Ex Vivo Cord Blood Manipulation: Methods, Data, and Challenges

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5.1 Introduction

Despite numerous advantages of umbilical cord blood (UCB) transplantation (UCBT), one of its major limitations is delayed engraftment of neutrophils and platelets as compared with peripheral blood progenitor cell (PBPC) or bone marrow (BM) grafts. After myeloablative conditioning, neutrophil engraftment is achieved in about 20–30 days with unmanipulated UCBT compared with 10–20 days after PBPC and 15–25 days after BM grafts. Similarly, platelet engraftment (>20 × 10^9/L) that occurs in about 10–20 days after PBPC and 15–30 days after BM HCT takes about 50–70 days after unmanipulated UCBT (Barker et al. 2015; Liu et al. 2014; Ruggeri et al. 2014; Sanz et al. 2012; Brunstein et al. 2010; Verneris et al. 2009; Barker et al. 2005; Takahashi et al. 2007; Eapen et al. 2010). This is explained by about tenfold fewer total nucleated cells (TNCs) in UCB graft compared with other grafts (Eapen et al. 2010; Laughlin et al. 2004; Rocha et al. 2004; Atsuta et al. 2009).

One of the ways to tackle the barrier of low cell dose is the use of two partially matched UCB grafts (Barker et al. 2005). Although the use of double-unit UCBT (dUCBT) increases the total cell content of the graft, it still does not hasten engraftment compared with adequately dosed single-unit UCBT (Ruggeri et al. 2014; Verneris et al. 2009; Wagner et al. 2014; Kindwall-Keller et al. 2012; Scaradavou et al. 2013). Instead, there is a suggestion that the use of dUCBT may in fact be associated with delayed and inferior platelet engraftment compared to that of single-unit UCBT (Wagner et al. 2014). Alternative strategies to enhance engraftment include ex vivo manipulation of UCB graft either to augment total cell dose or improve BM homing capacity of UCB progenitor cells (summarized in Table 5.1).

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Table 5.1 Ex vivo graft manipulation studies to augment engraftment

Study (sample size)	Post thaw/infused TNC, median (range), $\times 10^7$/kg			Post thaw/infused $CD34^+$ median (range), $\times 10^5$/kg			Median age (range), in year	Conditioning regimen	GvHD prophylaxis	Neutrophil engraftment time, in days, median (range)		Platelet engraftment time, in days, median (range)	
	Untreated cord	Treated cord	Total	Untreated cord	Treated cord	Total				Study group	Controls	Study group	Controls
Expansion studies													
Notch (Delaney 2010) $n = 10$	3.3 (1.5–6.3)	4.6 (0.6–9.1)		2.4 (0.6–5.4)	60 (9.2–133)		27.5 (3–43)	MA: Flu/Cy/TBI	CsA/MMF	16 (7–34).	26 (16–48); $p = 0.002$	*N.R.*	*N.R.*
MSC (De Lima 2012) $n = 24$	2.28 (1.57–4.82)	5.84 (0.03–14.37)	8.34 (1.66–19.19)	3.8 (0.7–17.4)	9.5 (16–93.4)	18.1 (0.9–988)	39 (18–61)	MA: Flu/MelThio	Tac/MMF + Rabbit ATG	15 (9–42)	24 (12–52); $p < 0.001$	42 (15–62)	49 (18–264); $p = 0.03$
StemEx (Stiff 2013) $n = 101$			2.2 *(N.R.)*			9.7 *(N.R.)*	37 (12.6–55.8)			21 *(N.R.)*	28 *(N.R.)*; $p < 0.0001$	54 (*N.R.*)	105(*N.R.*); $p = 0.008$
NAM (Horwitz 2014) $n = 11$			3.1	0.7 (0.3–4.8)	35 (9–183)		45 (21–61)	MA: Flu/TBI +/− Cy	Tac/ MMF	13 (7–26)	25 (13–38) $p < 0.001$	33 (26–49)	37 (20–66); $p = 0.085$
SR1 (Wagner 2016) $n = 17$	2 (2–5)	5 (1–12)	7 (3–17)	4 (2–9)	175 (14–483)	182 (23–485)	29.9 (12–54)	MA: Flu/Cy/TBI	CsA/MMF	15 (6–30)	24 (*N.R.*)	49 (28–136)	89 (*N.R.*); $p = 0.001$

Homing studies												
dmPGE2 (Cutler 2013) Cohort1: $n = 9$	1.8	2.17		0.68	0.7		43.0 (29–64)	Flu/Mel	Tac/Siro ATG	24 *(N.R.)*	21 *(N.R.)*; $p = 0.045$	72.5 *(N.R.)*	N.R.
Cohort 2: $n = 12$	1.7	1.8		0.56	0.74		57.5 (19–66)			17.5 (14–31)		43 (26–60)	
Fucosylation (Popat 2015) $n = 22$	2.59 (1.54–4.75)	1.75 (0.64–2.5)	4.26 (2.73–6.3)	–	–	–	42 (20–68)	MA: Bu/Flu/ Clo/ TBI RIC: Flu/Mel	Tac/ MMF + Rabbit ATG	17 (12–34)	26 (11–48); $p = 0.0023$	35 (18–100)	45 (27–120); $p = 0.05$

Abbreviations: *ATG* antithymocyte globulin, *Bu* busulfan, *Cy* cyclophosphamide, *CsA* cyclosporine A, *Clo* clofarabine, *dmPGE2* 16,16-dimethyl prostaglandin E2, *Flu* fludarabine, *GvHD* graft-*versus*-host disease, *MA* myeloablative, *Mel* melphalan, *MMF* mycophenolate mofetil, *MSC* mesenchymal stromal cells, *NAM* nicotinamide, *RIC* reduced intensity conditioning, *SR1* StemRegenin-1, *Tac* tacrolimus, *Thio* thiotepa, *TBI* total body irradiation, *TNC* total nucleated cells

5.2 Historic Methods of UCB Expansion Using Cytokine-Supported Culture Media Failed to Demonstrate Clinical Benefit

One of the earliest methods of ex vivo expansion was the use of "static culture," whereby purified UCB $CD34^+$ cells were cultured in medium containing stem cell factor (SCF), granulocyte-colony stimulating factor (G-CSF), and megakaryocyte growth and differentiation factor for 10 days (Shpall et al. 2002). Despite fourfold median expansion of $CD34^+$ cells, the median infused $CD34^+$ dose in adult patients was only 0.89×10^5/kg (median TNC, 0.79×10^7/kg). This was due to significant upfront cell loss from $CD34^+$ selection with the Isolex 300-i device (Nexell, Irvine, CA) and anti-CD34 antibody used in this method. The median time to neutrophil engraftment ranged from 26 to 31 days and 73–126 days for platelet engraftment, which was not different from results of unmanipulated UCBT trials.

Investigators from the Duke University Medical Center explored ex vivo UCB expansion using an automated device that perfused cultures with cytokines and maintained optimal culture conditions based on computerized monitoring of biological and physiological environment of the culture (Jaroscak et al. 2003). In a clinical trial using this system, CB samples were thawed on day 0 and a majority of cells were infused unmanipulated on the same day (median TNC count, 2.05×10^7/kg). A small fraction (TNC count, $1–2 \times 10^8$/kg) was expanded ex vivo and infused on day 12. The culture was supported by PIXY321 (a fusion protein of interleukin (IL)-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF)), Fms-like tyrosine kinase 3 ligand (FLT3L), and erythropoietin, which resulted in median TNC expansion of 2.4-fold, but the expansion of $CD34^+$ was rather feeble (median, 0.5-fold; range, 0.09- to 2.45-fold). The median time to neutrophil engraftment was 22 and that of or platelet was 71 days. Out of 22, 3 patients failed to achieve engraftment, and 2 died prior to engraftment.

5.3 Methods to Block *In Vitro* Differentiation of $CD34^+$ Cells to Expand Early Progenitor Cells (EPCs) Demonstrated Encouraging Results

One of the concerns with cytokine-supported culture system is potential loss of early progenitor cells (EPCs) as the cytokines stimulate $CD34^+$ cells to differentiate into mature cells (Koller et al. 1995). This provides rationale for exploring methods that block *in vitro* differentiation of $CD34^+$ cells by means of nicotinamide analogs (Horwitz et al. 2014), copper chelators such as tetraethylenepentamine (TEPA) (Stiff et al. 2013), or targeting *Notch* signaling pathway (Delaney et al. 2010).

In a study by Delaney et al. (2010), human UCB $CD34^+$ cells were transduced with an engineered Notch ligand ($Delta1^{ext\text{-}IgG}$) and then cultured for about 2 weeks in serum-free medium with IL-3, IL-6, thrombopoietin (TPO), SCF, and FLT3L. This led to an average 222-fold expansion of $CD34^+$ cells and rapid engraftment in non-obese diabetic-severe combined immunodeficiency (NOD-SCID) mice infused

with these cells as compared with control mice (Delaney et al. 2010). This method is now under investigation in a phase I clinical trial in patients with acute leukemia, where recipients receive myeloablative conditioning followed by infusion of one unmanipulated UCB unit followed 4 h later by the infusion of the UCB unit that has been expanded ex vivo for 2 weeks. In preliminary analysis, neutrophil engraftment occurred at a median of 16 days in ten patients and graft rejection occurred in one. At a median follow-up 354 days, 70% of the patients were in complete remission with sustained engraftment. Two patients had long-term engraftment from expanded cells for up to 240 days post UCBT, but it did not persist beyond 1 year after which the unexpanded CB unit contributed to engraftment (Delaney et al. 2010).

The use of a polyamine copper chelator, TEPA (StemEx®), was explored in a multicenter international trial, including 101 adult patients (median age 37 years, median weight 68 kg) with hematological malignancies (Stiff et al. 2013). In this study, $CD133^+$ cells were enriched from a fraction (20–50%) of single-unit cryopreserved UCB sample that was thawed on day −20. These were cultured in bags with TEPA, FLT3L, IL-6, TPO, and SCF. Three weeks of culture resulted in an impressive expansion of TNCs to 400×10^7/kg (baseline median 3.06×10^7/kg) and $CD34^+$ cells to 9.7×10^5/kg (baseline median 1.64×10^5/kg). Total median infused doses were 2.2×10^7 TNC/kg and 9.7×10^5 $CD34^+$/kg. The median times to neutrophil engraftment (21 *vs.* 28 days, $p < 0.0001$) and platelet engraftment (54 *vs.* 105 days, $p = 0.008$) were significantly faster in the study group compared to a control group of dUCBT recipients ($n = 295$) from the Center for International Blood and Marrow Transplant Research (CIBMTR) and the Eurocord registries. Even more interestingly, survival at day 100 was significantly superior in the study group compared with controls (84.2 *vs.* 74.6%; $p = 0.035$).

Another blocker of EPC differentiation is nicotinamide (NAM), which when cultured with UCB cells along SCF, TPO, IL-6, and FLT3L results in expansion not only of $CD34^+$ cells but also of $CD34^+Lin^-$ EPCs (Peled et al. 2012). These expanded cells also have enhanced BM homing potential compared with untreated cells (Peled et al. 2006). A phase I clinical trial using this strategy enrolled 11 adult patients (median age 45 years, median weight 83 kg) with hematological malignancies. For expansion, one UCB unit was thawed on day −21, selected for $CD133^+$ cells, and expanded ex vivo. The negative fraction was cryopreserved and later infused along with the expanded fraction and a second unmanipulated unit after myeloablative conditioning. The median doses of unmanipulated (2.6×10^7 TNC/kg and 0.12×10^6 CD34/kg) and expanded (2.5×10^7 TNC/kg and 0.17×10^6 CD34/kg) units were similar. Compared with a historical cohort of dUCBT, the time to neutrophil engraftment (13 *vs.* 25 days, $p < 0.001$), but not the platelet engraftment (33 *vs.* 37 days, $p = 0.085$), was significantly shorter in the study population. Engraftment was attained from the expanded cord in 50% of patients, while 25% engrafted with unexpanded cord and 25% had dual chimerism from both the cords. Further, the expanded cord provided long-term engraftment in eight of ten evaluable patients, which remained stable for up to 36 months (Horwitz et al. 2014).

Most recently, Wagner et al. reported the results of a phase I–II trial of UCB expansion using StemRegenin-1 (SR-1), which is an inhibitor of aryl hydrocarbon

receptor, which is crucial for differentiation of EPCs (Wagner et al. 2016). In this study, 17 patients (median age 29.9 years years) received myeloablative conditioning with fludarabine, cyclophosphamide, and total body irradiation (TBI), followed by dUCBT with one unmanipulated and one ex vivo expanded unit. For expansion, $CD34^+$ cells were enriched from the smaller of two UCB units on day −15 and cultured with SR1, SCF, FLT3L, TPO, and IL-6. The negative fraction was re-cryopreserved. After 15 days of culture, a median 330-fold expansion of a number of $CD34^+$ cells and an 854-fold expansion of TNCs were noted. On day 0, the unmanipulated unit was infused first followed 4 h later by the expanded unit, followed by infusion of the $CD34^-$negative fraction 4–24 h later. No graft failure was observed. Median times to neutrophil engraftment (15 *vs.* 24 days) and platelet engraftment (49 *vs.* 89 days, $p = 0.001$) were significantly faster than historical institutional controls. The expanded cord provided engraftment in 65% (11/17) of patients and the rest engrafted from the unmanipulated cord. Patients who engrafted from the expanded cord had durable myeloid engraftment (median follow-up, 272 days) with neutrophil engraftment occurring at a median of 11 days compared to 23 days (range, 14–30 days) in those who engrafted from the unmanipulated cord. There were no differences in other outcomes including acute or chronic GvHD, transplant-related mortality, and overall survival compared with controls. However, study population had significantly shorter duration of hospitalization (median 30 days) compared with controls (median 46 days), $p < 0.001$. The authors are evaluating the safety and efficacy of this approach as a "stand-alone" graft, in the setting of single-unit UCBT.

5.4 Coculture of UCB Cells with Mesenchymal Stromal Cells Simulates BM Niche and Provides Molecular Signals Crucial for UCB Expansion

A distinct technique of ex vivo expansion focuses on simulating the *in vivo* environment of EPC growth by coculturing UCB cells with mesenchymal stromal cells (MSCs) that form a BM "niche" and produce cytokines that regulate cell proliferation and homing (Robinson et al. 2011). Investigators from the M. D. Anderson Cancer Center tested this technique in 31 adult patients. Most common diagnoses were acute myeloid leukemia or myelodysplasia (68%) and acute lymphoblastic leukemia (16%), and about 60% of the patients had active disease at the time of transplantation. All patients underwent myeloablative conditioning with fludarabine (160 mg/m^2), melphalan (140 mg/m^2), thiotepa (10 mg/kg), and rabbit antithymocyte globulin (ATG 3 mg/kg), followed by dUCBT with one expanded and one unmanipulated UCB unit. The source of MSC was haploidentical donor in the first seven patients, while the rest of the patients received "off-the-shelf" MSC precursor cells (Mesoblast Limited, Melbourne, Australia) to circumvent the obvious logistic difficulties associated with obtaining haploidentical MSC. Smaller of the two UCB units was thawed on 14 days prior to transplantation and cocultured with MSCs along with SCF, FLT-3L, TPO, and G-CSF for 2 weeks. The culture resulted in

12.2-, 30.1-, and 17.5-fold expansions in TNC, $CD34^+$, and colony-forming unit-C populations, respectively, producing final median doses of 5.84×10^7/kg, 0.97×10^6/kg, and 3×10^6/kg, respectively. The unmanipulated unit was thawed on day 0, washed, and infused followed by infusion of the expanded UCB unit. Compared with 80 control patients from the CIBMTR, neutrophil engraftment was significantly faster (median 15 *vs.* 24 days, $p < 0.001$) and improved in the study group compared with the controls. The cumulative incidence of engraftment at day 42 was 96% (95% confidence interval (C.I.) 74–99%) in the study group compared with 78% (95% C.I. 67–86%) in controls, $p = 0.005$. The median time to platelet engraftment was a week shorter (42 *vs.* 49 days, $p = 0.03$) in the study group. Long-term engraftment beyond 1 year was provided primarily by the unmanipulated unit, and the expanded unit was present in only 13% of the patients at 6 months post UCBT (de Lima et al. 2012).

5.5 Ex Vivo Manipulation Can Also Improve BM Homing Capacity of UCB Cells Without the Need for Long-Term Cultures

Ex vivo treatment of EPCs with prostaglandin E2 (PGE2) up-regulates apoptosis-inhibiting protein survivin, proliferation genes such as cyclin D1 (leading to selective self-renewal capabilities of EPCs), and chemokine receptor CXCR4, collectively stimulating their growth and homing potential (Hoggatt et al. 2009). A study from the Dana-Farber Cancer Institute tested this hypothesis in a phase I clinical trial with a PGE2 derivative (dmPGE2) in adult patients (median age 57.5 years) undergoing dUCBT after a conditioning regimen of melphalan (100 mg/m^2), fludarabine (180 mg/m^2), and ATG (4 mg/kg). Both UCB units were thawed on day 0 – one of which was treated. Larger unit was infused first followed 4 h later by the smaller unit. In the first cohort of this study, UCB unit (smaller unit in six patients and larger unit in three patients) was incubated with dmPGE2 for 1 h at 4 °C. The median times to neutrophil (24 days) and platelet engraftment (72.5 days) were not improved compared to their historical controls. Plus, two patients experienced graft failure. The authors then optimized their culture conditions and enrolled additional 12 patients where the larger UCB unit was incubated with dmPGE2 for 2 h at 37 °C. With this modification, they observed significantly improved median time to neutrophil engraftment compared to historical controls (17.5 *vs.* 21 days, respectively, $p = 0.045$). No patient in this cohort experienced graft failure. Further, 10 of the 12 patients had sustained engraftment from the dmPGE2-treated cord, which could be seen for up to 27 months post UCBT for some patients. A randomized phase II trial is ongoing (Cutler et al. 2013).

Another method of enhancing BM homing potential of UCB cells was tested by researchers from the M. D. Anderson Cancer Center. This method was based on our understanding that successful homing of transplanted cells requires interactions between adhesion molecule receptors (E- or P-selectins) on vascular endothelial cells and selectin ligands on hematopoietic cells. It is known that UCB cells have

poor fucosylation of selectin ligands, such as P-selectin glycoprotein ligand 1, which likely contributes to their deficient BM homing (Xia et al. 2004). In mouse models, human CB $CD34^+$ cells treated with fucosyltransferase (FT)-VI and GDP-fucose demonstrated improved homing capability compared to untreated human CB $CD34^+$ cells, leading to faster and significantly higher rates of engraftment (Robinson et al. 2012). A phase I clinical trial included 22 adult patients (median age 42 years) who received dUCBT after either myeloablative conditioning with fludarabine (40 mg/m^2), clofarabine (120 mg/m^2), and busulfan (area under the curve of 16,000 μmol/min) and 2 Gy of TBI or reduced intensity conditioning with fludarabine (160 mg/m^2) and melphalan (140 mg/m^2). All patients received rabbit ATG (3 mg/kg), tacrolimus, and mycophenolate mofetil for GvHD prophylaxis. On the day of transplantation, the larger UCB unit was infused unmanipulated, while the smaller unit was treated ex vivo with FT-VI and GDP β-fucose for 30 min at room temperature, washed, and then infused. In the study population, median times to neutrophil engraftment (17 *vs.* 26 days, $p = 0.0023$) and platelet engraftment (35 *vs.* 45 days, $p = 0.05$) were significantly shorter than their historical controls. One patient had secondary graft failure. All evaluable patients had 100% chimerism on day 30 post UCBT – 40% from fucosylated cord, 40% from unmanipulated cord, and 20% having dual chimerism from both cords. Moreover, there were no differences in neutrophil and platelet engraftment among those who engrafted from treated *versus* untreated cord (Popat et al. 2015).

Investigators from Indiana University School of Medicine are investigating another method of improving homing of UCB cells without any ex vivo manipulation at all. In a phase II trial, patients with hematological malignancies undergoing UCBT are given sitagliptin 600 mg orally 1–3 times a day for 4–12 doses, starting day 1 [Clinicaltrials.gov, NCT00862719]. The drug works by inhibition of dipeptidyl peptidase-IV (DPPIV)/CD26, which is a membrane-bound peptidase that inhibits the migratory potential of $CD34^+$ cells by cleaving CXCL12/SDF-1α (stromal cell-derived factor 1) (Christopherson et al. 2002, 2004).

5.6 Graft Manipulation Techniques Advanced the Field of Adoptive Immunotherapy Tremendously

Disease relapse, GvHD, and infections are the leading causes of mortality after allogeneic hematopoietic cell transplantation (HCT) (Brunstein et al. 2010; Eapen et al. 2010; Scaradavou et al. 2013; Wagner et al. 2016; Popat et al. 2015; Pasquini and Zhu 2015). Relapse after UCBT is especially devastating as it excludes one of the potential therapeutic strategies, which is the use of donor lymphocyte infusion (DLI). Now with ex vivo expansion techniques, large-scale production of UCB T-cells and NK cells is feasible. Culturing UCB cells with anti-CD3/CD28 magnetic beads and IL-2 can expand UCB T-cells by a median of 100-fold while maintaining their polyclonal TCR repertoire (Parmar et al. 2006). Using this strategy, a phase I clinical trial is evaluating the role of UCB DLI in patients with relapse after UCBT [ClinicalTrials.gov:NCT01630564].

Several different methods exist for expansion of UCB NK cells. One of such methods is a two-step process where enriched UCB $CD34^+$ cells are first expanded in serum-free media with cytokine cocktail for 2 weeks, followed by differentiation and expansion of NK cells using a separate NK-cell differentiation medium for 5 weeks. This method resulted in 3- to 4-log expansion and generation of *functional* human NK cells that demonstrated activity against various leukemia and melanoma cell lines (Spanholtz et al. 2010). As it is technically tedious and a lengthy procedure, a different approach is to enrich $CD56^+$ $CD3^-$ NK cells from UCB mononuclear cells (MNCs) upfront and culture them in IL-2-containing media for 2 weeks. This method led to a median of 92-fold expansion of NK cells with enhanced *in vivo* antileukemia activity in NOD-SCID-IL2Rγ^{null} mouse (Xing et al. 2010). Another innovative technique includes culturing of MNCs with IL-2 with a feeder layer of irradiated artificial antigen-presenting cells (aAPC) that express membrane-bound IL-21, 4-1BBL, CD64, and CD86, which provide necessary signals for NK cell activation, maturation, and proliferation (Denman et al. 2012). After 2 weeks of culture, $CD3^+$ cells are depleted and the remaining cells are re-cultured for another week. This method resulted in an enormous expansion of NK cells from either fresh (mean 1848-fold) or cryopreserved (2389-fold) UCB samples, and the expanded NK cells demonstrated significant *in vivo* cytotoxicity against multiple myeloma target in mouse model (Shah et al. 2013). Two early phase clinical trials are evaluating the safety and efficacy of prophylactic UCB NK cell infusion (a) in the setting of UCBT in patients with chronic lymphocytic leukemia [ClinicalTrials.gov: NCT01619761] and (b) in conjunction with autologous HCT in patients with multiple myeloma [ClinicalTrials.gov:NCT01729091].

Similarly, antigen-specific chimeric antigen receptor (CAR) T-cells are being created from UCB directed against various antigens like CD19 (Kebriaei et al. 2013; Huls et al. 2013; Pegram et al. 2015) or carcinoembryonic antigen (CEA) (Yasmine van et al. 2015). What is more, CD19 CARs have been generated that not only have antitumor effect but also activity against multiple viruses including Epstein-Barr virus (EBV), cytomegalovirus (CMV), and adenovirus (Micklethwaite et al. 2010), which are the most common viral infections encountered after UCBT.

5.7 Use of Ex Vivo Expanded Lymphocytes to Treat Infections

Generation of virus-specific cytotoxic T lymphocytes (CTLs) from UCB is challenging given not only the finite numbers of but also the naivety of UCB T-cells. These barriers are overcome with ex vivo expansion and priming of UCB T-cells with specific pathogens (Hanley et al. 2009; Park et al. 2006; Sun et al. 1999). Several studies demonstrated successful generation of virus-specific CTL against EBV (Sun et al. 1999; Leen et al. 2013), CMV (Park et al. 2006; Leen et al. 2013), or adenovirus (Leen et al. 2013). More recently, CTLs specific against multiple viruses (EBV, CMV, and adenovirus) have been created and are currently under investigation in a clinical trial (Hanley et al. 2009; Hanley et al. 2013). In this study,

the ex vivo expansion was performed from only 20% fraction of UCB graft in the setting of single UCBT. Preliminary results ($n = 8$) showed that CTL infusion was able to clear reactivated CMV infections within a week of infusion in a majority of patients without the use of conventional treatment. Similarly, all patients with high EBV loads and almost everybody with adenovirus infection were able to clear the viruses (Hanley et al. 2013). No toxicities occurred in any of the patients treated so far (Barrett and Bollard 2015) [NCT00880789].

5.8 Risk of GvHD Can Be Reduced with Prophylactic Use of Ex Vivo Expanded Regulatory T-Cells

Although the risk of GvHD is lower after UCBT compared with mismatched PBPC or BM grafts, it still contributes to significant morbidity and mortality after transplantation (Brunstein et al. 2010; Takahashi et al. 2007; Eapen et al. 2010; Laughlin et al. 2004; Rocha et al. 2004; Atsuta et al. 2009; Brunstein et al. 2012; Chen et al. 2012; Le Bourgeois et al. 2013; Majhail et al. 2012; Majhail et al. 2008; Weisdorf et al. 2014). Regulatory T-cells (Tregs) are a subset of $CD4^+$ T-cells that modulate immune response and play crucial role in self-tolerance. A landmark dose escalation trial from the University of Minnesota (Brunstein et al. 2011) showed that UCB-derived ex vivo expanded Tregs could reduce the incidence of grade II–IV acute GvHD. In that study, $CD25^+$ cells were positively enriched from a 4 to 6/6 HLA-matched third UCB unit using anti-CD25 magnetic microbeads and then cultured with anti-CD3/CD28 monoclonal antibody-coated Dynabeads for about 18 days in the presence of 300 IU/mL IL-2. The culture method resulted in a median 211-fold (range, 13–1796-fold) expansion of Tregs. Twenty-three patients (median age 52 years) received nonmyeloablative regimen of fludarabine, cyclophosphamide, and TBI followed by dUCBT. Prophylaxis against GvHD was provided with mycophenolate mofetil plus either cyclosporine or sirolimus. All patients received expanded Tregs on day +1 at dose levels ranging from 1 to 30×10^5 Tregs/kg, and a second cohort received an extra dose of 30×10^5 Tregs/kg on day +15. No dose-limiting toxicities were observed. Although infused Tregs did not persist beyond 2 weeks, the risk of grade II–IV acute GvHD by day 100 (43%, 95% CI, 23%–64%) was reduced compared with historical controls (61%, 95% CI, 51%–72%, $p = 0.05$). The risks of grade III–IV acute GvHD (17% *vs.* 23%, p = NS), infection, relapse, nonrelapse mortality, and disease-free survival were similar in both the groups. With a median follow-up of 368 days (range, 226–388 days), no chronic GvHD was observed in the study group, compared with 26% (95% CI, 17%–35%) in the controls.

In their subsequent trial (Brunstein et al. 2016), instead of using anti-CD3/CD28 immunomagnetic beads, the authors modified their protocol by expanding positively selected $CD25^+$ cells with anti-CD3 monoclonal antibody plus K562 aAPC that expressed high-affinity Fc receptor (CD64) and CD86. This method resulted in impressive 13,000-fold (range, 1352 to 27,183-fold) expansion of Tregs allowing doses up to 100×10^6 Treg/kg. None of the 11 study

patients experienced dose-limiting toxicity. In contrast to their previous study where grade II–IV acute was seen in 43% of the study patients, cumulative incidence of grade II–IV acute GvHD in the current study was reduced to 9% (95% CI, 0–25), which was considerably lower compared with 45% (95% CI, 24–67, $p = 0.05$) in historical controls. One study patient developed grade III–IV acute GvHD compared with 27% (95% CI, 9–46, $p = 0.06$) in controls. With a median follow-up of 20 months, no study patient developed chronic GvHD compared with 14% in the controls. The density of bacterial, viral, and fungal infections (infections per 1000 patient days) was similar in study and control groups. Similar to the previous study, no Tregs persisted beyond 14 days, despite massive doses of products infused.

5.9 Expert Point of View

Graft engineering methods have evolved significantly over time – starting from culturing UCB cells with cytokines alone to the addition of supporting layer of MSCs to simulate the BM "hematopoietic niche" ex-vivo (de Lima et al. 2012) or blocking differentiation of EPCs leading to expansion of hematopoietic stem cells using nicotinamide analogs (Horwitz et al. 2014), copper chelators (Stiff et al. 2013), or targeting the *Notch* signaling pathway (Delaney et al. 2010). Alternative approach is to improve homing capacity of UCB cells with an aim to accelerate engraftment. This is achieved by treating UCB cells with prostaglandin E2 derivatives (Cutler et al. 2013), enforcing fucosylation of UCB progenitor cells (Popat et al. 2015; Robinson et al. 2014), or using dipeptidyl peptidase-IV (DPPIV) inhibitors (Christopherson et al. 2002, 2004). Graft manipulation has also permitted the generation and clinical use of antivirus and antitumor adoptive immunotherapies as well as cellular therapies for GvHD prevention.

With these novel strategies, the traditional challenge of UCBT – low graft dose – has now been ameliorated. This is expected to increase the available pool of potentially better human leukocyte antigen (HLA)-matched UCB grafts which would otherwise be deemed ineffectual due to inadequate cell content. All of these methods are currently performed in the setting of dUCBT. However with maturing experience, they are now being explored in the setting of single-unit UCBT also, which can potentially reduce the cost of transplantation.

5.10 Future Directions

Novel graft manipulation strategies have remarkably improved time to neutrophil engraftment after UCBT, which now approaches that of other grafts. Yet, time to platelet engraftment, although superior to unmanipulated UBCT, still remains prolonged as compared with PBPC or BM HCT. Therefore, studies focusing on manipulating megakaryocytes or their precursors to enhance platelet engraftment would be of interest. Further, almost all of the currently available expansion strategies

require extended period of incubation for about 2–3 weeks before the final product can be used. This not only delays treatment but also adds to the manufacturing cost and poses extra risk of graft contamination. Studies to shorten the expansion duration are warranted. Moreover, these techniques can currently be utilized only at specialized centers. Attempts to generate "off-the-shelf" products are ongoing which will extend the usability of these products to remote centers as well. Last but not least, the impact on these methods on immune reconstitution is yet to be determined, as none of the expansion techniques have shown reduction in risk of infections compared with unmanipulated UCBT.

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