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Immune Regulatory Cells in Umbilical Cord Blood and Their Potential Roles in Transplantation Tolerance

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Abstract

Umbilical cord blood (UCB) is a source of primitive hematopoietic stem (HSC) and progenitor cells, that served as an alternative to bone marrow (BM) for effective transplantation therapy. Success of HSC transplantation (HSCT) is limited in part by graft-versus-host disease (GVHD), graft rejection and delayed immune reconstitution, which all relate to immunological complications. GVHD after UCB transplantation is lower compared to that of BM HSCT. This may relate to the tolerogenic nature of T cells, mononuclear cells (MNCs) and especially immune regulatory cells existing in UCB. UCB contains limiting numbers of HSC or $CD34^+$ cell dose for adult patients resulting in delayed engraftment after UCB transplantation (UCBT). This needs to be improved for optimal transplantation outcomes. Approaches have been undertaken to promote HSC engraftment, including co-infusion of multiple units of UCB cells. These new methods however added additional immunological complications. Herein, we describe current knowledge on features of UCB immune cells, including regulatory T cells (Tregs) and mesenchymal stem/stromal cells (MSCs) and their potential future usage to reduce GVHD.

Keywords

Cord blood Transplantation; Transplantation Tolerance; T-lymphocytes; Dendritic Cells; Regulatory T cells; Mesenchymal Stromal/Stem Cells; Natural Killer Cells

1. Introduction

UCB offers an effective transplantation therapy to reconstitute the hematopoietic system in patients with malignant and non-malignant disorders. UCB HSCs cryopreserved for many years are functional [1] and ready to use. In general, UCB immune cells are less aggressive against allogeneic responses mainly due to their immaturity. In addition, UCB contains regulatory T cells (Tregs) and mesenchymal stem cells (MSCs) with potent functions for suppressing allogeneic immune responses. It is likely that such UCB immune regulatory cells contribute to less frequent GVHD in patients transplanted with UCB compared to the patients transplanted with BM. Conversely, UCBT often bears the disadvantage of insufficient HSC numbers in UCB for timely immune reconstitution particularly in adult patients and development of antigen-specific cellular immunity is affected by numerical and

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qualitative deficits of UCB T cells [2-4]. As a result, infections that afflict transplant patients occur frequently within the first 3-4 months after UCBT [5-6]. To overcome this limiting numbers of cells, double-unit UCBT has been used, but with additional immunological concerns [7].

This review focuses mainly on characteristics of immune regulatory cells, Tregs and MSCs in UCB, their potential modulating roles in GVHD and immune reconstitution, particularly after double-unit UCB transplantation for adult patients.

2. Umbilical Cord Blood Hematopoietic Stem Cell Transplantation

The first UBC-HSCT from an HLA-identical sibling was performed for a 6-year-old boy with Fanconi anemia over 20 years ago [8-9]. The first clinical efforts were based on laborattory studies suggesting the presence of HSCs in UCB [10]. The ability of UCB to reconstitute hematopoiesis after myeloablative conditioning was soon proven in large number of patients, being facilitated by numerous UCB banks worldwide. Prospective, multi-center studies of unrelated UCB-HSCT for pediatric patients clearly demonstrated the efficacy of UCB-HSCT [11].

2.1 UCB-HSCT allows a higher degree of HLA disparity

GVHD is a major cause of morbidity and mortality after allogeneic HSCT and donor lymphocyte infusions [7]. The main risk factor for development of acute GVHD is human leukocyte antigen (HLA) disparity of donor T cells with reactivity against recipient histocompatibility antigens [7]. The number of mismatched HLA class I antigens correlates with instance of GVHD and poor engraftment kinetics [12]. UCBT is characterized by lower incidence of acute and chronic GVHD compared to BMT [13-15]. In a matched-pair analysis, GVHD and survival of patients receiving unrelated UCB-HSCT with up to 3 HLA mismatches were comparable to those receiving HLA-matched unrelated donors-HSCT with 6/6 HLA matches [13,16]. Mechanisms of reduced acute GVHD in patients receiving unrelated donor UCB still remain poorly understood. T cells are primary cells responsible for GVHD. T cells from UCB have greater immune tolerance to HLA mismatch [13], and impaired allogeneic activation [17-18]. It is generally accepted that immaturity of UCB T cells is a major reason for permitting use of an HLA mismatched UCB unit as a graft for transplantation. Following in vitro priming with alloantigen, cytotoxicity of $CD8^+$ T cells from UCB is significantly diminished compared to that from adult peripheral blood (APB) and BM, suggesting that $CD8^+$ T cell cytotoxic defect may, in part, be related to the low incidence of GVHD thus far noted in human CB transplants [19-20]. Reduced incidence and severity of GVHD after allogeneic UCBT may also be due to a defect in activation of specific transcription factors and an impaired production of certain cytokines, although this has not been extensively proven. More recent studies indicate that immune regulatory cells, such as Tregs could be dominant factors in control of GVHD [21]. In this section, we describe the characteristic properties of UCB T cells and antigen presenting cells and their therapeutic implications for GVHD and graft rejection.

2.2. Defective Th1 responses of UCB T cells

In contrast to adult PB, UCB T cells are largely naïve ($CD45RA^+$). Thus, the primary reason for CB T cells being immunologically less responsive to alloantigens is an overall prevalent immaturity of immune cells [22]. This may be due to relatively low exposure of the fetus to environmental pathogens and vaccines as compared to the adult. As a result, T cells in UCB are activated significantly less readily than APB T cells in allogeneic response, producing less interferon-γ (IFN-γ) compared with APB $CD4^+$ T cells after allogeneic stimulation [18]. Donor $CD4^+$ T cell interactions with host antigen presenting cells (APCs) lead to activation

of donor T cells and their differentiation into T helper (Th) cells. However, the role of Th1, Th2, and Th17 cells in acute GVHD pathogenesis is still controversial. In general, Th1 cells play a critical role in mediating acute GVHD pathogenesis [23-24], and Th2 cells ameliorate GVHD mediated by Th1 cells [25-26]. Defective type Th1 immune responses by UCB T cells in comparison with APB T cells may be attributed to impaired expression and activation of transcription factors [27]. Expression of T-bet, a Th1-specific transcription factor, is low in UCB compared to APB T cells, whereas expression of GATA-3, a transcription factor correlating with expression of Th2 cytokines, is similar. Freshly purified UCB naive $CD4^+$ T cells show markedly lower expression of STAT4 at mRNA and protein levels than APB naive $CD4^+$ T cells. Since STAT4 plays a critical role in Th1 differentiation [28], low STAT4 expression might contribute to reduced IFN-γ production by UCB $CD4^+$ T cells after allogeneic stimulation. UCB naive $CD4^+$ T cells demonstrate substantially decreased expression of tyrosine phosphorylated-STAT4, an activated form of STAT4, after IL-12 treatment compared with similarly IL-12-treated APB naive $CD4^+$ T cells [18]. Transcription factors involved in the nuclear factor of activated T-cells (NFAT) pathway, including C/EBPβ and AP-1 are expressed at reduced levels in UCB compared with APB during primary stimulation. Reduced global expression of NFAT-associated genes, as well as Th1-related transcription factors in UCB $CD4^+$ T cells may contribute to the decreased GVHD observed after UCB transplantation [29].

Unexpectedly, donor T cells deficient in IFN-γ, a Th1 cytokine, induce exacerbated acute GVHD [30-31]. It was proposed that tissue damage mediated by IFN-$\gamma^{-/-}$ donor T cells was associated with the lack of an IFN-γ inducible protective mechanism [32]. More recently, the protective role of IFN-γ was identified. IFN-γ induces expression of a coinhibitory molecule, B7-H1 on host tissues [23]. B7-H1 plays an important role in inducing anergy and apoptosis of allogeneic T cells through programmed death-1 (PD-1) and contributes to protect tissue damage in recipients [33]. Th17 cells were shown to augment GVHD [34-35]. In contrast, it was shown that Th17 cells ameliorate acute GVHD in allogeneic recipients, and neutralizing IL-17 augments acute GVHD [24]. These seemingly contradictory reports remain to be resolved.

There are also conflicting reports regarding the ability of UCB T cells to produce IL-4, a Th2 cytokine. A majority of reports demonstrate that UCB T cells are more prevalent for skewing Th2 cytokine production compared to APB T cells in response to allogeneic stimulation. However, some investigators reported that UCB T cells are not different from ABL $CD4^+$ T cells in their ability to produce IL-4 in vitro [18]. IL-10 is an important anti-inflammatory cytokine and IL-10 produced by donor T cells has been known to reduce severity of acute GVHD [36] and to promote graft tolerance [37]. In contrast to tumor necrosis factor-α (TNF-α) and IFN-γ, IL-10 is readily produced by UCB T cells during T cell receptor (TCR) stimulation, while IL-10 is secreted by adult T cells only after repeated stimulation. Thus, the potential of UCB T cells to secrete IL-10 in abundance may contribute to reduced incidence of GVHD associated with UCBT [38], possibly through production of Treg cell populations [37].

2.3. Impaired activity of UCB-derived dendritic cells (DCs) in allogeneic T cell responses

Although the allogeneic response of donor T cells is associated with GVHD after HSCT, the antigen-presenting function of donor DCs may be also involved in chronic GVHD [39-40]. It has been speculated that the antigen presenting activity of DCs derived from UCB may be immunologically defective because UCB DCs are immature [41-42]. This hypothesis was validated by findings that UCB DCs were weakly stimulatory to T cells in a mixed lymphocyte reaction when compared to APB counterparts [40,43-45].

DCs can be developed from $CD14^+$ monocytes isolated from either APB or UCB by culture *in vitro* in the presence of GM-CSF and IL-4. GM-CSF/IL-4 derived immature DCs are converted to the mature form of DCs by various stimuli from pathogen related products such as bacterial lipopolysaccharides (LPS) [46], proinflammatory cytokines such as TNF-α and IL-1β [47], as well as CD40 ligation [48]. Studies performed with isolated $CD14^+$ monocytes, or *in vitro*-generated $CD14^+$ monocyte-derived DCs indicate that mononuclear cells (MNCs) and UCB DC are less susceptible to complete maturation compared to their counterparts in APB [49]. DCs generated from UCB $CD14^+$ monocytes produced lower amounts of TNF-α compared to DCs derived from APB [49]. UCB derived DC are characterized by a defect in IL-12 production, which is responsible for an impaired ability to elicit IFN-γ production by allogeneic T cells [50-51]. The low production of these pro-inflammatory cytokines does not result from low expression levels of LPS receptors, CD14 and toll-like receptor (TLR)-4 [49], suggesting that UCB MNCs have an intrinsic limitation in their differentiation into fully mature DCs [52]. Consistent with this, surface expression of HLA-DR, CD40, CD86 and CD83 was significantly lower in UCB DCs compared to APB DC after LPS stimulation [49]. These characteristics of UCB DC can delay activation of naive T cells, especially that of Th1 cell development. The defective APC activity of CB DCs can be restored with upregulation of CD86 and HLA-DR after prolonged stimulation with irradiated allogeneic T cells, suggesting that UCB APC activity can be gradually increased after transplantation and can contribute to delayed GVHD [40].

Human DCs consist of two subsets, $CD11c^+$ CD123- myeloid (mDC) and $CD11c^-$ $CD123^+$ plasmacytoid DCs (pDCs). pDCs tend to induce Th2 cell responses, involving the induction and maintenance of tolerance, whereas mDCs stimulate Th1 cell responses [53]. UCB DCs are characterized by a higher ratio of $CD11c^-$ $CD123^+$ pDCs to $CD11c^+$ $CD123^-$ mDCs than APB DCs [49,54]. In addition, UCB pDCs display low expression of costimulatory molecules and TNF-α compared to APB counterpart DCs, eliciting impaired responses to LPS and unmethylated cytosine-phosphate-guanosine (CpG) [49]. Taken together, weakly immunogenic UCB DCs to allogeneic T cells may be an additional important factor responsible for the relatively low incidence of severity of GVHD in UCB transplants.

2.4. Tolerogenic properties of UCB DCs

Immature DC are known to act as immune suppressor cells inducing antigen specific T-cell tolerance [55-58]. Allograft rejection has been partly prevented through tolerance inducing immature DCs in the periphery [59-60]. Immature DCs also induce regulatory properties to effector T cells [61-62] and expansion of naturally occurring $CD25^+$ Tregs [63]. In support of this, administration of DCs at an immature state into healthy volunteers in the absence of inflammatory signals induced Tregs capable of suppressing alloantigen-specific T cell responses [64]. The immunological features of DCs are affected by MSCs; DCs cultured in the presence of MSCs show increased production of IL-10 and reduced production of IL-12 [65]. Hence, MSCs in umbilical cord (UC) may induce DCs to enter an immature or a tolerogenic state, which might be an indirect mechanism responsible for inducing T-cell hyporesponsiveness.

Higher serum levels of macrophage colony-stimulating factor (M-CSF) have been detected in UCB than in APB [66], and pregnancy is associated with a skewed Th2 cell cytokine profile [67]. Thus, UCB $CD14^+$ monocytes might have a greater chance to be exposed to M-CSF and IL-4. We previously demonstrated that UCB $CD14^+$ monocytes cultured with M-CSF and IL-4 differentiated into DCs expressing high and low levels of IL-10 and IL-12, respectively with tolerogenic potential in allogeneic mixed lymphocyte reaction [68]. This suggests that M-CSF rich in UCB may play a role in maintaining or inducing immature tolerogenic DCs from UCB. Therefore, UCB DCs may be characterized by the lack of antigen presenting capacity but by the potential to induce and expand Tregs [56,61-63].

3. Immune modulator cells

UCB and umbilical cord (UC) are a source of both Tregs and MSCs. These cells have been scruinized intensively as potential cell mediators to control GVHD. Advances in Treg and MSC biology may be helpful for designing safer and more effective HSCT in treatment of a variety of malignant and non-malignant diseases [69]. The following discusses charateristics of Tregs and MSC in UCB, and the significance of these immune regulatory cells in terms of clinical implications in GVHD after UCBT. Also described are recent methods to expand these immune regulatory cells.

3.1. Tregs

Sakaguchi et al. [70] first reported that $CD25^+$-depleted $CD4^+$ T cells transferred into nude mice resulted in autoimmune disease, events reversed by adding back $CD25^+$ T cells. Since then, $CD25^+CD4^+$ T cells have been termed Tregs by their functional characteristics of suppressing T cell immune response [71]. Foxp3, a helix-loop-helix transcription factor, is considered to be one specific marker for Treg cells [72]. Treg cells are characterized as cells with constitutive expression of CD25 and Foxp3, and represent 5–10% of peripheral $CD4^+$ T cells. Tregs express other surface markers, including CTLA-4 (CD152) [73], glucocorticoid-induced TNF receptor (GITR) [74-75] and OX-40 (CD134) [76]. As Tregs modulate immune responses through selective migration and accumulative retention at tissue specific sites, they express integrin molecule CD62L (L-selectin) [77-78] and chemokine receptor CCR7 [79], crucial for homing to secondary lymphoid tissues. Treg cells also specifically express chemokine receptors CCR4 and CCR8 which may guide Treg cells to inflamed areas to attenuate T cell activation [80].

Transwell experiments demonstrate that the suppressive function of Tregs partially depend on cell–cell contact. Surface transforming growth factor-β (TGF-β) [81] and CTLA-4 might be involved in Treg mediated suppression [82]. TGF-β secreted by Tregs may further convert naive $CD4^+$ $CD25^-$ cells to $CD25^+$ $Foxp3^+$ Tregs referred to as induced Tregs (iTregs) [83]. Secretion of IL-10 and possibly other unknown cytokines action in a paracrine fashion may also be responsible for the suppressive activity of Tregs. In the presence of IL-6 and TGF-β, naive T cells differentiate into Th17 cells, whereas in the absence of IL-6, same cells are induced to become Tregs [84]. Thus, IL-6 occupies a unique position at the crossroads of Treg and proinflammatory Th17 cells. IL-6 is not indispensable for Th17 cell development because IL-21 may induce Th17 differentiation even in the absence of IL-6 [85].

Mammalian target of rapamycin (mTOR), a downstream target of the PI3K/AKT signaling pathway, acts as a negative regulator for Tregs. Rapamycin, an inhibitor of mTOR pathway promotes expression of Foxp3 and proliferation of Tregs, while it promotes activation-induced cell death of effector T cells [86-87]. *In vivo*, rapamycin administration reduces IL-6 production and promotes alloantigen-specific Treg cell activity as well as long-term, donor-specific heart graft survival in immunocompetent hosts [88]. These findings suggest that mTOR inhibition in Tregs may be an effective approach to control GVHD and promote long-term organ graft survival. It remains to be seen whether UCB Tregs are distinct from those from other tissue sources in the mTOR pathway or IL-6 production.

3.2. Human vs. mouse Treg

For more than two decades, immune suppressive properties of Tregs have been well-described mainly in mice [70,89-91]. Compared to mouse Tregs, human Tregs appeared to be difficult to study due to lack of specific surface markers. Initially, all $CD25^+$ cells in $CD4^+$ T cell populations was considered to represent Tregs in laboratory rodents [92], but

CD25 is also expressed on recently activated T cells [93]. Twenty–60% of $CD4^+$ T cells in human APB express CD25 [94], and a large proportion of these human T cells represent activated or memory cells generated as a result of previous encounter with foreign antigens [95]. Thus, the CD25 molecule is not a reliable marker for natural Tregs particularly in humans. $CD4^+$ $CD25^+$ T cells existing in human APB consist of two populations: $CD25^{int}$ and $CD25^{bright}$ cells. Only the 25^{bright} population in human $CD4^+25^+$ cells, which consist of only 1-2% of $CD4^+$ T cells, are largely $FoxP3^+$ Treg cells, and these populations may be relevant to murine $CD25^+$ $CD4^+$ T cells with regulatory ability [96-97].

Although Foxp3 is considered to be a reliable marker for murine Treg, in humans Foxp3 is also expressed in activated $CD4^+$ T cells [72,98-99]. Thus, Foxp3 expression detected in human $CD4^+$ $CD25^+$ T cells may not fully reflect the regulatory capacity of these cells. Other reliable markers have been sought to further specify human Tregs and to improve isolation and purification procedures. $CD4^+$ $CD25^+$ cells with low levels of CD127 (IL-7Ra chain) denote human Treg cells with increased suppressive activity. The $CD127^{lo}$ phenotype highly correlates with expression of FoxP3 within the $CD4^+CD25^+$ population [100]. Expression of CD45RA has also been reported to distinguish Tregs from activated T cells [101], but CD127 and CD45RA does not absolutely designate human Tregs. Booth et al. [102] report that the majority (approximately 80%) of natural Tregs in adults were $CD45RO^+$. The proportion of $CD45RO^+$ to $CD45RA^+$ Tregs increased significantly with age. $CD4^+CD25^+CD27^+$ Tregs have been identified as a potent regulatory subset in UCB and in a large-scale *in vitro* expansion system [103]. Combined expression of CD25 and CD27 allow the differentiation of highly suppressive $FoxP3^+$ regulatory T cells from activated effector T cells[104]. Rapamycin fosters dominance of $CD27^+$Tregs over $CD27^-$ Tregs after expansion of the $CD4^+CD25^+$ Treg pool upon allogeneic activation[104]. New discriminatory cell surface antigens have yet to be discovered to further specify human Tregs from $CD4^+CD25^+$ population.

3.3. Characteristics of UCB Tregs

UCB contains $CD4^+CD25^+$ cells which manifest similar suppressive activity to APB[97,105-106], but it remains controversial as to whether UCB possesses distinctive Treg functions compared to APB. It has been reported that the $CD4^+CD25^{bright}$ subset exists at relatively higher frequency in UCB compared to APB [97,106]. Consistent with this, UCB $CD4^+$ $CD25^+$ T cells have been shown to contain greater Foxp3 expression than their APB counterparts, suggesting the greater abundance of Tregs in UCB than APB [106]. Another study, however, showed that the expression of Foxp3 protein on naïve UCB $CD4^+$ T cells was lower compared with those of APB [18], which argues against increased Treg activity as a mechanism for decreased Th1 differentiation of UCB $CD4^+$ T cells. UCB $CD25^+$ cells suppress polyclonal T cell activation, but do not suppress antigen-specific responses [107]. It remains unclear what the causes are of the inconsistency among reports in respect to suppressive activity of CB Tregs. The inconsistent results on suppressive activities of CB Tregs may result from distinct sources of target $CD4^+$ T cells tested. UCB $CD4^+$ $CD25^+$ T cells show suppressive effects on APB $CD4^+$ T cells, but not UCB $CD4^+$ T cells, suggesting that target $CD4^+$ T cells from UCB are largely immature and therefore not as susceptible as target $CD4^+$ T cells from APB.

UCB $CD25^+$ cells are mainly naive [107]. Thus, a significant number of UCB Tregs remain as precursor cells and become capable of potent suppressor function after maturation [103]. It is likely that UCB $CD25^+$ $CD4^+$ T cells may require a certain type of stimulation to fully gain suppressive activity [108]. For example, UCB $CD4^+CD25^+$ cells function as Tregs after polyclonal stimulation [107]. IL-2 and IL-15 are known to increase expression of Foxp3, CTLA-4, GITR and membrane bound TGF-β, necessary elements for Treg, in UCB $CD4^+CD25^+$ T cells [106]. The number and activity of Treg cells in UCB have been

correlated with degrees of antigenic exposures of mothers during pregnancy [109]. Therefore, the inconsistency of UCB Treg activities reported may be rooted in the different isolation procedures used and degree of stimulation during Treg cell preparation.

3.4. UCB Tregs for clinical applications

Numerous studies from mice administered allogeneic HSCs demonstrated that donor or host Tregs are able to ameliorate GVHD [21,77,110-115]. In contrast, removal of Tregs from the donor allograft accelerated GVHD in recipients [111,115-117]. Although accumulated evidence suggests that donor Treg cell infusion is beneficial for allogeneic transplantation in mouse models, minimal data are reported on the function of UCB Tregs transplanted in humans. Further studies are needed to validate putative roles of UCB Tregs in preventing GVHD in humans. In fact, clinical trials of donor Treg infusions in humans have lagged behind mice mainly due to the difficulty in isolating definitive human Treg populations. The main difficulty in preparing adequate numbers of Tregs with high quality stems from the fact that unlike mouse Tregs of which separation is readily achievable, human Tregs are not clearly defined by surface expression of CD25. Although Foxp3 is the most reliable marker for Tregs, isolation of live $Foxp3^+$ population is not feasible, because intracellular staining of $FoxP3^+$ cells requires permeabilization of T cells, which is incompatible with their viability. Thus, establishment of a protocol to reliably and consistently identify and isolate human Tregs is necessary to fully exploit its therapeutic potential for controlling GVHD after UCB-HSCT. Tregs need to be at high frequency and present in the right microenvironment to exert sufficient suppressive function-dosing and localization. This requirement may render Treg therapy challenging. The suppressive effects of Tregs may be temporary and may be overridden by certain proinflammatory conditions and activating cell signaling [118]. Further research efforts are needed to ensure that infused Tregs retain immune suppressive activity and the capacity to home to local inflammatory sites and secondary lymphoid organs where GVHD is initiated. Since IL-2 is required for Treg cell function, paradoxically, immunosuppressive reagents such as steroids and calcineurin inhibitors, which are intended to block IL-2 production by preventing alogeneic T cell immune responses should be avoided when Tregs are infused to patients to suppress GVHD [119].

3.5. *Ex vivo* expansion of UCB Tregs

Tregs can be purified from UCB with a less cumbersome approach than from APB, because $CD25^+$ $CD4^+$ T cells in UCB do not contain activated or memory T cell populations [97]. Treg cells have been purified from frozen UCB units by positive selection using directly conjugated anti-CD25 magnetic microbeads [120]. Although $CD25^{bright}$ Tregs are more readily purified from UCB units than APB [103,121], only ~3–7.5 × 10^6 Tregs can be isolated from a single UCB unit [69,103]. The numbers of Tregs may not be enough for the patients who need multiple infusions for adoptive immunotherapy in GVHD prevention [69]. Therefore, many expansion procedures have been explored. Treg cells are cultured with beads coated with anti-CD3/28 monoclonal antibody (mAb) and artificial antigen-presenting cells (aAPCs) preloaded with anti-CD3/28 mAbs plus exogenous IL-2 and rapamycin for 18 to 21 days and cell cultures split every 2 to 3 days [120]. Tregs are successfully expanded approximately by ~200- to 1000-fold in <3 weeks under the conditions. These expanded $CD4^+CD25^+$ cells express Foxp3 mRNA at a level about 100-fold higher than that in isolated $CD25^-$ cells and can suppress allogeneic mixed lymphocyte culture by >80% (effector cells: $CD4^+CD25^+$ cells = 2:1) [122].

Costimulation promotes Treg cell proliferation as seen in conventional T cells. When additional costimulation with OX40 or 4-1BB, which belong to the tumor necrosis factor receptor family, is added to that with CD28, UCB Tregs expand to a significantly greater

extent, reaching mean expansion levels exceeding 1250-fold. Expanded UCB Treg cells (routinely ≥50% $CD4^+25^{++}Foxp3^+$) preserve suppressive potency and increase TGF-β secretion, as tested in a xenogeneic mouse GVHD lethality model [120]. IL-2 is important for expanding Treg cells [103,121]. As Tregs do not produce sufficient amounts of IL-2, exogenous IL-2 is critical for their expansion [123]. IL-15, whose receptor shares IL-2 receptor β and γ chains, is comparable or superior to IL-2 for optimal expansion of CD3/CD28-stimulated UCB Tregs [106], as well as APB derived Treg cells [124]. A combination of IL-15 and IL-2 enhances survival of Treg cells [123]. mTOR negatively regulates development of Treg [88]. Rapamycin, an mTOR inhibitor enhances Treg cell expansion and potency, while suppressing outgrowth of contaminating activated or memory T cells [125]. Improved techniques to expand Tregs *ex vivo* in high quality without losing their regulatory function are yet to be established for clinical applications.

3.6. *In vitro* development of Treg cells from UCB $CD34^+$ cells

$CD4^+CD25^+$ $CD127^{lo}FoxP3^+$ Treg cells can be developed *in vitro* from UCB $CD34^+$ cells by co-culture on Notch ligand, Delta-like 1 (DL1) expressing OP9 stromal cells [126]. These $CD34^+$ cell-derived Tregs are comparable in suppressive activity with natural Tregs existing in UCB, as tested *in vitro*. If this culture system can generate functional Tregs in sufficient numbers, UCB $CD34^+$ cell-derived Tregs may provide an alternative means to prepare Tregs for cell-based therapy to induce transplantation tolerance and treatment of GVHD. Recently, it has been reported that Tregs can be derived from human embryonic stem cells *in vitro* [127]. However, generation of Treg cells from UCB $CD34^+$ cells may be more efficient and less controversial for future clinical use than those from human embryonic stem cells.

4. Mesenchymal stem cells (MSCs)

Mesenchymal stem cells, also called mesenchymal stroma cells, were identified as undifferentiated stromal cells [128-129] originally from BM, and then from various other tissue sources [130]. MSCs can differentiate into several types of cells, including osteocytes, chondrocytes, adipocytes, cardiomyocytes and neurons [128-129,131]. They have been suggested as potential sources of cells for tissue engineering and cell-based therapy [128,132-134]. MSCs are able to self-renew with high proliferative capacity [135]. MSCs contain heterogeneous cell populations with multiple phenotypes [134,136], and thus, identity and functional complexity of MSCs *in vivo* remain to be better evaluated.

4.1. MSCs as immunomodulator cells

Importantly, MSCs are considered to have low immunogenicity and an ability to suppress immune responses [60,137-138], primarily suppressing T cell proliferation and cytokine production [139-147]. Because of their immune regulatory activity, MSCs are implicated in roles for controlling GVHD [148]. MSCs have been shown to exhibit immunosuppressive activity [60,138], MSCs do not express MHC class II molecules or costimulatory molecules (B7 and CD40) required for T cell activation [149]. Thus, MSCs do not initiate allogeneic responses *in vitro* [149], and may not be rapidly rejected *in vivo*. It is however unclear how long transplanted MSCs can be maintained in vivo and exactly where they home and reside.

Although MSC are able to prevent expansion of allogeneic T cells [144,149], exact mechanisms of MSC-mediated immunosuppression are largely unknown. Various immune suppressive factors such as IL-10 [150], TGF-β [151], nitric oxide [146], indoleamine 2,3-dioxygenase (IDO) [152], and prostaglandin (PG) E2 [144,153] have been suggested to be involved in MSC-mediated immunosuppression. In vitro addition of TNF-α, a proinflammatory cytokine, is sufficient to reverse the immunosuppressive effect of MSCs on T cell proliferation [154]. Interestingly, IFN-γ and inducible nitric oxide synthase (iNOS)

have been shown to be involved in MSC-mediated prevention of GVHD in mice, suggesting that proinflammatory cytokines are required to induce immunosuppression by MSCs [138]. In mice, BM MSCs inhibit proliferation of T and B lymphocytes via engagement of the inhibitory molecule PD-1 [143]. In addition to the suppressive effect on T cell function, MSCs induce *de novo* generation of antigen-specific $CD4^+CD25^+Foxp3^+$ Treg cells with the capacity to suppress effector T cell responses [155]. However, the clinical potential of these immunosuppressive properties is still a matter of speculation, because most data has been obtained from *in vitro* studies. If MSCs are immunosuppressive in controlled *in vivo* settings, then transplantation of MSCs together with allogeneic HSCs might facilitate acceptance of stem cell-based therapies, including possibly ES cell derivatives [60].

4.2. Umbilical cord (UC) and UCB MSCs

MSCs have been isolated from UCB [137,156-159], but their numbers are low and there is great variability in numbers of these cells in different UCB collections [160-163]. UCB-MSCs do not express costimulatory molecules, CD40, CD80, CD86 and MHC class II molecules commonly expressed on antigen presenting cells [137,164]. Parameters involved in MSC purification, including time from collection to isolation (less than 15 hours), a net volume (more than 33 ml), and MNC count (more than 1×10^8 MNCs) seem important for isolating MSCs from UCB [159]. More recently, instead of UCB, umbilical cord (UC) has been recognized as a source of MSCs [135,160,163,165-169]. In situ analysis showed that cells expressing mesenchymal markers CD44, CD105, CD73, and CD90 were enriched in UC. Jelly-like matrix and surrounding connective tissue, including vessels called Wharton's Jelly (WJ), in UC are tissue sources to obtain high numbers of MSCs [135,168-170]. Secco et al.[160] reported that they were able to isolate large amounts of multipotent MSCs from all UC samples, whereas MSCs were obtained from only 1 of the 10 UCB samples. MSCs isolated from WJ (UC-MSC) express MSC markers, including matrix receptors (CD44, CD105) and integrin markers (CD29, CD51), but not hematopoietic lineage markers (CD34, CD45) [164]. Unlike traditional MSCs derived from adult BM, small populations of UC-MSCs express endoglin (CD105) and CD49e. UC-MSCs are negative for CD14, CD33, CD56, CD31, CD34, CD45, and HLA-DR [170-171].

Perivascular niches are major sources of MSCs in several organs [168,172-173]. MSC-like cells have been obtained from the vasculature of the UC, called human umbilical cord perivascular cells (HUCPVCs) [174]. HUCPVCs have the same capacity as other MSCs to differentiate into an osteogenic phenotype with high proliferative potential. HUCPVCs have been considered as possible extra-embryonic MSC sources for cell-based therapies [175].

MSCs isolated either from UCB or UC are similarly multipotent for differentiation into various cell types and can undergo long-term expansion in culture [164,169,175]. However, UC- and UCB-MSCs appear to differ in gene expression profiles [167]. Genes related to cell adhesion, morphogenesis, secretion, angiogenesis and neurogenesis are expressed more dominantly in UC-MSCs, whereas genes related to osteogenesis and the immune system are preferentially expressed in UCB-MSCs [167]. These tissue-specific MSC gene expression patterns may reflect functional activities influenced by distinct niches. These findings highlight the consideration that banking UC as well as UCB may be of future clinical utility.

4.3. UC(B)- versus BM-MSC

UC-MSCs are different from BM MSCs in cytokine expression profile [171,176]. UC-MSCs secrete significantly higher levels of G-CSF, GM-CSF, HGF, LIF, IL-1α, IL-6, IL-8, and IL-11 compared with BM-MSCs, whereas BM-MSCs produce more VEGF and SDF-1β than UC-MSCs [177]. UC-MSCs act to significantly enhance numbers of granulocyte macrophage (CFU-GM), but not erythroid (BFU-E) or multi-potential (CFU-GEMM)

progenitor cells from UCB $CD34^+$ cells [177]. This UCB-MSC effect in relation to BM-MSCs has not been addressed. Recently, pluripotency of MSC was further extended by reprogramming into embryonic stem cell-like, induced pluripotent stem cells (iPS) [178]. Also, iPS cells have been generated from immature subpopulations of UCB, but the potential of these cells for clinical applicability is not clear [179].

MSCs derived from UCB and BM exhibit similar multilineage progenitor potential and cytokine expression profiles [180-182] and differentiate equally well to osteocytes and adipocytes [130,159,183-184]. However, there are studies indicating that UC-MSCs are more committed to angiogenesis, whereas BM-MSCs are more committed to osteogenesis [176]. Also, although MSCs derived from all three sources are morphologically and immunophenotypically similar, some studies suggest that UCB-derived MSCs produce less adipogenic differentiation with fewer and smaller lipid vacuoles, in contrast to those of BM- and adipose tissue (AT)-derived MSCs [185]. It was shown that although there are no obvious significant differences concerning morphology and immune phenotype of the MSCs derived from UCB, BM and AT, the success rate for isolating MSCs from UCB was low due to the low colony frequency compared to cells from BM and AT [130]. However, MSCs isolated from UCB have higher self-renewal capacity than those from BM [130,156]. UCB-MSC can be cultured for longer with a higher proliferation capacity compared with MSC derived from BM and AT [130,156-158,185-186]. This may reflect a superior “stemness” of UCB-MSC as newborn cells [187-190]. Of note, differentiation potential and maximum life span of human BM-derived MSC significantly decline with age of donor [191-193].

HUCPVCs are capable of osteogenic, chondrogenic, and adipogenic differentiation and show a higher proliferative potential than BM MSCs. Like UC-MSCs [176], HUCPVCs proceed to osteogenic differentiation more rapidly than BM MSCs [175]. HUCPVCs show a higher rate of growth relative to BM MSCs. BM MSCs tend to experience contact-inhibited growth, whereas HUCPVCs continue to grow in a multilayer form [175].

4.4. Preclinical possibilities of UCB-MSCs

MSCs have been suggested as potential clinically useful cells to prevent or treat autoimmune diseases, such as experimental autoimmune encephalomyelitis [194], and collagen-induced arthritis [154]. An important aspects of MSCs for clinical application is low immunogenicity and immunosuppressive activity [137]. The immunosuppressive property of MSCs is also attractive tool for modulating GVHD and HSC engraftment [140,195-196]. Most information on beneficial effects of UCB-MSCs on GVHD is available from mouse models. The ability of UCB-MSC to prevent and treat GVHD has been tested using sublethally irradiatiated non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice which were injected with human peripheral blood mononuclear cells (PBMCs) [148,197]. These mice exhibited pathologic signs of GVHD such as massive infiltrates of human T cells in mesenteric lymph node, liver, lung and peritoneal lavage [148]. Multiple systemic administration of UCB-MSCs to this NOD/SCID mouse model significantly reduced human T cell proliferation and tissue damage, improving survival of the animals [148]. No therapeutic effect was observed when UCB-MSCs were administered at the onset of GVHD, suggesting the clinical use of MSCs in HSCT as a prophylaxic treatment [148]. Multiple administration of UCB-MSC administration were required to prevent GVHD, suggesting that effects of MSCs may be transient. Rats that were not immune-suppressed have been used to test human UC-MSCs for therapeutic value. Transplantation of UC-MSCs into brains of hemiparkinsonian rats ameliorated the pathological symptoms without host immune rejection response [170]. This opens a possibility, far from proven, that UC-MSCs could be a cell source to treat neurodegenerative diseases.

Discordant reports have been reported on the efficacy of MSCs to treat GVHD in mouse models. Sudres *et al.* [198] demonstrated that infusion of MSCs was not sufficient to prevent the development of GVHD in mice, with infused MSCs being rapidly eliminated by an immune response from the recipient [199-200]. Chung *et al.* [201] showed that MSCs could prevent GVHD only when given to irradiated recipient mice. There have been numerous reports on preclinical human trials using MSCs isolated from BM to treat GVHD [202-204], but controversial results from preclinical models remain to be clarified. Thus far, the efficacy of MSCs on GVHD in preclinical settings, while encouraging, are not without controversy. Besides immune regulatory activity, *in vivo* administration of MSCs was found to be effective for promoting the engraftment of $CD34^+$ hematopoietic stem/progenitor and embryonic stem (ES) cells [196,205].

5. Natural killer (NK) cells as a regulatory cell in UCB

Recently, the impact of donor NK cells on autologous donor alloreactive T cells in GVHD induction has been investigated in a mouse model [206]. Animals receiving donor NK cells in addition to T cells showed improved survival and decreased GVHD compared to controls receiving donor T cells alone. NK cells exert a direct cytotoxic effect on autoantigen-specific, encephalitogenic T cells [207]. In another mouse model, NK cells recognize and kill syngeneic $CD4^+$ and $CD8^+$ T cells activated by APCs. Lysis required perforin and NKG2D in the NK cells [208]. These studies suggest that NK cells may play regulatory roles for down-modulating alloreactive T cells in GVHD by reducing donor T cell proliferation and increasing T cell apoptosis.

Human natural killer (NK) cells are defined as being membrane $CD3^-$, $CD16^+$, and/or $CD56^+$lymphocytes. Based on the surface expression of CD56 and CD16, NK cells can be subdivided into four subsets on the basis of CD56 and CD16 expression with distinct functions which are associated with expression of NKG2A, NKG2D, CD95 and the intracellular granzyme B and perforin. It was shown that the $CD56^-CD16^+$ subset is particularly abundant in UCB compared to APB and BM [209]. UCB $CD56^-CD16^+$ NK cells express equivalent levels of perforin and granzyme B to $CD56^+CD16^+$ NK cells [209-212]. Among different NK subsets, expression of NKG2A, an inhibitory receptor, is lower on $CD56^-CD16^+$ subset than the other NK cell subsets, while expression NKG2D, an activating receptor, is indistinguishably high on all the NK subsets [210]. $CD56^-CD16^+$ NKs have also been identified after allo-HCT using UCB, but not after BM or PB transplants [213]. $CD56^-CD16^+$ NKs are found at higher frequencies in immune compromised hosts, including those with chronic viral infections [214-216]. Culture of the UCB-derived $CD56^-CD16^+$ NK cells with IL-2 and/or IL-15 resulted in CD56 acquisition, possibly suggesting that $CD56^-CD16^+$ NK cells may be NK progenitors. These results suggest that UCB $CD56^+CD16^+$ NK cells are functionally immature but have potential for cell lysis. Whether alloreactive T cells are particularly susceptible to the $CD56^-CD16^+$ NK cell-mediated lysis remains unclear.

6. Summary

In summary, UCB is an alternative source of primitive HSC and progenitor cells to BM or G-CSF-mobilized APB stem cells. UCBT results in less incidence of GVHD compared to BM stem cell transplantation. Although reduced GVHD in patients receiving unrelated donor UCB transplantation is generally thought to be attributed to greater immune tolerance to HLA mismatch, the exact mechanisms remain unclear. In this review, we described the nature of immune regulatory cells in UCB. Tregs and MSCs, may eventually be of use for adaptive cellular therapy to suppress alloreactivity for GVHD. In light of the tolerogeneic nature of Tregs and MSCs, how to effectively expand these immune regulatory cells in UCB

is a key issue for clinical usage of these cells to treat GVHD in the near future. Delayed immune reconstitution is problematic in UCBT, largely due to the limited number of HSC infused, lack of prior priming against pathogens, and relative bias away from a protective Th1/Tc1 cytokine profile that in combination may delay emergence of protective antiviral immunity. Double unit UCBT [217-220] and inhibition of CD26 [221-223] may be feasible approaches to increasing the efficiency of immune reconstitution in UCBT.

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