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Multi-lineage expansion potential of primitive hematopoietic progenitors: Superiority of umbilical cord blood compared to mobilized peripheral blood

Ian D. Lewis and Catherine M. Verfaillie

Stem Cell Institute, Department of Medicine and Cancer Center, University of Minnesota, Minneapolis, Minn., USA

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Objective. **The majority of studies assessing ex-vivo expansion of primitive hematopoietic cells only address production of myeloid progeny whereas it may be more appropriate to maintain or expand progenitors that retain capacity for multilineage differentiation. In this study, we assessed the capacity of the murine fetal liver cell line AFT024 to expand primitive myeloid progenitors (LTC-IC) and lymphoid progenitors (NK-IC) from umbilical cord blood (CB) and mobilized peripheral blood (PB) $CD34^+lin^-38^-$ cells.**

Methods. **Sorted cells were established in expansion cultures in direct contact with the feeder or in a transwell above the feeder (noncontact culture) and various combinations of Flt-3L (FL), stem cell factor, interleukin 7, thrombopoietin (Tpo), and macrophage inflammatory protein-1α added. Frequency of LTC-IC and NK-IC was assessed at day 0 and following 2 and 5 weeks expansion culture.**

Results. **CB contained significantly more LTC-IC and NK-IC at day 0 and showed an enhanced capacity for expansion compared to PB. The combination of FL and Tpo showed maximal expansion of CB LTC-IC and NK-IC at 5 weeks in both contact and noncontact conditions. In contrast, expansion of PB LTC-IC and NK-IC was maximal at 2 weeks and required multiple cytokines.**

Conclusions. **These results demonstrate that AFT024 can expand primitive hematopoietic progenitors from CB and PB and expanded cells retain the capacity for myeloid and lymphoid differentiation. These findings emphasize the importance of assessing multi-lineage differentiation capacity following ex-vivo expansion. Elucidation of specific factors necessary for ex-vivo expansion will contribute to the development of a clinically applicable system.** **© 2000 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

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Introduction

Ex-vivo expansion of hematopoietic cells can be used to amplify defined cell populations for specific clinical purposes such as the abrogation of neutropenia post–bone marrow transplantation, expansion of immunomodulatory cells, or expansion of primitive hematopoietic cells. If the aim is to amplify primitive hematopoietic cells, then these ex-vivo expansion systems should generate lymphomyeloid progeny and maintain longevity and engraftment potential. Several assay systems are currently used to enumerate primitive hematopoietic cells. These include the cobblestone area-forming assay (CAFC) [1], long-term culture-initiating cell assay (LTC-IC) [2], and the severe combined immunodeficient (SCID) mouse repopulating cell assay (SRC) [3]. LTC-IC are defined as cells capable of producing colony-forming cells (CFC) after a minimum of 5 weeks culture. Our laboratory has recently described the NK-IC assay, which enumerates $CD34^+$ progenitor cells capable of initiating growth of lymphoid cells $\geq$5 weeks after plating [4].

In vivo, stem cells are maintained in close contact with the bone marrow microenvironment. Soluble as well as contact dependent signals are thought to be important in maintaining stem cell quiescence and self-renewal [5–8]. The recognition of a number of cytokines with important effects on early hematopoietic cells has led to the development of stroma-free cytokine-driven systems that are able to expand nucleated cells, hematopoietic progenitors, and $CD34^+$ cells. However, the ability of these systems to maintain

Offprint requests to: Catherine M. Verfaillie, M.D., Professor of Medicine, Box 806 UMHC, 420 Delaware Street SE, Minneapolis, MN 55455 USA; E-mail: verfa001@tc.umn.edu

Ian Lewis is currently at the Hanson Centre for Cancer Research, Adelaide, Australia.

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primitive hematopoietic cell function is less certain [9–11]. Thrombopoietin (Tpo), stem cell factor (SCF), and Flt3-ligand (FL) are all implicated in the regulation of primitive hematopoietic cells. Tpo supports hematopoietic stem cell (HSC) survival, induces HSC into cycle, and in combination with interleukin 3 (IL-3) or SCF, induces production of megakaryocyte, erythroid, and granulocyte-macrophage progenitors [12–14]. In synergy with other cytokines, SCF and FL promote the growth of primitive hematopoietic cells and FL also has a role in the production of B cells and dendritic cells [15,16]. Macrophage inflammatory protein-1α (MIP-1α) is a chemokine that inhibits primitive hematopoietic cell proliferation but may stimulate proliferation of more mature progeny [17].

BM stroma supports the maintenance and expansion of primitive human or murine progenitors [18,19]. This is thought to be multifactorial and may not be replaced by cytokines alone. We have shown that LTC-IC are maintained for up to 5 weeks when cultured either in direct contact or in a transwell above primary human stroma or cell lines such as M210-B4 (stroma noncontact), suggesting that stromal mediated influences on primitive hematopoietic cells are not directly dependent on stroma contact [20]. We use here the murine fetal liver cell line AFT024 that can maintain and expand murine competitive repopulating stem cells [21]. We and others have also shown that AFT024 maintains LTC-IC or CAFC from bone marrow (BM) or umbilical cord blood (CB) for up to 5 weeks when cultured in the presence of various cytokines and in contact with stroma [22,23].

CB and mobilized peripheral blood (PB) are sources of HSC that are amenable to ex-vivo manipulation. Compared with adult BM, CB contains a higher number of primitive hematopoietic cells that have greater proliferative potential [24]. However, the absolute cell number is a limiting factor in a clinical transplant setting. On the other hand, PB can relatively easily be obtained in large cell numbers from adult donors treated with hematopoietic growth factors.

Here we assessed the ability of the murine fetal liver cell line, AFT024, to expand primitive myeloid and lymphoid cells from CB and PB. Our results demonstrate critical differences in expansion potential and cytokine requirements, which may lead to the development of clinically applicable systems for ex-vivo expansion.

Methods

Isolation of cells

Umbilical CB cells were collected by standard procedures used for banking of CB after informed consent was obtained. Mononuclear cells (MNC) were collected by Ficoll-Hypaque (Sigma-Diagnostics, St. Louis, MO) density gradient. Samples were cryopreserved in 10% dimethylsulphoxide (Sigma-Diagnostics), 20% fetal calf serum (FCS, Hyclone Laboratories, Logan, UT), by controlled rate freezing. Following thawing, some samples were pooled because of low cell numbers. Mobilized PB was obtained from normal volunteer donors after informed consent was obtained. They received 5 μg/kg granulocyte colony stimulating factor (G-CSF) subcutaneously for 5 days prior to undergoing a single apheresis procedure. PB samples were generally used fresh.

$CD34^+$ cells were isolated either by selection on an avidin immunoaffinity system (Ceprate system; Cellpro, Bothell, WA) or by magnetic cell separation (MACS system; Miltenyi Biotec, Auburn, CA). Procedures were performed as per manufacturer's recommendations. $CD34^+$ selected cells were labeled with an anti-$CD34^+$ antibody, either conjugated to allophycocyanin (Becton Dickinson Immunocytometry Systems, San Jose, CA) or streptavidin (GIBCO-BRL, Grand Island, NY), R-Phycoerythrin (PE) anti-CD38 (Becton Dickinson), and a cocktail of fluorescein isothiocyanate (FITC) conjugated antibodies being anti-CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD14, CD15, CD16, and CD19 (all from Becton Dickinson). Cells were sorted on a FACS Star-Plus flow cytometry system equipped with a CONSORT 32 computer. Sort gates for $CD34^+lin^-38^-$ cells were based on the appropriate negative controls. Using this cell sorting strategy we were able to isolate populations consistently above 90% purity.

Stromal feeder

The murine fetal liver cell line, AFT024 (a kind gift from Dr. I. Lemischka, Princeton University), was maintained at 33°C in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) supplemented with 20% FCS, 50 μmol/l 2-mercaptoethanol (2-ME, Bio-Rad, Hercules, CA). Cells were subcultured in 24- or 96-well plates (Costar, Cambridge, MA), grown to confluency, and then irradiated at 2000 rads using a Mark 1 Cesium irradiator (Shepard, Glendale, CA) 24 hours prior to establishing cultures.

Expansion cultures

Cultures were established in 24 well plates with between 500 and 2000 sorted $CD34^+lin^-38^-$ cells per well. Cells were plated in direct contact with stroma (contact) or in collagen coated transwells with a 0.4 μm microporous filter (Transwell-COL, Costar) above the feeder (noncontact). Medium consisted of RPMI supplemented with 20% FCS, penicillin 1000 U/ml, streptomycin 100 U/ml (GIBCO-BRL), and 50 μmol/l 2ME. Different combinations of FL (Immunex, Seattle, WA), SCF (Amgen, Thousand Oaks, CA), IL-7 (R&D Systems, Minneapolis, MN), Tpo (Amgen), and MIP-1α (R&D Systems) were added, all at 10 ng/ml except IL-7, which was at 20 ng/ml. Medium was changed every 7 days. After 2 weeks or 5 weeks individual wells were harvested and contents split for establishment of LTC-IC assays, NK-IC assays, and CFC assays.

LTC-IC and NK-IC assays

Freshly sorted cell populations (day 0) and progeny after expansion culture of $CD34^+lin^-38^-$ cells were plated in limiting dilution in LTC-IC and NK-IC assays. LTC-IC assays were performed as previously described [25]. Briefly, cells were plated in 4 dilutions of 22 replicates on preirradiated AFT024 coated 96-well plates. Medium consisted of Iscove's modified Dulbecco's medium (IMDM) supplemented with 12.5% FCS, 12.5% horse serum (Stem Cell Technologies, Vancouver, Canada), penicillin 1000 U/ml, streptomycin 100 U/ml, 2 μmol L-glutamine (GIBCO-BRL), and 10^{-6} μmol hydrocortisone. Cultures were maintained for 5 weeks with a half-medium change weekly. Medium was then completely removed and replaced with clonogenic methylcellulose

medium consisting of 1.12% methylcellulose (Fisher, Chicago, IL), IMDM, 30% FCS, 3 U/ml erythropoietin (Amgen), and supernatant of the bladder cell carcinoma cell line 5637 (7.5%). After 2 weeks wells were evaluated for the presence or absence of hematopoietic colonies and scored as positive or negative, respectively. LTC-IC frequency was then calculated according to Poisson statistics [26].

NK-IC assay was established in limiting dilution on preirradiated AFT024 containing 96 well plates. Medium consisted of 2:1 vol/vol DMEM and Hams F12 (GIBCO-BRL) supplemented with 20% heat inactivated human AB serum (North American Biologicals, Miami, FL) supplemented with penicillin 1000 U/ml, streptomycin 100 U/ml, 25 μmol 2-ME, IL-2 1000 U/ml (Amgen), IL-3 5 ng/ml (R&D Systems), FL 10 ng/ml, SCF 10 ng/ml, and IL-7 20 ng/ml. Half-medium changes were performed weekly using the same media, with the exception of IL-3, which was omitted, and only 10% heat inactivated human AB serum. After 5 weeks culture, wells were scored visually for presence of mature progeny. Positive wells were harvested and stained with anti-CD56 PE (Becton Dickinson) and anti-CD1a FITC (PharMingen, San Diego, CA). NK-IC frequency was then calculated according to Poisson statistics based on the number of wells containing $CD56^+$ cells.

Evaluation of CFC generated per LTC-IC

CFC assays were established by plating $CD34^+lin^-38^-$ cells in methylcellulose containing IMDM supplemented with 30% FCS, 3 IU erythropoietin, 10 ng/ml each of IL-3, G-CSF, and granulocyte-macrophage-CSF (Immunex). Cultures were incubated in a humidified atmosphere at 37°C and 5% CO_2 and evaluated after 14 days for the presence of colony-forming units granulocyte-macrophage (CFU-GM) or burst-forming units erythroid (BFU-E). The CFC frequency was calculated per 100 cells plated at day 0. At weeks 2 and 5 the progeny of an equivalent number of cells were plated to calculate the CFC frequency. The number of colony-forming cells (CFC) per LTC-IC was calculated by dividing the frequency of CFC by the absolute frequency of LTC-IC at each time point.

Statistics

Results of experimental points obtained from multiple experiments are expressed as the mean ± SEM. Significance levels were determined by two-sided Student's *t*-test.

Results

Cord blood contains more primitive myeloid and lymphoid cells compared to peripheral blood

$CD34^+lin^-38^-$ cells were sorted from CB and PB and established in LTC-IC and NK-IC assays. CB $CD34^+lin^-38^-$ cells contained 6.5% ± 0.9% LTC-IC and 5.9% ± 1.2% NK-IC. This was significantly more than PB $CD34^+lin^-38^-$ cells, which contained 1.1% ± 0.2% LTC-IC ($p < 0.001$) and 1.9% ± 0.3% NK-IC ($p = 0.008$) (Fig. 1).

CB and PB LTC-IC expand after 2 and 5 weeks culture in direct contact with AFT024

Sorted populations were established in bulk culture in contact with AFT024 or in transwells above AFT024 (noncontact). Exogenous cytokines were added in various combinations based on their reported potential to stimulate expansion of primitive cells [27–31]. The combination of Flt3 L, SCF, and IL-7 (FS7) has been previously shown to expand BM LTC-IC for up to 5 weeks [32]. We used this as our standard combination and added MIP-1α (FS7M) or Tpo (FS7T). The combination of Flt3 L and Tpo (FT) was also tested. Cells were cultured for 2 weeks and 5 weeks and then harvested and their capacity to initiate LTC-IC and NK-IC assessed.

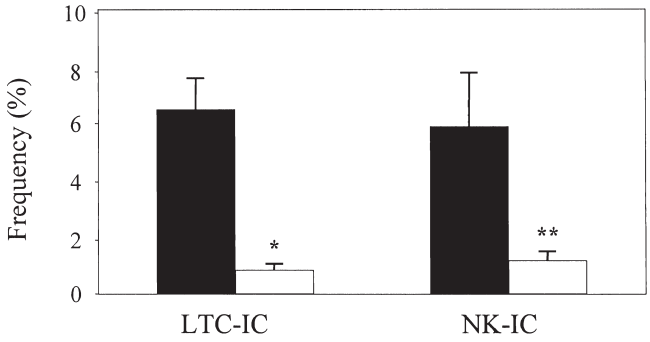

Figure 1. Frequency of $CD34^+lin^-38^-$LTC-IC and NK-IC in CB and PB. Five or ten thousand sorted cells were plated in limiting dilution in LTBMC or NK-IC medium in contact with AFT024 feeders. After 5 weeks culture, media was removed and wells were overlaid with clonogenic methylcellulose mixture. LTC-IC were scored 2 weeks later. For NK-IC enumeration, wells containing cells after 5 weeks were harvested individually and assessed for the presence of $CD56^+$ or $CD1a^+$ cells. LTC-IC and NK-IC frequencies were calculated according to Poisson statistics. Data represent the mean ± sem of 10 independent experiments for LTC-IC determination and 6 independent experiments for NK-IC determination. * $p < 0.0001$; ** $p < 0.01$. (■ cord blood, □ peripheral blood)

After 2 and 5 weeks culture, CB LTC-IC expanded 2.4- and 2.2-fold respectively when cultured in FS7 (Fig. 2). Addition of either MIP-1α or Tpo to this combination produced an improvement in expansion to 3.6-fold and 4.8-fold at 2 weeks and 2.3-fold and 3.5-fold at 5 weeks (Fig. 2). The best combination for expansion in contact conditions was FT, which induced expansion of 5.8-fold ($p = 0.002$ compared to FS7) at 2 weeks and 7.7-fold at 5 weeks ($p = 0.006$ compared to FS7). This was the only combination that showed superior expansion at 5 weeks compared to 2 weeks in contact conditions.

We next examined the effect of the same culture conditions on expansion of PB $CD34^+lin^-38^-$ LTC-IC. After 2 weeks, the FS7 containing cultures showed 1.2-fold expansion of LTC-IC in contact conditions (Fig. 2A) at 2 weeks. Addition of MIP-1α to FS7 produced a 3.6-fold expansion, whereas FS7T caused a 2.6-fold expansion at 2 weeks ($p = 0.04$ compared to FS7). FT was the best combination in contact at 2 weeks, producing 4.9-fold expansion of LTC-IC ($p = 0.03$ compared to FS7) (Fig. 2A).

After 5 weeks of culture, the number of LTC-IC remaining in PB $CD34^+lin^-38^-$ progeny was reduced compared to week 2 in all conditions tested. The FS7T combination was

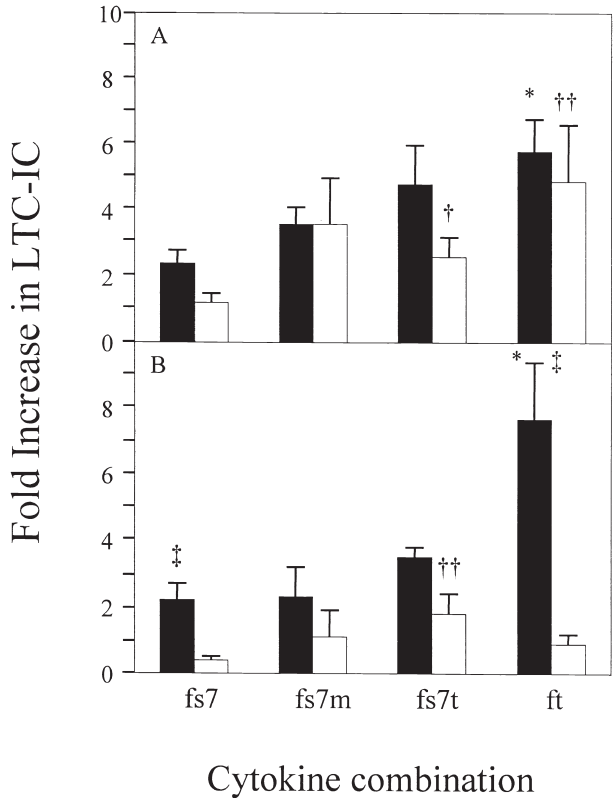

Figure 2. Cord blood and peripheral blood $CD34^+lin^-38^-$ LTC-IC expand after 2 weeks **(A)** or 5 weeks **(B)** culture in direct contact with AFT024. Sorted CB or PB $CD34^+lin^-38^-$ cells were plated in direct contact with stroma. Different combinations of FL (f), SCF (s), IL-7 (7), Tpo (t), and MIP-1α (m) were added. After 2 weeks or 5 weeks individual wells were harvested and contents split for establishment of LTC-IC and NK-IC assays. Fold expansion of LTC-IC was calculated by dividing the LTC-IC frequency at week 2 or 5 by the LTC-IC frequency of freshly sorted cells. Data represent the mean ± sem of 6–8 independent experiments **(A)** and 5 independent experiments **(B)**. * $p < 0.01$(comparison of CB LTC-IC expansion compared to FS7); † $p = 0.04$; †† $p = 0.03$ (comparison of PB LTC-IC expansion compared to FS7); ‡ $p < 0.01$ (comparison between CB and PB LTC-IC). (■ cord blood, □ peripheral blood)

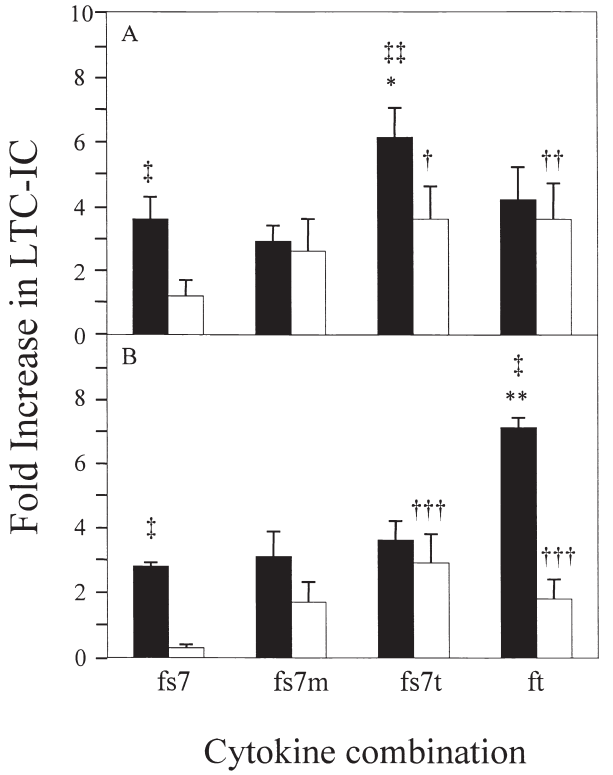

Figure 3. Cord blood and peripheral blood $CD34^+lin^-38^-$ LTC-IC expand after 2 weeks **(A)** or 5 weeks **(B)** in noncontact culture with AFT024. Sorted CB $CD34^+lin^-38^-$ cells were plated as described in the legend to Figure 2. After 2 weeks or 5 weeks individual wells were harvested and contents split for establishment of LTC-IC and NK-IC assays. Data represent the mean ± sem of 6–8 independent experiments **(A)** and 5 independent experiments **(B)**. * $p = 0.02$; ** $p < 0.01$ (comparison of CB LTC-IC expansion compared to FS7); † $p = 0.04$; †† $p = 0.03$; ††† $p < 0.01$ (comparison of PB LTC-IC expansion compared to FS7); ‡ $p < 0.01$; ‡‡ $p = 0.04$ (comparison between CB and PB LTC-IC). (■ cord blood, □ peripheral blood)

best, producing 1.8-fold expansion ($p = 0.03$ compared to FS7) (Fig. 2B).

Except for FS7M at 2 weeks, CB $CD34^+lin^-38^-$ LTC-IC showed superior expansion compared to PB in all cytokine combinations at 2 and 5 weeks. This was statistically significant at 5 weeks for FS7 and FT (Fig. 2).

AFT024 supports similar expansion of CB and PB LTC-IC in noncontact culture compared to contact conditions

We also examined whether AFT024 could support expansion of LTC-IC in noncontact conditions. After 2 weeks culture, a 3.6-fold expansion of CB LTC-IC was seen when cultured in FS7 (Fig. 3A). Addition of MIP-1α resulted in 2.9-fold expansion, whereas FS7T induced 6.1-fold expansion ($p = 0.02$ compared to FS7) (Fig. 3A). After 5 weeks, expansion of CB LTC-IC was similar in FS7 and FS7M but reduced to 3.6-fold in FS7T in comparison with week 2. However, a further increase in LTC-IC expansion was seen for FT at 5 weeks, with a 7.1-fold expansion ($p = 0.002$ compared to FS7), which was better than the 4.2-fold expansion seen at 2 weeks (Fig. 2B).

After 2 weeks, the FS7 containing cultures produced a 1.2-fold expansion of PB LTC-IC but only 0.3-fold expansion at 5 weeks (Figs. 3A and 3B). Addition of MIP-1α to FS7 produced a modest increase in expansion capacity, whereas the combination of FS7T caused a 3.6-fold expansion ($p = 0.04$ compared to FS7). FT was the best combination in contact, producing 4.9-fold expansion of LTC-IC ($p = 0.03$ compared to FS7) (Fig. 4A).

After 5 weeks of culture, the number of LTC-IC remaining in PB $CD34^+lin^-38^-$ progeny was reduced compared to week 2 in all conditions tested. The FS7T combination was best, producing 2.9- ($p = 0.009$ compared to FS7) fold expansion (Fig. 3B).

In all noncontact conditions tested, expansion of CB LTC-IC was superior to PB. At 2 weeks, expansion of CB LTC-IC was statistically superior in FS7 ($p = 0.006$) and

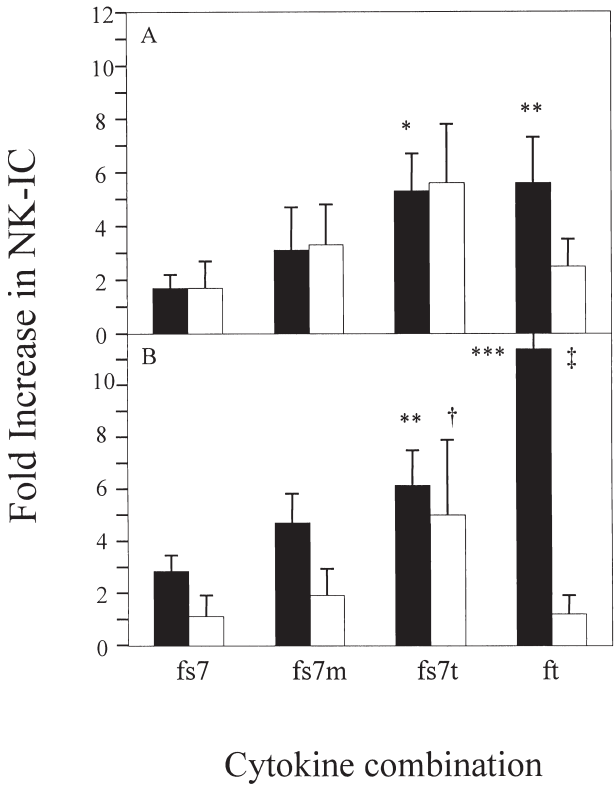

Figure 4. CB and PB CD34$^+$lin$^-$38$^-$ NK-IC expand after 2 weeks **(A)** or 5 weeks **(B)** in direct contact culture. Sorted CB CD34$^+$lin$^-$38$^-$ cells were plated as described in the legend to Figure 2. After 2 or 5 weeks of culture in expansion conditions, as in Figure 2, individual wells were harvested and contents split for establishment of LTC-IC and NK-IC assays. Data represent the mean ± sem of 5–6 independent experiments **(A)** and 5 independent experiments **(B)**. * $p = 0.01$; ** $p = 0.02$; *** $p = 0.04$ (comparison of CB NK-IC expansion compared to FS7); † $p = 0.05$(comparison of PB NK-IC expansion compared to FS7); ‡ $p < 0.01$ (comparison between CB and PB NK-IC). (■ cord blood, □ peripheral blood)

FS7T ($p = 0.04$), and at 5 weeks in FS7 ($p = 0.002$) and FT ($p = 0.0006$).

AFT024 supports expansion of CB and PB NK-IC in contact culture

We next examined if these culture conditions can expand primitive lymphoid progenitors or NK-IC. NK-IC are hematopoietic progenitor cells capable of initiating growth in lymphoid conditions to generate CD56$^+$ progeny. When CB CD34$^+$lin$^-$38$^-$ cells were cultured for 2 or 5 weeks in FS7 there was a 1.7-fold and 2.8-fold expansion of NK-IC, respectively. Addition of MIP-1α or Tpo to FS7 increased NK-IC expansion further, with greater expansion seen at 5 weeks (Figs. 4A and 4B). Again, the combination that gave the greatest expansion of NK-IC was FT, which resulted in a 5.6-fold expansion in contact culture ($p = 0.02$ compared to FS7) (Fig. 4A) at 2 weeks and 11-fold expansion at 5 weeks ($p < 0.001$ compared to FS7) (Fig. 4B).

After 2 weeks the number of NK-IC remaining in PB CD34$^+$lin$^-$38$^-$ progeny surpassed the number of LTC-IC. In contact conditions NK-IC expansion ranged from 1.7-fold for FS7 up to 5.6-fold for FS7T (Fig. 4A) at 2 weeks. At 5 weeks the number of NK-IC was almost identical to that seen at week 2 for most conditions tested. Of note, expansion in the four cytokine combinations FS7M and FS7T (1.9-fold and 4.9-fold in contact) was again greater than the two or three cytokine combinations (Fig. 4B).

Expansion of PB NK-IC with multiple cytokines was similar to that of CB NK-IC except in FT, where dramatic differences were noted ($p = 0.002$ at 5 weeks) (Fig. 4B).

CB and PB NK-IC expand in noncontact conditions on AFT024

When CD34$^+$lin$^-$38$^-$ cells were cultured for 2 or 5 weeks in AFT024-noncontact cultures with FS7, there was twofold and 2.7-fold expansion of NK-IC. Addition of MIP-1α or Tpo to FS7 increased NK-IC expansion further at both 2 and 5 weeks (Figs. 5A and 5B). Again, the combination that gave the greatest expansion of NK-IC was FT, which resulted in a 7.1-fold expansion at 2 weeks ($p = 0.04$ com-

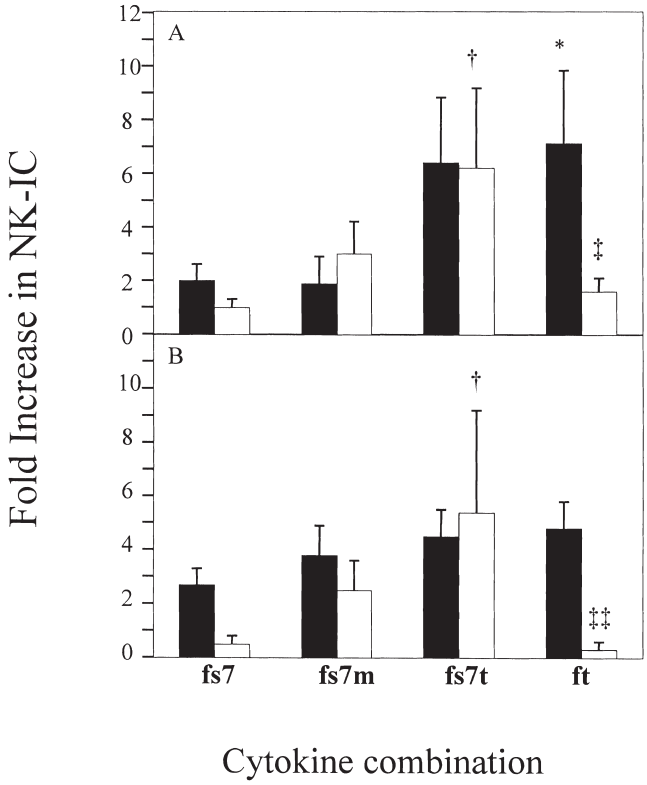

Figure 5. CB and PB CD34$^+$lin$^-$38$^-$ NK-IC expand after 2 weeks **(A)** or 5 weeks **(B)** in noncontact culture. Sorted CB CD34$^+$lin$^-$38$^-$ cells were plated as described in the legend to Figure 2. After 2 weeks or 5 weeks individual wells were harvested and contents split for establishment of LTC-IC and NK-IC assays. Data represent the mean ± sem of 5–6 independent experiments **(A)** and 5 independent experiments **(B)**. * $p = 0.04$ (comparison of CB NK-IC expansion compared to FS7); † $p = 0.05$ (comparison of PB NK-IC expansion compared to FS7); ‡ $p = 0.02$; ‡‡ $p = 0.05$ (comparison between CB and PB NK-IC). (■ cord blood, □ peripheral blood)

pared to FS7) (Fig. 5A) and 4.8-fold expansion at 5 weeks (Fig. 5B).

The expansion of PB $CD34^+lin^-38^-$ NK-IC in noncontact conditions was remarkably similar in magnitude and pattern compared to contact culture. At 2 weeks, expansion of NK-IC ranged from 1.0-fold in FS7 up to 6.2-fold for FS7T ($p = 0.05$ compared to FS7) (Fig. 5A). Of note, expansion in the four cytokine combinations FS7M and FS7T (threefold and 6.2-fold at 2 weeks and 2.5-fold and 5.4-fold at 5 weeks, respectively) was again greater than the two or three cytokine combinations (Figs. 5A and 5B). Similar to what was seen in contact culture, CB NK-IC showed superior expansion compared to PB NK-IC in FT at both 2 weeks ($p = 0.02$) and 5 weeks ($p = 0.05$).

Expanded LTC-IC from CB and PB retain CFC production potential

Finally, we determined the generative potential of LTC-IC after ex-vivo expansion by enumerating the number of CFC generated by each LTC-IC, calculated by dividing the number of CFC per 100 cells by the absolute frequency of LTC-IC per 100 cells. CFC generated per LTC-IC at day 0 was 4.2 for CB and 1.9 for PB. This increased at week 2 in CB to 10.6 and 9.4 CFC per LTC-IC in contact and noncontact conditions, respectively. Similarly, at week 5 these results were 11.2 and 10.7, respectively. There was a little more variability in the capacity of PB LTC-IC to generate CFC. At week 2 in contact conditions each LTC-IC produced 5.4 CFC, and 10.5 in noncontact conditions. At week 5 these results were 15 for contact and 11.6 for noncontact.

Discussion

In this study we compare the multi-lineage expansion potential of primitive hematopoietic progenitors present in CB and PB using the murine fetal liver cell line AFT024. We show that LTC-IC and NK-IC from either source can be expanded for ≥5 weeks but that important differences exist between the requirements of CB and PB progenitors to expand ex vivo.

We show that CB LTC-IC can be expanded with addition of various combinations of early acting cytokines in contact and noncontact conditions for a minimum of 5 weeks. At 2 weeks all combinations produced a twofold to sixfold expansion of LTC-IC in contact and noncontact conditions. Cultures were extended out to 5 weeks and at this time point all of the three- and four-factor combinations expanded LTC-IC twofold to fourfold. Of note, the two-factor combination, FT, induced a sevenfold expansion in both contact and noncontact conditions. Thus, in combination with early acting cytokines, AFT024 is able to expand primitive myeloid cells from CB $CD34^+lin^-38^-$ in both contact and noncontact conditions. In contrast to the three- and four-factor combinations, the combination of Flt-3L and Tpo added to AFT024 culture caused a greater expansion of LTC-IC at 5 weeks compared to 2 weeks. This may reflect the ability of the Flt-3L + Tpo combination to retain LTC-IC in an undifferentiated state while allowing them to divide. This is also consistent with published studies showing that Flt-3L + Tpo in the absence of stroma can support LTC-IC expansion [33,34]. A recent report has also shown that the combination of FL, SCF, and Tpo is a potent combination for expansion of BM $CD34^+38^-$ multipotent progenitors [29]. In our studies, the same combination supplemented with IL7 was less good at expanding LTC-IC from either UCB or PB. These discrepancies may be due to other differences in the culture conditions, or alternatively and less likely, that IL-7, added to the other combinations to promote lymphoid development, inhibits LTC-IC expansion. While it may be argued that expansion at 5 weeks is not relevant for clinical ex-vivo expansion protocols, studying this system may lead to insights into the mechanisms underlying the intrinsic capacity for primitive cells to divide and expand. Expansion was similar in contact and noncontact conditions, which is consistent with our earlier observation that culture in AFT024 requires cell-stroma interaction unless cytokines are added [23].

Successful stem-cell expansion will require that the culture also supports cells capable of differentiating into lymphoid cells. Most ex-vivo expansion studies have only assessed production of clonogenic myeloid progeny but not examined survival or expansion of lymphoid progenitors. Our group has described the NK-IC assay in which $CD34^+lin^-DR^-$ cells initiate lymphoid growth ≥5 weeks after plating. This is demonstrated by cells expressing the NK cell marker CD56 and can be confirmed in a cytotoxicity assay using K562 as a target [4]. We show that NK-IC can be expanded after 2 and 5 weeks culture in both contact and noncontact conditions in the different cytokine combinations. The majority of NK-IC generated were $CD56^+$ cells. We demonstrate that the combination of FL and Tpo is not only the best for LTC-IC expansion but also for CB NK-IC expansion. Again, expansion was greater at 5 weeks than at 2 weeks in contact culture. Addition of SCF and IL-7 to this resulted in expansion that was similar in both contact and noncontact culture. One possible explanation for these findings is the ability of FL and Tpo to preserve the primitive state of a cell and prevent its differentiation. Of note, a 3.5-fold expansion of NK-IC was also seen in AFT024 noncontact cultures. Thus we show that AFT024 has the unique capacity for supporting the expansion of NK-IC in CB $CD34^+lin^-38^-$ cells in a noncontact system. Several studies have shown that contact with stroma is essential for maintaining lymphoid progenitors and for commitment of primitive progenitors to the lymphoid lineage [35–37]. Our observation that AFT024 noncontact conditions can support the expansion of NK-IC may suggest that AFT024 cells produce a diffusible soluble factor required for lymphopoiesis. Elucidation of the nature of this factor(s) will contribute to our understanding of lymphopoiesis and may

further enhance our ability to manipulate lymphoid cell growth ex vivo.

Expansion of mobilized PB may be necessary when mobilization is unsuccessful or when tumor purging is needed. Consistent with previous studies from our laboratory and others, we show that the frequency of PB $CD34^+lin^-38^-$ LTC-IC [38–40] and NK-IC [41] is significantly lower than that found in CB. Here we also show that expansion of PB $CD34^+lin^-38^-$ LTC-IC was not as great as that seen in CB. Further, we found that maximal expansion generally required four cytokines, and that expansion was maximal at 2 weeks rather than 5 weeks. These differences compared to CB suggest that the capacity for PB to expand is more limited than that of CB. As maximal expansion of PB LTC-IC requires multiple cytokines, it is possible that induction of proliferation is associated with differentiation rather than maintenance and self-renewal. However, the LTC-IC that persisted, like those in CB cultures, continued to have a primitive phenotype (i.e., high numbers of CFC produced by LTC-IC).

Our AFT024 cultures supported expansion of NK-IC in PB to the same extent as from CB. The lymphoid potential of mobilized PB has only been studied in a limited fashion. Previous studies have shown that G-CSF mobilized PB contains fewer NK progenitors with reduced expansion capacity compared to adult BM [41], with culture on AFT024 being superior to M210-B4 [42]. Here we show that NK-IC in G-CSF mobilized PB from normal donors can expand when cultured with AFT024, which is maximal with the four-cytokine combination FS7T. This combination works in both contact and noncontact conditions, and expansion is seen out to 5 weeks. This further emphasizes the role of AFT024 in lymphopoiesis and supports our ongoing efforts to characterize AFT024 derived factors important for lymphopoiesis.

The interpretation of our data is based on changes in LTC-IC and NK-IC frequency rather than changes in absolute numbers. This is out of necessity because stromal cell contamination of the stromal contact cultures does not allow absolute values to be calculated. In fact, our method probably underestimates the difference between CB and PB as the CB population had around sixfold more LTC-IC and NK-IC at day 0 and all populations were visually noted to proliferate.

The quality of primitive progenitors (i.e., the ability of LTC-IC to generate CFC) may be equally important to judge the effect of an expansion system on primitive progenitors. Breems, Blokland, Siebel, et al. evaluated the capacity of mobilized PB $CD34^+$ CAFC to expand and produce CFC in various conditions. After 6 weeks culture they demonstrated loss of CAFC and reduction in CFC production per CAFC in all conditions tested [43]. We show, using a different stromal feeder, that CFC generation is increased at both 2 and 5 weeks from both CB and PB in both contact and noncontact. Thus AFT024 not only supports increases in number of LTC-IC but the expanded LTC-IC are functionally equal to or superior in regard to CFC production compared to uncultured LTC-IC.

We have previously extensively tested the $CD34^+lin^-HLA\text{-}DR^-$ population. We chose to study $CD34^+lin^-38^-$ cells in this study because this population may be more highly enriched for stem cell activity as measured by LTC-IC [38], extended-LTC-IC [44], and SRC [45]. Further, in UCB, LTC-IC are present in both the $HLA\text{-}DR^-$ and $HLA\text{-}DR^+$ positive population. Use of $CD34^+HLA\text{-}DR^-$ cells would therefore exclude a significant proportion of primitive UCB progenitors. Recent publications suggest that additional fractionation may uncover more primitive populations, such as $CD34^-lin^-$ [46,47] or $CD34^+KDR^+$ [48]. Whether conditions described here for $CD34^+lin^-CD38^-$ cells will be extendable to these other cell populations remains to be determined.

For practical reasons CB samples were cryopreserved prior to use, whereas PB samples were used fresh, and this may be a confounding factor in the interpretation of our results. Studies on the effect of cryopreservation on CB $CD34^+$ show CFC expansion is not impaired [49], although a recent study suggests the quality of recovered primitive progenitors may be impaired [50]. We did not formally test the effect of cryopreservation, but if there was any effect it would have been to reduce CB expansion and therefore our results may have underestimated the expansion capacity.

In summary, we have evaluated the capacity of AFT024 to maintain and expand primitive myeloid and lymphoid cells from CB or mobilized PB. We show that conditions favorable for expansion of these cells differ for each source. What this study does not address is whether the system retains stem cells in a multi-potent state, or retains cells that have partially differentiated along either the myeloid or lymphoid lineages. To resolve this question, gene marking studies will be necessary. The other important issue is whether these expanded cells retain multi-lineage engraftment potential, which is critical if this system is going to be applied to a clinical transplant setting. This is currently under investigation. The expansion capacity of CB is superior to PB and therefore CB may be a superior source of stem cells for ex-vivo manipulation. Understanding the molecular processes involved in stem cell self-renewal and proliferation and elucidating the differences between CB and PB may enable the design of specific ex-vivo systems for manipulation of stem cells.

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