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Ex vivo expansion of cord blood

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Abstract

A marked increase in the utilization of umbilical cord blood (UCB) transplantation has been observed in recent years; however, the use of UCB as a hematopoietic stem cell (HSC) source is limited primarily by the number of progenitor cells contained in the graft. Graft failure, delayed engraftment and profound delay in immune reconstitution lead to significant morbidity and mortality in adults. The lack of cells available for post transplant therapies, such as donor lymphocyte infusions, has also been considered to be a disadvantage of UCB. To improve outcomes and extend applicability of UCB transplantation, one potential solution is *ex vivo* expansion of UCB. Investigators have used several methods, including liquid suspension culture with various cytokines and expansion factors, co-culture with stromal elements and continuous perfusion systems. Techniques combining *ex vivo* expanded and unmanipulated UCB are being explored to optimize the initial engraftment kinetics as well as the long-term durability. The optimal expansion conditions are still not known; however, recent studies suggest that expanded UCB is safe. It is hoped that by *ex vivo* expansion of UCB, a resulting decrease in the morbidity and mortality of UCB transplantation will be observed, and that the availability of additional cells may allow adoptive immunotherapy or gene transfer therapies in the UCB setting.

Keywords

cord blood; *ex vivo* expansion; hematopoietic stem cells

Introduction

Umbilical cord blood (UCB) has become an important source of hematopoietic stem cell (HSC) support after myeloablative and non-myeloablative therapies.[1–6] UCB is rapidly available and seems to have a lower incidence of GVHD despite HLA disparity, which make it an attractive option when traditional HSC donors would not be optimal. In addition, because of the allowance of greater HLA disparity than BM or PBSC grafts, UCB has

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Conflict of interest
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provided a significantly higher chance of finding a donor for minority populations who are currently under-represented in donor registries.

Although the use of UCB as a stem cell source has observed a significant increase in recent years, especially in children and young adults, it is not without drawbacks. One major limitation of UCB as an HSC therapy is the low cell dose available for transplantation. It is now well documented that the total nucleated cell dose (TNC) transplanted per kg of body wt of the recipient correlates with outcomes. Patients >45 kg receiving a single UCB unit tend to have markedly prolonged time to neutrophil and plt engraftment and higher rates of engraftment failure.[7–11] As a consequence, UCB transplantation remains significantly more successful in children.[9,10,12] Even in small children receiving satisfactory cell doses, there is still a delay in engraftment on all cell lines when compared with traditional stem cell sources[13–15] and some delay in immune reconstitution,[16,17] suggesting that even in the optimal patient population, the low progenitor cell dose given with UCB transplantation has negative effects on the outcomes.

In general, there have been two approaches to overcome the obstacle of low TNC cell dose observed with UCB transplantation. One approach is the usage of more than one UCB unit to achieve a higher number of TNC available for infusion.[18–22] Many trials are currently underway in assessing efficacy in both adults and children. The second approach has been the attempt to expand UCB units *ex vivo*. *Ex vivo* expansion can be performed on either a portion of a UCB unit or on the unit in its entirety, with the expanded cells infused either at the time of transplant of ‘unmanipulated’ fraction, or can be given at a separate time. The manipulated UCB could be from either the same unit, or potentially a different UCB unit. The combination of *ex vivo*-expanded fractions and unmanipulated UCB fractions might prove to be a beneficial strategy.[23,24] Clinical protocols that explore these approaches are currently being assessed at the University of Texas MD Anderson Cancer Center and in other clinical centers[23–27] (Table 1).

Ex vivo expansion strategies are not unique to UCB, and could also be applied to HSC derived from BM and mobilized peripheral blood,[28–30] as there is evidence of functional and phenotypic heterogeneity within the HSC population.[31–35] Regardless of HSC source, there is a concern that during *ex vivo* expansion, one may selectively expand a particular subset, specifically a short-term reconstituting, lower ‘quality’ HSC at the expense of long-term reconstituting, higher ‘quality’ HSCs. If this occurs, it could potentially lead to earlier initial hematopoietic recovery with later graft failure, by diminishing the long-term reserve of the graft.[36] McNiece *et al*.[37] report compromised long-term repopulating activity after *ex vivo* expansion in a fetal sheep model; Von Drygalski *et al*.[38] report the loss of radioprotective and long-term engraftment potential with *ex vivo* expansion of murine BM; and in a clinical study, Holyoake *et al*.[39] report the absence of durable engraftment from *ex vivo*-expanded $CD34^+$ cells. On the contrary. Piacibello *et al*.[40] observe evidence of self-renewal and amplification of HSC during *ex vivo* expansion; Lewis *et al*.[41] report that UCB cells capable of engraftment in primary, secondary and tertiary xenogeneic recipients are preserved after *ex vivo* expansion;and Guenechea *et al*.[42] report a delay in engraftment in a mouse model, suggesting that potentially more primitive and less rapidly engrafting cells are preserved during *ex vivo* expansion. Homing of the cells after a short-term *ex vivo* expansion does not

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seem to be affected;[43] however, the overall durability of the grafts is an issue that will need to be clarified as more clinical expansion studies in humans progress.

Ex vivo-expanded products may possess an inherent reduction in the long-term hematopoietic reconstitution potential under certain conditions.[36,37,39] The potential skewing of the UCB product to a more rapidly reconstituting, but short-lived, HSC profile could potentially be exploited to provide a clinical advantage, especially when *ex vivo*-expanded and ‘unmanipulated’ UCB fractions are combined for transplantation. Clinical data have suggested that UCB that has been subject to *ex vivo* expansion does provide more rapid initial hematopoietic reconstitution, whereas ‘unmanipulated’ UCB is the source of the long-term, sustainable hematopoiesis.[24] There are other clinical data, however, that suggest that augmenting UCB with *ex-vivo* expanded cells may not provide any benefit in terms of outcomes,[24–26] a discrepancy that further studies may elucidate. The goal of *ex-vivo* expansion of cord blood is at least twofold. The primary focus of expansion has been to generate sufficient numbers of HSCs to optimize the graft available for transplant. Another important goal is to generate higher numbers of lineage-committed progenitor cells that, although transient, would allow rapid recovery from pancyotpenia, thus decreasing early morbidity and mortality. Combining expanded and unmanipulated products may provide the best product for transplantation. Currently, there are several different strategies used for *ex vivo* expansion.

Liquid suspension culture

In their natural microenvironment, HSCs are surrounded by marrow stroma that provides the required cytokines and growth factors to control hematopoiesis. In an effort to expand HSCs *ex vivo*, one method has been liquid culture, in which UCB cells are cultured with combinations of cytokines, growth factors and other growth-promoting compounds in various flasks, bags or containers. Before expansion in liquid culture, primitive hematopoietic progenitor cells (primarily $CD133^+$ or $CD34^+$) from UCB, BM or mobilized peripheral blood[30] must first be isolated from hematopoietic tissue, using one of the number of techniques available to perform this isolation at a clinical grade (including the Miltenyi CliniMACS system (Clinimacs-Milteny Biotec Inc., Auburn, CA, USA) and the Nexell Isolex device (Isolex-Baxter International Inc., Deerfield, IL, USA)). Once isolated, the hematopoietic progenitor cells are incubated in a culture medium. The centers have experimented with various ‘cocktails’ of growth factors and compounds targeted at stimulating the proliferation of primitive hematopoietic progenitors. Common components used in *ex vivo* HSC expansion protocols include: SCF, IL-3, IL-6 and G-CSF;[30] SCF, TPO and G-CSF;[24,29] and Flt-3 ligand (FL), SCF, IL-3, IL-6 and G-CSF,[44,45] although the concentration of the cytokines may not correlate with HSC expansion.[46] One of the concerns of expansion is telomere degradation with subsequent cycles of proliferation. It seems that FL and TPO may be important in preventing this from occurring and therefore protecting the self-renewal ability of primitive stem cells.[45,47] Increasing the proliferative potential of the HSC, and therefore the numbers of HSCs available, is a main goal of expansion, and SCF and IL-6 possibly enhance the proliferation of subpopulations.[48–50] IL-11 has also been incorporated into *ex vivo* expansion cocktails.[38,51–55] The optimal combination has yet to be defined.

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In a 37-patient study (25 adults and 12 children), Shpall *et al*.[23] showed the efficacy of *ex vivo* expansion of isolated $CD34^+$ UCB cells. UCB units were selected that matched at 4–6/6 HLA loci. In the majority of cases, the UCB was frozen in two fractions (40 and 60%). The patients received an ablative preparative regimen according to disease, age and previous treatment. One of the two strategies was adopted. (1) One fraction was thawed, $CD34^+$ cells isolated (Nexell Isolex 300-i device) and cultured *ex vivo* in defined medium (Amgen, Amgen Inc., Thousand Oaks, CA, USA) in the presence of SCF, TPO and G-CSF (each at 100 ng/ml) for 10 days. After 10 days, the remaining (unmanipulated) fraction of the UCB unit was thawed and administered with the *ex vivo*-expanded cells. (2) The whole CB unit was thawed, one fraction transplanted unmanipulated and the remaining fraction cultured *ex vivo* in defined medium (Amgen) in the presence of SCF, TPO and G-CSF for 10 days, before administration. The resulting expansion increased TNC 56-fold (median, range 1.03–278) and the total number of $CD34^+$ cells fourfold (median, range 0.1–20.0). There was no significant difference in the times to neutrophil and plt engraftment between groups.

McNiece *et al*.[28] subsequently developed a two-step, 14-day cord expansion protocol that they showed yields more effective *ex vivo* expansion than does the single-step 10-day protocol described above. An initial 7-day small-volume culture is followed by an additional 7-day larger-volume culture (total 14 days), yielding a >400-fold increase in TNC and a >20-fold increase in $CD34^+$ cells.[56] This two-step strategy was used at MD Anderson in a recent prospective randomized trial comparing double cord blood transplant with transplant using one unmanipulated UCB unit combined with one unit that was expanded *ex vivo*.[57] In all, 71 patients with advanced hematologic malignancies were randomized. Patients either received a myeloablative preparative regimen (n= 41) or non-myeloablative regimen (N=30), depending on the disease and clinical status. In patients who received an expanded UCB unit, the smallest unit was CD133 selected using the CliniMACS device (day –14). The T cell containing $CD133^-$ fraction was frozen. The $CD133^+$ fraction was cultured for 14 days in media containing SCF, G-CSF and TPO. On day 0, the unmanipulated UCB unit was infused, followed by both the $CD133^-$ and the *ex vivo*-expanded fractions of the second unit. The infused median TNC $\times 10^7$/kg was 3.5 and 3.6, and median CD34 $\times 10^5$/kg was 1.8 and 1.1, respectively, for expanded and unmanipulated patients, with a median TNC fold expansion of 23 (0.44–275) and for $CD34^+$ cells, 2.3 (0–957). The patients undergoing a reduced-intensity regimen who received an expanded UCB unit engrafted neutrophils in a median of 7 days (range 4–15 days; n = 14) vs 14 days (range 5–32 days; n = 12) in those receiving two unmanipulated units. (P=0.05). In total, 34 (48%) have survived for a median of 11.3 (range 2–49) months. Most of the patients on the expanded arm had some evidence of the expanded UCB chimerism after transplant (7–82%); however, by 14 months all patients had predominance of the unmanipulated cord. This suggests that expansion may affect the durability of engraftment by *ex vivo*-expanded cells.

Further modifications to this liquid *ex vivo* expansion technique have included attempts to further optimize *ex vivo* culture conditions;[46,48,52–55,58] the development of serum-free culture systems;[52,56,59] the use of tetraethylenepentamine, a copper chelator thought to modulate the proliferation and differentiation of primitive hematopoietic progenitors;[60–62] the use of histone deacetylases, thought to promote HSC self-renewal;[63] and the use of

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glycogen synthase kinase-3 inhibitors reported to maintain the pluripotency of stem cells.[64] A phase I/II trial was conducted by de Lima *et al.*[27] to analyze the potential therapeutic efficacy of tetraethylenepentamine added in liquid UCB expansion. Ten heavily pre-treated patients were allocated UCB units that were frozen in fractions. At 21 days before transplant, the smaller fraction was thawed, $CD133^+$ cells were isolated using the CliniMACS device and liquid culture expansion was performed in minimum essential medium α-medium containing 10% FCS (Hyclone, Thermo Fisher Scientific Inc., Waltham, MA, USA) and supplemented with SCF, FL, IL-6 and TPO and tetraethylenepentamine. Before transplant, the patients received myeloablative therapy, and on day 0 received the unmanipulated UCB fraction with the expanded fraction infused on day +1. In total, 9 of the 10 patients engrafted at a median of 30 days ($n = 9$; range 16–46 days) with 100% donor chimerism despite the low TNC/kg infused in this study (mean = 1.7×10^7/kg). Plt transfusion independence occurred at a median of 48 days (range 35–105). Nine patients were alive at day 100 and three died during the 180-day study period due to infectious complications. No grade III or IV GVHD occurred. The average fold expansion of TNC in the expanded fraction was 219 with a $CD34^+$ cell mean increase of sixfold over the CD34 + cell content of the entire unit. The small sample size and heterogeneous makeup of UCB units prohibited correlation between CFUs, CD34 cell dose or TNC count and engraftment. Additional studies will be required to analyze the efficacy of tetraethylenepentamine in the expansion of UCB.

As a variation on the liquid culture technique, Delaney *et al.* at the Fred Hutchinson Cancer Center recently used an immobilized, engineered form of the Notch ligand d–1 with recombinant cytokines (SCF, FL, IL-6, TPO and IL-3) to stimulate *ex vivo* UCB expansion.[65] Five patients with aggressive leukemias received fludarabine, cytoxan and TBI as a preparative regimen, followed by one unmanipulated UCB unit and a second unit that was CD34 enriched and cultured for 16 days with the combination of cytokine and ligand.[66] The median age of the patients was 28 years. The CD34 population increased at an average of 160-fold (range 41–382), with an average TNC fold increase of 660 (range 146–1496). The infused TNC/kg $\times 10^7$ average was 2.9 (range 1.9–5.8) for the unmanipulated cells and 4.6 (range 0.6–9.1) for the cultured cells, with an infused CD34 cells/kg ($\times 10^5$) of 2.2 (range 1.1–3.4) and 53.4 (range 9.3–133), respectively. All patients engrafted at a median of 14 days (range 7–34), as compared with 25 days (range 16–48) in patients (n =17) who underwent an identical transplant regimen with two unmanipulated UCB units. In patients who showed a trend toward early engraftment (ANC > 500 at days 7, 9 and 16), myeloid cells were mainly derived from the expanded unit at day 14, whereas in the other two patients who achieved ANC >500 at day 13 and 20, myeloid engraftment was derived from the unmanipulated cells. Two patients had persistence of expanded cells, one until day 280, now no longer present; the other still has persistence at day 75. Five of six patients are surviving in remission for an average of 277 days (range 70–632). These results further suggest that the expanded unit may provide short-term repopulating cells that may facilitate and improve speed of engraftment of the non-cultured unit. This is a promising study as expansion seems to have favorably affected outcomes. Regardless, the optimal combination of cytokines and growth factors has yet to be defined, and liquid culture is limited by smaller volumes and by the static nature of the culture.

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Stromal co-culture

The hematopoietic microenvironment is composed of hematopoietic and non-hematopoietic (cellular and extracellular) components.[67–69] Complex molecular cues that direct hematopoiesis are provided by the stem cell 'niche', and are, at least in part, re-responsible for the regulation of differentiation and maturation of HSCs.[70–86] When cells are expanded *ex vivo*, they lose the support and regulation provided by the microenvironment, and receive only the specific cytokines and growth factors provided in the culture media, thus relying on exogenous direction, and potentially driving differentiation at the expense of self-renewal. MSC can be isolated as plastic adherent cells from a variety of fetal and adult tissues.[87–96]

The third-party (neither donor nor recipient) allogeneic MSCs have been shown in NOD-SCID mice to promote engraftment of UCB CD34$^+$ when co-administered[93,97] and also to possess immunomodulatory activity.[96,98–104] In culture, MSCs are characterized by a spindle-shaped and plastic-adherent morphology and are phenotypically characterized as HLA-I (ABC), CD105, CD73, CD90 and CD166 positive, and HLA-DR (II), CD80, CD31, CD34 and CD45 negative. Unfortunately, UCB and mobilized peripheral blood are poor sources of MSCs,[105] although a recent study did suggest that MSCs from the Wharton's jelly of umbilical cords showed surface receptors similar to other MSCs, and may be able to support UCB expansion.[106] Co-culture of UCB with MSCs (even allogeneic) can restore some of the interaction that occurs between the microenvironment of the marrow stroma and the HSC.[80–84] The foci of hematopoiesis and cobblestone areas are visible during co-culture,[92] showing that direct HSC-MSC interactions are occurring and that the MSCs are not simply acting as a feeder layer.

For stromal co-culture, MNCs are isolated by density separation and co-cultured with established MSC monolayers in a medium containing FBS and a growth factor cocktail (for example, SCF, TPO and G-CSF, as with liquid culture expansion).[92] The non-adherent cells are removed from the co-culture after 7 days and subjected to a secondary expansion on an additional MSC monolayer. The original adherent layer, which is then composed of MSC and HSC, is re-fed with fresh medium containing growth factors. Culture is then continued for an additional 7 days (total 14 days).[92] A 10- to 20-fold increase in total nucleated cells, 7- to 18-fold increase in committed progenitor cells (GM-CFC), two- to five-fold increase in primitive progenitor cells (high proliferative potential-CFC) and a 16- to 37-fold increase in CD34$^+$ cells, have been reported using co-culture expansion.[92] Co-administration of third-party MSC with the UCB-derived HSC may aid engraftment[93,97] and provide immunomodulatory benefits;[98–103,107] therefore, it may prove clinically beneficial to re-infuse both non-adherent and adherent cells from the expansion process.

A clinical trial is underway at MD Anderson using UCB expanded on related donor MSCs combined with an unmanipulated UCB unit. A family member (minimum of 2/6 HLA match) serves as the BM-derived MSC donor. Approximately 100ml of BM is aspirated and confluent MSCs are generated over approximately 21 days. The UCB unit with the lowest TNC dose is then thawed, washed and divided into 10 equal fractions. Each fraction is placed into one flask containing >70% of the confluent MSC and cultured in *ex vivo* expansion medium. After an incubation for 7 days at 37 °C, the non-adherent cells are

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collected from each flask. The content of a single flask is then placed into a 1 l Teflon-coated culture bag and cultured for an additional 7 days (14 days total). The flasks are then re-fed and incubated as well. The myeloablative therapy for this protocol is antithymocyte globulin and fludarabine, melphalan and thiotepa, and the non-myeloablative therapy is antithymocyte globulin and fludarabine, CY and 200 cGy TBI. On day 0, the unmanipulated UCB unit is infused, followed by the expanded UCB cells (from both the bags and the co-culture flasks). A median fold expansion of 12 was observed in both the TNC and the $CD34^+$ subsets. For the six recipients of myeloablative therapy, the median time to neutrophil engraftment has been 14.5 days (range 12–23) and 30 days (range 25–51) for plt engraftment. In total, two of six patients developed grade II acute GVHD that resolved with steroids; One patient died of pneumonia in remission at day 150; and five of the six patients are alive and in complete remission at a median follow-up of 1 year with accrual continuing.[108]

As with the development of liquid *ex vivo* expansion, optimization of culture conditions for this approach will continue, including the growth factor cocktail used, the length of MSC and hematopoietic cell co-culture for most effective HSC expansion and the development of potentially more effective stromal cell lines to support the HSC expansion.[109]

Continuous perfusion culture systems

Automated, continuous perfusion culture systems, or 'bioreactors', are also being analyzed for the *ex vivo* expansion of HSC, rather than the use of 'static' culture (culture flasks or bags).[24–26,110–114] These systems were designed to allow larger volumes as well as to provide improved nutrient delivery and gas exchange. The secreted products of mature granulocytes and macrophages are toxic to progenitors,[115] and mature macrophages can directly damage cultured stroma and hematopoietic pro-genitors.[116] Therefore, a continuous perfusion of culture medium that removed mature cells could protect the cultured cells from toxic byproducts. In one phase I trial,[25] fractions of UCB were expanded *ex vivo* using Aastrom Replicell bioreactor technology (Aastrom Biosciences, Inc, Ann Arbor, MI, USA) and a growth factor cocktail (PIXY321, FL and EPO). The expanded cells were administered 12 days after the transplant of unmanipulated fractions of UCB. No difference in the time to myeloid, erythroid or plt engraftment was observed. In a second study with two patients, *ex vivo*-expanded UCB cells (Aastrom Replicell bioreactor), generated to augment unmanipulated UCB, seemed to facilitate hematopoietic recovery.[24] A newer bioreactor that uses serum-free medium, the Dideco 'Pluricell System' (DIDECO srl, Mirandola, MO, Italy) was used in recent preclinical and murine studies, in which Astori *et al*.[117] showed an MNC fold expansion of 230.4±91.5 and a $CD34^+$ fold expansion of 21.0±11.9 at 12 days, as well as improved engraftment in NOD-SCID mouse model. Other technologies, such as rotating wall vessels that decrease sheer stress while maintaining consistent environment, are being evaluated.[118] The effect of bioreactor-expanded UCB remains uncertain, and further clinical trials are necessary to establish its safety and efficacy.

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Summary

Current clinical trials have shown that the use of expanded UCB can be safe and recent results suggest the potential for improved outcomes; however, the optimal expansion conditions have yet to be identified. The ongoing trials are addressing the clinical implications of expansion of all or part of an UCB unit. New data suggest that perhaps engraftment and outcomes can be favorably altered. Although current trials are primarily using expansion of UCB to either increase the progenitor number or to facilitate and/or accelerate engraftment, *ex vivo* expansion technology could have additional clinical applications. Through cell sorting and manipulation of culturing techniques, it is possible to expand particular subsets of UCB-derived cells, such as T cells[119] or natural killer cells.[120] The *ex vivo*-expanded cells could then be available as a platform for adoptive immunotherapy to target either tumor or infectious pathogens. In addition, *ex vivo* expansion could allow gene transfer technologies to be available in the UCB setting.

It is hoped that expansion of the UCB populations responsible for engraftment could favorably alter the kinetics of neutrophil and plt recovery and possibly even immune reconstitution, depending upon the expansion conditions used. Shortening the time to engraftment and reducing graft failure should reduce the morbidity and mortality of UCB transplantation. Expansion techniques could also allow adoptive immunotherapy or gene transfer therapy in the UCB setting. With the rapidly evolving expansion technologies described, important improvements in the safety, efficacy and application of UCB transplantation may be observed in the near future.

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Table 1

Summary of clinical trials evaluating UCB that has been expanded ex vivo

Type of expansion	*Authors*	*Subjects*	*Cytokines*	*Days in culture*	*Fold expansion*		*Days to ANC >500*	*Days to plts >20000*	*Survival (median length) and GVHD*
					TNC	*CD34⁺*			
Liquid suspension	Shpall *et al.*	*n*=37, adults and children	SCF, TPO, G-CSF	10	56	4	28	106	32% survival (minimum 17 months) 67% grade II–IV aGVHD 40% grade III and IV GVHD
	de Lima and Shpall	*n*=35 adults and children	SCF, TPO, G-CSF	14	23	2.3	14	34	48% survival (11 months) 43% grade II–IV aGVHD 7% grades III and IV
	de Lima and Shpall	*n*=10 adults and children	SCF, FL, IL-6, TPO, TEPA	21	219	6	30	48	30% survival (25 months) 44% grade II aGVHD No grade III and IV aGVHD
	Delaney *et al.*	*n*=5 adults and children	Notch ligand δ–1, SCF, FL, IL-6, TPO, IL-3	16	660	160	14		83% survival (277 days)
Stromal co-culture	de Lima and Shpall	*n*=6 adults and children	SCF, TPO, G-CSF	14	12	12	14.5	30	83% survival (12 months) 33% grade II aGVHD No grade III or IV aGVHD
Continuous perfusion system	Jaroscak *et al.*	*n*=27 children, few adults	PIXY321, FL, EPO	12	2.4	0.5	22	71	39% survival (41 months) 36% grade II–IV aGVHD 22% grade III and IV aGVHD
	Pecora *et al.*	*n*=2 adults	PIXY321, FL, EPO	12	2.2	1.6, second did not expand	28	56	100% survival (13 months) No aGVHD

Abbreviations: aGVHD =acute GVHD; FL =Flt-3 ligand; TEPA=tetraethylenepentamine; TNC =total nucleated cell; UCB= umbilical cord blood.