termed the 3-cytokine (3CK) combination. The addition of interleukin 3 (20 ngr/mL; Genzyme) and interleukin 6 (20 ngr/mL; Genzyme) produced the 5-cytokine (5CK) combination.

Transplantation of CD34+ cells into NOD/SCID mice

Ex vivo-expanded mobilized peripheral blood CD34+ cells were injected into the tail vein of pre-irradiated 8 to 12-week old

Table 1. Parameters of proliferation history after *ex vivo* expansion with different cytokine combinations.

Cytokines	*Undivided cells %*	*Non proliferative fraction*	*Number of generations (median)*	*Proliferative index*	*Precursor frequency*
3CK Day +4	7.3±4.6	0.21±0.1	5±0.2	3.7±0.5*	0.7±0.1
5CK Day +4	3.9±2.3	0.18±0.08	6±0.2	5.7±0.8*	0.8±0.1
3CK Day +6	1.8±0.7°	0.11±0.03	7±0.4	7.4±0.9#	0.87±0.03
5CK Day +6	0.4±0.2°	0.05 ±0.2	8±0.4	16.5±2.5#	0.93±0.02

*3CK: Thrombopoietin, stem cell factor and Flt3-ligand; 5CK: the 3CK combination plus, interleukin-3 and interleukin-6. Statistical differences: *p=0.08, °p=0.01 and # p=0.04. Comparisons of the remaining parameters (3CK vs. 5CK) were not statistically significant.*

NOD/SCID mice, as described elsewhere.[8,9] Human chimerism was assessed 8 weeks after the transplant.

Tracking of division kinetics

PKH26-labeled cells were harvested at day +4 and +6 post-expansion. Acquisition was performed in a FACScalibur (Becton Dickinson Immunocytometry Systems San Jose, CA, USA) flow cytometer and data files were analyzed with Modfit LT 3.0 Mac Software (Verity House, Topsham, Maine, USA) and the Proliferation Wizard Model. The data collected on day +4 and day +6 of culture included: (i) the number of generations, (ii) the proliferation index, which is the sum of the cells in all generations divided by the computed number of original parent cells, used to measure the increase in cell number, (iii) the non-proliferate fraction, which is the number of cells in the parent generation at the time of data collection divided by the computed number of cells present in the original culture and represents the fraction of cells that did not proliferate during the experiment; (iv) undivided cells, the percentage of cells which remain with the same PKH26 intensity as that of the parental population; and (v) the precursor frequency, which is the proportion of the total cells calculated to have been present at the start of the experiment which underwent true proliferation during the culture. We also computed the sum of percentages of cells contained within generations 1, 2 and 3.

Analysis of G_0 quiescent and cycling $CD34^+$ cells

Steady state and expanded $CD34^+$ cells were first stained with fluorescein isothiocyanate (FITC)-conjugated CD34 (BDIS). To facilitate identification of different phases of cell cycle, $CD34^+$ cells were stained with Hoechst 33342 (Hst; Molecular Probes, Eugene, OR, USA) and Pyronin Y (Polysciences, Warrington, PA, USA) as described elsewhere (14,15). Fifty thousand events were acquired in a FACS Vantage SE (BDIS) device with three-laser capability and UV tuned at 350 nm.

Results

Proliferation history and quiescent status after ex vivo expansion of PKH26-labeled mobilized peripheral blood CD34 cells using different cytokine combinations

The results of high resolution cell tracking after *ex vivo* expansion of mobilized peripheral blood $CD34^+$ cells with different cytokine combinations are detailed in Table 1. At day +6, the percentage of undivided cells was 1.8 ± 0.7% and 0.4±0.2% for cells cultured with the 3CK and 5CK combination, respectively, (p=0.04).

The sum of percentages of the first three generations was 20.3±3.9% of cells expanded using the 5CK combination and 43.1±5.1% of those expanded with the 3CK cocktail (p<0.01). Likewise, the predominant generation, which encompasses the highest percentage of cells, was 1.7 vs. 5.7 (p<0.01) at day +4 and 3.8 vs. 5.6 (p=0.02) at day +6 for the 3CK and 5CK combination, respectively. A representative experiment of high reso-

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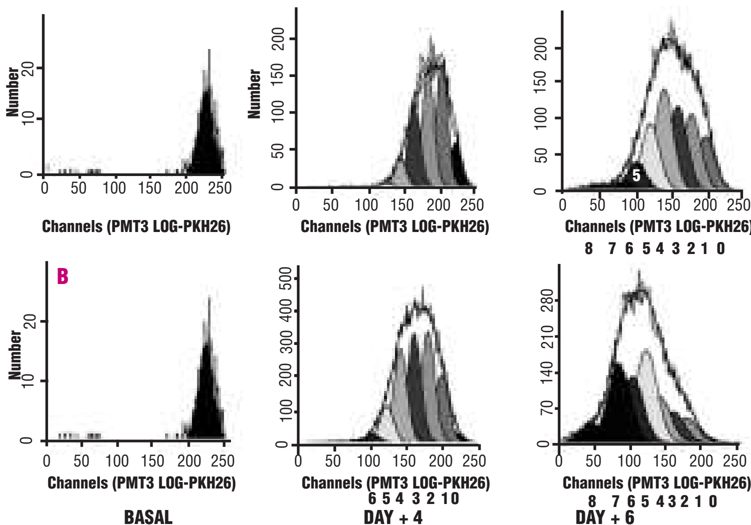

Figure 1. High resolution cell tracking of $CD34^+$ cells after *ex vivo* culture with single cytokine combinations. Histograms obtained after analyses of the proliferation wizard model of MODFIT software of a representative experiment. The X axis represents the linear intensity of PKH26 dye at day 0 (Basal), and after 4 days and 6 days of culture with thrombopoietin (TPO), stem cell factor (SCF), Flt3-Ligand (FL) in row A and after culture with TPO, SCF, FL, interleukin-3 and interleukin-6 in row B. The Y axis represents total events. Numbers under the X axis represent the number of generations after *ex vivo* expansion, generation 0 being the parental cells with the same PHK26 intensity as day 0.

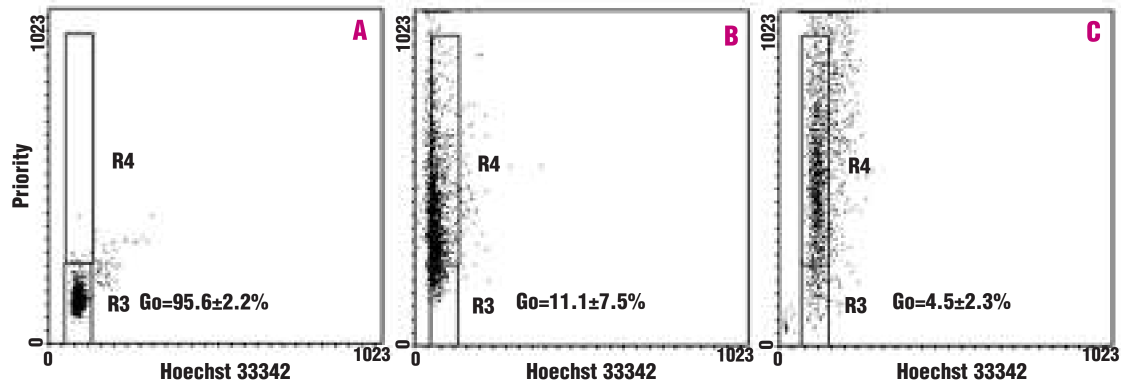

Figure 2. Cell cycle analysis after dual DNA/RNA staining of CD34+ cells undergoing *ex vivo* expansion. CD34+ cells were stained with Hoechst 33342 for DNA and pyronin-Y for RNA. Quiescent G_0 cells have a low content of RNA and are the predominant fraction before *ex vivo* culture (A). After 6 days of culture with thrombopoietin (TPO), stem cell factor (SCF) and Flt3-Ligand (FL), CD34+ cells progress in the cell cycle with acquisition of RNA (B). The percentage of quiescent cells (G_0) was significantly lower after *ex vivo* expansion with TPO, SCF, FL, interleukin-3 and interleukin-6 (C).

lution cell tracking analysis using both cytokine combinations is displayed in Figure 1. Freshly isolated mobilized peripheral blood CD34+ cells mostly reside in the G_0 phase of the cell cycle (95.6±2.2%) and only a minor population in G_1 (3.2%±1.7%). Importantly, the use of the 5CK cocktail to generate CD34+ cell expansion resulted in a significantly lower percentage of cells remaining in the G_0 cell cycle phase (measured as Hoechst-positive and pyronin Y-negative cells) as compared to the 3CK cocktail (4.5±2.3 vs. 11.1±7.5%, $p<0.01$) (Figure 2). No statistical differences were observed when comparing cells in G_1 (60.3±5.2 vs. 57.2 ± 3.4%), in S-phase (25.3±5.4 vs. 19.3±6.7) and in G_2M-phase (9.2±6.5 vs 8.6±3.6%) according to whether they had been cultured with the 5CK or 3CK combination, respectively.

SCID-repopulating capacity of expanded CD34+ cells with cytokine combinations

Groups of ten pre-irradiated mice were injected with 1×10^5, 1×10^6 and 4×10^6 unlabeled bulk CD34+ cells obtained from cultures with 3CK or 5CK. No human engraftment was observed in mice injected with human mobilized peripheral blood CD34+ cells after *ex vivo* expansion with the 5CK cocktail. However, freshly isolated or *ex vivo*-expanded CD34+ cells with the 3CK combination still retained measurable levels of SCID-repopulating capacity. Thus, human engraftment levels for mobilized peripheral blood expanded with 3CK were 0.018±0.01%, 1.0±0.1% and 8.5±5.4% (mean±SEM) for 1×10^5, 1×10^6 and 4×10^6 injected cells, respectively, versus undetectable human cells in the bone marrow of each mouse injected with any dose tested after 5CK expansion.

Impact of IL-3 and IL-6 on proliferative history and quiescent status after ex vivo expansion of CD34+ cells

We performed a set of experiments to differentiate the effect of IL-3 and IL-6 on proliferation kinetics. Thus, the percentage of undivided cells at day +6 was 1.3%±0.8% for IL-6 and 0.8%±0.6% for IL-3 and the non-proliferative fraction was 0.2%±0.03% and 0.8%±0.2% for 3CK+IL-3 or IL-6, respectively. The percentage of cells contained within the first three generations was 38%±3.4% for 3CK+IL-6 and 27%±4.3% for 3CK+IL-3. None of these differences reached statistical significance. We did not observe statistical differences in the number of generations, proliferative index or precursor frequency produced by expansion with 3CK+IL-3 or 3CK+IL-6 (*data not shown*).

Discussion

In this study we separately analyzed the percentage of cells remaining in a quiescent status and the percentage of cells with a short proliferative history after *ex vivo* expansion with different cytokine combinations. Both fractions are widely considered of great importance because they have been proven to be responsible for the maintenance of long-term repopulating capacity.[10-15] Our most important finding was that the percentage of undivided cells (retaining the same PKH26 initial staining), the percentage of cells encompassed in the three first generations and the percentage of cells in the G_0 phase were significantly reduced when mobilized peripheral blood CD34+ cells were expanded with early-acting cytokines plus IL-3 and IL-6. The role of IL-3 in *ex vivo* expansion protocols has been a matter of controversy. Our results suggest that IL-3 induced more proliferation than IL-6 and are in strong agreement with those recently reported by Levac *et al.*[16] and others.[17,18] However, there are also reports of successful expansion of murine primitive hematopoietic stem cells or human cord blood CD34+ cells with early cytokines plus IL-3 preserving repopulating capacity.[19,20] These contradictory results could be explained either by the negative effect of IL-3 in serum-supplemented cultures when compared to serum-free cultures or by

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the different IL-3 concentrations or culture conditions used in the studies.

According to the divisional history assessed by surface tracking dyes, it seems plausible that primitive pluripotent hematopoietic stem cells might undergo asymmetric cell division to sustain self-renewal of the stem cell pool and to generate lineage-committed progeny. Long-term repopulating capability would be maintained by cells that undergo fewer than three divisions and return to G_0 cell cycle phase. It is also tempting to hypothesize that only truly undivided hematopoietic stem cells will retain that repopulating potential. We also found that 10-fold more cells remain in the G_0 cell phase than truly undivided (PKH26^{bright}) cells after *ex vivo* expansion with either three or five cytokines. These findings strongly support the theory of asymmetric cell divisions to generate self-renewal of the stem cell pool, especially during the first divisions after *ex vivo* expansion. Collectively, our data suggest that *ex vivo* expansion of mobilized peripheral blood CD34$^+$ cells with early-acting cytokines could be safely used in clinical practice because of the persistence of enough cells with long-term repopulating capability.

All persons designated as authors qualified for authorship by contributing to the design and development of the study as well as the interpretation of data. All of them approved the final version of the manuscript.

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