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STEM CELL TRANSPLANTATION (R MAZIARZ, SECTION EDITOR)

Thinking Out of the Box—New Approaches to Controlling GVHD

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Abstract Graft-versus-host disease (GVHD) remains a major limitation of allogeneic hematopoietic cell transplantation (allo-HCT). Despite major advances in the understanding of GVHD pathogenesis, standard GVHD prophylaxis regimens continue to be based on the combination of a calcineurin inhibitor with an antimetabolite, while first line treatments still rely on high-dose corticosteroids. Further, no second line treatment has emerged thus far in acute or chronic GVHD patients who failed to respond with corticosteroid treatment. After briefly reviewing current standards of GVHD prevention and treatment, this article will discuss recent approaches that might change GVHD prophylaxis/treatment for decades to come, with a special focus on recently developed immunoregulatory strategies based on infusion of mesenchymal stromal or regulatory T-cells, or injection of low-dose interleukin-2.

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Introduction

Allogeneic hematopoietic cell transplantation (allo-HCT) remains the best treatment option for selected patients with severe primary immune-deficiencies, hemoglobinopathies, severe aplastic anemia, or hematological malignancies [1]. In the latter case, its anti-tumoral efficacy depends not only on high-dose (myeloablative conditioning) or lower-dose (reduced-intensity or nonmyeloablative conditioning) chemo/radiotherapy given during the conditioning regimen, but also on immune-mediated graft-versus-tumor effects [2]. Although mechanisms of graft-versus-tumor effects are not yet fully elucidated, destruction of recipient tumor cells by donor T-cells contained in the graft (and directed against minor or major histocompatibility antigen mismatches between donor and recipient or against tumor-associated antigens) is thought to play a primordial role [3–6]. Unfortunately, donor T-cells contained in the graft can also target healthy recipient tissues and cause graft-versus-host disease (GVHD), a potentially life-threatening complication of allo-HCT [7]. GVHD has been historically separated into two syndromes: acute GVHD, occurring within 100 days after transplantation, and chronic GVHD developing thereafter [8]. However, GVHD with characteristics of the chronic form can occur before day 100 after allo-HCT, while GVHD with characteristics of the acute form may occur beyond day 100, particularly in patients transplanted after nonmyeloablative conditioning and in those given donor lymphocyte infusions. These observations are the basis of a new GVHD classification (NIH classification) that recognized two categories of GVHD: ***acute GVHD***, defined as GVHD without signs consistent with

chronic GVHD and comprising *classic acute GVHD* occurring before day 100, and *late acute GVHD* occurring beyond day 100; and ***chronic GVHD*** including *classic chronic GVHD,* defined as chronic GVHD without signs of acute GVHD, and *overlap syndrome,* in which features of both acute and chronic GVHD coexist [9]. Interestingly, despite a strong association between GVHD and graft-versus-tumor effects [10–12], only milder forms of acute GVHD and NIH-defined chronic GVHD have been associated with improved survival, because of a strong association between severe forms of GVHD and nonrelapse mortality [13, 14]. In this article, we will review current practice and perspectives in GVHD prevention and treatment, with a special focus on innovative cellular therapies.

GVHD Prevention

Current Practice

The most efficient way to prevent GVHD consists of in vitro T-cell depletion of the graft. Based on data observed in murine experiments by Dicke *et al.* in the late 1960 s [15], hundreds of T-cell depleted allogeneic bone marrow transplantations were performed in the 1980 s [16]. Methods of T-cell depletion were mainly based on negative selection techniques (by physical separation or antibody-based purging). For example, a number of groups of investigators performed T-cell depletion by combining murine monoclonal antibodies and rabbit complement. This technique allowed a T-cell reduction of 2–3 logs and a low incidence of grade II–IV acute (10–20 %) GVHD, even without any postgrafting immunosuppression [17–19]. However, this benefit was offset by higher incidences of graft rejection/failure, infection (including post-transplant lymphoproliferative disease), and leukemia relapse [16, 19]. In the 1990s, techniques of positive immune-magnetic selection of hematopoietic stem cells (CD34+ cells) were developed concomitantly to the use of G-CSF mobilized peripheral blood stem cells (PBSC) as allogeneic stem cell source. This method allows for a 3–4 log T-and B-cell depletion and a significant reduction in the incidence of acute and chronic GVHD, without increasing the risk of post-transplant lymphoproliferative disease (probably due to concomitant B-cell depletion) [20–22, 23•]. This approach allowed doctors to perform successful allo-HCT across HLA barriers by infusing megadoses of HLA-haploidentical CD34+ selected cells [24]. In the HLA-identical setting, although a recent study suggested similar leukemia-free survival but less chronic GVHD in patients given CD34-selected PBSC in comparison to unmanipulated PBSC [23•], large randomized studies are needed before this approach may become a standard of care for GVHD prophylaxis.

Thus, despite progress in T-cell depletion methods, the current standard of care for GVHD prevention in patients receiving grafts from HLA-matched donors in most transplant centers remains the combination of a calcineurin inhibitor (cyclosporine or tacrolimus) and an antimetabolite [short methotrexate in case of myeloablative transplantation or mycophenolate mofetil (MMF) in case of reduced-intensity or nonmyeloablative transplantation, or in the setting of cord blood transplantation] [25–31]. Although these combinations have been relatively successful at preventing the most severe forms of acute GVHD, they failed to prevent the development of moderate/severe chronic GVHD in a large proportion of patients, and particularly in those given PBSC [32], and/or in those given grafts from unrelated donors [33].

In order to decrease the incidence of moderate/severe chronic GVHD in patients at high risk for this complication, a number of groups have added anti-thymocyte globulin (ATG) to standard GVHD prophylaxis regimens. Recent studies have suggested that the addition of ATG successfully decreased the incidence of chronic GVHD without increasing the risk of relapse both in patients receiving grafts from HLA-matched unrelated donors after myeloablative conditioning and given 60 mg/kg ATG Fresenius® or 4.5–6.0 mg/kg ATG Thymoglobulin® [34–36, 37••], and in those receiving PBSC after reduced-intensity conditioning and given ≤5 mg/kg of ATG Thymoglobulin® [38]. In contrast, the use of ≥10 mg/kg ATG Thymoglobulin® was associated with higher risks of disease relapse and infections in patients transplanted after reduced-intensity conditioning [39–41].

New Pharmacological Approaches

A number of new pharmacological approaches for GVHD prevention are being evaluated. These include in vivo T-cell depletion with alemtuzumab, or the post-transplant use of azacitidine, sirolimus, cyclophosphamide, anti-TNF agents, or bortezomib.

In vivo T-cell depletion with alemtuzumab, a humanized anti-CD52 IgG1 monoclonal antibody with broad lymphocyte-depleting properties, has been extensively studied in patients given grafts after reduced-intensity conditioning, generally combined with cyclosporine [42]. Administration of 100 mg alemtuzumab during the conditioning regimen was very successful at preventing acute and chronic GVHD, but was also associated with higher risks of infection and relapse in patients transplanted for hematological malignancies [14, 43, 44]. Current efforts to prevent relapse with this approach are focusing on decreasing the dose of alemtuzumab, or administering pre-emptive DLI in patients with evidence of poor donor chimerism of minimal residual disease [45, 46].

Another recent approach consisted at administering the hypomethylating agent azacitidine post-transplantation. Post-

transplant azacitidine increased regulatory T-cell (T_{reg}, vide infra) numbers, and thus potentially prevented GVHD, and also induced a cytotoxic CD8+ T-cell response to several tumor antigens including WT1 [47•].

Sirolimus (rapamycin) is a mTOR inhibitor that prevents activation of T-cells by inhibiting their response to IL-2 without affecting T_{regs} that respond to IL-2 stimulation mainly by the Stat-5 pathway [48]. Based on encouraging data observed in phase II studies [49, 50], the Blood and Marrow Transplant Clinical Trials Network conducted a large randomized study (n=304) comparing postgrafting immunosuppression with tacrolimus and methotrexate versus tacrolimus plus sirolimus in patients given grafts from HLA-identical siblings after TBI-based myeloablative conditioning. No difference in 114-day acute GVHD-free survival (primary endpoint) was observed, while relapse, nonrelapse mortality and progression-free survival were also similar in the two arms [51].

Taking advantage of the observations that cyclophosphamide is highly cytotoxic to proliferating T-cells but spares resting T-cells and hematopoietic stem cells, the John Hopkins group investigated post-transplant administration of cyclophosphamide as sole immunosuppressive treatment in patients given bone marrow from HLA-matched donors or combined with tacrolimus and MMF administration in those given HLA-haploidentical grafts. Administration of cyclophosphamide (50 mg/kg) on days +3 and +4 after transplantation allowed a low incidence of each grade III–IV acute (10 %) and chronic (10 %) GVHD in patient given bone marrow from HLA-matched donors [52], while administration of cyclophosphamide (50 mg/kg) on day +3 (with or without a second dose on day +4) combined with tacrolimus and mycophenolate mofetil started 1 day after cyclophosphamide administration also produced a low incidence of grade III–IV acute (6 %) and chronic (25 %) GVHD in patients receiving bone marrow from HLA-haploidentical donors [53]. However, given that post-transplant cyclophosphamide might also kill donor T-cells involved in graft-versus-tumor effects, the impact of post-transplant cyclophosphamide on the relapse risk needs to be further assessed.

TNF-α plays an important role in the initiation of GVHD [54•]. Further, following myeloablative and even nonmyeloablative conditioning regimens, the magnitude of change in the TNF-receptor-1 (TNFR1; a good surrogate for TNF-α) ratio (day +7 after transplantation/pretransplantation baseline values) has been correlated with later occurrence of acute GVHD [55, 56]. These observations prompted the development of clinical studies aimed at assessing the impact of TNF-α inhibition on GVHD prevention [57, 58]. Unfortunately, administration of the TNF-α inhibitors infliximab (10 mg/kg one day prior to conditioning and then on days 0, +7, +14, +28, and +42) or etanercept (25 mg twice weekly from start of conditioning to day +56) in addition to standard GVHD prophylaxis failed to prevent acute GVHD [57, 58].

Finally, based on extensive murine experiments [59], the proteasome inhibitor bortezomib (on days 1, 4 and 7 after allo-HCT) has been assessed in combination with tacrolimus and methotrexate as GVHD prophylaxis in 1–2/10 HLA-mismatched unrelated graft recipients in a phase I–II study including 45 patients. Indeed, bortezomib immunomodulatory properties include selective depletion of proliferating alloreactive T-cells while sparing T_{regs}, and inhibition of antigen-presenting cell activation. Incidences of both grade II–IV (22 %) and chronic (29 %) GVHD were very encouraging [60].

Taken together, these studies suggest that a combination of tacrolimus and sirolimus might be equivalent to standard tacrolimus plus methotrexate in patients given TBI-based conditioning, while in vivo T-cell depletion with alemtuzumab as well as post-transplant azacitidine, cyclophosphamide or bortezomib are promising approaches that should be further investigated in multicenter randomized studies.

New Cellular Approaches

During the last decade, important advances have been made regarding our knowledge of immunoregulatory cells such as mesenchymal stromal cells (MSC) or T_{reg}. MSC are multipotent progenitors that can found within the bone marrow, but also within several connective tissues such as adipose tissue, fetal membranes and the umbilical cord [61, 62, 63•]. Minimal criteria for MSC definition according to the International Society for Cellular Therapy (ISCT) include: 1) plastic adherence when maintained in standard culture conditions; 2) expression of CD105, CD73 and CD90, and lack of expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules; and 3) ability to differentiate into osteoblasts, adipocytes and chondrocytes in vitro [64]. MSC have important immunoregulatory properties both in vitro and in vivo, such as inhibition of T-cell proliferation and dendritic cell differentiation, and prolongation of skin or cardiac transplant survival [63•, 65, 66]. Our understanding on how MSC impact immune cells has considerably improved over the last decade [63•, 66]. As a result of these studies, MSC are now considered to respond to their immediate environment and adapt their response accordingly through the release of soluble factors, such as prostaglandin E2 (PGE2), IL-10, transforming growth factor beta (TGF-β), nitric oxide (NO), heme oxygenase-1 (HO-1), HLA-G5, hepatocyte growth factor (HGF), bone morphogenetic protein 2 (BMP2), or galectin-1, through induction of indoleamine 2,3 dioxygenase (IDO), and/or through cell contact signaling via Notch and CD95/Fas [63•, 66–68]. Further, context-dependent modification of T-helper (Th)1/Th2 ratio has been shown in several inflammatory disease models [69], while a number of additional studies have demonstrated the ability for MSC to promote/expand T_{regs} in vitro and in vivo (mainly

through TGF-β and IDO) and to inhibit the differentiation of pro-inflammatory Th-17 cells (through PGE2) [70]. Finally, In addition to having a plethora of immunoregulatory properties, MSC are hypoimmunogenic, allowing transfer across HLA barriers [63•], which is mandatory for "off-the-shelf" cellular therapy.

A number of studies have assessed the ability of MSC infusion at preventing GVHD in various murine models of GVHD [71–76], in humanized murine models of xenogeneic GVHD [77, 78], as well as in a preclinical canine model of GVHD [79]. Taken together, these studies suggested that a single injection of (non-activated) MSC given on day 0 failed to prevent GVHD in most models [71, 73, 77, 78], while a single infusion of activated MSC on day 0 [73], or repeated MSC injections at the time of and after transplantation showed clinical benefit in some [72, 76, 77] but not all [74, 75, 78] studies, depending on the GVHD model, the timing of MSC infusion, the dose of MSC infused, as well as the origin of MSC. However, repeated MSC injection failed to prevent lethal GVHD in a pre-clinical canine model of dog leukocyte antigen-haploidentical transplantation [79].

A number of small clinical studies have studied the impact of MSC infusion at the time of allo-HCT on engraftment and GVHD [80–85]. These pilot studies suggested that MSC co-transplantation did not prevent graft rejection in the setting of T-cell repleted transplantation [83, 84], while it might do so in the setting of HLA-haploidentical T-cell depleted allo-HCT [81]. Interestingly, three studies observed a lower incidence of acute GVHD in patients co-transplanted with MSC compared to historical [83, 84] or concurrent [82] controls, suggesting that MSC might help reducing GVHD. However, these results should be taken with caution given the small number of patients included in these pilot studies. Prospective, multicenter, double-blind randomized studies are ongoing in order to assess more definitely the impact of MSC co-transplantation on GVHD (ClinicalTrials.gov Identifier: NCT01045382).

T_{reg} play a critical role in the maintenance of tolerance to self-antigens [86•]. Their development and function requires the transcription factor FoxP3. In the last decade, much attention has been paid to the potential role of T_{regs} after allo-HCT. In murine experimental GVHD models, administration of high doses of T_{regs} (conventional T cells /T_{regs} ratio of 1 or 2) at the time of transplantation prevented acute GVHD without apparently impairing graft-versus-tumor effects [87–89]. Similarly, a number of studies demonstrated that T_{reg} infusion was able to prevent xenogeneic GVHD in humanized mice [90, 91]. In humans, Di Ianni *et al.* investigated the ability of T_{reg} to allow for infusion of relatively large numbers of conventional T-cells in the HLA-haploidentical setting [92••]. The trial included 28 high-risk adult patients with advanced hematological malignancies. Patients received a myeloablative conditioning regimen, followed by the infusion of freshly isolated donor CD25+ T_{regs} ($2x10^6$/kg, enriched using a two-step procedure, simultaneous CD8 and CD19 depletion followed by CD25-positive selection) on day-4 before transplantation. Following this preconditioning, patients were transplanted on day 0 with megadoses of CD34+ hematopoietic stem cells to reconstitute the bone marrow and conventional T-cells ($0.5–4.0\times10^6$ cells/kg) to reconstitute the immune system. Immune reconstitution was prompt with CD4+ and CD8+ donor T-cell counts reaching the normal range within 2–3 months. Most remarkably, of the 28 patients only two developed at least grade II acute GVHD and none chronic GVHD, despite not using postgrafting immunosuppression and a relatively low T_{reg} purity (80+/−22 % FoxP3-positive cells [93]). In addition, Brunstein *et al.* investigated the ability of umbilical cord blood-derived T_{regs} ($0.1–30\times10^5$ T_{regs}/kg) to decrease the incidence of acute GVHD in 23 adult patients given double umbilical cord blood transplantation [94]. T_{regs} were obtained from a third cord blood unit that was 4–6/6 HLA-matched with the recipient and expanded in vitro over 18 days with anti-CD3/anti-CD28 monoclonal antibody-coated Dynabeads® and 300 IU/mL Il-2. Importantly, the authors observed a lower incidence of grade II–IV acute GVHD in patients given T_{regs} than in a group of similarly treated 108 historical patients not given T_{regs} (43 % versus 61 %, P=0.05). Although very promising, the results should be confirmed in prospective randomized studies.

Treatment of Acute GVHD

Current Practice

The standard of care for first line treatment of grade II–IV acute GVHD consists of methylprednisolone (or prednisone) starting at 2 mg/kg for 7 days with slow subsequent tapering according to GVHD response [31, 95, 96••]. Nonabsorbable oral corticosteroids (budesonide, 9 mg/day) are often added to systemic corticosteroids in patients with gastrointestinal acute GVHD [97]. Methylprednisolone produces complete responses in 25 to 69 % (53 % in the largest study reported thus far [98]) of patients with grade II–IV acute GVHD [99–102]. Interestingly, one retrospective study suggested that initial treatment with 1 mg/kg methylprednisolone did not compromise disease control or mortality in patients with grade II acute GVHD [101].

Outcomes for patients with corticosteroid-refractory acute GVHD are dismal, and unfortunately, there is no standard of care for second line treatment of acute GVHD (vide infra) [96••, 103].

New Pharmacological Approaches

First Line Treatment

Many attempts at intensifying the front line treatment for grade II–IV acute GVHD failed. Specifically, the addition of horse ATG, antibodies against IL-2 [basiliximab, daclizumab

or denileukin diftitox (denileukin)], antibodies against TNF-α (etanercept or infliximab), or pentostatin to (methyl)prednisolone failed to improve response rates, and were often associated with higher nonrelapse mortality [104–108]. A phase II four-arm randomized study was recently conducted with the aim of identifying the most promising agent in addition to standard methylprednisolone for initial therapy for grade II–IV GVHD [109]. Patients were randomized to receive 2 mg/kg methylprednisolone per day plus etanercept, MMF, denileukin diftitox, or pentostatin. Day 28 complete response rate was the highest with MMF (60 %). Based on this study, the BMT-CTN conducted a multi-center, randomized, double-blind phase III study comparing corticosteroids plus placebo versus corticosteroids plus MMF as initial treatment for acute GVHD [110]. Unfortunately, 56-day GVHD-free survival (primary endpoint) and 6-month survival were similar in the two arms.

In an attempt at avoiding morbidity associated with high doses of corticosteroids, Pidala *et al.* assessed the ability of sirolimus as initial treatment for grade I (n=4), II (n=24), or III (n=4) acute GVHD [111]. Sixteen of 32 patients (50 %) achieved sustained, complete resolution of acute GVHD, a rate similar to what was observed in matched historical controls treated with standard 1 mg/kg steroids, suggesting that sirolimus had comparable activity to that of high-dose glucocorticoids in the primary therapy of grade I–III acute GVHD. While encouraging, these results should be confirmed in prospective randomized trials before sirolimus could become a standard of care for initial treatment of moderate acute GVHD.

Second Line Treatment

Antimetabolites (mycophenolate mofetil or methotrexate), extracorporeal photopheresis, antibodies against IL-2 (basiliximab, daclizumab, inolimomab or denileukin diftitox (denileukin)), antibodies against TNF-α (etanercept or infliximab), alemtuzumab and ATG have been assessed as second line therapy for steroid-refractory acute GVHD (nicely reviewed in reference [96••]). Combining data from 25 clinical studies, 6-month overall survival from initiation of second line therapy (a good surrogate for GVHD response) ranged from 0 to 86 % (weighted average 6-month survival of 49 %), without evidence that any of these treatments were better or worse than another [96••]. This was confirmed by a single-center retrospective study (n=93) showing similar outcomes in patients with steroid-refractory acute GVHD treated with mycophenolate mofetil, versus inolimomab, or etanercept [112]. In contrast, a multicenter comparative analysis suggested better survival for patients with corticosteroid-refractory grade II acute GVHD treated by extracorporeal photopheresis compared to those treated by anticytokine therapy [113].

New Cellular Approaches

Currently, cellular approaches for acute GVHD treatment consist primarily of MSC infusion, although a few patients with steroid-refractory acute GVHD have been treated with (in vitro expanded) T_{regs} on a compassionate basis by Edinger *et al* [114]., and in a patient with grade IV acute GVHD by Trzonkowski *et al* [115]..

First Line Treatment

A phase II randomized multicenter study has evaluated two different doses of MSC (Prochymal®, Osiris Therapeutics, Columbia, MD) given in combination with standard methylprednisolone for the initial therapy for acute GVHD [116]. Thirty-two adult patients with grade II (n=21), grade III (n=8) or grade IV (n=3) acute GVHD were randomized to receive, in addition to standard corticosteroids, two doses of either two or eight million MSC/kg each. The first MSC infusion was given within the 48 hr following diagnosis of grade II–IV acute GVHD, and the second 3 days later. Ninety-four percent of patients achieved complete (77 %) or partial (16 %) responses. Interestingly, the response rates were comparable in patients given two or eight million MSC/kg. Based on these encouraging results, a randomized, multicenter, phase III trial compared 2×10^6 MSC/kg (Prochymal®) versus placebo in addition to standard corticosteroid therapy as primary treatment for 184 patients with grade II–IV acute GVHD (ClinicalTrials.gov: NCT00562497). Although the results of the final analysis are not yet published in a peer-reviewed journal, Osiris reported that the trial failed to reach the primary endpoint of durable complete response lasting ≥ 28 days without any increase in corticosteroid dose nor second line therapy and survival of ≥ 90 days [117].

Second Line Treatment

The use of MSC as treatment for steroid-refractory acute GVHD was pioneered by Le Blanc *et al.* in 2004 [118]. Based on the impressive impact of MSC in a child with steroid-refractory grade IV acute GVHD, the Developmental Committee of the European Group for Blood and Marrow Transplantation developed a phase II study assessing MSC therapy in patients with steroid-refractory acute GVHD [119]. The study included 55 patients with steroid-refractory grades II (n=5), III (n=25) or IV (n=25) acute GVHD (Table 1). Twenty-seven patients received one, 22 two, 4 three, 1 four and 1 five MSC infusions (total of 92 MSC infusions). MSC donors were either HLA-identical siblings (n=5), HLA-haploidentical relatives (n=18), or third-party HLA-

Table 1 Phase I-II studies assessing MSC infusion(s) in patients with steroid-refractory acute GVHD.

	MSC origin	MSC administration	# of patients (# with grade II/III/IV acute GVHD)	# of patients (%) with CR/PR	Outcomes
Le Blanc *et al.* [119]	BM, expanded in FBS-containing media.	Median of 1.4×10^6 cells/infusion; 1–5 (median 2) infusions.	55 (5/25/25)	30 (54)/9 (16)	2-yr OS (from allo-HSCT): 35 %
Fang *et al.* [120]	Adipose tissue-derived, expanded in FBS-containing media.	1×10^6 cells/infusion; 1–2 (median 1) infusions.	0/2/4	5/0	2-yr OS : 66 %
von Bonin *et al.* [121]	BM, expanded on platelet-lysate.	Median of 0.9×10^6 cells/infusion; 1–5 (median 2) infusions.	13 (0/2/11)	1 (8)/1 (8)	NR
Prasad *et al.* [122] †	BM, expanded in FBS-containing media.	$2–8\times10^6$ cells/infusion; 2–21 (median 8) infusions.	12 (0/5/7)	1 (8)/8 (67)	2-yr OS : 40 %.
Introna *et al.* [147]	BM, expanded on platelet-lysate.	1.5×10^6 cells/infusion; 1–8 (median 3) infusions.	40 (NR)	11 (28)/16 (40)	2-yr OS : 39 %
Ball *et al.* [148] †, ‡	BM, expanded in FBS-containing media.	$1–2\times10^6$ cells/infusion; 1–13 (median 2) infusions.	37 (NR; all 3–4)	24 (65)/8 (22)	6-yr OS: 37 %
Resnick *et al.* [149]	BM, expanded in FBS-containing media.	$1.1\pm0.5\times10^6$ cells/infusion ; 1–4 infusion.	50 (0/8/42)	17 (34)/16 (32)	3.6-yr DFS: 56 %.

† only children; ‡ patients were reported also in the Le Blanc et al. study [119]. BM, bone marrow; FBS, fetal bovine serum; OS, overall survival; DFS, disease-free survival; NR, not reported; allo-HSCT, allogeneic hematopoietic stem cell transplantation

mismatched individuals (n=69), and the median dose of MSC infused was 1.4 (range 0.4–9) x 10^6 MSC/kg. No side effects were seen after MSC infusions. Among the 55 patients, 30 had complete and nine had partial responses. Median time from MSC infusion to complete response was 18 (range, 3–63) days, and responses were more frequently observed in children than in adults. Following this report, a number of phase II studies assessed the impact MSC in patients with steroid-refractory acute GVHD (Table 1) [120–122]. While the majority of these studies suggested efficacy of MSC therapy, it remains to be demonstrated whether MSC infusion provides better results than other second line treatments for acute GVHD.

In an attempt at answering this question, the potential role of MSC (Prochymal®) was evaluated in addition to standard of care, including institutionally selected second line treatment, in a randomized (2:1) multicentre trial in 244 patients with steroid-refractory grade II–IV gastrointestinal (n=179), skin (n=144), and/or liver (n=61) acute GVHD (ClinicalTrials.gov: NCT00366145). Patients received eight infusions of placebo or 2×10^6 MSC/kg each over 4 weeks, with an additional four infusions administered weekly after day 28 in patients who had partial responses. Although the study was completed in 2009, results have been published only in abstract form thus far [123]. Unexpectedly, the study did not observe a significant difference in the rate of overall complete and durable (≥28 days) responses between the two groups [primary endpoint; 35 in the MSC versus 30 in the placebo groups (P=0.3)]. Incidence of relapse, infection, and toxicities were also comparable in the MSC and placebo arms.

Potential explanations for the discrepancies between phase II studies and the Prochymal® randomized study might be due to differences in immunoregulatory properties between Prochymal and "EBMT" MSC products [124]. Another potential explanation could be that adding MSC to other efficient second line therapy for acute GVHD failed to further improve GVHD responses (indeed the response rates in the second line plus placebo arm of the Prochymal study were as high as 77 % and 68 % for skin and gastrointestinal GVHD, respectively [123]).

Treatment of Chronic GVHD

Current Practice

Mild chronic GVHD is generally managed by topical treatments. In contrast, first line treatment for moderate/severe forms of chronic GVHD is generally based on corticosteroids, often combined with cyclosporine or tacrolimus [125]. Unfortunately, with these regimens only 20–50 % of patients achieve complete resolution of GVHD and withdrawal of all systemic treatment within 2–3 years [126, 127]. Further, long-

term corticosteroid treatment causes numerous complications such as infections, diabetes, myopathy, avascular necrosis, osteoporosis, weight gain with changes in body habitus, cataracts, and emotional lability. These observations led Martin *et al.* to assess the addition of MMF to standard initial treatment of chronic GVHD in a double-blind multicenter study [126]. The study, unfortunately, did not demonstrate any benefit of adding MMF, with a trend for higher risk of death in patients given MMF (HR 1.9, 95 % CI:0.9–4.3).

Even though several immunosuppressive/immunomodulating approaches have demonstrated therapeutic activity in steroid-refractory chronic GVHD, the prognosis of patients with such GVHD remains unsatisfactory with 2-year survival ranging from 41 to 85 % with salvage therapy [128–130]. There is no standard second line treatment for steroid-refractory chronic GVHD and the "trial-and-error" approach has remained the usual way to identify the drug effective in the individual patient [130]. Second line treatments include immunosuppressors (such as mycophenolate mofetil, methotrexate, alemtuzumab), immunomodulating approaches [such as photopheresis, mTOR inhibition (sirolimus or everolimus), thalidomide], thoraco-abdominal irradiation, antifibrotic agents (imatinib, nilotinib), and rituximab [130].

New Pharmacological Approaches

As mentioned above, T_{regs} play a critical role in the maintenance of tolerance to self-antigens [86•]. Indeed, absence of T_{regs} causes fatal autoimmunity in mice and humans [131, 132], while deficits in T_{reg} function/number have been observed in various autoimmune/inflammatory disorders [86•, 133], as well as in GVHD [134, 135]. Interleukin-2 (IL-2) is the critical cytokine regulating T_{reg} homeostasis [136, 137]. Following allo-HCT (and particularly in patients with chronic GVHD), there are high amounts of IL-7 and IL-15 that activate conventional CD4+ T-cells [138, 139, 140••], combined with a relative deficit of IL-2 favoring T_{reg} apoptosis [140••]. These observations prompted Koreth *et al.* to conduct a phase 1 dose-escalation study aimed at determining the maximum tolerated dose of daily low-dose subcutaneous IL-2 in patients with steroid-refractory chronic GVHD [141]. Twenty-nine patients were included, and the maximum tolerated dose of IL-2 was 1×10^6 IU/m^2. Remarkably, 12 of 23 evaluable patients achieved an objective partial response during the 8 weeks of IL-2 treatment. Further, IL-2 therapy improved T_{reg} homeostasis by increasing T_{reg} proliferation, T_{reg} resistance to apoptosis, and T_{reg} thymic export [140••]. Based on these promising results, low-dose (1×10^6 IU/m^2/day) IL-2 is currently being assessed in patients with steroid-refractory chronic GVHD in a phase II study (ClinicalTrials.gov Identifier: NCT01366092).

New Cellular Approaches

There are few studies published thus far on the impact of MSC infusion in patients with steroid-refractory chronic GVHD. Zhou *et al.* reported data from four patients with severe sclerodermic chronic GVHD who were given MSC injected directly into the bone marrow at total doses of $1–2\times10^7$ cells for four to eight infusions within a 22–52 day period [142]. Following MSC infusions, the doses of immunosuppressive medications were tapered significantly, while GVHD symptoms gradually improved in all four patients. More recently, Weng *et al.* assessed the impact of i.v. MSC infusions in 19 patients with steroid-refractory chronic GVHD [143]. Patients received a median of two (range, one to five) MSC infusions given approximately 6 months apart at a median dose of 0.6 (range, 0.2–1.4) x 10^6 MSC/Kg. Fourteen of the 19 patients achieved partial (n=10) or complete (n=4) responses. Two-year survival rate from the first MSC infusion was 78 %. These two reports might serve as the basis for prospective, double-blind, randomized studies assessing the impact of MSC infusions in patients with steroid-refractory chronic GVHD.

Although T_{reg} homeostasis after allo-HCT is not fully understood, it has been demonstrated that even in younger patients, thymic generation of T_{regs} was minimal while T_{regs} reconstituted by peripheral expansion of mature T_{regs} present in the graft and exhibited a predominantly activated/memory phenotype [144]. This was particularly true in older patients given grafts after nonmyeloablative conditioning [145, 146]. Further, it has been observed that T_{regs} from patients with extensive GVHD had low telomerase activity, thus restricting their proliferative capacities [134], and were more susceptible to apoptosis due to a higher expression of Bim and CD95 and a lower expression of Bcl-2, stressing the interest for administering fresh T_{reg} as GVHD therapy. These observations are the basis of ongoing protocols aimed at assessing the feasibility (and efficacy) of T_{reg} infusion alone (ClinicalTrials.gov Identifier: NCT01911039) or in combination with sirolimus (ClinicalTrials.gov Identifier: NCT01903473) or low-dose IL-2 (ClinicalTrials.gov Identifier: NCT01937468) in patients with steroid-refractory chronic GVHD.

Conclusions

A number of promising approaches are being evaluated for their potential role in GVHD prophylaxis or treatment. These approaches are focused on depleting alloreactive donor T-cells (alemtuzumab, post-transplant cyclophosphamide) or modulating donor T-cell responses against the recipients (sirolimus, MSC, T_{regs}, IL-2, bortezomib, azacitidine). With results of phase 2 studies being promising, large multicenter

randomized studies are needed to help better defining their optimal role in GVHD prevention and/or treatment.

Compliance with Ethics Guidelines

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