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EXPERT OPINION

Emerging drugs for prevention of graft failure after allogeneic hematopoietic stem cell transplantation

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Introduction: Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the treatment of choice for many patients suffering from hematological malignancies, severe hemoglobinopathies, bone marrow failures or severe primary immunodeficiencies. Graft rejection/failure (GF) is a life-threatening complication following allo-HSCT that is most commonly caused by the reactivity of recipient T cells, natural killer (NK) cells or antibodies against donor grafted hematopoietic cells. The increasing use of allo-HSCT following reduced-intensity conditioning (RIC) and the increasing use of alternative donors (unrelated cord blood and human leukocyte antigen (HLA)-mismatched donor) have resulted in higher frequency of GF.

Areas covered: This review describes the pathogenesis and current prevention and treatment of GF as well as agents in development for GF prevention or treatment.

Expert opinion: The risk of GF may be reduced in the future by optimizing the conditioning regimens and post-grafting immunosuppression, increasing the number of hematopoietic stem cells (HSCs) and/or immune cells transplanted, optimizing HSC homing and better detecting patients at high risk of GF by searching for pre-transplant donor-specific anti-HLA antibodies in patients given grafts from HLA-mismatched donors, or by closely monitoring donor T- and/or NK-cell chimerism after allo-HSCT following RIC.

Keywords: alemtuzumab, anti-thymocyte globulin, chimerism, costimulation, CTLA4, fludarabine, graft failure, graft rejection, hematopoietic cell transplantation, mesenchymal stromal cells, reduced-intensity conditioning, total body irradiation, treosulfan

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1. Background

1.1 Allogeneic hematopoietic stem cell transplantation: current modalities

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) has therapeutic potential for a wide range of life-threatening diseases [1]. First, it is an efficient treatment for a variety of hematological malignancies such as leukemias, lymphomas and multiple myeloma. For these indications, its curative potential relies on two principles: i) the cytotoxic effect of radio/chemotherapy (conditioning) given as preparation for transplantation and ii) the immunotherapeutic effect of donor immune cells contained in the graft against recipient malignant cells, called graft-versus-tumor effects [2-4]. Allo-HSCT is also an effective treatment for a number of non-malignant diseases such as severe primary or secondary bone marrow (BM) failures, hemoglobinopathies, severe primary immune deficiencies and some rare inborn errors of metabolism [5-7].

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The conditioning regimen given before allo-HSCT has three aims: i) reduction or ablation of the host defective/malignant cells (myeloablation), ii) clearance of BM space allowing engraftment of donor hematopoietic stem cells (HSCs) and iii) suppression of host immunity to prevent graft rejection (immunosuppression). Over the years, a number of conditioning regimens have been developed (Figure 1). The decision to use one conditioning regimen or another is based on several factors, including the type and status of the underlying disease, the fitness of the patient, the type of donor, the stem cell source and the center preferences. Myeloablative conditionings are generally based on maximally tolerated doses of either total body irradiation (TBI) or busulfan (an alkylating agent) combined with high-dose cyclophosphamide (another alkylating agent with potent immunosuppressive properties).

The modalities of allo-HSCT have changed significantly over the last decades. One of the most important advances has been the development of reduced-intensity conditioning (RIC) regimens [8-10]. In comparison with conventional myeloablative regimens, RIC have been developed to reduce the toxicity and the mortality related to the conditioning, allowing performing allo-HSCT in older patients and in those with medical comorbidities [9,10]. RIC regimens exert only low or moderate myelosuppression but they provide sufficient immune suppression to allow donor cell engraftment and to promote graft-versus-tumor effects for hematological malignancies [3,4].

In the past, BM has been the most common source of HSC for allo-HSCT. However, mobilization by recombinant human granulocyte colony-stimulating factor (G-CSF) of marrow HSC into the peripheral blood has allowed the use of peripheral blood stem cells (PBSCs) as stem cell source. In comparison with BM grafts, PBSCs grafts contain three-fold more $CD34^+$ (a marker for HSC) cells, and 10- to 30-fold more T cells, B cells, natural killer (NK) cells and monocytes [11]. Prospective randomized studies have demonstrated that using stem cells derived from G-CSF primed PBSCs instead of BM was associated with faster hematopoietic recovery, higher incidences of grade III – IV acute and extensive chronic graft-versus-host disease (GVHD, a serious complication of allo-HSCT consisting of the attack of host healthy tissues by donor immune cells) and increased overall survival in patients transplanted for high-risk hematologic malignancies [12].

Cord blood (CB) has also been used as a source of transplantable HSC [13,14]. Potential advantages include more rapid availability and, because CB is relatively deficient in mature T cells, a greater degree of human leukocyte antigen (HLA) mismatching is acceptable. Disadvantages of CB include slower engraftment and an increased risk of graft failure (GF), both probably due to the low number of cells (and particularly of HSC) contained in one CB unit.

Allo-HSCT is usually performed with a donor who is matched with the recipient for major histocompatibility complex loci (MHC, termed HLA in humans), with optimal donors being 10/10 allelic HLA-matched (HLA-A, -B, -C, -DR and -DQ) with the recipient. The best donor is a HLA-identical sibling. However, such a donor is available for only 30% of patients in need for allo-HSCT [14]. In patients without HLA-matched sibling donor, alternative donors are searched for. These alternative donors include HLA-matched unrelated donors or CB, and if not available, HLA-mismatched donors (either unrelated CB, related donor sharing only 1 of the 2 HLA haplotypes with the recipients (haploidentical donor), or unrelated donors who are matched for only 8 – 9/10 HLA alleles with the recipient) [14]. While outcomes after allo-HSCT with HLA-matched unrelated donors are currently close to what is achieved with HLA-identical siblings [3,4], HLA-mismatched allo-HSCT remains associated with worse outcomes due to higher incidences of infection, graft rejection and/or GVHD [14].

T-cell depletion of the graft has been evaluated by several groups of investigators as an approach to reduce GVHD [15]. Disadvantages of T-cell depletion of the graft include higher risk of disease relapse, higher risk of graft rejection and delayed immune recovery [15,16]. Currently, the most common procedure for T-cell depletion is based on positive selection of HSC based on their expression of the cell surface antigen CD34 [17].

1.2 Biology of allogeneic HSC engraftment

Successful sustained engraftment of donor HSC relies on three principles: i) clearance of BM space for donor HSC implantation, ii) donor HSC ability to home recipient BM niches and to proliferate and iii) host acceptance of grafted cells mainly through limitation of host-versus-graft alloreactivity [18]. These three biological mechanisms are studied for potential pharmacological modulation to prevent and treat GF after allo-HSCT.

1.2.1 Clearance of BM spaces for donor HSC implantation

Successful donor HSC engraftment is dependent on HSC making their way to free BM niches where they can find optimal conditions to survive and proliferate. Although it has been initially thought that creation of BM spaces required administration of chemotherapeutic agents or BM irradiation, a number of findings have demonstrated that donor T cells present in the graft are able to create BM spaces through graft-versus-host reactions [19]. Indeed, attempts at preventing GVHD by depleting the graft from donor T cells have led to a high incidence of graft rejection [15]. The Perugia group also demonstrated, both in preclinical MHC-mismatched murine models and in patients given T-cell-depleted grafts from HLA-haploidentical donors, that donor alloreactive NK cells could ablate both host hematopoiesis and host residual immune cells, thus promoting donor HSC engraftment [20].

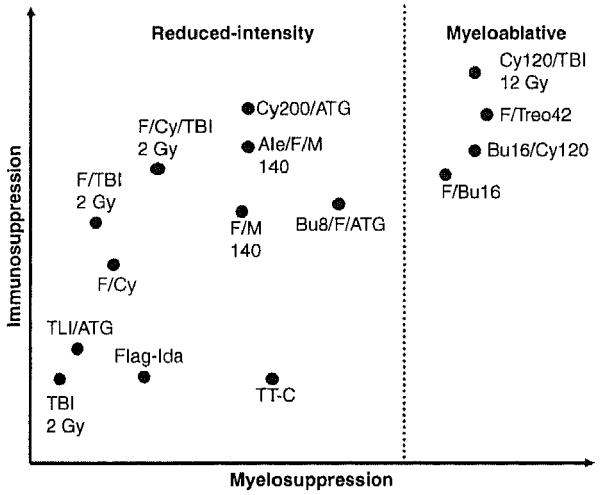

Figure 1. Commonly used conditioning regimens in relation to their immunosuppressive and myelosuppressive properties. Please note that this classification is not based on direct experimentation, and is thus hypothetical.

Adapted from [124].

Ale: Alemtuzumab; ATG: Anti-thymocyte globulin; Bu8: Busulfan 8 mg/kg; Bu16: Busulfan 16 mg/kg; Cy: Cyclophosphamide; Cy 120: Cyclophosphamide 120 mg/kg; Cy 200: Cyclophosphamide 200 mg/kg; F: Fludarabine; Flag-Ida: Fludarabine/cytosine arabinoside/idarubicin; M: Melphalan, M 140; melphalan 140 mg/m^2; M 180; melphalan 180 mg/m^2; TBI: Total body irradiation; TLI: Total lymphoid irradiation 8 Gy; Treo42: Treosulfan 14 g/m^2 for 3 days; TT: Thiotepa.

1.2.2 HSC homing and proliferation

The biology governing HSC homing is still emerging [21]. The first step in HSC homing to BM involves interactions between HSC and BM sinusoidal endothelial cells and transmigration through the endothelium. The interaction between chemokine receptor type 4 (CXCR4) expressed on circulating HSC and its ligand, the chemokine stromal-derived factor 1 (SDF-1) appears to be one of the key axes in HSC homing to the BM [22]. SDF-1 is secreted by BM stromal cells and its secretion is up-regulated in allo-HSCT recipients after the conditioning regimen. Circulating CD34$^+$ HSC navigate and engraft in the BM niches toward a SDF-1 gradient. Other molecules involved among interactions between HSC receptor and niche stroma ligands include very late antigen-4 (VLA-4) and vascular cell adhesion molecule-1 (VCAM-1), lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule (ICAM), E-selectin ligand-1 and endothelial-selectin and P-selectin glycoprotein ligand-1 and P-selectin. Importantly, appropriate carbohydrate modification (fucosylation) of selectin ligands is critical for the rolling of HSC on P- and E-selectins expressed by the hematopoietic microvasculature [23].

Once they have reached the BM microenvironment, HSC have to proliferate and to generate all hematopoietic cell subsets. The molecular regulation of HSC proliferation and self-renewal is governed by complex interactions between HSC and BM niches [21,24,25]. Among HSC niche interactions, activation of c-Kit receptor by membrane stem cell factor expressed on osteoblasts plays an important role in long-term HSC maintenance and HSC self-renewal, while activation of the thrombopoietin receptor MPL by thrombopoietin, as well as activation of Tie2 by angiopontin-1 and activation of Notch by Notch ligands play an essential role in maintaining HSC quiescence and adhesion to bone [24].

1.2.3 Host-versus-graft reactivity and immune graft rejection

Immune graft rejection is due to reactivity of immune recipient cells against grafted cells. It is commonly admitted that it is mainly mediated by recipient alloreactive T cells in case of MHC-matched transplantation, and by both alloreactive T cells and alloreactive NK cells in case of MHC-mismatched transplantation [18]. The role of NK cells in MHC-mismatched HSC rejection is well illustrated by the observation that patients with severe combined immunodeficiency (SCID) who lack both NK- and T-cell functions engraft more readily than those who lack only T-cell function when given grafts from HLA-mismatched donors [18].

1.3 Clinical assessment of allogeneic HSC engraftment

Methods to determine engraftment after allo-HSCT are based on assessment of the donor origin of hematopoietic cells in the recipient BM and peripheral blood (termed chimerism analysis) [26]. Chimerism analyses are commonly performed for both myeloid and lymphoid (T- and sometimes NK-) cell subsets [27-29]. Full replacement of recipient hematopoiesis by donor hematopoiesis usually occurs after myeloablative allo-HSCT. A proportion of hematopoietic cells of recipient origin may sometimes persist after allo-HSCT (particularly in patients given grafts after RIC [26-29]) and is defined as a state of mixed chimerism. Currently, chimerism analyses are generally carried out with molecular techniques assessing short tandem repeats (STR, the number of repeats varying among individuals and being inherited as co-dominant Mendelian traits) by multiplex polymerase chain reaction (PCR) [26].

1.4 Graft failure and graft rejection after allo-HSCT

GF is an increasing problem after allo-HSCT given the increasing use of RIC regimens, HLA-mismatched grafts and CB grafts [30]. GF corresponds to either a lack of initial engraftment of donor hematopoietic cells (primary GF) or a loss of donor hematopoietic cells after initial engraftment (secondary GF). Immune graft rejection, caused by host immune responses against donor immune-hematopoietic cells, is the main cause of GF. In that case, chimerism analyses typically demonstrate the absence of donor cells in the recipient's BM and the presence of > 95% of T lymphocytes of recipient origin [26]. Other causes of GF include viral

infections (particularly cytomegalovirus, human herpes virus type 6 or parvovirus infections), septicemia, drug toxicity or relapse of the underlying disease (hematologic malignancies or BM failures).

The incidence of GF varies from 0.1 to 30% of patients, depending on transplantation modalities. Risk factors for GF include donor/recipient HLA disparities (and particularly HLA class 1 disparities) [31], use of an unrelated donor [27], use of CB as stem cell source (in comparison with BM or PBSC) [32], T-cell depletion of the graft [33], recipient sensitization by blood transfusions (that can be largely prevented by leukodepletion and irradiation of blood products) or pregnancy [5], transplantation for non-malignant disease or absence of prior chemotherapy [5,27,34], use of RIC regimens [35,36], low number of transplanted cells [5,32,37] and presence of donor-directed HLA-specific allo-antibodies (Table 1) [38].

GF is associated with considerable morbidity and mortality mainly related to infectious and hemorrhagic complications. It is particularly true for patients who experienced prolonged marrow aplasia without autologous hematopoiesis recovery [39].

2. Existing treatment

Several strategies have been developed to prevent primary or secondary GF. These strategies include: development of optimal pre-transplant conditioning and post-grafting immunosuppressive regimens, optimization of graft composition and early identification of patients at high risk of secondary GF through chimerism monitoring. Unfortunately, rescue treatments for patients with GF are limited and consist of the administration of hematopoietic growth factors, boost of donor immune cells or purified donor HSC and second allo-HSCT.

2.1 Conditioning regimens and post-grafting immunosuppression

2.1.1 Myeloablative conditioning regimens

Conditioning regimens for allo-HSCT usually combine a potent immunosuppressive agent (to avoid host-versus-graft reactions) with a potent myeloablative agent (to clear BM niches and to eradicate malignant hematopoiesis) [1]. Potent immunosuppressive agents include TBI, high-dose cyclophosphamide (an alkylating agent) and fludarabine (a purine analog). Potent myeloablative agents include TBI (when used at high dose), and high-dose alkylating agents such as busulfan, melphalan and thiotepa.

The two most widely used myeloablative conditioning regimens combine cyclophosphamide with high-dose fractionated TBI (12 Gy total dose; Cy-TBI regimen), or with high-dose busulfan (Bu-Cy regimen) [40]. Over the last decades, improvements to these conditioning regimens have included i) adjustment of subsequent doses of oral busulfan based on pharmacokinetics of the initial dose, thereby achieving a targeted busulfan plasma concentration preventing both under treatment with increased relapse rates and excessive toxicity [41], and ii) the use of i.v. busulfan instead of p.o. busulfan.

Immunotherapy with anti-thymocyte globulins (ATG, depleting both host and donor T cells given its persistence several days after transplantation) has been evaluated in combination with radio-chemotherapy conditioning since the late 70s. In patients with aplastic anemia conditioned with cyclophosphamide, addition of ATG in the conditioning regimen prevented GF and improved survival, at least in part by overcoming transfusion-induced sensitization in these patients [5].

2.1.2 RIC regimens

In the 90s, Storb *et al.* hypothesized that optimizing post-grafting immunosuppression could reduce both host-versus-graft and graft-versus-host reactions, thereby enabling a decrease in intensity of the pre-transplant conditioning regimen needed for sustained engraftment and prevention of severe GVHD [42]. This hypothesis was demonstrated in a preclinical canine model of transplantation [42]. In that model, whereas a single TBI dose of 9.2 Gy was sufficiently immunosuppressive to allow engraftment of BM from dog leukocyte antigen (DLA)-identical littermate in 95% of transplanted dogs, only 41% of dogs had stable engraftment when the dose was decreased to 4.5 Gy. However, the addition of post-grafting immunosuppression with cyclosporine (a calcineurin inhibitor) led to 100% engraftment. When the TBI dose was further decreased to 2 Gy, more intense post-grafting immunosuppression combining cyclosporine and mycophenolate mofetil (a purine synthesis inhibitor) allowed the establishment of stable mixed chimerism. When the dose of TBI was decreased to 1 Gy, stable long-term engraftment could no longer be achieved even by maximizing post-grafting immunosuppression [43]. In contrast to what has been observed in aplastic anemia patients conditioned with high-dose cyclophosphamide, the addition of ATG to 1 Gy TBI failed to prevent graft rejection [44], perhaps because donor T cells were needed to clear BM spaces through subclinical graft-versus-host reactions [19].

Based on these results, a pilot clinical trial of allo-HSCT after conditioning with 2 Gy TBI and post-grafting immunosuppression with cyclosporine and mycophenolate mofetil was performed in older patients ineligible for classical allo-HSCT [8]. Non-fatal GF occurred in 20% of the patients. This could be later prevented by adding 3 days of fludarabine (30 mg/m^2/day) to TBI [27], and by increasing the dose of mycophenolate mofetil [45]. This low-intensity RIC regimen is currently largely used in patients ineligible for high-dose chemo/radiotherapy [45].

Other (but more intense) RIC regimens, also developed in the late 1990s, have combined fludarabine with intermediate-dose alkylating agents (busulfan (8 mg/kg) or melphalan (140 mg/m^3); **Figure 1**) [46,47].

2.1.3 HLA-mismatched (haploidentical) transplantation

Several strategies have been assessed to facilitate engraftment after HLA-mismatched allo-HSCT. Among these, infusion

Table 1. Main transplant-related risk factors for GF after allo-HSCT.

Risk factors
Donor/recipient HLA disparities [31]
Use of an unrelated donor [27]
CB transplantation [14,32]
T-cell depletion of the graft [33]
Recipient sensitization by multiple blood transfusion or pregnancy [5]
Transplantation for non-malignant disease or absence of prior chemotherapy [5,27,34]
Use of RIC regimen [35,36]
Low number of transplanted cells [5,32,37]
Presence of donor-directed HLA-specific allo-antibodies [38]

Allo-HSCT: Allogeneic hematopoietic stem cell transplantation; CB: Cord blood; GF: Graft failure; HLA: Human leukocyte antigen; RIC: Reduced-intensity conditioning.

of megadoses of purified $CD34^+$ HSC appeared to increase engraftment rate [48]. Further, administration of high-dose cyclophosphamide on days 3 and 4 after transplantation has been associated with a low incidence of both graft rejection and GVHD in the haploidentical RIC allo-SCT setting [49-51], by specifically killing T cells clones that are activated directly after transplantation such as both host-versus-graft and graft-versus-host reactive T cells.

2.2 Graft composition

Over the last 20 years, a number of studies in animal models and in humans have analyzed the impact of graft composition on engraftment and on transplantation outcomes. These studies indicated that higher numbers of transplanted HSC ($CD34^+$ cells) [13,37,48,52] and T cells [37,53] were both associated with lower risks of GF. Specifically, in mice, transplantation of megadoses of MHC-mismatched T-cell-depleted HSC allowed stable engraftment after sublethal irradiation [54]. In the canine preclinical model of engraftment, whereas all dogs given MHC-identical BM after 1 Gy TBI and post-grafting immunosuppression with cyclosporine and mycophenolate mofetil experienced graft rejection, the addition of PBSC to BM (but not of T-cell-depleted PBSC) allowed stable engraftment in 62% of dogs, demonstrating the important role of donor T cells for securing engraftment [53].

Similar to what was observed in mice, transplantation of megadoses of T-cell-depleted HSC allowed stable engraftment in patients given HLA-haploidentical grafts after myeloablative or RIC regimens [48,55]. Similarly, higher doses of transplanted $CD34^+$ HSC and $CD8^+$ T cells were both associated with higher donor chimerism levels and a lower incidence of GF in patients given unrelated HLA-matched unmanipulated grafts after RIC with fludarabine and 2 Gy TBI [37], while higher numbers of transplanted HSC correlated with a lower incidence of GF in patients receiving unrelated CB transplantation [13,52]. The latter observation led to the development of protocols of double unrelated CB allo-HSCT that permitted robust engraftment (even in the RIC setting) in patients who lacked a single unrelated CB unit containing sufficient numbers of cells to ensure stable engraftment [56].

2.3 Early detection of patients at risk of secondary GF through chimerism monitoring

In patients given grafts after 2 Gy TBI with or without fludarabine, day-14 T- and NK-cell donor chimerism levels below 50% were each associated with an increased risk of subsequent graft rejection ($p = 0.003$ and 0.004, respectively) [27]. Similar observations were reported by Breuer *et al.* who observed graft rejection in 9 out of 10 children (given various RIC regimens) with < 50% donor NK-cell chimerism on day 28 after allo-HSCT versus in 10 out of 76 children (13%) with ≥ 50% donor NK-cell chimerism ($p < 0.0001$) [29].

2.4 Management of GF after allo-HSCT

Rescue strategies for patients with GF after allo-HSCT are limited. They are based on the administration of high doses of hematopoietic growth factors, although usually with little success. Reinfusion of cryopreserved autologous HSC, if stored before allo-HSCT, has also been performed by some centers [57]. In patients with persistent poor graft function without evidence of immunologic graft rejection, reinfusion (boost) of donor stem cells without conditioning may improve graft function [58,59]. Because unmanipulated BM or PBSC boost can induce GVHD, CD34 selection or other forms of T-cell depletion of the graft have been assessed in that setting [60]. By contrast, in patients with low or decreasing donor T-cell chimerism levels but still good marrow function, donor lymphocyte infusions have been used in an attempt at preventing impending graft rejection [61,62]. While this approach was successful in patients given T-cell-depleted grafts or *in vivo* T-cell depletion [62], donor lymphocyte infusions failed to prevent graft rejection in the majority of patients given unmanipulated grafts [61], as observed in the preclinical canine model of transplantation [63]. Further, donor lymphocytes can induce GVHD or marrow aplasia (when residual hematopoiesis is mainly host-derived) [2].

In patients with primary GF or fulminant graft rejection leading to profound aplasia, re-transplantation is necessary, using either the same or another donor [64]. Conditioning regimens for the second allo-HSCT should be immunosuppressive rather than myeloablative (to avoid unacceptable cumulative toxicities of two successive high-dose conditionings given in a short interval of time), thus most commonly based on cyclophosphamide, purine analogs, low-dose TBI or a combination of these agents. ATG or alemtuzumab are also frequently used [65,66]. Finally, because it is associated with faster engraftment in comparison with any other HSC source, PBSC might be the best stem cell source for salvage transplantation [67].

A recent survey from the US National Marrow Donor Program analyzed outcomes of salvage transplantation with HLA-matched unrelated donors performed between 1990 and 2005 in 122 patients with primary GF [64]. Conditioning regimens for the salvage transplantation were myeloablative in 38%, RIC in 47% and none in 15% of patients. The outcome was dismal, with a 100-day incidence of non-relapse mortality of 75% and survival of 11% at 1 year. Despite the cumulative incidence of engraftment at day 100 being 74%, GF was the main cause of death (34%). Better results (30% overall survival at 3 years) were observed by Guardiola *et al.* in patients given mainly grafts from HLA-identical siblings for salvage transplantation [68]. More recently, Waki *et al.* analyzed the clinical outcomes of 80 patients with GF who underwent salvage CB allo-HSCT following fludarabine-based RIC regimens [66]. The 1-year overall survival was 33%, with non-relapse mortality, especially caused by infections, as the major cause of death.

3. Market review

Allo-HSCT is performed worldwide and over the last two decades, the number of allo-HSCT per year has dramatically increased. A recent review of the Worldwide Network for Bone and Marrow Transplantation registry has reported a total of 21,156 allo-HSCT performed in 1327 centers in 71 countries worldwide during the year 2006 [69]. The median rate of allo-HSCT ranged from 0.2 to 434.9 per 10 million inhabitants, with the majority of them performed in Europe and in America. The European Group of Blood and Marrow Transplantation (EBMT) also analyzed recently its activity from 1990 to 2010 [70]. A total of 12,276 allo-HSCT were reported during this period and the median rate ranged from 0 to 458 per 10 million inhabitants. Interestingly, the number of allo-HSCT performed by EBMT-affiliated centers has increased by 37% from 2005 to 2010 [70]. Unfortunately, as mentioned above, the incidence of GF has also increased due to the introduction of RIC for allo-HSCT, CB and HLA-mismatched transplantation.

Allo-HSCT is a complex, resource-intense and costly procedure. From 2004 to 2007, hospital costs for allo-HSCT have increased by 85% [71]. In 2012, median cost estimates for allo-HSCT for the first year ranged from US$96,000 to US$204,000 [71]. Factors associated with higher costs included advanced disease, use of high-dose myeloablative conditioning (as opposed to RIC), use of unrelated donors or unrelated CB as stem cell source, longer duration of hospitalization and transplant complications [71]. Given that GF predisposes to infections (and the need for costly anti-infectious agents) and implies the use of growth factors, transfusions and second transplants, it is not surprising that the occurrence of GF is associated with high costs. This has been demonstrated in a recent study by Majhail *et al.* who evaluated costs of allo-HSCT within the first 100 days among patients transplanted either from matched related donor or unrelated CB, after either RIC or myeloablative conditioning [72]. The median cost per day survived, excluding the cost of graft acquisition, was US$1016 and US $612 for myeloablative and RIC-based matched-related allo-HSCT, respectively, and US$2082 and US$1156 for myeloablative and RIC-based CB allo-HSCT, respectively. The occurrence of GF was significantly associated with higher costs within the first 100 days after allo-HSCT, with US $6976 median cost per survived day in patients with GF [72].

4. Current research goals

Given that second transplantation is currently the sole option to manage patients who experienced severe GF after allo-HSCT, current challenges are mainly focused on identifying new therapeutic agents aimed at preventing this complication. Specifically, current research is based on two main strategies: improving HSC engraftment and preventing immune-mediated GF.

5. Scientific rationale and competitive environment

5.1 Advances in the field of promotion of HSC engraftment

Emerging strategies to enhance donor HSC engraftment are summarized in Table 2.

5.1.1 New methods for BM niches clearance

New approaches aimed at clearing HSC niches with limited toxicity are currently under investigation. A promising approach consists of antibody-mediated myeloablation. Among monoclonal antibodies used for this purpose, those directed against the CD45 panleukocyte antigen are particularly appealing because CD45 is expressed at high density in BM and its expression is restricted to hematopoietic cells. Further, given that CD45 is expressed not only on hematopoietic progenitors but also on all leucocytes, anti-CD45 targeted therapies may provide both myelosuppression and immunosuppression in preparation for allo-HSCT. Straathof *et al.* assessed a conditioning regimen combining two rat anti-CD45 monoclonal antibodies (YTH 24.5 and YTH 54.12) for myelosuppression, and alemtuzumab, fludarabine and low-dose cyclophosphamide for immunosuppression, in a cohort of 16 high-risk patients suffering from primary immunodeficiencies [73]. Remarkably, 15 of 16 patients achieved sustained engraftment. Other anti-CD45 antibodies have also been conjugated with radionuclides (radioimmunoconjugates) with the aim of delivering high-dose radiation to the BM while limiting toxic exposure of other organs [74]. Currently, several Phase I – II studies are ongoing to evaluate the combination of the radiolabeled anti-CD45 antibody BC8 with fludarabine and low-dose TBI as RIC preparative regimen before allo-HSCT for patients with high-risk acute leukemia, myelodysplatic syndrome or multiple myeloma (ClinicalTrials.gov #NCT00119366, #NCT00589316, #NCT01300572, #NCT01503242). To date, most

Table 2. Emerging strategies to enhance donor HSC engraftment.

Category	Compound	Company	Chemical name	Indication/Aim	Stage of development	Mechanism of action
Lytic anti-CD45 mAb	YTH 24.5 YTH 54.12	Generated by Therapeutic Antibody Centre, Oxford, UK	Rat IgG1 mAb	To facilitate donor HSC engraftment by clearing BM niches	Phase I – II [73]	Depletion of host hematopoiesis mainly through complement-dependant cytotoxicity
Anti-CD45 radioimmunoconjugate	^{131}I-BC8 Ab	Generated by the Biologics Production Facility at the FHCRC, Seattle, USA	Murine IgG1 mAb	To facilitate donor HSC engraftment by clearing BM niches	Phase I – II [74] Phase II (NCT00119366, NCT00589316)	Depletion of host hematopoiesis through radiation-induced cytotoxicity
Anti-CD45 radioimmunoconjugate	^{90}Y-BC8 Ab	Generated by the Biologics Production Facility at the FHCRC, Seattle, USA	Murine IgG1 mAb	To facilitate donor HSC engraftment by clearing BM niches	Phase I (NCT01300572, NCT01503242)	Depletion of host hematopoiesis through radiation-induced cytotoxicity
Anti-CD45 radioimmunoconjugate	Not available for human use yet	NA	NS	To facilitate donor HSC engraftment by clearing BM niches	Preclinical [75]: canine model with ^{211}At-CA12.10C12-B10 (^{211}At anti-canine CD45)	Depletion of host hematopoiesis through radiation-induced cytotoxicity
c-Kit antagonist mAb	Not available for human use yet	NA	NS	To facilitate donor HSC engraftment by clearing BM niches	Preclinical [76]: mouse model with ACK2 (anti-mouse CD117 mAb)	Depletion of host hematopoiesis by blocking vital c-Kit interaction with its ligand, stem cell factor (aka, kit ligand or steel factor)
CXCR4 antagonist	Plerixafor (AMD 3100, JM 3100, SDZ SID 791)	Genzyme (Sanofi)	1,4,8,11-Tetraazacyclotetradecane, 1,11-(1,4-phenylenebis(methylene)) *bis*-, octahydrochloride (CAS)	To facilitate donor HSC engraftment by clearing BM niches	Phase I – II (NCT01182675, NCT01280955, NCT0165587, NCT01068301)	Mobilization of host HSC by disrupting HSC interaction with BM niches through inhibition of SDF1-CXCR4 axis
CD26/DPPIV inhibitor	Sitagliptin (MK-0431)	Merck & Co.	(*R*)-4-Oxo-4-[3-(trifluoromethyl)-5, 6-dihydro[1,2,4]triazolo[4,3-a] pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine	To facilitate donor HSC engraftment by enhancing HSC homing	Phase I – II (NCT00862719, NCT01720264)	Promotion of CB HSC homing to BM niches through prevention of SDF-1 truncation by CD26/DPPIV and protection of SDF-1/CXCR4 axis
Cellular therapy	*ex vivo* fucosylated CB HSC	NA	Cellular product	To facilitate donor HSC engraftment by enhancing HSC homing	Phase I (NCT01471067)	Facilitation of interaction between CB HSC with selectins expressed in the BM microvasculature

Allo-HSCT: Allogeneic hematopoietic stem cell transplantation; BM: Bone marrow; CB: Cord blood; cGMP: Current good manufacturing practice; CXCR4: Chemokine receptor type 4; DPPIV: Dipeptidylpeptidase IV; HSC: Hematopoietic stem cells; mAb: Monoclonal antibody; MSC: Mesenchymal stem cells; NA: Not applicable; NMA: Nicotinamide; NS: Not specified; PGE2: Prostaglandin E2; TEPA: Tetraethylenepentamine.

Table 2. Emerging strategies to enhance donor HSC engraftment (continued).

Category	Compound	Company	Chemical name	Indication/Aim	Stage of development	Mechanism of action
PGE2	Dimethyl-PGE2 (FT-1050)	Fate Therapeutics	16,16-Dimethyl-PGE2	To facilitate donor HSC engraftment by enhancing HSC homing To optimize the dose of CB HSC to infuse	Phase I (NCT01527838)	Enhancement of CB HSC homing properties through up-regulation of CXCR4 *ex vivo* expansion of CB HSC by promoting cell proliferation and inhibiting cell apoptosis
Immobilized engineered Notch ligand	Delta-1$^{ext-IgG}$ Jagged-1	NS	Extracellular domain of Delta-1 fused to the Fc domain of human IgG1	To optimize the dose of CB HSC to infuse	Phase I [83]	*ex vivo* expansion of CB HSC through Notch signaling pathway
Cellular therapy	MSC MSC from mesenchymal precursor cell product (Mesoblast)*	NS Mesoblast Ltd., Melbourne, Australia*	Cellular product	To optimize the dose of CB HSC to infuse	Phase I (NEJM 2012, de Lima) [84] (NCT00498316)	*ex vivo* expansion of CB HSC by co-culturing them with MSC
Copper chelator	TEPA TEPA-expanded CB HSC (StemEx®)*	Sigma-Aldrich NovaSep Cellular product from Gamida Cell*	*N*-(2-Aminoethyl)-*N'*-[2-[(2-aminoethyl)amino]ethyl}-1,2-ethanediamine	To optimize the dose of CB HSC to infuse	Phase I – II [86] (NCT00469729, NCT01484470)	*ex vivo* expansion of CB HSC by inhibiting cell differentiation and promoting cell expansion through reduction of cellular copper concentration
SIRT1 inhibitor	NMA NMA-expanded CB HSC (NiCord®)*	NS Cellular product from Gamida Cell*	Pyridine 3-carboxamide	To optimize the dose of CB HSC to infuse	Phase I (NCT01221857, NCT01590628)	*ex vivo* expansion of CB HSC by inhibiting cell differentiation and promoting cell expansion through inhibition of SIRT1 deacetylase and enhancement of CB HSC homing properties
Cellular therapy	Co-infusion with purified $CD34^+$ HSC from third-party donor	NA	Cellular product	To shorten the neutropenic phase after CB allo-HSCT	Phase I – II [88]	To act as a 'bridge allo-HSCT' through an early and rapid engraftment of HSC from third-party donor before CB HSC engraftment

Allo-HSCT: Allogeneic hematopoietic stem cell transplantation; BM: Bone marrow; CB: Cord blood; cGMP: Current good manufacturing practice; CXCR4: Chemokine receptor type 4; DPPIV: Dipeptidylpeptidase IV; HSC: Hematopoietic stem cells; mAb: Monoclonal antibody; MSC: Mesenchymal stem cells; NA: Not applicable; NMA: Nicotinamide; NS: Not specified; PGE2: Prostaglandin E2; TEPA: Tetraethylenepentamine.

anti-CD45 radioimmunoconjugates have been coupled to β-emitters (i.e., ^{131}I, ^{90}Y) for practical reasons. However, because α-emitters have more favorable physical characteristics such as long path lengths, stronger biological effects and low linear energy transfer, they might prove to be more suitable for conditioning. Confirming this hypothesis, Chen *et al.* recently reported high engraftment rates in dogs conditioned only with an anti-CD45 antibodies coupled with the α-emitter astatine-211 [75].

Other minimally toxic myelosuppressive therapies that are under investigations are based on the inhibition of the interactions between HSC and BM niches. Among these strategies, the pre-transplant administration of monoclonal antibodies blocking c-Kit function [76] or of CXCR4 antagonists such as plerixafor have been shown to promote both BM niche clearance and donor HSC engraftment in murine models [77]. Based on this concept, a Phase I – II trial at the University of California in San Francisco is currently assessing the combination of plerixafor, filgastrim and alemtuzumab as minimally toxic conditioning regimen for children with severe combined immunodeficiency (ClinicalTrials.gov #NCT01182675). Another Phase I – II study of plerixafor in myeloablative allo-HSCT is now recruiting at Duke University with the aim of promoting donor engraftment (ClinicalTrials.gov #NCT01280955).

5.1.2 *Improvement of HSC homing*

As mentioned above, SDF-1/CXCR4 axis is crucial for HSC homing to BM niches. It has been shown recently that a subpopulation of HSC isolated from CB expressed on its surface the CD26/dipeptidylpeptidase IV (CD26/DPPIV) enzyme [22]. CD26/DPPIV is a membrane-bound extracellular peptidase that cleaves N-terminal dipeptides from diverse chemokines including SDF-1. Functional studies have shown that N-terminal truncated SDF-1 lacked the ability to induce migration of HSC and inhibited HSC migration toward SDF-1. Therefore, inhibition of the endogenous CD26/DPPIV activity on $CD34^+$ HSC cells (i.e., with sitagliptin, MK-0431) might be another strategy to enhance HSC homing and engraftment after CB allo-HSCT (ClinicalTrials.gov #NCT00862719, #NCT01720264). Other strategies include CB fucosylation prior to infusion (fucosylation of specific cell surface ligands is required before effective interaction of HSC with selectins expressed by the BM microvasculature can occur) [23] (ClinicalTrials.gov #NCT01471067), or *ex vivo* CB treatment with prostaglandin E2 (PGE2 might increase homing of HSC through up-regulation of the CXCR4) [78] (ClinicalTrials.gov #NCT00890500). Finally, another approach to promote engraftment of CB allo-HSCT consists of injecting CB HSC directly intrabone instead of intravenously [79].

5.1.3 *Optimization of graft content*

5.1.3.1 *Increasing the number of HSC transplanted for CB allo-HSCT*

Research on improvement of HSC graft content is mainly performed in the field of CB allo-HSCT since low numbers of HSC within a single CB unit has remained a limiting factor for this transplantation modality, particularly in adult recipients [80].

Besides transplantation of two unrelated CB units that has allowed performing safe unrelated CB transplantation when an adequately dosed single unrelated CB unit was not available [81], research has been focused on *ex vivo* HSC expansion. Several groups of investigators have demonstrated the expression of the Notch receptors (Notch-1 and Notch-2) by human HSC and of Notch ligands (Delta-1 and Jagged-1) by human BM stromal cells, endothelial cells and osteoblasts [24]. While signaling through Notch-1 inhibited HSC differentiation and therefore enhanced HSC proliferation, addition of a soluble form of Jagged-1 in the culture media induced the expansion of human HSC *in vitro* [82]. Based on this concept, Delaney *et al.* assessed the feasibility and safety of co-infusing a first unmanipulated unrelated CB unit with a second CB unit that was expanded *ex vivo* using an engineered form of the Notch ligand Delta-1 [83]. While long-term engraftment originated from the non-expanded unit, the expanded graft contributed to initial myeloid engraftment and allowed a median time to neutrophil recovery (absolute neutrophil count > 500/μl) of 16 days (compared with 26 days for concurrent patients given double unmanipulated unrelated CB transplantation after a similar conditioning regimen) [80]. de Lima *et al.* investigated a similar strategy by expanding the second CB unit in co-culture with mesenchymal stromal cells (MSC, a multipotent cell type that resides in the BM, differentiates into multiple mesodermal tissues, participates in the formation of HSC niches and supports hematopoiesis by secreting various hematopoietic growth factors) [84]. Median time to neutrophil engraftment was 15 days in the recipients of the MSC-expanded CB unit, as compared with 24 days in controls who received unmanipulated CB units only ($p < 0.001$). As observed by Delaney *et al.*, long-term engraftment (> 1 year) originated primarily from the unmanipulated CB unit in all patients. PGs have also been shown to enhance HSC expansion [85]. A clinical Phase I trial is currently assessing the safety and efficacy of transplantation of a single PGE2 expanded CB unit in adult patients with hematological malignancies (ClinicalTrials.gov #NCT01527838). Other strategies to support *ex vivo* HSC expansion include culturing CB with growth factors in conjunction with the copper chelator tetraethylenepentamine (TEPA) [86] (ClinicalTrials.gov #NCT00469729, #NCT01484470) or with nicotinamide (which also seems to enhance homing properties of CB HSC toward SDF-1 gradient) [87] (ClinicalTrials.gov #NCT01221857, #NCT01590628).

To overcome the frequent problem of a suboptimal number of HSC infused in CB allo-HSCT, Bautista *et al.* reported another strategy consisting in the co-infusion of a limited number of highly purified mobilized HSC from a HLA-unrestricted third-party donor with the CB unit [88]. They observed an early and initially predominant engraftment of

HSC from the third-party donor (providing early neutrophil recovery), while full CB engraftment (providing long-term hematopoiesis and immune recovery) was achieved within 100 days in 90% of patients. The shortened neutropenic phase resulted in a significant reduction in the incidence of severe infections.

5.1.3.2 Graft manipulation for HLA-haploidentical allo-HSCT

As mentioned above, positive $CD34^+$ HSC selection was proposed as an approach to limit GVHD occurrence and transplant-related mortality after HLA-haploidentical allo-HSCT [48]. However, this procedure is associated with significant risks for GF and delayed immune recovery. Recently, the Tuebingen group has reported robust engraftment, low incidence of GVHD and prompt immune reconstitution following transplantation of TcR$\alpha\beta$/CD19-depleted PBSC from HLA-haploidentical donor [89].

5.2 Improvements in the prevention of immune-mediated GF

Emerging strategies to prevent immune-mediated graft rejection are summarized in Table 3.

5.2.1 Conditioning regimen

5.2.1.1 New chemotherapeutic agents with potent immunosuppressive characteristics

Given the demonstrated efficacy of fludarabine as an immunosuppressive agent in RIC regimens for allo-HSCT [27], other purine analogs have also been evaluated. Among them, pentostatin has a unique mechanism of action. It inhibits adenosine deaminase, thereby leading to lymphocyte apoptosis due to the cytotoxic accumulation of deoxyadenosine triphosphate [90]. Using a mice model, Mariotti *et al.* described that a RIC regimen combining cyclophosphamide and pentostatin was able to induce durable host T-cell depletion and to prevent rejection of T-cell-depleted fully MHC-mismatched HSC allografts more consistently than the combination of cyclophosphamide and fludarabine [91]. However, pentostatin failed to promote engraftment in the preclinical canine model of engraftment (Table 4) [90]. Several clinical trials have been opened to assess pentostatin as part of a RIC regimen before allo-HSCT (ClinicalTrials.gov #NCT00496340, #NCT0057162) or as a therapeutic approach in combination with DLI for GF prevention in case of low donor T-cell chimerism after allo-HSCT (NCT00096161). Unfortunately, investigators of the University of Arizona were forced to prematurely terminate a clinical study evaluating conditioning with pentostatin and alemtuzumab for allo-HSCT, as preliminary data indicated that it was associated with a higher incidence of GF than standard RIC regimen (ClinicalTrials.gov #NCT00698685).

Another new chemotherapeutic agent currently investigated for allo-HSCT conditioning is the alkylating agent treosulfan (dihydroxybusulfan) [92]. In comparison with busulfan, treosulfan might induce more prominent B- and T-cell depletion, more profound myelosuppression through broad effects against both primitive HSC and committed precursors, and less toxicity (particularly less veno-occlusive disease of the liver) [92]. Based on these promising properties, a RIC regimen combining treosulfan and fludarabine has been assessed in patients suffering from severe primary immunodeficiency [7]. Preliminary results suggest that this regimen was associated with lower toxicity and improved T-cell chimerism compared with the association of melphalan and fludarabine [93]. Treosulfan-based regimens are currently assessed in Phase II trials in patients with non-malignant (ClinicalTrials.gov #NCT00919503) or malignant (ClinicalTrials.gov #NCT00253513, #NCT00796068) hematological disorders. An open-label, randomized Phase III trial in Europe is also currently comparing the efficacy and safety of a treosulfan-based regimen with a busulfan-based RIC regimen (ClinicalTrials.gov #NCT00822393).

5.2.1.2 New anti-T-cell targeted therapies

As mentioned above, ATG has been used as part of the conditioning regimen to overcome both host and donor T-cell alloreactivity. Another antibody increasingly used for GF prevention is alemtuzumab. Alemtuzumab is a non-T-cell-specific humanized monoclonal antibody directed against CD52. It induces efficient T-cell but also B- and NK-cell depletion through complement-mediated lysis and antibody-dependent cellular cytotoxicity. Alemtuzumab-based RIC are particularly evaluated in the setting of allo-HSCT for non-malignant disorders (ClinicalTrials.gov #NCT00920972, #NCT01499888, #NCT01626092, #NCT00849745, #NCT01652092, #NCT00578266, #NCT00578292, #NCT00455312, #NCT00176852). A RIC regimen combining fludarabine, melphalan and alemtuzumab has shown promising results in terms of both long-term engraftment and survival in patients with primary immunodeficiencies [93]. Alemtuzumab has also been shown to allow T-cell-repleted HLA-haploidentical RIC transplantation, by preventing both GF and severe GVHD [94].

Investigators from the Fred Hutchinson Cancer Center have assessed a more specific T-cell antibody directed against TCR$\alpha\beta$. This antibody has shown promising activities in the preclinical canine model of allo-HSCT [95], particularly when linked to the α-emitter bismuth-213 [96]. However, no such reagents have been used in human clinical trials yet.

5.2.1.3 Costimulation blockade

As discussed above, when transplant pairs are fully HLA-matched, immune-mediated GF is mainly caused by T-cell immunity, while immune-mediated GF is caused by both recipient T and NK cells in cases of HLA-mismatched transplant pairs. Clonal expansion and activation of naïve T cells are not only dependent on antigen-specific binding of T-cell receptor (TCR) to the HLA–peptide complex but also require a second costimulatory signal. One of the best characterized costimulatory signals is through the interaction

Table 3. Emerging strategies to prevent immune-mediated GF.

Category	Compound	Company	Chemical name	Indication/Aim	Stage of development	Mechanism of action
Purine analog	Pentostatin	Parke-Davis (Pfizer)	Imidazo[4,5-d][1,3]diazepin-8-ol, 3-(2-deoxy-β-D-erythro-pentofuranosyl)-3,6,7, 8-tetrahydro-, (*R*)- (CAS)	To limit host-versus-graft immune reactions	Phase I – II (NCT00496340, NCT00096161)	Lymphocyte depletion through cytotoxic accumulation of deoxyadenosine triphosphate
Alkylating agent	Treosulfan	Medac	1,2,3,4-Butanetetrol, 1,4-dimethanesulfonate, [*S*-(*R**,*R**)]- (CAS)	To limit host-versus-graft immune reactions and to enhance donor HSC engraftment	Phase II (NCT00919503, NCT00253513, NCT00796068) Phase III (NCT00822393)	B and T cells depletion and myelosuppression through cytotoxicity due to lethal DNA damages
Anti-CD52 mAb	Alemtuzumab	Genzyme (Sanofi)	Humanized rat IgG1 mAb	To limit host-versus-graft immune reactions	Phase I – II (NCT00920972, NCT01499888, NCT01626092, NCT00849745, NCT01652092, NCT00578266, NCT00578292, NCT00455312, NCT00176852)	Depletion of $CD52^+$ cells (including T, B and NK cells) through complement-mediated lysis and antibody-dependent cellular cytotoxicity
^{213}Bi-Anti-TcRαβ mAb	Not available for human use yet	NS	NS	To limit host-versus-graft immune reactions	Preclinical canine models [96]	Selective depletion of T cells
CTLA4-Ig	Abatacept Belatacept ASP-2408	Bristol-Myers Squibb Perseid Therapeutics	Fusion protein composed of the extracellular domain of CTLA4 fused to the Fc portion of IgG1 heavy chain	To limit host-versus-graft immune reactions	Preclinical canine model [98]	Induction of T-cell hyporesponsiveness through T-cell costimulation blockade
mTOR inhibitor	Sirolimus	Pfizer	(3*S*,6*R*,7*E*,9*R*,10*R*,12*R*,14*S*,15*E*, 17*E*,19*E*,21*S*,23*S*,26*R*,27*R*,34a*S*) 9,10,12,13,14,21,22,23,24,25, 26,27,32,33,34,34a-hexadecahydro-9,27-dihydroxy-3-[(1*R*)-2[(1*S*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23, 27-epoxy-3H-pyrido[2,1-c][1,4] oxaazacyclohentriacontine1,5,11, 28,29 (4H,6H,31H)-pentone	To limit host-versus-graft immune reactions	Phase I – II (NCT01499888, NCT00061568, NCT00426517, NCT00923364, NCT00352976)	Inhibition of effector T cells and dendritic cells and induction of Tregs

Allo-HSCT: Allogeneic hematopoietic stem cell transplantation; BM: Bone marrow; cGMP: Current good manufacturing practice; HSC: Hematopoietic stem cells; mAb: Monoclonal antibody; MSC: Mesenchymal stem cells; mTOR: Mammalian target of rapamycin; NK: Natural killer; NS: Not specified; Tregs: Regulatory T cells.

Table 3. Emerging strategies to prevent immune-mediated GF (continued).

Category	Compound	Company	Chemical name	Indication/Aim	Stage of development	Mechanism of action
mTOR inhibitor	Everolimus (RAD001)	Novartis	(1*R*,9*S*,12*S*,15*R*,16*E*,18*R*,19*R*, 21*R*,23*S*,24*E*,26*E*,28*E*,30*S*,32*S*,35*R*)-1, 18-dihydroxy-12-{(1*R*)-2-[(1*S*,3*R*,4*R*)-4-(2-hydroxyethoxy)-3-methoxycyclohexyl]-1-methylethyl}-19,30-dimethoxy-15,17,21,23,29,35-hexamethyl-11, 36-dioxa-4-aza-tricyclo[30.3.1.04,9] hexatriaconta-16,24,26,28-tetraene-2,3,10,14,20-pentaone	To limit host-versus-graft immune reactions	Preclinical canine model [108]	Inhibition of effector T cells and dendritic cells and induction of Tregs
Cellular therapy	MSC	NS	Cellular product	To limit host-versus-graft immune reactions and to enhance donor HSC engraftment	Phase I – II [110-112] (NCT01045382)	Modulation of alloreactive immune responses and enhancement of HSC expansion and survival

Allo-HSCT: Allogeneic hematopoietic stem cell transplantation; BM: Bone marrow; cGMP: Current good manufacturing practice; HSC: Hematopoietic stem cells; mAb: Monoclonal antibody; MSC: Mesenchymal stem cells; mTOR: Mammalian target of rapamycin; NK: Natural killer; NS: Not specified; Tregs: Regulatory T cells.

of CD28 on T cells and CD80/CD86 (B7-1/B7-2) molecules at the surface of antigen-presenting cells (APC). Cytotoxic T-lymphocyte antigen 4 (CTLA4, also called CD152) is another ligand for CD80/CD86 and is also expressed on T cells. CD28 promotes T-cell activation and proliferation by enhancing cytokine production, promoting cell division and up-regulating anti-apoptotic proteins, whereas CTLA4 inhibits T-cell activation through several mechanisms including delivery of inhibitory signals to T cells and ligand competition with CD28 for CD80/86 [97]. Antigen recognition through TCR while CD28 costimulation is blocked by CTLA4-Ig, a recombinant protein composed of the Fc region of an IgG fused to the extracellular domain of CTLA4, results in T-cell anergy. Preclinical canine studies have demonstrated that, in the setting of MHC-matched allo-HSCT, pre-transplant infusions of donor peripheral blood mononuclear cells in the presence of CTLA4-Ig could reduce host T-cell responsiveness to donor antigens and therefore could further prevent graft rejection [98]. However, this strategy was not efficient in the setting of MHC-haploidentical transplantation [99], probably because recipient residual NK cells are not inhibited by CTLA4-Ig. Several CTLA4-Ig agents are currently commercially available as treatment for autoimmune diseases (abatacept, belatacept and ASP-2408). However, to the authors' knowledge, there are no current registered clinical trials assessing CTLA4-Ig for GF prevention after allo-HSCT.

CD40:CD154 interaction is another pathway implicated in immune responses. CD40 is expressed on all conventional APC while CD154 (also called CD40 ligand or GP39) is preferentially expressed on $CD4^+$ T cells. The CD40:CD154 costimulation pathway is important for $CD4^+$ T-cell priming. Further, CD40:CD154 interactions also increase costimulatory activity on APC through induction of high CD80 and CD86 expression. In mice, transplantation of high doses of fully MHC-mismatched allogeneic BM cells, followed by an injection of both anti-CD154 monoclonal antibodies and CTLA4-Ig, resulted in stable multi-lineage chimerism [100]. Further, preclinical canine studies have demonstrated that GF could be avoided in a fraction of dogs after recipient exposure to donor PBMC in the presence of CD154 blocking agents when donor and recipients pairs were MHC-matched (Table 4) [101]. Several companies have developed anti-CD154 agents for clinical use (i.e., toralizumab and ruplizumab). Unfortunately, their development was suspended when these agents were found to precipitate arterial and venous thromboembolism, likely due to aggregation of CD154-expressing activated platelets [102].

Finally, Lambert *et al.* reported a strategy that allowed obtaining stable donor chimerism without any irradiation/chemotherapy in a MHC-mismatched murine model of transplantation [103]. Specifically, they observed that the establishment of an initial state of microchimerism after infusion of high dose of HSC with costimulatory blockade (CD40-ligand antibody blockade) alone induced tolerance and allowed the

Table 4. The preclinical canine model of engraftment/graft rejection.

Refs.	Conditioning (TBI dose (Gy))/other	Stem cell source	Post-grafting immunosuppression	# of dogs with stable engraftment (%)*/# of dogs transplanted
[120]	9.2	Marrow	None	20/21 (95%)
[121]	8.0	Marrow	None	4/5 (80%)
[121]	7.0	Marrow	None	3/5 (60%)
[121]	6.0	Marrow	None	12/23 (52%)
[121]	4.5	Marrow	None	16/39 (41%)
[122]	4.5	Marrow	CSP‡	7/7 (100%)
[42]	2.0	Marrow	CSP‡	0/4 (0%)
[42]	2.0	Marrow	MTX§ + CSP‡	2/5 (40%)
[42]	2.0	Marrow	MMF¶ + CSP‡	11/12 (92%)
[42]	2.0	Marrow	Rapa# + CSP‡	6/7 (86%)
[108]	2.0	Marrow	RAD001** + CSP‡	4/9 (44%)
[98]	1.0/PBMC CTLA4-Ig‡‡	Marrow	MMF¶ + CSP‡	4/6 (67%)
[101]	1.0/PBMC anti-CD154 Ab§§	Marrow	MMF¶ + CSP‡	3/6 (50%)
[42,106]	1.0	Marrow	MMF¶ (or Rapa#) + CSP‡	0/11 (0%)
[43]	1.0	Marrow	MMF¶ + CSP‡ + Rapa#	1/8 (12%)
[53]	1.0	Marrow + PBSC	CSP‡	5/8 (63%)
[123]	1.0/FTY720¶¶	Marrow	MMF¶ + CSP‡	0/5 (0%)
[44]	1.0/ATG##	Marrow	MMF¶ + CSP‡	1/5 (20%)
[90]	1.0/Pentostatin***	Marrow	MMF¶ + CSP‡	4/10 (40%)
[113]	1.0	Marrow + MSC‡‡‡	MMF¶ + CSP‡	0/4 (0%)
[63]	1.0	Marrow + DLI§§§	MMF¶ + CSP‡	3/9 (33%)
[96]	0.0/TcRαβ¶¶¶	Marrow	MMF¶ + CSP‡	4/4 (100%)

Effect of conditioning and post-grafting immunosuppression on engraftment of DLA-identical grafts.
Adapted from [124].
*Mixed or full chimerism.
‡CSP, 15 mg/kg b.i.d. p.o., days -1 to 35.
§Methotrexate, 0.4 mg/kg i.v. on days 1, 3, 6 and 11.
¶MMF, 10 mg/kg b.i.d. s.c., days 0 – 27.
#Rapamycin (sirolimus), 0.05 mg/kg b.i.d. s.c., days 0 – 27.
**Everolimus 0.25 mg twice a day orally from day 0 to +27.
‡‡10^6 PBMC/kg/day i.v. days -7 to -1; CTLA4-Ig, 4 mg/kg/d i.v. days -7 to -1.
§§Single intravenous injection of 5 mg/kg anti-CD154 antibody (on day -5), followed 1 day later by donor PBMC.
¶¶FTY720, 5 mg/kg/day, days -5 and -4.
##Anti-thymocyte globulins, 3.5 – 5.0 mg/kg total dose administered from day -12 to day -7.
***Pentostatin 6 × 4 mg/m^2 (n = 8) or 3 × 4 mg/m^2 (n = 2).
‡‡‡MSC were injected i.v. at concentrations of 1.2 – 1.8 × 10^6 cells/kg on days 0 and 35 after transplantation.
§§§A single DLI was given 28 – 36 days after HCT, either with (n = 5) or without (n = 4) preceding treatment with pentostatin.
¶¶¶Administration of 0.13 – 0.46 mg/kg TcRαβ mAb labeled with 3.7 – 5.6 mCi/kg (137 – 207 MBq/kg) ^{213}Bi on days -3 and -2.
CSR: Cyclosporine; DLA: Dog leukocyte antigen; DLI: Donor lymphocyte infusion; HCT: Hematopoietic cell transplantation; MMF: Mycophenolate mofetil; MSC: Mesenchymal stromal cells; PBSC: Granulocyte-colony stimulating factor mobilized peripheral blood mononuclear cells; TBI: Total body irradiation.

establishment of high donor chimerism (macrochimerism) through subsequent HSC infusions.

5.2.1.4 Prevention of NK-cell-mediated graft rejection

As mentioned above, residual host NK cells play a major role in the pathogenesis of graft rejection after HLA-mismatched transplantation. Thus, targeting host NK-cell function could be useful in that setting. It is well established that NK cells require direct cell-to-cell interactions with targeted cells to mediate cytotoxicity. One of the most important molecules for NK/target cell interactions is LFA-1, a heterodimer of integrins that interact with ICAM-1 [104]. Based on these concepts, Kean *et al.* investigated whether LFA-1/ICAM-1 blockade could prevent NK-cell-mediated graft rejection. Interestingly, blockade of LFA-1/ICAM-1 increased HSC engraftment in a murine model of MHC-mismatched allo-HSCT with CD28/CD40 costimulation blockade as the sole conditioning regimen [104]. Therefore, combining anti-host NK-cell treatments with costimulation blockade agents may become a promising strategy for minimally toxic conditioning regimens enabling HSC engraftment after HLA-mismatched allo-HSCT, for example in cases of non-malignant diseases.

5.2.2 Post-grafting immunosuppression

Sirolimus (formerly rapamycin) is an inhibitor of the mTOR (mammalian target of rapamycin). mTOR inhibition decreases effector T-cell proliferation and activities through

several biochemical pathways [105]. It also inhibits dendritic cells and promotes the induction of regulatory T cells (Tregs) through FoxP3 expression. The impact of sirolimus on GF prevention has been assessed in a preclinical canine model of non-myeloablative transplantation. While all dogs given MHC-identical BM after conditioning with 2 Gy TBI and cyclosporine alone as post-grafting immunosuppression experienced graft rejection, 83% of dogs given cyclosporine with sirolimus as post-grafting immunosuppression achieved stable long-term engraftment [106]. Since, sirolimus has been assessed in Phase II clinical trials in patients given grafts following RIC regimens and has shown encouraging results in terms of engraftment, GVHD and anti-tumor responses [107]. The role of sirolimus in non-myeloablative allo-HSCT is currently under investigation in a multicenter Phase II randomized study (ClinicalTrials.gov #NCT01428973). Further, sirolimus-based peritransplant immunosuppression is under investigation in the setting of RIC allo-HSCT for non-malignant disorders, with the aim of favoring stable engraftment while minimizing the toxicity of the preparative regimen (ClinicalTrials.gov #NCT01499888, #NCT00061568, #NCT00426517, #NCT 00923364, #NCT00352976).

Everolimus (RAD001) is a novel mTOR inhibitor. In the preclinical canine model of RIC transplantation, the combination of cyclosporine with RAD001 was less efficient than the combination of cyclosporine and sirolimus to promote stable long-term engraftment [108].

5.2.3 Cellular therapies

A number of studies have demonstrated that MSC not only supports hematopoiesis but also exhibits strong immunosuppressive properties both *in vitro* and *in vivo* [109]. These observations led to the development of a number of Phase I – II clinical trials aimed at assessing the impact of MSC co-transplantation on engraftment in patients given unrelated HLA-mismatched PBSC grafts after RIC regimens [110], HLA-haploidentical T-cell-depleted transplants [111] or unrelated CB grafts [112]. While MSC co-transplantation was efficient to promote engraftment in patients given HLA-haploidentical T-cell-depleted grafts [111], they failed to prevent graft rejection in patients given unrelated HLA-mismatched PBSC after RIC and in those given unrelated CB [110,112]. These results are in agreement with those observed in the preclinical canine model of RIC transplantation [113].

Preclinical studies in mice have demonstrated that co-transplantation of donor-derived veto cells (defined as cells able to selectively suppress T cells directed against their antigens) [114] or of facilitating cells (phenotypically characterized as a $CD8\alpha^+$ cell population devoid of any conventional surface $\alpha\beta$- and $\gamma\delta$-TCR) [115] could promote engraftment. However, to the authors' knowledge, these cells are not yet under investigation in clinical trials, although one might argue that transplantation of megadoses of purified stem cells act in part by a 'veto' effect [114].

5.2.4 Prevention of antibody-mediated graft rejection

The role of pre-existing antibodies in the pathogenesis of graft rejection is increasingly recognized. In 2007, Taylor *et al.* demonstrated, in a murine model of allo-HSCT, that preformed antibodies were the main driver of MHC-disparate HSC graft rejection in allosensitized recipients [116]. Among pre-existing antibodies, anti-HLA antibodies have been widely studied because of their implication in solid organ graft rejection [117]. The development of Luminex™ testing for anti-HLA antibodies has allowed to assess their impact in GF. Importantly, the presence of donor-specific anti-HLA antibodies (DSHAs) was associated with a dramatically higher risk of graft rejection in the setting of HLA-mismatched unrelated allo-HSCT (hazard ratio (HR) = 23, $p < 0.001$) [38], unrelated CB transplantation (HR = 4.3, $p = 0.001$) [118] and HLA-haploidentical transplantation ($p = 0.008$) [119]. The clinical consequence of these observations is that Luminex testing for DSHAs should be performed before all HLA-mismatched allo-HSCT [117]. Immunoadsorption, plasmapheresis and perhaps rituximab administration might be efficient to desensitize the recipient when there is no alternative donor.

6. Potential developments

Future efforts to enhance homing and proliferative properties of HSC will likely need more in-depth understanding of intracellular signaling pathways involved in HSC self-renewal, proliferation, survival, differentiation and migration. Further knowledge on the BM microenvironment and its interactions with HSC will also probably permit the development of effective therapeutic interventions to enhance engraftment. Similarly, further insights about immune cell modulation remain crucial to identify new targets to prevent graft rejection without compromising immune recovery and graft-versus-tumor effects or increasing the risk of GVHD after allo-HSCT. Despite several decades of studies, HSC and immune cell expansion cultures are still challenging as cells may prove unstable or lose their physiological properties after *in vitro* expansion. New protocols and new technologies will be needed to optimize cell culture.

A new field for allo-HSCT is the induction of immune tolerance for solid organ transplantation and for some autoimmune disorders. However, the routine application of allo-HSCT in such indications remains currently elusive as conditioning regimens are still too toxic. Thus, development of new drugs that will allow allogeneic HSC engraftment with minimally toxic conditioning in patients with non-malignant diseases will also be a challenge for the future.

7. Conclusion

The risk of GF might be reduced in the future by optimizing the conditioning regimens, increasing the dose of cells (and particularly of HSC) transplanted, improving post-grafting immunosuppression and better detecting patients at high

risk of GF by searching for pre-transplant DSHAs in patients given grafts from HLA-mismatched donors, or by closely monitoring donor T-cell chimerism after allo-HSCT following RIC.

8. Expert opinion

Promising approaches for optimizing the conditioning regimen include the use of monoclonal antibodies (such as anti-CD45) conjugated with radionuclides to clear BM niches and the induction of recipient-versus-donor anergy through costimulation blockade. Most improvements in the field of post-grafting immunosuppression include the development of new mTOR inhibitors. The Luminex technology has allowed the systematic detection of DSHAs in patients given grafts from HLA-mismatched donors, while improvements in STR PCR kits allow close monitoring of donor chimerism in virtually all patients. Finally, the main hot topic for increasing the dose of infused HSC in CB allo-HSCT include double unrelated CB transplantation with one of the two units expanded *ex vivo*, for example, with the engineered form of the Notch ligand Delta-1 or with MSC.

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