Haematologica 2000; 85:25-30
original paper

Hematopoiesis and Growth factors

Interleukin-6 and interleukin-11 act synergistically with thrombopoietin and stem cell factor to modulate *ex vivo* expansion of human CD41+ and CD61+ megakaryocytic cells

LORENZA LAZZARI,° REINHARD HENSCHLER,* LUCILLA LECCHI,° PAOLO REBULLA,° RONALD MERTELSMANN,* GIROLAMO SIRCHIA°

*°Milano Cord Blood Bank, Centro Trasfusionale e di Immunologia dei Trapianti, IRCCS Ospedale Maggiore, Milan, Italy, and *Department of Hematology-Oncology, Albert-Ludwigs University Medical Center, Freiburg, Germany*

ABSTRACT

Background and Objectives. **Thrombopoietin (TPO), the ligand for the *c-mpl* receptor, regulates *in vivo* platelet production and increases the number of colony-forming unit megakaryocytes (CFU-MK). Other cytokines including interleukin (IL) -3, IL-6, IL-11 and stem cell factor (SCF) can stimulate megakaryopoiesis. The aim of this study was to evaluate the effects of different combinations of cytokines involved in megakaryocytopoiesis on stroma-free liquid cultures of purified human CD34+ cells.**

Design and Methods. **Peripheral blood cells were collected after mobilization with granulocyte colony-stimulating factor (G-CSF). Purified CD34+ cells were then cultured with different combinations of TPO, SCF, IL-3, IL-6 and IL-11.**

Results. **The addition of TPO and SCF alone generated a population positive for the antigens CD41 (5.5±2.9%) and CD61 (6.1±2.2%) but induced a low amplification of cell number (8.1±0.9 fold expansion). The presence of IL-6 or IL-11 was associated with MK progenitor cell expansion, and up to 7-10% of cultured cells were found to be CD41 and CD61 positive by flow cytometry. Conversely, the addition of IL-3 to this cytokine combination was associated with a prominent expansion of the myeloid lineage (70±10% of CD33+ cells) but only 0.9% and 2% of cultured cells were positive for CD61 and CD41 respectively.**

Interpretation and Conclusions. **Our study supports the idea that IL-6 and IL-11 play crucial roles in the proliferation of MK progenitors and the use of SCF, TPO, IL-6 and IL-11 for *ex vivo* expansion of this cell population.**

Key words: *ex vivo* expansion, megakaryocyte progenitors, hematopoietic growth factors, thrombopoietin, interleukin-6, interleukin-11, interleukin-3, stem cell factor

Correspondence: Lorenza Lazzari, DSc, Milano Cord Blood Bank, Centro Trasfusionale e di Immunologia dei Trapianti, IRCCS Ospedale Maggiore, via Francesco Sforza 35, 20122 Milan, Italy. Phone: international +39-02-55034053 – Fax: international +39.02.5458129 – E-mail: cbbank@polic.cilea.it

Megakaryopoiesis is a cellular process, characterized by proliferation of megakaryocytic precursors and platelet production, which can be critically impaired in patients undergoing hematopoietic stem cell transplantation.[1] Cytokine-driven *ex vivo* expansion of multipotent and lineage-committed hematopoietic progenitors can be used to overcome this impairment.[2-5] In addition to data concerning the effect of the megakaryocyte (MK) specific cytokines, several studies have shown that megakaryopoiesis could be regulated by other factors, including erythropoietin (EPO),[6] interleukin (IL)-3,[7,8] IL-6[9-11] and IL-11.[12-14]

In this context, we were interested in defining a cytokine combination capable of supporting the expansion of MK progenitors in stroma-free liquid cultures. To this aim, we used several factors, including the c-mpl receptor ligand, also known as thrombopoietin (TPO), which is a major regulator of MK and platelet production *in vivo*.[15-19] In liquid cultures of murine progenitors, TPO can synergize with the early acting cytokine stem cell factor (SCF) and stimulate both MK progenitor proliferation and maturation.[19] Since other cytokines such as IL-3, IL-6 and IL-11 are known to modulate MK progenitor proliferation and maturation at different levels,[20] we evaluated the effect of different combinations of TPO with these cytokines on *ex vivo* expansion of MKs in liquid cultures of purified human CD34+ cells collected from cancer patients undergoing autologous hematopoietic stem cell transplantation.

Design and Methods

CD34+ cell purification

Nine patients undergoing peripheral blood progenitor cell (PBPC) autologous transplantation were treated with conventional chemotherapy and subsequent administration of recombinant human granulocyte-colony stimulating factor (G-CSF). PBPCs were collected by apheresis on 10-12 days after chemotherapy. Mononuclear cells from the apheresis unit were separated by density gradient centrifugation (1.077 g/mL; Lympholyte-H, Cedarlane Laboratories, Ontario, Canada) at 400 g for 30 minutes at room temperature. The interface cells were collected and washed twice with Ca^{2+}-, Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS) containing 1% bovine

serum albumin (BSA). CD34+ cells were purified through avidin-biotin immunoaffinity columns (CellPro Inc, Bothell, WA, USA). An aliquot of the CD34+ target cell fraction was analyzed to determine purity by flow cytometry. The final recovery of CD34+ cells ranged from 70% to 98% of the initial CD34+ population and the analysis of the enriched cell fraction, performed with an anti-CD34+ monoclonal antibody (Becton Dickinson, Mountain View, CA, USA) revealed a purity of 75% to 88% CD34+ cells. The trypan-blue dye exclusion test showed a viability of 96-99%.

Growth factors

The following human recombinant factors were purchased from Genzyme (Cambridge, Mass., USA): IL-3 (used at 10 ng/mL), IL-6 (100 ng/mL), IL-11 (10 ng/mL), SCF (10 ng/mL). TPO (10 ng/mL) was kindly provided by Genentech, San Francisco, CA, USA. Five different combinations were tested: 1. TPO+SCF (T+S); 2. TPO+SCF+IL-3 (T+S+3); 3. TPO+SCF+IL-6 (T+S+6); 4. TPO+SCF+IL-11 (T+S+11); 5. TPO+SCF+IL-3+IL-6+IL-11 (T+S+3+6+11).

Study #1: effect of TPO on megakaryocyte colony formation from CD34+ cells

In this study we determined the effect of different TPO concentrations on the formation of MK colonies from CD34+ cells.

Megakaryocyte colony assay

Colony-forming units-megakaryocyte (CFU-MK) were assayed in plasmaclot culture as previously described.[21,22] Briefly, 5×10^3 purified CD34+ cells were cultured in 1 mL of Iscove's modified Dulbecco's medium (IMDM) (GIBCO, Grand Island, New York, USA), containing 10% BSA, 10% heat-inactivated pooled human AB serum, 10% citrated bovine plasma (GIBCO), 20 µg of L-asparagine (Sigma, St. Louis, MO, USA), 3.4 mg/mL $CaCl_2$ and different concentrations of TPO (2, 5, 10, 50 ng/mL). MK colonies were identified after 12 days of culture by fixing the plasmaclot *in situ* with methanol-acetone (1:3) for 20 min, washing with PBS (GIBCO) and double-distilled H_2O. The sample was then air-dried and stored at –20°C until immunofluorescence staining was performed. MK colonies were composed of cells displaying bright fluorescence after incubation with anti-CD41 (Immunotech Inc., Westbrook, Maine, USA), a monoclonal antibody directed against the glycoprotein (GP) IIb/IIIa complex. MK colonies were scored as aggregates of 3 or more brightly fluorescent cells.

Study #2: effect of TPO with different combinations of cytokines on CD34+ cells

TPO and SCF, alone or in combination with IL-3, with IL-6, with IL-11 and with IL-3+IL-6+IL-11, were added to stroma-free liquid cultures of purified CD34+ PBPCs.

Cells harvested after 12 days were counted and assayed for surface antigen expression and for clonogenic potential.

Liquid cultures of CD34+ cells

Isolated CD34+ cells were plated at 3×10^4/mL, 1 mL final volume, in wells of tissue culture plates, 24 wells (Sterilin Limited, Feltham, UK). The medium was IMDM supplemented with 10% fetal bovine serum (FBS) (GIBCO) containing different concentrations of growth factors as indicated. Cells were incubated for 12 days at 37ºC in fully humidified atmosphere in 5% CO_2 in air.

Clonogenic assay

The purified CD34+ cells and those after liquid culture were incubated in duplicate in 35 mm tissue culture plates at the concentrations of 250 and 5,000 cells/mL respectively. The medium contained 0.9% methylcellulose, 30% FBS, 1% BSA, 10^{-4} M2-mercaptoethanol, 3 U/mL erythropoietin, 50 ng/mL SCF, 10 ng/mL GM-CSF, 10 ng/mL IL-3 (StemCell Technologies, Vancouver, Canada). After 14 days of culture at 37°C in a 5% CO_2 fully humidified atmosphere, cultures of more than 50 cells were scored by microscopy as colony-forming cells (CFCs), i.e. the sum of colony-forming units (CFU)-granulocyte, –macrophage (GM), burst forming unit-erythrocyte (BFU-E) and CFU-granulocyte, -erythrocyte, -monocyte, and -megakaryocyte (CFU-GEMM).

Flow cytometry

CD34+ cultured cells were stained with one or more of the following monoclonal antibodies: anti-mouse IgG (to establish the background level of non-specific staining), anti-CD41 (GPIIb-IIIa), -CD61 (GPIIIa), -CD34, -CD33 (Becton Dickinson). Anti-CD41 and anti-CD61 were used to identify MK cells. For flow cytometry analysis 3×10^5 cells were exposed to monoclonal antibodies for 30 min at 4ºC and washed three times. Cells were analyzed by flow cytometry using a FACScan analyzer (Becton Dickinson) equipped with a filter set for FITC-PE dual-color fluorescence. The percent of stained cells was determined as compared to PE- and FITC-conjugated mouse IgG1 isotypic control (Becton Dickinson). Cell viability was evaluated by staining cells with ethidium bromide and acridine orange, and dead cells were gated out based on orange fluorescence.[23]

The *ex vivo* expansion was determined for each cytokine cocktail by the ratio between the total numbers of output and input cells.

Statistical analysis

Statistical analysis was performed using the Wilcoxon signed-rank sum test. Values of p lower than 0.05 were considered as statistically significant. The results are reported as mean ± standard deviation (SD) from nine separate experiments performed with CD34+ cells isolated from nine PBPC collections.

Results

Study 1: effect of TPO on megakaryocyte colony formation from CD34+ cells.

When human CD34+ cells were incubated with different concentrations of recombinant human TPO alone, maximum MK colony formation was observed with concentrations of 10 ng/mL or greater (Figure 1). Only MK colonies were formed. Based on the above observations, we used TPO at 10 ng/mL in Study #2.

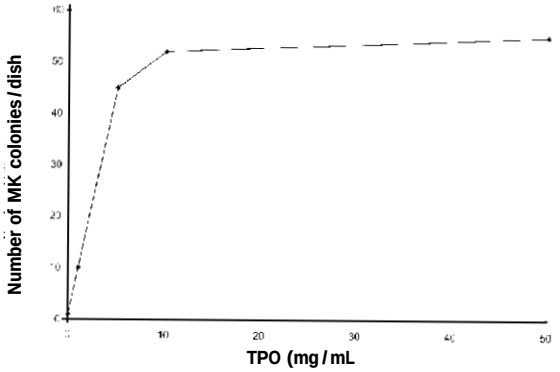

Figure 1. Effect of TPO on megakaryocyte colony formation from human CD34⁺ cells (Study #1).

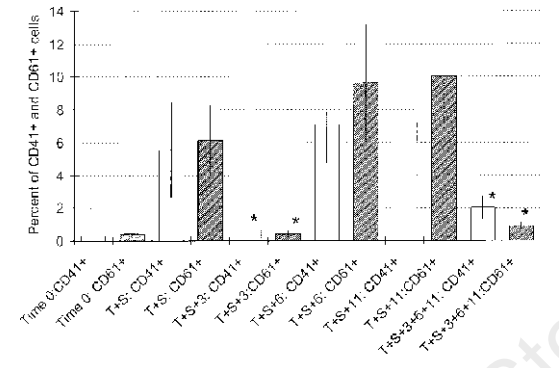

Figure 2. Effect of different combinations of cytokines on percentages of CD61⁺ and CD41⁺ cells before (dashed columns) and after 12 days of liquid culture (white columns). Data are shown as mean ± SD from nine separate experiments. *p<0.001 when TPO+SCF+IL-3 or TPO+SCF+IL-3+IL-6+IL-11 were compared with other combinations.

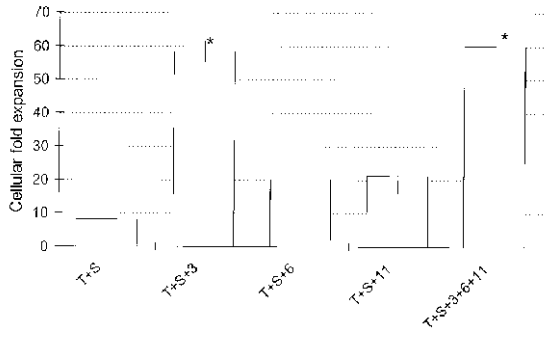

Figure 3. Effect of different combinations of cytokines on the cellular fold expansion after 12 days of liquid culture. Data are shown as mean ± SD from nine separate experiments. *p<0.05 when TPO+SCF+IL-3 or TPO+SCF+IL-3+IL-6+IL-11 were compared with other combinations.

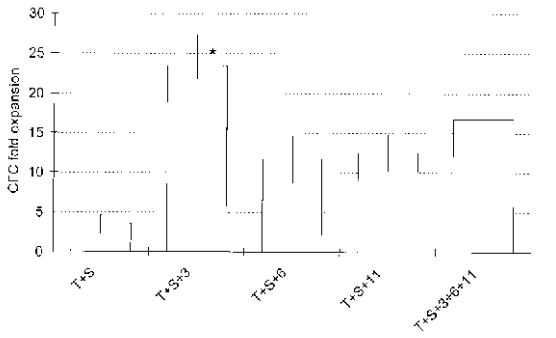

Figure 4. Effect of different combinations of cytokines on fold expansion of colony-forming cells (CFC) after 12 days of liquid culture. Data are shown as mean ± SD from nine separate experiments. Median (and range) absolute numbers of CFC with the different cytokine combinations were as follows: T+S: 166.3/5,000 cells (122-201); T+S+3: 154.5/5,000 cells (141-172.4); T+S+6: 224.1/5,000 cells (188.1-252.5); T+S+11: 227/5,000 cells (215-247); T+S+3+6+11: 107.3/5,000 cells (102-122). *p<0.05 for the comparison TPO+SCF+IL-3 vs other combinations.

Study #2: effect of TPO with different combinations of cytokines on CD34⁺ cells.

The percentage of CD41⁺ and CD61⁺ cells generated after cultures in the presence of IL-3 was significantly lower than in the presence of TPO+SCF+IL-6 and TPO+SCF+IL-11 (Figure 2; p<0.001 for the comparison of TPO+SCF+IL-3 or TPO+SCF+IL-3+IL-6+IL-11 vs combinations without IL-3). TPO+SCF+IL-6 and TPO+SCF+IL-11 were associated with the highest numbers of CD41⁺ and CD61⁺ cells.

Conversely, the addition of IL-3 alone or in combination with IL-6 and IL-11 to cultures in the presence of TPO and SCF was associated with a significant increase of cellular fold expansion (Figure 3; p<0.05 for the comparison of TPO+SCF+IL-3 or TPO+SCF+IL-3+IL-6+IL-11 vs other combinations).

The presence of IL-3 provided maximal stimulation for the production of CFC (Figure 4; p<0.05 for the comparison of TPO+SCF+IL-3 vs other combinations) and generated a significantly larger number of myeloid progenitors when compared to results obtained by the addition of IL-6 or IL-11 (Figure 5; p<0.05 for the comparison of TPO+SCF+IL-3 or TPO+SCF+IL-3+IL-6+IL-11 vs other combinations).

Discussion

Prolonged thrombocytopenia resulting from delayed MK progenitor cell reconstitution is an important complication after stem cell transplantation. In this regard, the infusion of expanded MK progenitors could be clinically useful.

In this study we examined the ability of TPO and SCF in combination with different cytokines (IL-3, IL-6, IL-11) to expand MK progenitors from human hematopoietic progenitors of peripheral blood origin. We used antibodies against GP IIIa (CD61) and

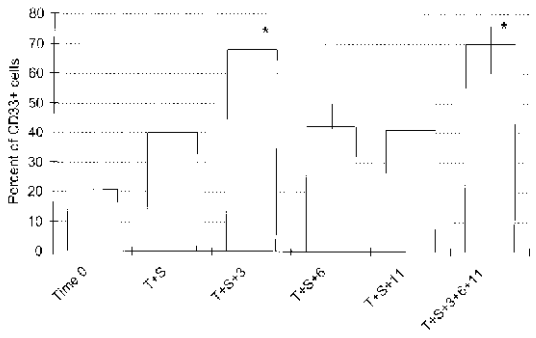

Figure 5. Effect of different combinations of cytokines on percentage of CD33+ cells after 12 days of liquid culture. Data are shown as mean ± SD from nine separate experiments. *$p<0.05$ for the comparison TPO+SCF+IL-3 or TPO+SCF+IL-3+IL-6+IL-11 vs other combinations.

GP IIb-IIIa (CD41) to identify MK in our cultures, as these glycoproteins show early expression during megakaryocytopoiesis.[24-26]

The addition of TPO and SCF alone produced higher percentages of MK progenitors than those obtained in cultures with added IL-3, but the cell growth under these conditions was limited and at the end of the expansion the absolute numbers of MK progenitors were smaller. At the same time, IL-3 showed little effect on *ex vivo* expansion of MK progenitors, which was offset by a higher fold expansion of cell growth.

Moreover, in agreement with previous studies,[9-14] our data support the hypothesis that IL-6 and IL-11 have critical roles in modulating cytokine-driven proliferation of MK progenitors. The data indicate that the addition of TPO and early-acting human hematopoietic growth factors, such as SCF, supports MK growth. Apparently, there is no significant difference between the effects of IL-6 and IL-11, because peripheral blood $CD34^+$ cells generated very similar percentages of $CD41^+$, $CD61^+$ and $CD33^+$ cells. Moreover, $CD34^+$ cells showed very similar clonogenic potentials in the presence of TPO+SCF+IL-6 and TPO+SCF+IL-11.

Results similar to ours were obtained by Williams *et al.*,[27] who used combinations of five cytokines (TPO+SCF+IL-1, -6 and -11). Their data showed a marked expansion of early MKs from $CD34^+$ cells. Moreover, these authors demonstrated that TPO requires additional MK-active cytokines for optimal *ex vivo* expansion of MK precursor cells.

The opinions in regard to IL-3 as an MK-active cytokine are controversial. Some authors reported a negative effect of IL-3 on MK differentiation.[8,19,27] According to these authors, it is unclear whether the pleiotropic actions of IL-3 support the commitment to other lineages or directly inhibit terminal megakaryocytopoiesis. Williams *et al*.[27] did not include IL-3 in their cultures because their data indicated that this cytokine is not essential in the early phases of megakaryocytopoiesis and its presence is inhibitory to MK differentiation.

Additional information was recently collected through animal studies performed in the murine model by Broudy *et al.*[19] These investigators proposed a new model of thrombopoiesis regulation based on data documenting the effects of TPO on proliferation of the more differentiated MK progenitor cell, CFU-Meg, and the ability of optimal levels of IL-3 or TPO to stimulate the growth of similar numbers of CFU-MK. Moreover, these growth factors were shown to exert an additive effect on MK *in vitro*.[28] Thus, it seems that IL-3 maintains steady-state proliferation of MK progenitors, while TPO, IL-6 and IL-11 augment the proliferative effects of IL-3 and induce MK progenitor cell maturation.

Human studies by Kobayashi *et al*.[29] investigating these concepts showed that TPO can function as an early-acting cytokine capable of regulating proliferation of multipotential progenitors in bone marrow and acts more synergistically with IL-3 than with IL-6 and IL-11 in supporting progenitor production. Interestingly, Piacibello *et al.*[30] demonstrated the key role played by TPO and Flt-3 ligand in sustaining proliferation and self-renewal of primitive stem cells in cord blood for more than 6 months.

Some authors showed that the combination of early-acting cytokines and TPO provides optimal MK growth, as evaluated in terms of MK purity and yield.[31,32] In the study by Guerriero *et al*.[32] the addition of TPO alone generated a virtually pure MK progeny, but also induced a rather low amplification of cell number. Moreover, although the addition of early growth factors without TPO caused extensive MK proliferation, it was associated with prevailing growth of granulocytic cells. These results suggest that TPO induces the differentiation of MK-committed hematopoietic progenitor cells, whereas IL-3, SCF and IL-6 trigger extensive growth of progenitor cells which are then channeled by TPO into the megakaryocytic differentiation pathway, as also described by Laluppa *et al.*[33]

In conclusion, our results support the idea that IL-3 has a critical role in the complex cytokine network regulating hematopoiesis and the use of early-acting cytokines, such as SCF, TPO, IL-6 and IL-11, for *ex vivo* expansion of MK progenitors before autologous or allogeneic hematopoietic progenitor cell transplantation. Moreover, our methodology for the expansion of MK hematopoietic progenitors of peripheral blood origin compares favorably with other procedures in terms of MK purity and yield.[34-37] Further work aimed at the optimization of the *ex vivo* expansion procedure in the absence of animal-derived substances will be necessary to facilitate the development of protocols suitable for clinical application. Encouraging observations have been published in this regard by Ramsfjell *et al.*,[38] who showed that the synergistic effect of TPO was observed both in fetal calf serum-supplemented and serum-depleted media.

Contributions and Acknowledgments

LLa and RH were the principal investigators and designed the study. LLa and PR prepared the manuscript and managed the statistical data. LLe, RM and GS were involved in critically revising the intellectual content of the manuscript and

RM and GS gave the final approval for its submission. The order of the names reflects the importance of the contribution of each author to the work and their hierarchical position in the Institutions.

Funding

This work was supported in part by a grant from Fondazione Cariplo, Italy.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received April 8, 1999; accepted September 15, 1999.

Potential implications for clinical practice

- The positive effect of IL-6, IL-11, thrombopoietin and stem cell factor on the expansion of megakaryocyte precursors present in autologous peripheral blood stem cell collections may translate into decreased platelet transfusion requirements during the early post-transplantation phase of expanded autologous PBSC recipients.
- The use of this static, stroma-free expansion system may facilitate the clinical use of expanded hematopoietic progenitor cells. In addition, this system may prove useful in cord blood transplantation, which is associated with delayed platelet reconstitution.

References

1. Armitage JO. Bone marrow transplantation (review). N Engl J Med 1994; 330:827-38.
2. Koller MR, Emerson SG, Palsson BO. Large-scale expansion of human stem and progenitor cells from bone marrow mononuclear cells in continuous perfusion cultures. Blood 1993; 82:378-84.
3. Henschler R, Brugger W, Luft T, Frey T, Mertelsmann R, Kanz L. Maintenance of transplantation potential in ex vivo expanded CD34(+) selected human peripheral blood progenitor cells. Blood 1994; 84:2898-903.
4. Verfaillie CM. Can human hematopoietic stem cells be cultured ex vivo? Stem Cells 1994; 12:466-76.
5. Zandstra PW, Eaves CJ, Piret JM. Expansion of hematopoietic progenitor cell populations in stirred suspension bioreactors of normal human bone marrow cells. Biotechnology 1994; 12:909-14.
6. Hoffman R. Regulation of megakaryocytopoiesis. Blood 1989; 74:1196-212.
7. Bruno E, Cooper RJ, Briddell RA, Hoffman R. Further examination of the effects of recombinant cytokines on the proliferation of human megakaryocyte progenitor cells. Blood 1991; 77:2339-46.
8. Debili N, Hegyi E, Navarro S, et al. In vitro effects of hematopoietic growth factors on the proliferation, endoreplication, and maturation of human megakaryocytes. Blood 1991; 77:2326-38.
9. Navarro S, Debili N, Le Couedic JP, et al. Interleukin-6 and its receptor are expressed by human megakaryocytes: in vitro effects on proliferation and endoreplication. Blood 1991; 77:461-71.
10. Ishibashi T, Kimura H, Uchida T, Kariyone S, Friese P, Burstein SA. Human interleukin 6 is a direct promoter of maturation of megakaryocytes in vitro. Proc Natl Acad Sci USA 1989; 86:5953-7.
11. Koike K, Nakahata T, Kubo T, et al. Interleukin-6 enhances murine megakaryocytopoiesis in serum-free culture. Blood 1990; 75:2286-91.
12. Teramura M, Kobayashi S, Hoshino S, Oshimi K, Mizoguchi H. Interleukin-11 enhances human megakaryocytopoiesis in vitro. Blood 1992; 79:327-31.
13. Burstein SA, Mei RL, Henthorn J, Friese P, Turner K. Leukemia inhibitory factor and interleukin-11 promote maturation of murine and human megakaryocytes in vitro. J Cell Physiol 1992; 153:305-12.
14. Yonemura Y, Kawakita M, Masuda T, Fujimoto K, Kato K, Takatsuki K. Synergistic effects of interleukin 3 and interleukin 11 on murine megakaryopoiesis in serum-free culture. Exp Hematol 1992; 20:1011-6.
15. de Sauvage FJ, Hass PE, Spencer SD, et al. Stimulation of megakaryocytopoieis and thrombopoieis by the c-Mpl ligand. Nature 1994; 369:533-8.
16. Lok S, Kaushansky K, Holly RD, et al. Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. Nature 1994; 369:565-8.
17. Bartley TD, Bogenberger J, Hunt P, et al. Identification and cloning of a megakaryocyte growth and development factor that is a ligand for the cytokine receptor Mpl. Cell 1994; 77:1117-24.
18. Zeigler FC, de Sauvage FJ, Widmer RH, et al. In vitro megakaryocytopoietic and thrombopoietic activity of c-mpl ligand (TPO) on purified murine hematopoietic stem cells. Blood 1994; 84:4045-52.
19. Broudy VC, Lin NL, Kaushansky K. Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin, stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy in vitro. Blood 1995; 85:1719-26.
20. Metcalf D. Hematopoietic regulators: redundancy or subtlety? Blood 1993; 82:3515-23.
21. Bagnara GP, Zauli G, Giovannini M, Re MC, Furlini G, La Placa M. Early loss of circulating hemopoietic progenitors in HIV-1 infected subjects. Exp Hematol 1990; 18:426-30.
22. Zauli G, Re MC, Davis B, et al. Impaired in vitro growth of purified ($CD34^+$) hematopoietic progenitors in human immunodeficiency virus-1 seropositive thrombocytopenic individuals. Blood 1992; 79:2680-7.
23. Gee AP. Preparation of samples for flow cytometric analysis. J Hematother 1993; 2:413-9.
24. Vainchenker W, Deschamps JF, Bastin JM, et al. Two monoclonal antiplatelet antibodies as markers of human megakaryocyte maturation: immunofluorescent staining and platelet peroxidase detection in megakaryocyte colonies and in vivo cells from normal and leukemic patients. Blood 1982; 59:514-21.
25. Breton-Gorius J, Vainchenker W. Expression of platelet proteins during in vitro and in vivo differentiation of megakaryocytes and morphological aspects of their maturation. Semin Hematol 1986; 23:43-67.
26. Debili N, Issaad C, Massè JM, et al. Expression of CD34 and platelet glycoproteins during human megakaryocytic differentiation. Blood 1992; 80:3022-35.
27. Williams JL, Pipia GG, Datta NS, Long MW. Thrombopoietin requires additional megakaryocyte-active cytokines for optimal ex vivo expansion of megakaryocyte precursor cells. Blood 1998; 91:4118-26.
28. Bertolini F, Battaglia M, Pedrazzoli P, et al. Megakaryocytic progenitors can be generated ex vivo and safely administered to autologous peripheral blood progenitor cell transplant recipients. Blood 1997; 89:2679-88.
29. Kobayashi M, Laver JH, Kato T, Miyazaki H, Ogawa M. Thrombopoietin supports proliferation of human

primitive hematopoietic cells in synergy with steel factor and/or interleukin-3. Blood 1996; 88:429-36.
30. Piacibello W, Sanavio F, Garetto L, et al. Extensive amplification and self-renewal of human primitive hematopoietic stem cells from cord blood. Blood 1997; 89:2644-53.
31. Koizumi K, Sawada K, Yamaguchi M, et al. In vitro expansion of $CD34^+/CD41^+$ cells from human peripheral blood $CD34^+/CD41^-$ cells: role of cytokines for in vitro proliferation and differentiation of megakaryocytic progenitors. Exp Hematol 1998; 26:1140-7.
32. Guerriero R, Testa U, Gabbianelli M, et al. Unilineage megakaryocytic proliferation and differentiation of purified hematopoietic progenitors in serum-free liquid cultures. Blood 1995; 86:3725-36.
33. Laluppa JA, Papoutsakis ET, Miller WM. Evaluation of cytokines for expansion of the megakaryocyte and granulocyte lineages. Stem Cells 1997; 15:198-206.
34. Kaushansky K, Lok S, Holly RD, et al. Promotion of megakaryocyte progenitor expansion and differentiation by c-Mpl ligand thrombopoietin. Nature 1994; 369:568-71.
35. Choi ES, Nichol JL, Hokom MM, Hornkohl AC, Hunt P. Platelets generated in vitro from proplatelet-displaying human megakaryocytes are functional. Blood 1995; 85:402-13.
36. Nichol JL, Hornkohl AC, Choi ES, et al. Enrichment and characterization of peripheral blood-derived megakaryocyte progenitors that mature in short-term liquid culture. Stem Cells 1994; 12:494-505.
37. Mazur EM, Basilico D, Newton JL, et al. Isolation of large numbers of enriched human megakaryocytes from liquid cultures of normal peripheral blood progenitors cells. Blood 1990; 76:1771-82.
38. Ramsfjell V, Borge OJ, Veiby OP, et al. Thrombopoietin, but not erythropoietin, directly stimulates multilineage growth of primitive murine bone marrow progenitor cells in synergy with early acting cytokines: distinct interactions with the ligands for c-kit and FLT3. Blood 1996; 88:4481-92.