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Immune Regulatory Cell Biology and Clinical Applications to Prevent or Treat Acute Graft-Versus-Host Disease

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ABSTRACT

The most common approaches to prevent and treat graft-versus-host disease (GVHD) are intended to deplete or suppress the T cells capable of mediating or supporting alloresponses; however, this renders the recipients functionally T cell deficient and hence highly susceptible to infections and tumor recurrence. Depletion is often accomplished through the use of broadly reactive antibodies, while functional impairment is typically achieved by pharmacological agents that require long-term administration (usually six months or more), have significant side effects, and may not result in tolerance (i.e., non-responsiveness) of donor T cells to conditioning regimen-resistant host alloantigen-bearing cells. As our knowledge of immune system homeostasis has increased, cell populations with immune regulatory function have been identified and characterized. Although such cell populations are typically present in low frequencies, methods to isolate and expand these cells have permitted their supplementation to the donor graft or infusion late post-transplant in order to stifle GVHD. This review discusses the biology and preclinical proof of concept of GVHD models, along with GVHD outcomes that focus exclusively on immune regulatory cell therapies that have progressed to clinical testing.

1. Introduction

The first successful bone marrow transplant (BMT) to correct immune deficiency was reported in 1968 [1]. Today, more than 1 million patients have received hematopoietic stem cell transplants [2]. However, despite extensive preclinical research and clinical trials over the past five decades, graft-versus-host disease (GVHD) remains a leading cause of morbidity and mortality (~20%) after allogeneic hematopoietic stem cell transplant (HSCT), even taking into account improvements that have been made over the years in the frequency (20%–70% of allogeneic patients) and severity of acute GVHD [3].

2. GVHD biology, prevention, and therapy

2.1. GVHD etiopathogenesis

GVHD is an iatrogenic complication caused by the reaction of donor T cells to host target tissues, especially epithelial rich organs and those that are in direct contact with or scavenge foreign environmental antigens and pathogens. These predominantly include the skin, gut, liver, and lung. During acute GVHD, tissue infiltration and destruction by pathogenic cytolytic donor T cells occurs, most often but not always in the early (1–3 months) post-transplant time period [3]. Acute GVHD, which is known as secondary or runting disease in mice, was first reported by Barnes and Loutit in 1955 [4]. Acute GVHD generation, as has been eloquently stated by Billingham [5], has three principle requirements: ① The graft must contain immunologically competent cells; ② the recipient must express tissue antigens that are not present in the transplant donor; and ③ the recipient must be incapable of eliminating the transplanted cells.

2.2. Prevention of acute GVHD by donor graft T cell depletion

Mouse BMT studies have indicated that donor T cells are primarily responsible for acute GVHD [6]. This led to a series of trials beginning about 40 years ago using soybean lectins, sheep red blood cell (erythrocyte) rosettes, antibody and complement depletion, and antibody conjugated to toxins [6]. In aggregate, these studies demonstrated that *ex vivo* graft-depleting regimens achieving 2–4 $\log_{10}$ T cells significantly lowered acute GVHD rates.

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However, complications recognized in the 1980s included host anti-donor mediated graft rejection with a high mortality rate, infectious complications caused by the slow recovery of donor T cells in the periphery due to thymus and lymphoid organ injury, and—especially for myeloid leukemia—increased relapse rates [6]. Other techniques have included physical separation of T cells from the donor graft based on binding to lectins or reaction with cells expressing T cell ligands, or *ex vivo* exposure to T cell cytolytic drugs [6].

2.3. Pharmacological approaches to prevent acute GVHD

Methotrexate, a dihydrofolate antagonist, became a mainstay of acute GVHD prevention in the mid-1970s, and continues to be so today. *In vivo* anti-T cell antibodies (e.g., antithymocyte or antilymphocyte globulin; anti-cluster of differentiation (CD)52 monoclonal antibody (mAb)) and prednisone, given alone or in combination, have been popular for preventing acute GVHD. Beginning in the early 1980s, a calcineurin inhibitor, cyclosporine, entered the scene and has remained as an often-used preventive therapy [7]. FK506, another calcineurin inhibitor, yielded an outcome similar to that of cyclosporine in allogeneic HSCT patients [8]. More recently, the antiproliferative mycophenolate mofetil, when given in combination with calcineurin inhibitors (cyclosporine A; FK506, tacrolimus) or rapamycin (sirolimus), has become one of the preferred drug regimens [9]. Lastly, cyclophosphamide (Cytoxan) given in two doses in the first week post-allogeneic HSCT has substantially reduced the rate of severe acute and chronic GVHD in recipients of haploidentical T cell-replete grafts and other graft sources [10–13]. Although overall acute GVHD severity has been reduced by incorporating these combinatorial drug regimens, toxicities are frequently observed, and uniform efficacy has not been achieved.

2.4. Rationale for immune cell therapies for acute GVHD prevention

In the 1980s, studies using mixed donor and host sources of bone marrow (BM) in mice showed that the host marrow component suppressed the otherwise immune competent grafts from causing acute GVHD [14], and that elimination of the grafted host cells later, post-BMT, restored a GVHD response [15]. In other studies, donor anti-host alloreactive T cells were found to be suppressed by interleukin (IL)-10-producing CD4 T cells in recipients of haploidentical or fetal liver stem cell transplants [16]. These suppressor cells were subsequently identified as type 1 regulatory T (Tr1) cells [17]. Together, these data provide the foundational information that a lack of GVHD and tolerance induction in patients may not be dependent upon the deletion of donor anti-host alloreactive T cells, but may rather be an active, ongoing cellular immune regulatory process.

In addition, in some patients receiving human leukocyte antigen (HLA) mismatch or fractionated total lymphoid irradiation, regulatory cell populations (invariant natural killer T cells, iNKTs) were identified that could suppress donor anti-host alloreactive T cells, leading to acute GVHD prevention [18,19]. The implications of these studies are twofold: ① The persistence of donor anti-host alloreactive T cells makes it possible that inciting triggers (e.g., viral infection or ultraviolet light) may increase their frequency and result in acute GVHD; and ② cellular immune mechanisms are powerful and provide continuous protection against detrimental alloresponses without requiring global suppression or the depletion of donor T cells. Such cellular mechanisms of tolerance induction allow for the greater possibility of anti-tumor and anti-pathogen responses and for the avoidance of the frequent side effects that are seen with most drugs in this high-risk patient population.

3. Adaptive immune system regulatory cell products in the clinic

Although the rationale for cell therapies to prevent or treat GVHD has been based upon their immune regulatory properties, several of these products have the dual function of immune regulation and tissue repair. For example, adaptive immune system cells such as regulatory T cells (Tregs) that inhibit productive alloresponses also secrete a protein, amphiregulin, which is mitogenic for epidermal growth factor (EGF) receptor-expressing epithelial cells, and thus stimulates their repair from conditioning and GVHD-induced tissue injury, especially in the gut [20]. In contrast, Tr1 cells are believed to suppress GVHD by IL-10 and transforming growth factor beta (TGF-β) secretion, rather than by direct tissue repair [17]. Non-hematopoietic cells, such as mesenchymal stromal cells (MSCs), also have immune regulatory and tissue-reparative properties [21].

3.1. Thymus-derived regulatory T cells

One of the most significant discoveries in the field of immunology in the last 25 years has been the identification and characterization of specific $CD4^+$ T cell subsets that are critical for regulating immune responses [22]. Also known as natural Tregs, thymus-derived Tregs (tTregs) co-express CD4, CD25, and the master regulator, forkhead box P3 (FOXP3) transcription factor, which encodes scurfin, a protein belonging to the forkhead/winged-helix family [23]. These $CD4^+CD25^+$ Tregs are necessary in order to suppress the activation of self-reactive lymphocytes and autoimmunity [24], and limit the immune response to chronic pathogens and commensal bacteria in the gut [25]. Tregs are essential in maintaining immune homeostasis; the adoptive transfer of Tregs is able to restore immune homeostasis in rodent models in which tolerance to self-antigens has been broken and autoimmune disease occurs. These seminal studies have led to the testing of Tregs in murine models of transplantation tolerance.

Tregs regulate T cell responses to alloantigens, and are critical for *ex vivo* tolerance induction [26]. Mechanisms by which Tregs may attenuate GVHD include the release of regenerative cytokines (e.g., amphiregulin) [20], antigen-presenting cell (APC) function inhibition (e.g., via cytotoxic T-lymphocyte-associated protein 4 (CTLA4)), and the inhibition of T-conventional cells (Tcons) by the release of inhibitory molecules (e.g., adenosine, TGF-β, IL-35, and IL-10) [27] and/or by IL-2 consumption [28] and homeostasis [28]. Three reports appeared in 2002 showing that the infusion of *ex vivo* expanded, isolated, and infused Tregs could suppress GVHD in mice [29–31]. In two studies, freshly purified donor Tregs given at the time of BMT modestly inhibited GVHD when administered in equal numbers with T cells, while large numbers of Tregs could be obtained by *ex vivo* activation and expansion, which increased not only Treg numbers, but also suppressor function [29,31]. When administered in equal numbers with T cells, a significant inhibition of rapidly lethal GVHD was observed [29]. Alternatively, Treg activation and expansion could take place *in vivo* in allogeneic murine BMT recipients by Treg infusion after conditioning induced lymphopenia and several days prior to BMT [30].

Challenges in the isolation of extremely pure Tregs created a practical problem that precluded a more rapid clinical development of Treg cell therapy. A large and overlapping population of $CD25^{dim}$ effector/memory T cells exists in humans; thus, tTregs isolated from peripheral blood (PB) using CD4 and CD25 antibody conjugated immunomagnetic beads also contained $CD4^+CD25^+$ $FOXP3^-$ cells, and did not consistently maintain FOXP3 expression or suppressive function when expanded *in vitro* [32,33]. In contrast to magnetic-bead-purified PB Tregs, tTregs can be readily purified

from umbilical cord blood (UCB) due to the relative paucity of $CD25^{dim}$ non-Tregs in UCB, as a fetus is exposed to fewer environmental antigens than an adult [32,34]. Cells purified from UCB contained fewer $CD4^+CD25^{dim}$ cells and could be expanded *ex vivo* using anti-CD3/CD28 mAb-beads and IL-2, while maintaining FOXP3 expression and suppressive function [32,33].

In 2009, initial clinical studies reported that Tregs, which were isolated from family donors by flow cytometry sorting as $CD4^+CD25^+CD127^-$ and *ex vivo* expanded for 2–4 weeks, were given to a patient with severe, treatment refractory acute GVHD. The patient was stated to have had a transient and moderate improvement of his clinical condition, despite ultimately succumbing to GVHD [35]. Our clinical trial, which started in 2007, included 23 patients who received a double UCB transplant to provide allogeneic hematopoietic stem cells and mature T cells, as well as tTregs expanded from a third UCB unit [36] in doses from 1×10^5 to 3×10^6 kg^{-1}. This resulted in a ratio of 1 Treg to about 6 T cells, which was far below the optimal 1:1 ratio that is needed to protect mice against lethal GVHD. UCB tTregs from a separate unit were tracked *in vivo* in seven patients [36], and were detected in circulation for only about 14 d, with the highest frequency being observed on day 2 after UCB transplant. No infusional toxicity, the primary endpoint, was observed. The secondary endpoints suggested that the outcome results in patients with tTreg infusion(s) were superior to historical controls, as they reduced the incidence of steroid-requiring acute GVHD (43% versus 61%, $P = 0.05$) compared with 108 historical controls that were treated identically except without supplemental Tregs. There was no increased risk of infection, relapse, or early mortality [36].

In subsequent studies, changes in tTreg expansion, which included the time of re-stimulation of the cultures and the use of KT64/86-expanded UCB Tregs instead of anti-CD3/CD28 bead-based artificial APCs, increased the yields dramatically to a mean of greater than 13 000-fold expansion, versus 200–400-fold expansion with beads [37]. In a trial of 12 double UCB transplant patients who received a rapamycin-containing GVHD preventive regimen and a single supplemental dose of tTregs, where the doses ranged from 3×10^6 to 1×10^8 kg^{-1}, there was a significant reduction in acute GVHD. Only one patient had possible acute GVHD (the biopsy was inconclusive and the patient was treated for only three weeks), in contrast to the 48% incidence in 19 contemporary controls who received the same conditioning and GVHD prophylaxis regimen without tTregs.

Using fresh, bead-purified tTregs, investigators in Italy [38] assessed the effect of adding tTreg pre-transplant on GVHD prevention and immunologic reconstitution in allogeneic HSCT recipients. The tTregs were infused into patients 3 d prior to HLA-haploidentical $CD34^+$ cells supplemented with frozen/thawed mature donor T cells in the absence of any post-transplant immunosuppression. Purification was very consistent, and only two of the 28 patients enrolled in the study did not receive tTregs due to low purity ($\geq$ 50% $FOXP3^+$). These studies confirmed the safety of *ex vivo* purified tTregs, and found that they promoted lymphoid reconstitution and did not overtly weaken the graft-versus-leukemia effect of the co-transferred mature T cells [38]. Indeed, in a follow-up study in myelodysplastic and acute myeloid leukemia patients, the relapse rates were significantly lower, likely due to the absence of GVHD- and treatment-induced immune suppression. While no GVHD was observed for doses of 5×10^5 or 1×10^6 T cells·kg^{-1} plus 2×10^6 tTregs·kg^{-1}, two of the five patients receiving 2×10^6 T cells·kg^{-1} plus 4×10^6 tTregs·kg^{-1} developed GVHD. This finding indicates that 1×10^6 T cells·kg^{-1} is the maximum dose, unless increased numbers of Tregs are given [39].

Clinical testing is likely to begin in the relatively near future on new approaches to better target IL-2 to Tregs using mutated IL-2 receptor beta chains expressing Tregs and mutated IL-2 protein [40], or IL-2/anti-IL-2 complexes that preferentially bind the IL-2 receptor beta chain [41] and hence stimulate the high-affinity IL-2 receptor complex expressed on Tregs. A second approach, involving tumor necrosis factor (TNF) receptor-2 agonists, has been shown to expand recipient rather than donor Tregs in order to attenuate acute GVHD [42]. An approach involving inhibition of cytokines that subvert Treg differentiation [43] (e.g., IL-6) is currently in clinical trials [44]. Although future randomized trials are required to assess tTreg efficacy and the effects on anti-pathogen and anti-tumor responses, these studies in aggregate hold promise for the future.

3.2. Induced regulatory T cells

Induced Tregs (iTregs) are named as such because they exit the thymus as naïve T cells; FOXP3 expression and suppressive function are then induced in the periphery. iTregs are required not only for peripheral tolerance, but also to prevent lymphoproliferative disease [25]. Two types of iTregs exist. One type of iTregs is $CD8^+$ and HLA class I-restricted [45], and does not express FOXP3 at the steady state but can do so after stimulation *in vivo* [46,47] or *in vitro* in the presence of IL-2 and TGF-β [45]. Although these CD8 iTregs can suppress effector T cell (Teff) responses *in vitro*, they are inherently unstable and can revert to Teffs to exacerbate murine GVHD [45]. However, since these iTregs are not yet in the clinic, they will not be further discussed here. The second type comprises Tregs that can be induced and expanded *in vitro* by stimulating $CD4^+CD25^-$ T cells in the presence of TGF-β or all-trans retinoic acid (ATRA); like tTregs, the adoptive transfer of these iTregs suppresses disease [48,49]. TGF-β or ATRA also induce FOXP3 expression after the stimulation of naïve human T cells; however, while one study showed these cells to be suppressive [50], other studies have observed modest or no suppression [51–53], although $CD4^+CD25^-CD45RA^+$ T cells stimulated in the presence of TGF-β plus ATRA acquired stable suppressive function [54]. Similarly, rapamycin enhanced TGF-β-dependent FOXP3 expression, induced potent suppressor function in naïve T cells [55], and induced suppressive function in unfractionated T cells, which is therapeutically advantageous because it increases yield and decreases cost. Rapamycin/TGF-β iTregs express CD25 at levels that are equal to or higher than expanded tTregs; furthermore, they contain few IL-2, interferon gamma (IFNγ), or IL-17 secreting cells, and suppress disease in a xenogeneic model of GVHD, in a manner that is comparable to that of tTregs [55].

Due to the higher abundance of the starting $CD4^+CD25^-$ T cell population in a non-mobilized PB apheresis unit, and the fact that PB contains far more cells than UCB, iTregs should permit the infusion of large numbers of Tregs that would achieve the desired Treg:T cell ratios of 1:1 or higher, which are especially useful for recipients of PB stem cell (PBSC) transplants that contain high non-Treg T cell numbers [56]. Large-scale experiments have shown that about 2.2×10^{11} iTregs can be generated from a single apheresis product, which is more than 50 times more than available in initial tTreg clinical trials. Similar to how tTreg expands in the presence of rapamycin, less than 4% of the cells in rapamycin/TGF-β iTreg cultures secrete IFNγ or IL-17. A concern in the field has been whether tTregs—and iTregs in particular—can become unstable and be reprogrammed to become Teffs with loss of suppressor function [57]. In some disease models, tTregs can produce pathogenic Teff cytokines such as IFNγ or IL-17, and have a methylated Treg-specific demethylation region associated with tTreg but not with iTreg or with peripheral Treg suppressor function [58–60]. Instability likely requires a highly inflammatory local environment, Treg persistence that is sufficiently long and in the right environment to become unstable, and strong indications that the effector

cytokines that are produced are pathogenic and that suppressor function is reduced or lost, which is not a uniform finding [61]. For iTregs generated from $CD4^+CD25^-$ T cells and expanded to high numbers *in vitro*, no evidence for conversion into Teff has been found, despite the postulated higher likelihood compared with tTregs due to methylation of the Treg-specific demethylation region in the xenogeneic GVHD model throughout the 82-day assay [56]. With this efficacy data in hand, we recently completed and are currently analyzing an iTreg phase I dose escalation (3×10^8–1×10^9 kg^{-1}) study in 14 non-myeloablated recipients of matched sibling donor granulocyte-colony stimulating factor (G-CSF) mobilized PBSCs who received iTregs and mycophenolate mofetil plus cyclosporine prophylaxis (the standard of care for GVHD prevention at our institution for this patient population).[†]

3.3. Type 1 regulatory T cells

Tr1 cells arise in the periphery and do not require FOXP3 expression for suppression. Phenotypically, Tr1 cells have now been characterized as co-expressing integrin alpha-2 (CD49b) and lymphocyte-activating gene-3 (LAG-3) [62]. Tr1 cells produce IL-10, TGF-β, and IFNγ, and suppression has been shown to be conferred by IL-10 and TGF-β secretion; this secretion is highest in $CD49b^+LAG\text{-}3^+$ cells, which also are the most suppressive [63]. Tr1 cells are triggered via engagement of the antigen-specific T cell receptor, and can be generated in response to potent alloantigen stimulation by recipient dendritic cells (DCs) in the presence of IL-27, which is secreted mostly from donor monocytes/macrophages in mice [64]. The stable persistence of Tr1 cells is dependent on the transcription factor Eomesodermin [64]. Mechanistically, Tr1 cells can directly suppress T helper (Th)17 and Th1 effector cells in an antigen-specific fashion as well as via contact-dependent processes, CD8 T cell proliferation, and IFNγ production. Tr1 cells also can indirectly suppress Teffs by modulating or killing APCs (DCs or macrophages) that are key in priming GVHD-causing T cell response. Alternatively, Tr1 cells can render DCs tolerogenic and skew macrophages toward anti-inflammatory M2 macrophages that themselves support *in vivo* Tr1 cell and peripheral Treg generation [63].

In acute GVHD, donor tTregs are profoundly deficient, and Tr1 cells are the dominant Treg population post-transplant in mice [64]. Moreover, Tr1 deficiency exacerbates GVHD [64]. These data and the findings described in Section 2.4 demonstrate the capacity of Tr1 cells to suppress donor anti-host alloreactive T cells in patients. A clinical trial of Tr1 cell infusion was performed in high-risk malignancy patients who were given haploidentical transplants of a high median CD34-enriched graft dose with a low number of supplemental T cells (10^4 kg^{-1}) isolated from a family donor G-CSF mobilized apheresis product [65]. To generate Tr1 cells, recipient monocyte-derived DCs obtained from a non-mobilized apheresis unit were treated with exogenous IL-10 in order to induce DCs that produce IL-10, which were then co-cultured with donor PB mononuclear cells for 10 d [65]. At that time, phenotypically about 1/7 of the culture is $CD49b^+LAG^+$ and is effective in suppressing donor anti-host but not anti-third-party T cell responses *in vitro*. Tr1-containing cultured cells were given as fresh or frozen products in escalating doses from 1×10^5 to 3×10^6 kg^{-1} in a semi-log fashion not earlier than 1 month post-transplant and only after neutrophil engraftment. Of the 19 patients enrolled, 17 received an allogeneic HSCT and 12 received the Tr1-containing product. Of the 11 patients receiving 1×10^5 kg^{-1}, four had relapse or graft rejection, three were not immune reconstituted and succumbed to infections, and four were immune reconstituted and were alive and well at the time of publication. The single patient who received 3×10^5 kg^{-1} was immune reconstituted but developed severe GVHD. The five patients in total who were immune reconstituted had T cells that were proliferative but poorly responsive to host alloantigen-bearing stimulators. An ongoing trial is testing the safety and tolerability of this approach to prevent GVHD in adult and pediatric patients receiving mismatched related or mismatched unrelated unmanipulated donor HSCT for hematological malignancies.

4. Non-hematopoietic system immune regulatory cell products in the clinic

The first cellular product that was successfully used to treat severe acute GVHD in the clinic was MSCs. Multi-potent adult progenitor cells (MAPCs) are distinct from MSCs, but have commonality with them and have higher proliferation capacity [66]. Both are adherent BM-derived progenitor cells with stromal cell features that fall within the consensus definition of MSCs and that possess immune modulatory and tissue repair properties. However, MAPCs have a wider range of differentiation potential encompassing all three germ cell layers [66].

4.1. Mesenchymal stromal cells

Under appropriate inductive conditions, MSCs can differentiate into mesenchymal lineage cells including chondrocytes, osteoblasts, and adipocytes [67,68]. The consensus definition includes their adherence, their capacity to differentiate into several mesenchymal lineages (e.g., bone, cartilage, muscle, adipocytes, tendon, and stroma), and their phenotyping ($CD105^+$, $CD166^+$, $CD73^+$, $CD90^+$, and $CD29^+$ and, without expression of hematopoietic antigens, CD34, CD45, and CD14) [69]. MSCs reside in a differentiated state in most tissues, albeit at low frequency (about 1:10 000 cells in BM) [70]. Their widespread distribution (e.g., BM, fat, fetal tissues) and rapid proliferation suggest that these cells can be called upon to protect the tissue, organ, or organism from injury.

MSCs are immunosuppressive, rather than immunostimulatory, and therefore may function to prevent rather than support an overly aggressive immune response that might be detrimental to the organism. For example, MSCs express low levels of class I and low/absent major histocompatibility complex (MHC) class II and costimulatory molecules. Multiple mechanisms have been proposed to suppress adaptive and innate immune responses, and have been summarized in a recent review [21]. These include upregulation of the inhibitory enzyme indoleamine 2,3-dioxygenase (IDO), which metabolizes the essential amino acid tryptophan; upregulation of nitric oxide, which suppresses T, B, and NK cell function; and the elaboration and secretion of inhibitory molecules including TGF-β, soluble HLA-G, and prostaglandin E2 (PGE2) [71], which also participate in supporting Treg generation, and which inhibit Th17 generation with IL-10 [72–74]. IL-6 works in conjunction with PGE2 to generate anti-inflammatory M2 macrophages [75] and inhibit DC maturation [76,77]. MSCs express co-inhibitory molecules such as the IFNγ-inducible programmed cell death ligand-1 (PD-L1), and also produce soluble PD-L1 and PD-L2 [78,79]. MSCs limit Teff migration into target tissues by downregulating chemokines and chemokine receptor expression on T cells and monocytes/macrophages [80].

MSC exosomes, which are small (50–200 nm) in size and are derived from endosomes, contain cytokines, growth factors, signaling lipids, messenger RNAs (mRNAs), and regulatory microRNAs that can influence cell signaling, communication, and metabolism [81]. MSC exosomes exert immunosuppressive effects on T, B,

[†] See clinicaltrials.gov study NCT01634217.

and NK cells through their CD73, which has been shown to suppress human/mouse xenogeneic GVHD by increasing $CD39^+$ Th1 cell apoptosis, PD-L1, and IL-10 mRNA, among other inhibitory molecules [82–85]. Investigators have also observed that donor $CD8^+$ cytotoxic T cells induce apoptotic MSCs, the frequency of which correlates with acute GVHD response [86]. The result is recipient phagocytic engulfment of apoptotic MSCs, IDO production, and host immune suppression. Together, such data may explain how GVHD amelioration can take place, even though it is challenging to find MSCs in the tissues. MSCs can contribute to tissue repair through regeneration, remodeling, and angiogenesis via connective tissue growth factor, vascular endothelial growth factor (VEGF)-α, keratinocyte growth factor, angiopoietin-1, and stromal derived factor-1 [87–89].

Le Blanc et al. [90] were the first to administer haploidentical MSCs to a pediatric patient with severe steroid-refractory acute GVHD, which resulted in a rapid and significant decrease in GVHD symptoms. This finding led to a plethora of clinical studies and reports using autologous, haploidentical, or third-party HLA mismatched MSCs for steroid-refractory acute GVHD [91–98]. The results have been mixed. For example, in a large collaborative European phase II study, in which 49 adult and pediatric patients received one or two doses and six received 3–5 doses as adjunctive therapy, an overall response rate of 70.9% was reported, including complete responses and the disappearance of all symptoms in 54.5% [93]. The Prochymal made by Osiris Therapeutics, Inc. has been approved for GVHD therapy in pediatric patients [99] in the United States, Canada, and New Zealand. In addition, a phase III trial treating children with steroid-refractory acute GVHD with MSCs (100 million cells per dose) met the primary day 28 endpoint of overall response that was higher than the historical controls.[†] However, a different phase III randomized double-blind study[‡] using the Prochymal product showed no significant difference in clinical outcomes between the control and allogeneic MSC groups. An ongoing double-blind placebo-controlled multi-center phase III trial is ongoing in Europe on the use of MSCs for the treatment of steroid-refractory acute GVHD. A recent meta-analysis of MSC treatment studies favored MSCs for overall survival in patients with steroid-refractory acute GVHD [100].

Finally, it is notable that in the BM itself, the stromal cell features of MSCs suggest a role in supporting hematopoiesis, as confirmed by the co-transplantation of MSCs, which can enhance the engraftment of human cord blood hematopoietic cells in immunodeficient mice [101]. These findings have led to several clinical trials testing the *in vivo* capacity of MSCs in speeding hematopoietic recovery. Depending on the context, graft rejection has appeared to be reduced and hematopoietic or lymphocyte recovery has been augmented in settings of parental haploidentical $CD34^+$ PB stem cell grafts [101]. Such benefits were not seen in one study, which involved parental haploidentical MSCs in the recipients of UCB, although severe acute GVHD was significantly reduced [102]; however, in another study, the median time to neutrophil recovery appeared to be faster [103]. Both studies had small numbers of patients (13 and 8, respectively). The contrasting results have been ascribed to differences in the mechanisms of graft rejection.

4.2. Multi-potent adult progenitor cells

MAPCs are CD45 negative cells isolated from adult sources and cultured in low serum and supplemental growth factors (e.g., EGF, platelet-derived growth factor (PDGF), leukemia inhibitory factor (LIF)). Their reported phenotype is negative for CD34 and c-kit, low/absent for MHC classes I and II, and positive for Oct4 and Rex1. MAPCs are non-immunogenic expanded BM-derived adult stem cells with immunomodulatory, immunosuppressive, and tissue-regenerative capacity that exhibit a broader differentiation capacity than MSCs, including mesenchymal, endothelial, and endodermal lineages [104,105], and that have a greater expansion potential. Thus, they permit the development of large-scale off-the-shelf products from a single donor, which reduces product variability [106,107]. MAPCs are able to suppress allogeneic T cell response contact-independent mechanisms through PGE2 and IDO-mediated suppression of proliferation and pathogenic cytokine-producing cells [75,108,109] and the production of IL-10 or TGF-β, leading to Treg generation [75,108]. In a phase I dose escalation study (1×10^6–1×10^7 cells·kg^{-1} for 1, 3, or 5 doses) using MultiStem, a commercial MAPC product (Athersys, Inc.), feasibility and safety were established. Furthermore, encouraging GVHD outcomes were reported, with 37% for grades II–IV GVHD (n = 36), 14% for severe GVHD, and even lower rates for the highest doses (11% and 0, respectively; n = 9) [110].

5. Concluding statements

Less than 15 years have passed since the first-reported cell therapy—non-hematopoietic cell product MSCs for treating steroid-refractory acute GVHD [90]. At the present time, three distinct Treg products (tTregs, iTregs, and Tr1 cells) have completed phase I studies, along with a second non-hematopoietic cell product, MAPCs. Collectively, these trials highlight the possibility of using the body's natural immune regulatory mechanisms to provide a source of cells that can be isolated, expanded, and differentiated as needed. Intriguing similarities between these varied products are the low frequency (for iTregs, we consider peripheral Tregs as the *in vivo* counterpart) and the dual function of immune suppression/regulation and tissue repair. Moreover, after infusion, there have been challenges in detecting long-term persistence within the blood in GVHD patients.

Using Tregs for the purpose of illustration, current limitations to broader applications include access to Current Good Manufacturing Practices (CGMPs) to produce cells for clinical trials or use as treatment [111]; the requirement for personalized products that require patient-specific generation [112]; manufacturing costs; short-term persistence of infused cells in PB, along with the inability to accurately track infused cells in GVHD tissue sites precluding selection of the precise timing for multiple infusions using infused cell nadir as the trigger [36]; and unknown risks for relapse and infection. For non-hematopoietic cells, there is also the theoretical potential for oncogenic conversion with prolonged culture, as seen in rodents [113]. At the same time, the BMT community is deeply engaged in the development and testing of additional immune regulatory/reparative products that have already shown efficacy in preclinical acute GVHD models, and have recently been reviewed [114]. These products include CD8 Tregs [45,47,115–117]; myeloid-derived suppressor cells (MDSCs) [118–120]; invariant NK T cells [18,19,121], which rapidly release anti-inflammatory and immune modulatory cytokines and can stimulate MDSCs [122] and Tregs [19,123]; innate lymphoid cells (e.g., type 2 innate lymphoid cells, which have been shown to both prevent and treat gut GVHD) [124]; tolerogenic DCs [125–128]; and monocytes/macrophages [129].

The immediate goals in the field of cell therapy of acute GVHD are to determine the therapeutic index, patient population(s), and venues that may benefit by cell infusion. Intermediate goals would include optimizing cell distribution to key GVHD target organs, extending longevity while maintaining lineage fidelity and

[†] See clinicaltrials.gov study NCT00366145.
[‡] See clinicaltrials.gov study NCT02652130.

function, and augmenting immune regulatory potency and tissue-reparative properties. Longer-term goals would include creating off-the-shelf, exportable, and less costly products; assessing efficacy and long-term outcomes; developing products that have suppressor functions specific for the desired target antigen(s); and defining the best setting to harness the power of these cells in treating patients with the otherwise poor prognosis of steroid-refractory acute GVHD. In closing, much progress has been made. The future is now bright, with proof of concept for the utility of cell therapies in patients who are in desperate need of new treatments after failing drug and antibody therapy.

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