Clinical Advancement and Challenges of ex vivo Expansion of Human Cord Blood Cells

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ABSTRACT

Apart from peripheral blood stem cell (PBSC), umbilical cord blood (UCB) is now a recognized source of stem cells for transplantation. UCB is an especially important source of stem cells for minority populations, which would otherwise be unable to find appropriately matched adult donors. UCB has fewer mature T lymphocytes compared with peripheral blood, thus making a UCB transplantation (UCBT) with a greater degree of HLA mismatch possible. The limited cell dose per UCB sample is however associated with delayed engraftment and a higher risk of graft failure, especially in adult recipients. This lower cell dose can be optimized by performing double unit UCBT, ex vivo UCB expansion prior to transplant and enhancement of the capabilities of the stem cells to home to the bone marrow. UCB contains naive and immature T cells, thus posing significant challenges with increased risk of infections, graft versus host diseases (GVHD) and relapse following UCBT. Cell engineering techniques have been developed to circumnavigate the immaturity of the T cells, and include virus-specific cytotoxic T cells (VSTs), T cells transduced with disease-specific chimeric antigen receptor (CAR T cells) and regulatory T cell (Tregs) engineering. In this article, we review the advances in UCB ex vivo expansion and engineering to improve engraftment and reduce complications. As further research continues to find ways to overcome the current challenges, outcomes from UCBT will likely improve.

1. INTRODUCTION

Umbilical cord blood (UCB) is now a recognized source of stem cells for transplantation [1]. The first umbilical cord blood transplantation (UCBT) was performed on a child with Fanconi anemia (FA) and was reported 30 years ago [2]. The UCB donor was an HLA-identical sister who was found to be compatible while she was still in utero. Following on, UCBT was performed for a malignant condition (juvenile chronic myeloid leukemia) in a child after myeloablative conditioning (MAC) with busulfan and cyclophosphamide. The donor was also an HLA-identical sibling [3]. A few years later, UCBT was performed with UCB from an unrelated donor for an adult with chronic myeloid leukemia (CML) [4].

In contrast to peripheral blood (PB) stem cell transplantation (PBSCT), UCB has fewer mature T lymphocytes, thus making possible a UCBT with a greater degree of HLA mismatch [5]. Also, hematopoietic stem and progenitor cells (HSPCs) derived from UCB have greater telomere length which confers a better proliferation potential [6].

There are certain limitations with UCBT, the first of which is the limited cell dose per UCB sample. This limitation is associated with the delayed engraftment and a higher risk of graft failure. The limited cell dose is of particular importance in adult UCBT recipients, because of their greater body weight [5]. In Japanese adult patients, with lower body weight compared with North Americans, mismatched UCBT and 8/8 HLA matched unrelated bone marrow transplant (BMT) had comparable outcomes [7].

A retrospective review was performed by Eapen et al. to assess the impact of graft source on outcomes in acute leukemia patients after MAC. Engraftment was slower with UCBT compared with PBSCT or BMT: the incidence of neutrophil engraftment on day 42 was 80% in UCB, 93% in BM and 96% in PBSCT recipients (p < 0.001). The incidence of acute graft versus host disease (GVHD) was lower after UCBT compared to PBSCT. Chronic GVHD incidence was lower in UCB recipients compared with BM or PBSCT recipients. Leukemia-free survival (LFS) was similar among recipients in each of the three sources of stem cells, although non-relapse mortality (NRM) was higher in UCB recipients [8].

Due to the limitation posed by the reduced cell dose in UCB, it has been determined that the number of total nucleated cells (TNC) is an important predictive factor for NRM, LFS and overall survival (OS) after adult UCBT [9]. The adequate cell dose in a single-unit of UCB, depending on the indication for UCBT, has been defined as: >3 × 10^7 TNC/kg for 6/6 HLA-matched units, >4 × 10^7 TNC/kg for 5/6 HLA-matched units and >5 × 10^7 TNC/kg for 4/6 HLA-matched units [5]. Newer guidelines from the National Marrow Donor Program (NMDP) and the Center for International Blood...
and Marrow Transplant Research (CIBMTR) recommend that for single-unit UCBT, cell doses of at least $2.5 \times 10^7$/kg TNC and 1.5 $\times 10^7$/kg CD 34+ cells are adequate [10]. For double unit UCBT, the acceptable cell doses are at least $1.5 \times 10^7$/kg TNC per unit and $1 \times 10^7$/kg CD 34+ cells per unit [10]. HLA-A, HLA-B, HLA-C and HLA-DRB1 all need to be considered in the HLA high-resolution matching of UCB prior to transplantation [10].

Some of the strategies to overcome the limitation posed by the low UCB cell dose and consequent delayed engraftment include double UCBT, ex vivo UCB expansion and techniques to improve homing of the stem cells to the bone marrow [11,12]. UCB contains naïve and immature T cells, thus posing significant challenges with increased infections, GVHD and disease relapse following UCBT. Engineering virus-specific cytotoxic T cells (VSTs), T cells transduced with disease-specific chimeric antigen receptor (CAR T cells) and regulatory T cells (Tregs) are some of the approaches to overcome these challenges.

In this article, we review the advances in UCB ex vivo expansion and the modalities for UCB engineering to improve engraftment and reduce complications.

2. UCB EX VIVO EXPANSION MODALITIES

2.1. Cytokine Expansion

Cytokines that are naturally found in hematopoietic stem cell (HSC) niches have been used to expand UCB ex vivo. The most potent cytokine stimulators for ex vivo HSPC expansion are stem cell factor (SCF), thrombopoietin (TPO) and Flt3 ligand (flt3l). However, interleukin (IL)-3, IL-6, IL-11 and granulocyte colony stimulating factor (GCSF) tend to result in differentiation of the HSC [13]. In contrast, the combination of IL-6 and both SCF and flt3l has been shown to have synergistic effect on ex vivo HSC expansion [13].

The addition of mesenchymal stromal cells (MSCs) to the culture medium has also been found to improve ex vivo expansion and prevent differentiation of the HSC [14,15]. These findings were applied to UCBT in a clinical study using unrelated UCB expanded ex vivo to treat patients with hematologic malignancies or breast cancer. Between 40 and 60% of each patient’s UCB was CD 34- selected and expanded ex vivo for 10 days in a culture medium to which SCF, G-CSF and TPO were added. After ex vivo expansion, the number of CD 34+ cells were only slightly greater than that in the unmanipulated fraction; the number of TNC was even lower post-expansion compared to the unmanipulated unit. Neutrophil engraftment occurred after a median of 28 days (range, 15–49 days) while platelet engraftment happened after a median of 106 days (range, 38–345 days) after UCBT. The OS at 30-months follow up was 35% (n = 13) and there was no graft failure. Grade III-IV acute GVHD occurred in 40% of patients while 63% of patients had chronic GVHD. This study has paved the way for ex vivo expansion of UCB by showing that it is clinically feasible (Table 1) [16].

2.2. Ex vivo Expansion with Copper Chelation

Preclinical studies in mice showed that copper chelation attenuates the differentiation of HSC, thus prompting a phase I/II clinical trial to assess the feasibility of ex vivo expansion of UCB-derived HSCs using this system [23]. The copper chelator tetraethylenglycolenamine (TEPA) was used; SCF, flt3l, IL-6, TPO were added to the culture medium in addition to TEPA. CD133+ HSC were selected from a fraction of the UCB and were cultured ex vivo for 21 days. The unmanipulated fraction of the UCB was transplanted on day 0 while the expanded fraction was transplanted on day 1. Up to 620-fold (median 161, range 2–620) TNC and 19-fold (median 2.2, range, 0.7–19.2) CD 34+ expansions were observed. Ninety percent (n = 9) of the patients achieved engraftment. Neutrophil engraftment occurred at a median of 30 days (range, 16–46 days) while that of platelets happened at a median of 48 days (range, 35–105 days) after UCBT. There was no case of severe (grade III-IV) acute GVHD, but chronic GVHD was reported in 60% (n = 6) of the patients who survived beyond 6 months [17].

These studies, however, did not demonstrate improved engraftment after ex vivo expansion of UCB-derived HSC. In a systematic review of ex vivo expansion in UCBT, higher cell doses in the transplanted units were associated with faster engraftment of neutrophils and platelets, but this did not translate into improved survival or reduction in TRM [24].

2.3. Notch-Mediated Ex vivo Expansion

HSPCs express Notch receptors (Notch-1 and Notch-2) while bone marrow stromal cells express the Notch ligands Delta-1 and Jagged-1 [25]. Constitutively active intracellular Notch-1 domain inhibited myeloid differentiation and enhanced precursor cell

| Table 1 | Types of Umbilical Cord Blood (UCB) expansion. |
|---------|-----------------------------------------------|
| Type of UCB Expansion | Study | CD-34-Fold Expansion (Median) | Days of Expansion | Days to Neutrophil Engraftment (Median) |
| Cytokine expansion | Shpall et al. [16] | 1 | 10 | 28 |
| Copper chelation | de Lima et al. [17] | 2.2 | 21 | 30 |
| Notch-mediated | Delaney et al. [18] | NA | 16 | 16 |
| Mesenchymal stem cells (MSCs)-mediated | de Lima et al. [19] | 30.1 | 21 | 15 |
| Nicotinamide-mediated | Horwitz et al. [20] | NA | 21 | 11.5 |
| StemRegenin 1-mediated | Wagner et al. [21] | NA | 21 | 15 |
| Lysine-specific histone demethylase 1A (LSD1)-mediated | Subramaniam et al. [22] | 10 | | |

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2.4. Mesenchymal Stem Cell-Mediated Expansion

Mesenchymal stem cells (MSCs) are normal components of the human bone marrow niche. They support hematopoiesis and also modulate immune response, thus playing important roles in GVHD [30,31]. Co-culture of unmanipulated UCB with bone-marrow-derived MSCs was compared with ex vivo expansion of CD133+ HSC selected from UCB. In both groups the culture media contained SCF,flt3l, TPO and G-CSF. The unmanipulated unit which was co-cultured yielded 8- and 31-fold higher numbers of CD34+ and CD133+ HSC, as compared to a 3- and 6-fold increase, respectively, in the manipulated units [32].

A clinical trial in patients with hematologic malignancies using double UCBT with the first unit unmanipulated and the second co-cultured ex vivo with MSCs showed promising results. The co-culture medium also included SCF,flt3l, TPO and G-CSF. CD34+ expansions ranged from 0- to 138-fold (median 30.1-fold); neutrophil and platelet engraftment occurred after a median of 15 (range, 9–42 days) and 42 days (range 15–62 days), respectively. Severe (grade III–IV) acute GVHD occurred in 13% of participants, while chronic GVHD was reported in 45% [19]. The final follow-up data for this study are yet to be published, but it appears that the unmanipulated unit of the allograft was responsible for long term engraftment (ClinicalTrials.gov NCT00498316) [19].

A recent study was performed to identify the molecular pathways responsible for the roles of MSCs in co-culture with UCB-derived HSCs. This was performed with a system using BM-derived MSCs as feeders, with and without SCF, TPO,FLT3-L, G-CSF and IL6. Increased viability of UCB was reported regardless of the presence of growth factors (SCF, TPO,FLT3-L, G-CSF and IL6). Higher numbers of CD45+CD34+CXCR4+ and CD45+CD34+EphB4+ UCB cells were noted after co-culture with MSCs. The MSCs led to higher expression of CXCR4, EphB4, FOXO1, Myc and HPRT1, and a lower expression of HOXCH8, SDF-1, SOX17 and SOX9 genes in the UCB cells. FOXO expression represses the cell cycle via the P66hs-ch-Akt-FOXO pathway, thereby preventing HSC differentiation and maintaining stemness in UCB. MSCs are probably able to maintain stemness in UCB [33].

2.5. Nicotinamide-Mediated Expansion

Nicotinamide (NAM) is a form of vitamin B3 that was found to increase ex vivo expansion of UCB-derived HSPC. It exerts its effects by enhancing HSPCs homeing to the bone marrow by increasing migration toward stromal cell-derived factor-1 (SDF1), and inhibiting the differentiation of CD34+ HSPCs by inhibiting sirtuin (SRT-1) [34].

In a phase I clinical trial using double UCBT for patients with hematologic malignancies following MAC, 11 patients received UCB-derived HSPCs expanded for 21 days with NAM and an unmanipulated T cell fraction. Neutrophil engraftment occurred after a median of 13 days compared with 25 days in historical controls (p < 0.001). Platelet engraftment happened after a median of 33 compared with 37 days (p = 0.09) in historical controls.

In contrast to ex vivo expansion of UCB with MSCs, NAM-mediated expansion resulted in engraftment of the manipulated unit. The NAM-expanded unit (Nicord) was responsible for long term hematopoiesis in 6 of the study participants. In clinically measurable terms, the OS and progression-free survival (PFS) were 82% and 73% respectively at 1 year [35]. In a follow-up study, the rates of infections in the Nicord recipients was compared with that in standard UCBT recipients at Duke University. The initial cohort of 11 patients in the Nicord arm was expanded to 18, and the rates of infection in the first 100 days post-transplant were evaluated. The risk for any infection was reduced in the Nicord arm compared to the standard UCBT recipients (RR 0.69, p = 0.01); moderate/severe infections were also fewer in the Nicord recipients (RR 0.36, p < 0.001). These effects were still present after adjusting for confounders like age, disease stage, and grade II–IV acute GVHD. In the first 100 days, the Nicord recipients also spent fewer days in the hospital compared with controls (69.9 days versus 49.7 days, p = 0.005) [36].

Another study using the NAM-expanded unit (Nicord) only was subsequently carried out. This was a Phase 1/II clinical trial using NAM-expanded UCB (Nicord) for 36 patients with hematologic malignancies after MAC. The UCB grafts used were 4–6/6HLA matched. The UCB unit underwent selection for CD133+ cells prior to ex vivo expansion: the CD 133-, T cell replete portion was left unmanipulated and cryopreserved, while the CD133+ fraction was cultured ex vivo for 21 days. The comparator arm was a historical group of 146 patients who received unmanipulated UCBT as reported to the CIBMTR. Neutrophil recovery occurred after a median of 11.5 days (95% CI, 9 to 14 days) in Nicord recipients versus 21 (95% CI, 20 to 23 days) in historical controls (p < 0.001). Platelet recovery occurred after a median of 34 days (95% CI, 32 to 42 days) in Nicord recipients versus 46 days (95% CI, 42 to 50 days) in historical controls (p = 0.001).

Acute grade 2–4 GVHD after 100 days occurred in 44% of study participants while chronic GVHD was reported in 40% after 2 years. NRM and relapse were 24% and 33%, respectively, in Nicord recipients. NRM was lower in Nicord recipients compared with the CIBMTR controls. This study confirms that a NAM-mediated
ex vivo expanded UCB can be transplanted without a second unmanipulated UCB unit [20].

2.6. StemRegenin 1-Mediated Expansion

StemRegenin 1 (SR1) is a purine derivative which was found to be a potent inhibitor of HPSC differentiation that acts by antagonizing the aryl hydrocarbon receptor (AHR). Twenty-one days of HPSC culture with SR1 in a medium supplemented with growth factors (SCF, flt3l, TPO and IL-6) resulted in 11-fold and 73-fold increases in TNC and CD34+ cells, respectively, compared to control cultures without SR1 [37].

This was followed by a Phase I/II clinical trial with SR1-expanded UCB (referred to as HSC835). Seventeen patients received double UCBT using one unmanipulated unit and a second SR1-expanded unit (HSC835), while 2 patients received HSC835 allograft (with the CD34-unexpanded portion of the single UCB unit). As in the Nicord study, the CD34+ portion of the allograft that was meant for ex vivo expansion was separated and left unmanipulated in order to preserve the T cell function and prevent rejection of the expanded unit. HSC835 had a very high CD34+ cell dose (median = 12.3 × 10^6 CD34+ cells/kg; range, 2.3–48.5 × 10^6 CD34+ cells/kg). Neutrophil engraftment occurred after a median of 15 days (range 6–30 days) in HSC835 recipients compared to a median of 24 days in historic controls (p = 0.001). For platelet recovery these numbers were 49 days (range 28–136 days) and 89, respectively (p = 0.001). HSC835 was for the main contributor to hematopoiesis in 11 patients, while the unmanipulated unit gave rise to hematopoiesis in 6 recipients. The CD34+ cell dose correlated with the speed of neutrophil engraftment in HSC835 recipients. Among the 11 patients who engrafted with HSC835, neutrophil recovery occurred earlier, at a median of 36 days (range 16–64 days). There were no cases of severe (grade III–IV) acute GVHD [42]. The outcomes of 87 patients who received intra-bone UCBT (IB-UCBT) was compared with that of 149 who received double UCBT (dUCBT) in a retrospective study. Although the number of TNC was significantly higher in dUCBT than in the IB-UCBT recipients, platelet and neutrophil engraftment occurred earlier in the latter cohort. After 180 days, platelet engraftment had occurred in 74% of IB-UCBT recipients compared with 64% in dUCBT recipients (p = 0.003). The median time to neutrophil engraftment was 23 days in IB-UCBT compared with 28 days in dUCBT recipients (p = 0.001) [43].

2.7. UM171-Mediated Expansion

UM171 is a small molecule that was found to be a potent stimulator of ex vivo expansion of human UCB HSPCs that retained their self-renewal capability when transplanted into immunodeficient mice. UM171's mechanism of action is separate from antagonism of AHR but is yet to be assessed in a clinical trial [39].

2.8. Lysine-Specific Histone Demethylase 1A-Mediated Expansion

Lysine-specific histone demethylase 1A (LSD1) is an epigenetic regulator of gene expression that acts by removing methyl groups. Murine models have shown that LSD1 inhibition results in bone marrow HSPC expansion. Agatheeswaran et al. showed that LSD1 inhibition in vitro resulted in expansion of UCB-derived HSPCs. LSD1 inhibitors (2-PCPA, GSK-LSD1 and RN1) were used in culture media to which SCF, TPO, flt3L were added. A 10-fold increase in CD34+ cells was demonstrated, as compared with 2-fold increase in control cultures. Combination of LSD1 inhibition with other modalities for UCB expansion is still being investigated in preclinical models [22].

3. PROMOTION OF UCB-DERIVED HSPC HOMING TO THE BONE MARROW

Apart from ex vivo expansion of UCB to overcome the limitation posed by limited cell dose, techniques to increase homing of UCB HSPCs to bone marrow niches have also been developed. Homing techniques are less costly and are easier to deploy compared to ex vivo expansion. A chemokine called stem cell-derived factor 1 (SDF-1) is important in directing the homing of HSPCs to the bone marrow [40]. SDF-1 binds to the CXCR4 receptor on HSPCs and attracts HSPCs to the endothelium in the bone marrow. This is followed by trans-endothelial movement of the HSPCs and repopulation of the bone marrow niches [41].

Some of the techniques to improve UCB homing to the bone marrow include injection of the UCB directly into the bone, inhibition of dipeptidyl peptidase 4 (DPP-4), pulse treatment of UCB with 16,16-dimethyl prostaglandin E2 (dmPGE2), enforced fucosylation of UCB, and priming of UCB complement fragment 3a.

Injection of the UCB allograft directly into the bone is a physical measure to improve homing of the HSPC to the bone marrow niches. In a phase I/II trial, a single unit of UCB allograft was injected into the bone marrow of 32 patients with acute leukemia after MAC. This study demonstrated the feasibility of direct intra-bone injection of UCB. Neutrophil recovery occurred at a median of 23 days (range 14–44 days) while that of platelet occurred after a median of 36 days (range 16–64 days). There were no cases of severe (grade III–IV) acute GVHD [42]. The outcomes of 87 patients who received intra-bone UCBT (IB-UCBT) was compared with that of 149 who received double UCBT (dUCBT) in a retrospective study. Although the number of TNC was significantly higher in dUCBT than in the IB-UCBT recipients, platelet and neutrophil engraftment occurred earlier in the latter cohort. After 180 days, platelet engraftment had occurred in 74% of IB-UCBT recipients compared with 64% in dUCBT recipients (p = 0.003). The median time to neutrophil engraftment was 23 days in IB-UCBT compared with 28 days in dUCBT recipients (p = 0.001) [43].

CD26/DPP-4 is an extracellular peptidase that is expressed in the cell membranes of some CD34+ UCB-HSPCs, and its main role is to inactivate SDF-1. DPP-4 cleaves dipeptides off the N terminus of the SDF-1 alpha chain, thereby making it inactive and leading to an impairment in the ability of the HSPCs to home to their bone marrow niches [44]. Inhibition of DPP-4 activity by treating the UCB with diprotin A prior to transplantation into murine models showed improved engraftment [45]. A clinical trial using sitagliptin, a DPP-4 inhibitor that had been FDA approved for another indication, was performed. Twenty-four patients with hematological malignancies were enrolled. After MAC, they were treated with sitagliptin at a dose of 600 mg daily on days −1 to day +2 and a single-unit UCB was infused on day 0. Neutrophil engraftment occurred in 88% (95% CI 74–100%) of patients by day 30. There was a significant association between the area under the curve (AUC) for sitagliptin and speed of engraftment: higher AUC led to slower engraftment. This calls for further studies to identify the most appropriate dosing regimen for sitagliptin and to evaluate other DPP4 inhibitors that are already available [46].

Preclinical work showed that the SDF-1 receptor, CXCR4, is activated by priming the UCB-derived HSPCs with complement...
fragment 3a (C3a), thereby improving engraftment in mouse models. In a clinical trial using double UCBT, one of the two units was primed with C3a while the other was unmanipulated. All the patients received non-MAC, so it was difficult to determine the speed of engraftment of the individual UCB units. Cells derived from the primed UCB unit were only detectable in 6 patients beyond 100 days. There was no clinically demonstrable benefit from C3a priming in this study [47].

The 16,16-dimethyl prostaglandin E2 (dmPGE2) causes increase in HSPCs in vivo. The mechanism through which dmPGE2 exerts its effect is via Wnt signaling mediated by cAMP, and through increased cyclinD1 expression leading to reduced apoptosis. dmPGE2 also increases CXCR4 expression on the HSPC membrane, increases binding to SDF-1 and improves HSPC homing [48,49]. In a clinical trial, 12 patients received double UCBT, with the larger of the two units modulated with dmPGE2 while the other was unmanipulated. After reduced intensity conditioning, the modulated unit was transplanted first followed by the unmanipulated unit after 4 hours. Neutrophil recovery occurred after a median of 17.5 days compared with 21 days in historical controls (p = 0.04). The dmPGE2-modulated unit was also preferentially engrafted long term in 10 out of 12 patients. These results are very promising, but it should also be pointed out that dmPGE2 has at least 2 modes of action: it increases the number of HSPCs and promotes homing of the HSPCs. In addition, the larger of the two UCB units was selected for dmPGE2 modulation and this might have contributed to the preferential engraftment of the that unit [50]. A randomized phase II trial tagged “PUMA trail” (Clinicaltrials.gov NCT01627314) is still underway.

Endothelial cells interact with HSPCs via E- and P-selectins expressed on the endothelial membrane. HSPCs also express ligands such as P-selectin glycoprotein ligand-1 (PSGL-1) through which they bind to the selectins on the endothelial membrane. This interaction between the HSPCs and the endothelium is important in the homing process after UCBT. Hidalgo et al. found that relative to PB CD34+ cells, a larger fraction of UCB-derived CD34+ cells have non-functional PSGL-1 [51]. The selectin ligands on HSPCs also require alpha 1, 3-fucosylation in order to bind to the selectins on endothelial cells. In murine models, treatment of UCB cells with alpha1–3 fucosyltransferase V1 and guanosine diphosphate (GDP) fucose improved engraftment [52]. In a phase I trial, following MAC, 22 patients received double UCBT, with the smaller of the two units treated with fucosyltransferase V1 and GDP fucose, while the other was unmanipulated. Compared to 31 historical controls who received unmanipulated dUCBT, neutrophil engraftment occurred after a median of 17 days in trial participants versus 26 days in controls (p = 0.0023), while for platelet engraftment those numbers were 35 and 45 days, respectively (p = 0.0520). Hematopoiesis derived solely from the fucosylated unit in 40% of recipients, while 20% of recipients showed hematopoiesis from both the fucosylated and the unmanipulated units [53].

To reduce major complications (such as relapse, infections and GVHD) contributing to post-transplant morbidity and mortality, T cell manipulation techniques are being developed.

4.1. Prevention of Post-UCBT Relapse with Chimeric Antigenic Receptors Engineering on UCB-Derived T Cells

CAR-T cell therapy has proven efficacy in a number of hematologic malignancies including diffuse large B cell lymphoma, follicular lymphoma, refractory multiple myeloma, acute lymphoblastic leukemia and chronic lymphocytic leukemia [55]. Typically, CAR-T cells are able to recognize antigens independent of HLA presentation. They are highly specific because the extracellular immunoglobulin-derived fragment is engineered for a specific ligand like CD 19 for B cell leukemias and lymphomas. The intracellular portion of the CAR-T consists of an activating domain (CD3ζ) in first generation chimeric antigenic receptors (CARs), while a co stimulatory domain such as CD28 is added in the second-generation CARs [56]. Given the limited cell dose per unit of UCB, obtaining adequate number of UCB-derived T cells for CAR engineering is also a challenge necessitating ex vivo expansion.

Donor UCB-derived T cells were engineered to express second-generation CAR (CD19RCD28) specific for CD 19-expressing tumors and using the CD28 signaling and CD3-ζ endo-domain. The T cells were engineered using the Sleeping Beauty (SB) transposon/transposase system to induce CAR expression. Following transfection, the T cells were cultured for 28 days in the presence of artificial antigen presenting cells (APCs) (IL-2 and IL-21). More than 1000-fold expansion of CAR+ T cells resulted from the engineering technique employed, with the CAR T cells showing markers of T cell activation [56].

An alternative technique for CAR T cell engineering via the use of cytokines was described by Pegram et al. UCB-derived T cells were cultured with IL-12 and IL-15, followed by retroviral transduction to express CD19-specific CAR and to secrete IL-12. This resulted in a more than 150-fold T cell expansion, and expression of memory markers in the T cells. In SCID mice with CD19+ tumors, these engineered T cells improved survival owing to the graft versus tumor effect exerted by the engineered T cells [57].

4.2. Ex vivo Expansion of Tregs to Reduce GVHD

Tregs are CD4+ cells that are crucial in preventing autoimmunity by maintaining a state of tolerance to self- and non-self-antigens. Tregs express nuclear forhead box P3 (FOXp3) and CD25 (the alpha chain of the heterotrimeric IL-2 receptor) on their cell membrane [58]. In the seminal work done by Sakaguchi et al., it was shown that mice with inhibition of Tregs had various autoimmune phenomena, including GVHD, and these autoimmune features improved with reintroduction of Tregs [59].

In a phase I clinical trial, low dose IL-2 was administered to 29 patients with steroid refractory chronic GVHD for 8 weeks. FOXp3+ Tregs were significantly increased in all recipients, with peak levels noted at 4 weeks. Twelve of the 23 evaluable patients
had major responses at multiple sites (including skin, joints, liver and peripheral nerves) and the steroid dose could be significantly tapered. Chronic GVHD did not relapse or progress in any of the study participants. Renal failure requiring dialysis occurred in 2 cases, but may have been due to the patients’ other medications; 3 patients had grade 3 bacterial infections and grade 1 constitutional symptoms, necessitating an IL-2 dose reduction [60]. This has been followed up by a phase II study in which 35 patients with steroid refractory chronic GVHD received IL-2 for 12 weeks. Sixty-one percent (n = 20) of the 33 evaluable patients had a clinical response at multiple sites, and Tregs increased significantly by >5-fold compared with pretreatment levels. Patients with clinical response continued IL-2 therapy indefinitely; at the 2-year follow up, IL-2 was well tolerated and the Tregs increase was sustained [61]. Constitutive phosphorylation of signal transducer and activator of transcription 5 (Stat5) in conventional CD4+ T cells (Tcons) is a major feature of chronic GVHD, resulting in IL-2 deficiency. The mechanism by which daily IL-2 therapy improves chronic GVHD was shown to be via increased Stat5 phosphorylation of Tregs, thus increasing the Tregs:Tcons ratio and shifting the balance in favor of immune tolerance [62].

The low cell dose of UCB also limits the number of Tregs that can be obtained. PB Tregs were used as a source for ex vivo expansion. PB-derived Tregs were expanded ex vivo using artificial APCs, with resulting Treg yields of up to 3000-fold after a single re-stimulation. In murine models, the expanded Tregs were able to reduce the severity of GVHD [63].

UCB-derived Tregs were expanded ex vivo and used in a phase I clinical trial for patients receiving UCBT. Tregs from cryopreserved third party UCB were cultured ex vivo for 18 days using anti-CD3, anti-CD28 antibody-coated beads and IL-2. Twenty-three double UCBT recipients received UCB-derived Tregs. These cells persisted in the recipients for up to 14 days. In comparison to 108 historical UCBT recipients, the incidence of acute GVHD was lower in UCB-Tregs recipients (43 versus 61%, p = 0.05), and there was no significant difference in infection or relapse rates [64]. In another study using UCB-derived Tregs which were cultured ex vivo, there was a lower incidence of acute GVHD in UCBT patients who received the Tregs infusion compared to controls (9% versus 45%, p = .05). Chronic GVHD was similarly lower in Tregs recipients compared to controls (chronic GVHD at 1 year 0 versus 14%) [65].

Human bone marrow-derived MSCs have been shown to have a role in the ex vivo expansion of Tregs. In an IL-2-driven ex vivo 21-day culture, MSCs not only increased the number of Tregs (80.2 × 10^6 in MSC medium versus 39.3 × 10^6 in control medium p < 0.01), but the MSC co-cultured Tregs were also more potent in their immunomodulatory role compared with Tregs cultured in control medium lacking MSC (79% versus 35% inhibition of T cell proliferation ratio, p < 0.01). The mechanism by which the MSCs enhance ex vivo Tregs growth was found to be via direct cell to cell contact with the MSC. This was further evaluated with flow cytometry and optical sectioning microscopy, and was found to be via mitochondrial transfer via tunneling nanotubes, because the MSC effect was inhibited by cytochalasin B, which blocked the mitochondrial transfer [66,67].

The choice of culture media for Tregs expansion also affects the expression of homing markers. A group evaluated the effect of different culture media (XVIVO and SCGM) on expression of the homing markers CD62 ligand and cutaneous lymphocyte antigen (CLA) by Tregs. CLA expression was 40–60% in SCGM-cultured Tregs compared with 7–10% in the Tregs cultured in XVIVO. The higher CLA expression also correlated with binding to selectin [68].

### 4.3. Natural Killer Cell Expansion

Natural killer (NK) cells are an important component of the innate immune system that function to control tumors and infections; they also have some regulatory function [69]. They possess killer immunoglobulin-like receptors (KIRs) which are recognized by class I HLA alleles which leads to inhibition of NK activity. The practice of administering T cell depleted allografts to prevent GVHD increases the risk of tumor relapse, because the NK donor versus tumor effect is lost by T cell depletion. Ruggeri et al. also showed that donor versus recipient NK cell reactivity provides a balanced effect and prevents GVHD. Infusing NK cells before transplant was demonstrated to obviate the need for MAC in murine models [70]. In unrelated donor HPSCT transplant and in UCBT recipients, studies have shown better outcomes (including improved OS, DFS and relapse rates) associated with NK cell alloreactivity [71,72].

Like other types of UCB components, the number of NK cells in UCB is also limited, so ex vivo expansion of UCB-derived NK cells has been studied. UCB-derived CD56+ cells were expanded in the presence of feeder cells and IL-2 with a yield of up to 181-fold after 22 days of culture. The expanded CD56+ cells showed potent antitumor activity against K562 cells in vitro, resulting in 36.5–51.5% apoptosis [73]. Ex vivo expansion of NK cells from a small volume of UCB (1 mL) has also been demonstrated. The CD3-fraction of the 1-mL UCB was cultured with IL-2 media after stimulation with Epstein-Barr virus-transformed lymphocytes. Compared to NK cells derived from PB, these UCB-derived NK cells were slightly less cytotoxic [74].

Clinical studies of UCB-derived NK cells will be needed to better understand their effectiveness in UCBT.

### 4.4. CARs Engineering on UCB-Derived NK Cells

There is ongoing preclinical and clinical work to develop CAR-NK cells because they may be safer immunotherapy agents compared to CAR-T cells, given the lower risk of cytokine release syndrome (CRS) with CAR-NK cells [75]. UCB is a good source of NK cells for CAR-NK cell engineering. Preclinical work at the MD Anderson Cancer Center has resulted in the development of a CAR-NK therapy using NK cells derived from UCB. The UCB-derived NK cells were transduced with a retroviral vector containing genes for CAR-CD19, IL-15 and inducible suicide gene (iC9). A phase I/II trial clinical trial (NCT03056339) using this CAR-NK cell for CD19 positive B-lymphoid malignancies is underway [76].

### 4.5. Generation of VSTs from UCB

Following UCBT, recovery of immune cells (B and T lymphocytes and NK cells) including T lymphocytes specific for different viruses (e.g. adenovirus, Epstein-Barr virus (EBV), cytomegalovirus (CMV), BK virus (BKV), respiratory syncytial...
virus (RSV), and influenza) is very slow [77]. Given the already slow rate of immune recovery following UCBT, the use of alternative MAC regimens excluding antithymocyte globulin (ATG) has been considered, because ATG has been shown to further slowdown the rate of immune recovery. Bosch et al. showed that ATG worsened CD4 T cell recovery after HSCT [78]. This delay in immune reconstitution is of great concern, because it increases the risk of viral infections post-transplant. A phase II trial compared immune reconstitution post-transplant in patients who had an ATG-containing conditioning regimen versus a regimen without ATG. They found that the ATG group had a higher rate of CMV reactivation ($p < 0.001$) and a higher relapse rate ($p = 0.01$) compared to the non ATG group [79]. Furthermore, in a retrospective review of 91 patients who received single-unit UCBT, the use of an ATG-containing MAC regimen was associated with reduced OS (hazard ratio (HR) = 1.99, $P = 0.02$), reduced event-free survival (EFS) (HR = 1.83, $P = 0.02$) and higher incidence of NRM (HR = 2.54, $P = 0.04$) [80].

UCB is not only low in T cell numbers, but the few T cells in that source are also naïve, thus making them ineffective in combating viral infections post-transplant. This translates into a higher morbidity and mortality from infections (especially from CMV, adenovirus (Adv), and EBV) in the post-transplant period. To address the problem of UCB-derived T cell naïvety, T cell engineering to produce multispecific cytotoxic T cells (VSTs) has been studied. Cytotoxic T lymphocytes (CTL) derived from mononuclear of lymphocytes obtained from the PB of CMV-seropositive donors have been used to combat CMV, EBV and adenovirus infections after HSCT [81]. It is challenging to generate VSTs from UCB because, given their naïvety, UCB-derived T cells first have to be primed before being expanded. Hanley et al. developed a protocol in which EBV-infected cells were transduced with clinical-grade Ad5f35CMVpp65 adenoviral vector and VSTs from UCB-derived naïve T cells were expanded. These VSTs were shown to be cytotoxic against EBV, CMV and adenovirus [82]. The technical complexity of the protocol described by Hanley et al. is a major limitation for its clinical usefulness. Another approach using a single step for VST generation from PB mononuclear cells (PBMCs) from healthy donors has been developed. These PBMCs were stimulated with peptide libraries including 12 antigens from EBV, adenovirus, CMV, BKV and human herpes virus 6 (HHV6). The generated VSTs were safely infused into 11 post-HSCT patients and successfully treated 8 of the patients with sustained long-term virologic response [83].

In conclusion, there have been major advances in UCB expansion, and these have been translated into clinical improvement. There are still major challenges and more research is ongoing to overcome them.

**CONFLICT OF INTEREST**

The authors declare they have no conflicts of interest.

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