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REVIEW

Novel approaches for preventing acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation

Sophie Servais[a,b], Yves Beguin[a,b], Loic Delens[b], Grégory Ehx[b], Gilles Fransolet[b], Muriel Hannon[b], Evelyne Willems[a], Stéphanie Humblet-Baron[c,d], Ludovic Belle[b] and Frédéric Baron[a,b]

[a]Division of Hematology, Department of Medicine, University and CHU of Liège, Liège, Belgium; [b]GIGA I[3], University of Liège, Liège, Belgium; [c]Translational Immunology Laboratory, VIB, Leuven, Belgium; [d]Department of Microbiology and Immunology, KUL-University of Leuven, Leuven, Belgium

ABSTRACT

Introduction: Allogeneic hematopoietic stem cell transplantation (alloHSCT) offers potential curative treatment for a wide range of malignant and nonmalignant hematological disorders. However, its success may be limited by post-transplant acute graft-versus-host disease (aGVHD), a systemic syndrome in which donor's immune cells attack healthy tissues in the immunocompromised host. aGVHD is one of the main causes of morbidity and mortality after alloHSCT. Despite standard GVHD prophylaxis regimens, aGVHD still develops in approximately 40–60% of alloHSCT recipients.

Areas covered: In this review, after a brief summary of current knowledge on the pathogenesis of aGVHD, the authors review the current combination of a calcineurin inhibitor with an antimetabolite with or without added anti-thymocyte globulin (ATG) and emerging strategies for GVHD prevention.

Expert opinion: A new understanding of the involvement of cytokines, intracellular signaling pathways, epigenetics and immunoregulatory cells in GVHD pathogenesis will lead to new standards for aGVHD prophylaxis allowing better prevention of severe aGVHD without affecting graft-versus-tumor effects.

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1. Introduction

Allogeneic hematopoietic stem cell transplantation (alloHSCT) offers potential curative treatment for a wide range of hematological disorders.[1] In patients with hematological malignancies, tumor eradication depends both on the conditioning regimen given before alloHSCT, and on graft-versus-tumor effects mediated by donor immune cells (mainly T cells) contained in the graft.[2] Several conditioning regimens have been developed ranging from high-dose myeloablative regimens to reduced-intensity conditioning (RIC) or truly nonmyeloablative conditionings.[3,4] Myeloablative conditionings result in complete ablation of host hematopoiesis. Nonmyeloablative and RIC regimens exert only moderate myelosuppression but provide sufficient immune suppression to allow sustained donor T-cell engraftment and tumor eradication through graft-versus-tumor effects. They have been developed to reduce transplant-related mortality, allowing to perform alloHSCT in older patients and in those with medical comorbidities.

Three different sources of stem cells can be used for alloHSCT: bone marrow (BM), granulocyte-colony stimulating factor (G-CSF) mobilized peripheral blood stem cells (PBSCs), and umbilical cord blood (UCB).[5] The ideal donor is a human leukocyte antigen (HLA)-identical sibling. When such a donor is not available, alternative donors can be HLA-matched or 1–2/10 HLA-mismatched unrelated donors, HLA-haploidentical-related (haplo) donors (mismatched for 1 of the 2 HLA haplotypes), or UCB. While outcomes after alloHSCT with HLA-matched unrelated donors are currently similar to those achieved with HLA-identical siblings, HLA-mismatched alloHSCT as well as UCB-alloHSCT remain associated with higher transplant-related and mortality.[5] HLA-haploidentical alloHSCT has historically been limited by high transplant-related mortality.[6] However, several recent approaches, including effective *ex vivo* and *in vivo* T-cell depletion strategies (see later), enabled achieving very favorable outcomes in that setting.[6]

Although donor T cells contained in the graft are the main driver of graft-versus-tumor effects, they can also mediate harmful damages to host healthy tissues causing graft-versus-host disease (GVHD). GVHD is one of the main complications of alloHSCT. GVHD has been historically separated into two syndromes: acute GVHD (aGVHD), occurring within 100 days after transplantation, and chronic GVHD (cGVHD) developing thereafter. This classification was simple but did not rely on actual biological or clinical bases. Currently, it is widely accepted that aGVHD and cGVHD have specific clinical presentation and pathogenesis.[7–9] In this article, we will focus on aGVHD.

Clinically, aGVHD manifests as strong inflammatory lesions mainly of the skin, gut, and liver. AGVHD can be scored from grade I to IV, according to the severity of organ signs and dysfunctions.[10] Clinically significant grade II–IV aGVHD is a major cause of transplant-related morbidity after alloHSCT while mild aGVHD (grade I) is associated with better survival due to lower risk of disease relapse (translating higher graft-

CONTACT Frédéric Baron f.baron@ulg.ac.be Division of Hematology, Department of Medicine, CHU Sart-Tilman, University of Liège, 4000 Liège, Belgium

Article highlights

- Three randomized studies have demonstrated that ATG (combined with CSA and MTX) might be the new standard of care for patients transplanted with HLA-matched PBSC.
- New understanding of aGVHD pathogenesis has led to the development of new targets for aGVHD prophylaxis.
- Most promising pharmacological approaches include post-transplant Cy administration, JAKs inhibitors, HDAC inhibitors, bortezomib, hypomethylating agents, as well as IL-6 blockade.
- Most promising cellular approaches include co-transplantation of Tregs.
- Risk-stratification directed strategies for aGVHD prevention will likely help to improve aGVHD-related morbidity and mortality.

This box summarizes key points contained in the article

versus-tumor effects).[11–13] Thymic and BM niches can also be significantly damaged by aGVHD,[14,15] further compromising efficient T- and B-cell reconstitution and predisposing patients to cGVHD.[16] Hence, prevention strategy to avoid aGVHD occurrence remains a major challenge of alloHSCT.[17]

2. Pathogenesis of aGVHD

AGVHD immunopathophysiology is complex. Insights from experimental models and clinical translational research have enabled the identification of a complex immune network of triggers, sensors, mediators and effectors of aGVHD reactions, as well as tolerogenic actors that can mitigate the process. [7,8,17–20] Here is presented a brief summary of some key mechanisms, in the attempt to better understand the rationale of novel approaches for aGVHD prevention (Figure 1).

It is commonly accepted that aGVHD results from the detection of host disparate antigens by donor T cells. The primordial role of T cells in aGVHD pathogenesis is demonstrated by the low incidence of GVHD observed in patients given T-cell depleted alloHSCT.[21] Under most circumstances, including aGVHD, adaptive T-cell responses are primed by the innate immune system. By expressing pattern recognition receptors (such as Toll-like and nucleotide oligomerization domain (NOD)-like receptors), innate immune cells may recognize danger signals released by damaged tissues

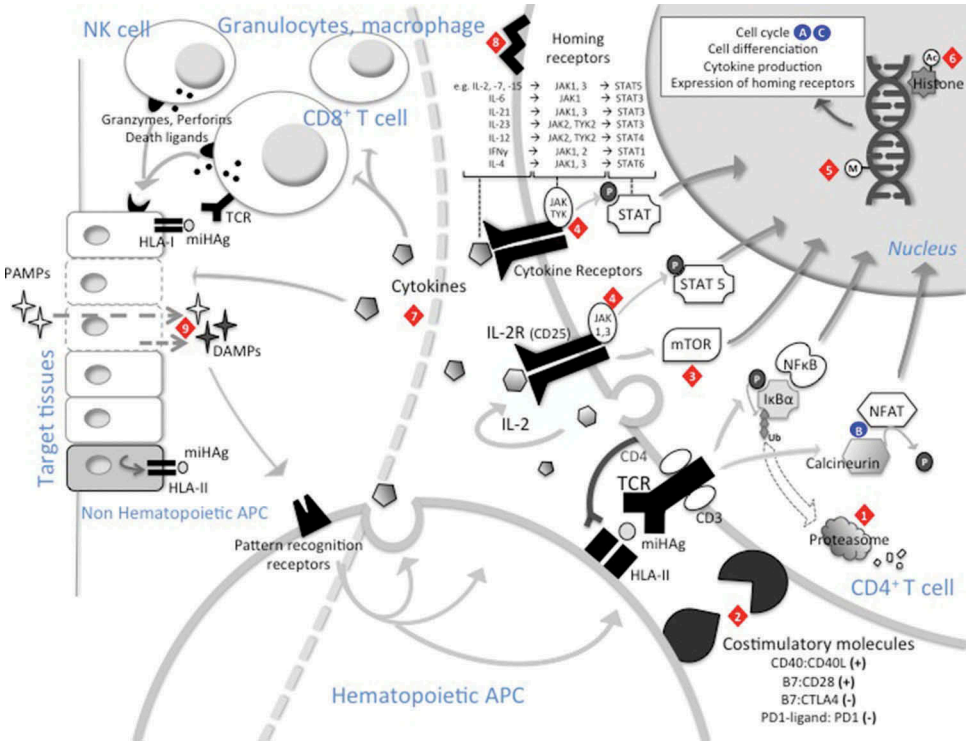

Figure 1. Targets of standard and emerging therapies for the prevention of aGVHD.
Events presented on the left side of the central discontinued grey line mostly occur in peripheral tissues, whereas those presented on the right side mostly occur in secondary lymphoid organs.
Sites of action of standard prophylaxis regimens are depicted in circles (A = methotrexate; B = calcineurin inhibitors; C = mycophenolate mofetil) while sites of action of emerging strategies are depicted in losanges (1 = proteasome inhibitors, 2 = CTLA4-Immunoglobulin; 3 = mTOR inhibitors; 4 = JAK inhibitors; 5 = hypomethylating agents; 6 = histone deacetylase inhibitors; 7 = Cytokine antagonists, such as anti-IL-6 receptor antibody; 8 = homing receptor antagonists, such as anti-CCR5; 9 = strategies aiming at decreasing the production of DAMPs or PAMPs, such as modulation of gut microbiota or alpha-1 anti-trypsine supplementation. Note that hypomethylating agents and histone deacetylase inhibitors can also act as epigenetic modulators in other immune cells than $CD4^+$ T cells, such as in $CD8^+$ T cells, Tregs, and APCs. Concerning JAK/STAT pathways, STAT3 is a key transcription factor for type 17 cell differentiation whereas STAT4 and STAT6 are more implicated in type 1 and type 2 T-cell differentiation, respectively.
Ac: acetyl; APC: antigen-presenting cell; DAMPs: damage-associated molecular patterns; HLA: human leukocyte antigen; IL: interleukin; IL-2R: IL-2 receptor; M: methyl; miHAg: minor histocompatibility antigen; NK cells: Natural killer cells; P: phosphate; PAMPs: pathogen-associated molecular patterns; TCR: T-cell receptor; TYK2: tyrosine kinase 2; Ub: ubiquitine.

(damage-associated molecular patterns [DAMPs]) and/or by pathogens (pathogen-associated molecular patterns [PAMPs]) that further activate them. In humans, single nucleotide polymorphisms of some pattern recognition receptors (such as NOD-like receptor NOD2/CARD15 variants) in the recipient and/or the donor have been reported to be associated with aGVHD.[22] Further, several DAMPs and PAMPs have also been identified in aGVHD. The most well known are heparan sulfate (an extracellular matrix component), adenosine triphosphate (ATP) and uric acid (both released by dying cells), and lipopolysaccharide (mostly from the gut microbiota). These molecules can be released during conditioning regimen. In this way, conditioning regimens may prime host innate immune cells before donor T-cell infusion.

There is also increasing evidence of roles of the commensal microbiota in aGVHD pathophysiology. Indeed, several studies demonstrated that early microbiome shifts and loss of microbiome diversity after alloHSCT were associated with higher risks of aGVHD.[23–25]

Activated antigen presenting cells (APCs) present host alloantigens on HLA molecules to donor T cells. They also provide them costimulatory signals that are necessary to induce their proliferation and differentiation after T-cell receptor (TCR) activation by antigens. In most cases, TCR stimulation without second signal through costimulatory molecules leads to T-cell anergy. Many costimulatory ligand/receptor interactions have been reported to be involved in aGVHD.[26] The most extensively studied involve interactions between B7 molecules (CD80 and CD86) on APCs and CD28 on T cells, as well as between CD40 on APCs and CD40 ligand on activated T cells. The former create a more complex system, with both positive (B7: CD28) and inhibitory (B7: cytotoxic T lymphocyte antigen 4 (CTLA-4)) pathways. T-cell activity may also be counter-regulated by inhibitory molecules, such as programmed cell death protein 1 (PD1). Finally, APCs provide a third proliferative signal to alloreactive T cells through the release of cytokines, such as IL-7 and IL-15, and are also implicated in helper T (Th) cell polarization.[27]

Interactions between T cell and APC trigger multiple and complex intracellular signaling pathways in both cells. In T cell, TCR stimulation induces calcium-dependent signal transduction leading to the activation of calcineurin. Calcineurin dephosphorylates the nuclear factor of activated T cell (NFAT) proteins and enables their import to the nucleus, where they induce transcription of interleukin (IL)-2 and other cytokines that ultimately lead to T-cell proliferation. The nuclear factor kappa B (NFκB) pathway is also activated downstream to TCR stimulation. NFκB is normally located in the cytosol in an inactivated state, complexed with the inhibitory protein IκBα. TCR activation induces phosphorylation of IκBα, which results in its ubiquitination and degradation by the proteasome. Dissociated from IκBα, activated NFκB then translocates into the nucleus where it binds to specific sequences of DNA leading to transcription of cytokines (predominantly IL-2), the high-affinity IL-2 receptor (CD25), and of costimulation molecules. Hence, T-cell activation through TCR leads to the autocrine secretion of IL-2 and to the expression of IL-2 receptor. IL-2 is a key cytokine for T-cell proliferation, differentiation, and survival. The stimulation of IL-2 receptor at the surface of activated conventional T cells (Tconvs) can activate the mammalian target of the rapamycin (mTOR) as well as Janus kinases (JAK) and signal transducers and activators of transcription (STAT) (precisely JAK1/STAT5 and JAK3/STAT5), which both regulate cell cycle and lead to T-cell proliferation. JAK/STAT pathways may also be critical for determining T-cell differentiation (Figure 1).[28] Further, importantly, the mTOR pathway is constitutively inactivated in regulatory T cells (Tregs), that depends mainly on the STAT5 pathway for signaling upon IL-2 receptor stimulation.[29]

Both donor $CD4^+$ and $CD8^+$ T cells are crucial in the pathogenesis of GVHD. Studies in mice suggested that naive T cells are the primary drivers of aGVHD reactions, while effector memory T cells are less prone to mediate GVHD but participate in transfer of anti-pathogen functional memory.[30] Naive T cells can differentiate into various lineages (such as the well-established type 1, type 2, and type 17 T-cell lineages) in the presence of specific cytokines (i.e. IL-12, IL-4, and IL-6, respectively). Recent data suggest that aGVHD within individual organs is preferentially generated by specific T-cell subsets, in part because of their respective chemokine profiles and the relative sensitivity of targeted tissues to their effector cytokines. Specifically, type 1 cells seem to be preferentially implicated in gastrointestinal, type 2 cells in cutaneous and hepatic, and type 17 cells in cutaneous and pulmonary aGVHD.[18,31,32] Newly identified T-cell subsets (including type 9, type 22, and T follicular helper cells) add further complexity.

Tissue homing molecules and receptors are important in aGVHD immunobiology, since they orchestrate alloreactive T-cell (as well as Treg) trafficking toward target organs.[33] Among them, CCR5 was reported to be involved in T-cell migration to both lymph nodes and gastrointestinal tract in murine models.

Activated APCs and T cells initiate an immune response and a cytokine storm that further recruit additional immune cells, amplifying the phenomenon and leading to the effector phase of aGVHD in target organs. The effector phase involves cellular effectors (such as cytotoxic $CD8^+$ T cells, natural killer (NK) cells, neutrophils, and activated macrophages) and inflammatory molecules (mainly IL-1β, tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and reactive oxygen species) that lead to antigen-dependent and -independent destruction of host tissues. It is well demonstrated that regulatory/tolerogeneic cells can control this destructive machinery of immune cells. Intense investigations have focused on Tregs. Tregs represent a fraction of $CD4^+$ T cells that are indispensable for maintaining immunological self-tolerance.[34,35] They express the forkhead box protein 3 factor (FoxP3) in their nuclei, and CD25 (the high-affinity component of the trimeric form of the IL-2 receptor) on their surface.[34,35] Tregs are also featured by a dependency on IL-2 for their homeostasis.[35,36] Importantly, several studies have demonstrated that Treg co-transplantation at a Treg/$CD4^+$Tconv ratio of 1/1 or 1/2 prevented aGVHD in mouse to mouse [37,38] as well as in humanized mice [39,40] models of aGVHD. In humans, Treg infusion also resulted in promising results for preventing aGVHD (see later).

Mesenchymal stem cells (MSCs) are multipotent progenitor cells with fibroblastic-like morphology that exert several

immunomodulatory effects on both adaptive and innate immune cells.[41–43] MSC can inhibit T-cell proliferation by secretion of soluble mediators (i.e. TGF-β, IL-10, IDO, etc.) and by cell contact interactions with molecules expressed on their surface (i.e. PD1–ligand, etc.). Furthermore, MSC might also promote the generation of tolerogenic dendritic cells and of Tregs. Finally, MSC are hypoimmunogenic, allowing transfer across HLA barriers.

Several other cells have also been reported to mitigate GVHD, such as invariant natural killer T (iNKT) cells,[44–46] myeloid-derived suppressor cells (MDSCs),[47,48] and more recently $CD34^+$ regulatory monocytes.[49]

Finally, controversies still remain about implication of B cells in the pathogenesis of aGVHD, with data from preclinical and clinical studies showing both potential pathogenic as well as protective effects.[50] In mice, the B-cell protective activity appears to be mediated by their secretion of IL-10. Recently, impairment in IL-10-producing B cells (regulatory B cells, Breg) was also described in patients with cGVHD.[51]

3. Current approaches for aGVHD prevention

3.1. Standard approaches: antimetabolites and calcineurin inhibitors

Since the 1980s, standard GVHD prophylaxis for patients given myeloablative alloHSCT from HLA-matched sibling or unrelated donor has consisted of the combination of the folate antagonist methotrexate (MTX, which deletes proliferating lymphocytes) with a calcineurin inhibitor (either cyclosporin A, CSA or tacrolimus, FK506, tacro).[52,53] There is no consensus about what it the best calcineurin inhibitor between CSA and tacro. Compared to the association of MTX and CSA, the combination of MTX and tacro is associated with a 40% lower risk of aGVHD, but similar incidence of cGVHD and comparable mortality.[54] Mycophenolate mofetil (MMF) has also shown synergistic activities with calcineurin inhibitors for preventing aGVHD.[55] Its metabolite, mycophenolic acid, inhibits lymphocyte proliferation by blocking *de novo* synthesis of guanosine nucleotides. MMF is currently commonly used instead of MTX in combination with either CSA or tacro for GVHD prophylaxis in the settings of nonmyeloablative/RIC-alloHSCT [52,56,57] and UCB-alloHSCT.[58] Although GVHD prevention does not seem to be improved by the use of MMF rather than MTX in RIC-alloHSCT, MMF-based regimens have demonstrated a more favorable toxicity profile.[52,56] Further, in the UCB-alloHSCT setting, the combination of CSA and MMF is associated with a lower incidence of cGVHD than the association of CSA and steroids.[58] Mechanisms of action of these standard molecules are schematized in Figure 1.

3.2. T-cell depleting approaches

Although combinations of calcineurin inhibitors with MTX or MMF have been relatively successful at preventing the most severe forms of aGVHD, these regimens are not uniformly effective and mostly fail to prevent the development of moderate/severe cGVHD, particularly after alloHSCT with PBSC or with unrelated donors.[59,60] This prompted number of groups to investigate the addition of pre-transplant anti-T-cell antibodies to standard prophylaxis regimens, in the attempt to induce *in vivo* depletion of donor T cells. Anti-T-cell globulins (ATG) are polyclonal IgG antibody preparations from horses or rabbits (the later being the most frequently used for GVHD prevention) that were immunized with human thymocytes or the T-cell line Jurkat. Rabbit ATG induces *in vivo* T-cell depletion through several cell-death mechanisms (complement-dependent lysis and activation-associated apoptosis) and induce the generation of Tregs.[29,61] Several studies have reported that ATG more specifically induces profound and prolonged depletion in the naive compartment of T cells.[46,62] Three phase III randomized trials have reported that pre-transplant ATG successfully decreased the incidence of both a- and cGVHD without increasing the risk of relapse after myeloablative alloHSCT with related [63] or unrelated PBSC.[64,65] In RIC setting, the role of ATG has not been formally established yet. Indeed one registry study including data from patients transplanted for various hematological malignancies found a higher risk of relapse and of overall mortality in patients given ATG,[66] while another study focusing on patients with acute myeloid leukemia given PBSC from HLA-identical siblings found lower incidence of cGVHD but similar risks of relapse and mortality in patients given ATG in comparison to patients not given *in vivo* T-cell depletion.[67] Finally, recent reports have observed that ATG increased the risks of infection and mortality after UCB-alloHSCT.[68,69]

In vivo T-cell depletion with alemtuzumab, a humanized anti-CD52 IgG1 monoclonal antibody with both T- and B-cell-depleting properties, has also been studied, mostly in the setting of RIC-alloHSCT.[70,71] CD52 antigen is expressed on T, NK, and B cells, but not on hematopoietic stem cells. Pre-transplant infusion of alemtuzumab was shown to very efficiently prevent both a- and cGVHD.[66,67,70,71] However, potential limitations of alemtuzumab included delayed immune recovery, increased risk of infections, delayed achievement of full donor T-cell chimerism and the need to give pre-emptive donor lymphocyte infusions to prevent disease relapse in patients with persisting mixed donor chimerism or with evidence of minimal residual disease. One registry study including data from patients transplanted for various hematological malignancies found a higher risk of relapse and of overall mortality in patients given alemtuzumab compared to patients not given *in vivo* T-cell depletion,[66] while another study focusing on patients with acute myeloid leukemia given PBSC from HLA-identical siblings found lower incidence of cGVHD but similar risks of relapse and mortality in patients receiving alemtuzumab compared to patients not given *in vivo* T-cell depletion.[67]

In a related approach, *ex vivo* T-cell depletion of the graft has been evaluated to prevent GVHD. Although initial studies in BM-alloHSCT suggested that the beneficial effect of such an approach on GVHD prevention was offset by increased rates of graft failure, relapse, and infections,[72] two multicenter prospective phase II trial with PBSC as stem cell source, and with optimized techniques for *ex vivo* T-cell depletion (i.e. immunomagnetic positive $CD34^+$ cell selection) have suggested that this strategy efficiently prevented GVHD without apparently increasing the relapse incidence.[73,74] However, large

randomized studies are needed before this approach might become a standard of care for GVHD prophylaxis in the HLA-matched setting, and specifically to assess the impact of profound T-cell depletion of the graft on the incidence of relapse. Finally, infusion of megadoses of purified $CD34^+$ cells has been the basis for T-cell depleted HLA-haploidentical transplantation approaches.[21,75]

4. Novel approaches for aGVHD prevention

Despite GVHD prophylaxis with standard regimens, aGVHD still develops in approximately 40–60% of patients after alloHSCT, underlying the need for developing new approaches aimed at better preventing aGVHD. It is the matter of intense research since several years. Considering the large body of recent work in this field, citing all original studies in this review is elusive. Here, we are reviewing only a selection of them, which we feel are the most promising.

4.1. T-cell depleting approaches

4.1.1. Depletion of donor T cells

As mentioned in Table 1, strategies for *in vivo* lymphocyte depletion with ATG or alemtuzumab are now recognized as potent approaches to prevent both a- and cGVHD. Other anti-T-cell antibodies, such as anti-CD3 or anti-CD2 monoclonal antibodies (visilizumab and siplizumab, respectively) are currently under investigation (ClinicalTrials.gov#NCT00113646 with siplizumab), but does not seem to be superior to rabbit ATG for aGVHD prevention (#NCT00720629).

Pentostatin, a purine analog, may also induce *in vivo* lymphocyte depletion through inhibition of adenosine deaminase (ADA), thereby leading to lymphocyte apoptosis due to the cytotoxic accumulation of deoxyadenosine triphosphate. Since ADA enzymatic activity is greater in T cells than in B cells, pentostatin mostly depletes the T-cell compartment. Recently, a phase I–II controlled study was performed where patients undergoing unrelated or mismatched related donor alloHSCT received increasing doses (0–2 mg/m^2) of post-transplant pentostatin (on days 8, 15, 22, and 30 after alloHSCT) in combination with tacro/MTX for GVHD prevention.[76] The lowest aGVHD incidence was observed in patients receiving the 1.5 mg/m^2 dosing regimen (35.7% versus 55.6% in patients who did not receive pentostatin, $P = 0.085$).

Other approaches for deleting donor T cells in case of aGVHD rely on engineering T cells through the expression of suicide genes before their infusion into patients. Hence, if GVHD occurs, the *in vivo* selective destruction of donor T cells may be pharmacologically induced by specific molecules. The advantage of this technique is that it allows the occurrence of T-cell mediated graft-versus-tumor effects. This approach was initially developed by inducing the expression of the herpes simplex 1 virus thymidine kinase (HSV-TK) in T cells and by using Ganciclovir as a prodrug for elimination of HSV-TK-expressing T cells. A phase I–II multicenter non-randomized study of HSV-TK cell infusions after T-cell-depleted haploidentical alloHSCT suggested that this therapy was feasible and enhanced immune recovery while cases of GVHD could be controlled by activating the suicide gene.[77] Concern about the potential immunogenicity of HSV-TK protein led to the development of alternative suicide gene therapies. These include gene transfer of human CD20 and truncated human EGFR polypeptide into T cells, which confer them sensitivity to anti-CD20 and anti-EGFR antibodies, respectively. An inducible system based on a fusion protein comprised of an extracellular binding domain linked to human caspase-9 signaling domains to deliver apoptotic signals in response to a chemical inducer of dimerization (i.e. AP1903/Rimiducid) was also recently

Table 1. T-cell depleting approaches.

Drug or pathway	Main mechanism of action	Level of clinical evidence	Ongoing clinical studies for novel therapies (ClinicalTrials.gov)
Anti-T-cell globulin (ATG)	Donor T-cell depletion	Phase III [63,64]	Standard approach in the PBSC setting
Alemtuzumab	Depletion of $CD52^+$ cells (including donor T cells)	Phase I–II [71]	–
Ex vivo $CD34^+$ cell selection	*Ex vivo* depletion of graft's immune cells	Phase II [74]	–
Anti-CD2 monoclonal antibody (siplizumab)	Donor T-cell depletion	–	Phase II: #NCT00113646
Pentostatin	Apoptosis of donor T cells through inhibition of adenosine deaminase	Phase I–II [76]	Phase I: #NCT00096161
Suicide gene therapies: transducing T cells with herpes simplex 1 virus thymidine kinase (HSV-TK), CD20, EGF-R, inducible caspase (iCasp)	Donor T-cell depletion with specific molecules, i.e. ganciclovir, anti-CD20 antibodies, anti-EGFR antibodies, AP1903.	HSV-TK: Phase I–II [77] iCasp: phase I [78]	iCasp: phase I–II: #NCT01744223, #NCT02065869
Post-transplant cyclophosphamide (Cy)	Depletion of early proliferating alloreactive donor T cells after graft infusion	Phase II [79]	Cy combined with standard prophylaxis: phase II: #NCT01349101, #NCT01374841, #NCT02065154 Single-agent postransplant Cy: phase III: #NCT02345850
Ex vivo photodepletion of anti-host reactive donor T cells (TH9402, Kiadis)	Depletion of anti-host alloreactive donor T cells	Phase II [80]	Phase II: #NCT01794299
Ex vivo depletion of $TCR\alpha\beta^+$ donor T cells	Depletion of mature $TCR\alpha\beta^+$ donor T cells (preservation of donor-derived NK cells and $TCR\gamma\delta^+$ T cells)	$TCR\alpha\beta^+/CD19^+$ depletion: phase I–II [81]	$TCR\alpha\beta^+/CD19^+$ depletion: phase II–III: #NCT02323867, #NCT02600208 $TCR\alpha\beta^+$ depletion: phase I–III: #NCT02327351, #NCT02193880
Ex vivo depletion of $CD45RA^+$ naive T cells	Depletion of naive donor T cells, based on the postulate that they are the main T-cell subset mediating GVHD	Phase II [82]	Phase II: #NCT 00914940, #NCT02220985

developed and provided promising results in a phase I clinical trial in patients given T-cell depleted HLA-haploidentical grafts.[78]

4.1.2. Depletion of proliferating, activated, alloreactive donor T cells

Cyclophosphamide (Cy) is a nitrogen mustard alkylating agent that mostly kills rapidly proliferating cells. Hence, Cy administration early after transplant can be an efficient method to specifically delete proliferating alloreactive T-cell clones while sparing resting T cells and hematopoietic stem cells. Recent findings also suggested that Tregs are relatively preserved under Cy exposure, partly due to their higher expression of aldehyde dehydrogenase.[83] Based on this evidence, the John Hopkins University group investigated post-transplant administration of high-dose Cy as a novel strategy for GVHD prevention. They explored high-dose Cy (50 mg/kg, on days +3 and +4) in combination with tacro/MMF (both started after Cy administration) in patients transplanted with HLA-haploidentical BM after a non-myeloablative conditioning regimen combining fludarabine and 2 Gy total body irradiation (TBI). Remarkably, they reported a very low incidence of grade III–IV aGVHD (6%).[84] Similar results (10% incidence of grade III–IV aGVHD) were recently observed using similar postgrafting immunosuppression in the setting of myeloablative haploidentical BM-alloHSCT.[85]

Investigators at the John Hopkins further assessed the efficacy of high-dose Cy (50 mg/kg on days +3 and +4) as the sole post-transplant GVHD prophylaxis after myeloablative HLA-matched BM-alloHSCT, and reported a low incidence of grade III–IV aGVHD (10%).[79] A 3-arm phase 3 study is currently ongoing, comparing post-transplant Cy to standard tacro/MTX and to a $CD34^+$ cell graft selection approach for GVHD prophylaxis in HLA-matched myeloablative BM- or PBSC-alloHSCT (ClinicalTrials.gov#NCT02345850) (Table 1).

Another approach to selectively deplete anti-host reactive donor T cells has been developed.[86] It consists of *ex vivo* donor T-cell activation against host antigens, and their subsequent selective elimination through a photodynamic purging method. Specifically, donor T cells are cultured with allogeneic cells *in vitro* in the presence of the photosensitizer 4,5-dibromorhodamine methyl ester (TH9402). If donor T cells are activated, they retain TH9402, which becomes highly cytotoxic after exposure to a fluorescent-light scanning device. Interestingly, this method preserved pathogen- and tumor-specific T cells as well as Tregs. A multicenter phase II study is currently evaluating post-transplant adoptive transfer of donor lymphocytes selectively allodepleted of host-reactive T cells using this photodynamic therapy, in the setting of haploidentical donor alloHSCT (ClinicalTrials.gov#NCT01794299). Preliminary results were presented at the 2015 meeting of the American Society of Hematology and suggested that this approach is safe, and associated with a very low incidence of aGVHD (none of the 23 patients experienced grade III-IV aGVHD).[80]

4.1.3. Depletion of T-cell subtype(s)

Based on the observation that aGVHD could be prevented by CD8-depletion of the graft in a number of murine models, Nimer et al. assessed CD8-depletion of the graft as a way to prevent aGVHD without promoting infections or compromising graft-versus-tumor effects.[87] In a randomized study including 38 patients, CD8-depletion of BM from HLA-identical siblings effectively prevented the incidence of grade II–IV aGVHD (20% versus 80%, $P = 0.004$). Unfortunately, these findings were not confirmed in another phase II randomized study performed in patients given PBSC from HLA-matched related or unrelated donors after nonmyeloablative conditioning.[88]

Attempts to improve immune reconstitution and graft-versus-tumor effect after T-cell depleted alloHSCT recently led to the development of a new method of T-cell depletion based on the specific elimination of mature T cells carrying α and β chains of the TCR ($\alpha\beta^+$ T cells) and preservation of donor-derived NK cells and $TCR\gamma\delta^+$ T cells in the graft.[89] This approach was recently reported to efficiently prevent aGVHD after HLA-haploidentical alloHSCT, without any need for post-transplant pharmacologic prophylaxis [81] (Table 1).

Based on the postulate that GVHD-promoting T cells mainly reside within the naive compartment (see above), several groups have studied *ex vivo* graft depletion of naive donor T cells to limit aGVHD. One of them relies on $CD45RA^+$ cell immunomagnetic depletion. This strategy was recently assessed in a pilot trial in the setting of myeloablative PBSC-alloHSCT.[82] Unfortunately, no reduction in the incidence of aGVHD was observed as compared with historical controls, but the incidence of cGVHD was remarkably infrequent (9% versus 50% in historical controls). This approach is currently further assessed in a phase II study (ClinicalTrials.gov#NCT02220985).

4.2. Other pharmacological approaches

Main targets of the approaches represented in Table 2 are also shown in Figure 1.

4.2.1. Functional inhibition of donor T-cell activation

4.2.1.1. Inhibition of TCR-mediated signaling pathways.

Blockade of the calcium-dependent TCR signal transduction pathway with calcineurin inhibitors is universally used as standard GVHD prophylaxis. As mentioned above, signal transduction downstream to TCR activation also mediates through the NFκB pathway. Proteasome inhibitors, such as bortezomib, can block NFκB activation (by inhibiting the degradation of its inhibitory protein IκBα) and were reported to efficiently mitigate GVHD in preclinical studies.[99] Moreover, by inhibiting degradation of many other intracellular proteins, proteasome blockade may also affect T-cell chemotactism, inflammatory cytokine secretion, APC functions and promote Tregs.[99] Based on these observations, the early addition of short-course bortezomib (on days +1, +4, and +7 after alloHSCT) to standard tacro/MTX has been assessed in phase I–II clinical trials and provided encouraging results.[90,100] Combination of bortezomib with other agents, such as tacro and sirolimus (#NCT00670423) or post-transplant Cy (#NCT01860170), as well as use of the novel proteasome inhibitor carfilzomib (#NCT01991301) are currently under investigation.

A selective inhibitor of NFκB, PS-1145, has also been reported to efficiently prevent experimental aGVHD and to

Table 2. Other (non T-cell depleting) pharmacological approaches.

	Drug or pathway	Main mechanism of action	Level of clinical evidence	Ongoing clinical studies for novel therapies (ClinicalTrials.gov)
Standard approaches	Methotrexate (MTX)	Inhibition of T-cell proliferation by acting as folate antagonist	Phase II–III, in combination with a calcineurin inhibitor (reviewed in [52])	–
	Cyclosporin A	Inhibition of TCR-induced T-cell activation by blocking calcineurin	Phase II–III, in combination with MTX or MMF (reviewed in [52])	–
	Tacrolinus	Inhibition of TCR-induced T-cell activation by blocking calcineurin	Phase II–III, in combination with MTX or MMF (reviewed in [52])	–
	Mycophenolate mofetil	Inhibition of T-cell proliferation by blocking *de novo* synthesis of guanosine nucleotides	Phase II–III, in combination with a calcineurin inhibitor (reviewed in [52])	–
Novel approaches	*Functional inhibition of donor T-cell activation*			
	Proteasome inhibitors (bortezomib)	Inhibition of T-cell activation downstream TCR stimulation through inhibition of NFkB (additional effects on cytokine secretion, chemotactism, APC functions and Treg differentiation)	Early administration after alloHSCT: phase II [90]	Bortezomib: phase II: #NCT02208037 Carfilzomib: phase I: #NCT01991301
	CTLA-4 Ig (abatacept, belatacept)	Blockade of T-cell CD28 positive costimulatory signal	Phase II [91]	Phase II: #NCT01012492, #NCT01743131
	mTOR inhibitors (sirolimus)	Inhibition of cell-cycle progression in response to IL-2 in T cells	Phase III [92]	Phase II: #NCT01428973, #NCT01251575
	JAKs inhibitors (JAK1/2 inhibitor ruxolitinib, JAK3 inhibitor tofacitinib)	Blockade of cytokine-induced signal transduction in T cells	Retrospective study [93]	Ruxolitinib: phase I: #NCT02528877
	Epigenetic modulation in immune cells			
	Demethylating agents (5-azacytidine, decitabine)	Inhibition of activation and proliferation of alloreactive donor T cells, induction of Treg	Phase I–II [94]	5-Azacitdine: phase I–II: #NCT01747499, #NCT02204020, #NCT02458235, #NCT01541280, #NCT01835587, #NCT02017457 Decitabine: phase I–II: #NCT01758367
	Histone deacetylase inhibitors (vorinostat, givinostat)	Decreased secretion of inflammatory cytokines, increased expression of IDO by dendritic cells, suppression of innate and allo-stimulating functions of APCs, increase in Treg numbers	Phase I–II [95]	Vorinostat: phase II: #NCT01789255, NCT01790568
	Inhibition of signals mediated by extracellular mediators			
	Anti-IL-6 receptor antibody (tocilizumab)	Inhibition of IL-6-mediated effects	Phase I–II [96]	Phase II: #NCT02206035
	CCR5 inhibitor (maraviroc)	Inhibition of T-cell trafficking towards target organs	Phase I–II [97]	Phase II: #NCT01785810, #NCT02208037
	Recombinant urate-oxidase (rasburicase)	Oxidation of the uric acid (that acts as a DAMP) into an inactive soluble metabolite	Phase I [98]	–
	B-cell depletion			
	Anti-CD20 antibody (rituximab)	B-cell depletion	–	Phase II: #NCT01044745, #NCT01810926

have a superior therapeutic index compared with bortezomib, enabling prolonged administration. Hence, this molecule may constitute a viable therapeutic approach to reduce GVHD severity.[101]

4.2.1.2. Costimulation blockade. Costimulatory signal blockade to induce T-cell anergy has been the subject of several preclinical studies.[26] Most of them were dedicated to inhibition of B7:CD28 and CD40:CD40L interactions.

CTLA4-Ig (abatacept, belatacept) are recombinant proteins composed of the extracellular domain of CTLA4 with the Fc fragment of human immunoglobulin G1 (IgG1). CTLA4-Ig binds to B7 proteins (CD80 and CD86) expressed on the surface of APCs, thus making them unavailable for interaction with CD28 on T cells. Preclinical studies suggested that CTLA4-Ig could reduce GVHD.[26] Further, addition of abatacept to CSA/MTX standard aGVHD prophylaxis was investigated in a pilot clinical trial involving 10 patients transplanted from unrelated donors.[91] Only two patients developed grade II–IV acute GVHD. A phase II multicentre randomized trial of abatacept combined with calcineurin inhibition and MTX after unrelated donor alloHSCT is currently ongoing in the United States (ClinicalTrials.gov#NCT01743131).

Preclinical studies demonstrated that blockade of CD40: CD40L interactions could reduce GVHD.[102] However, the clinical application of anti-CD40L antibodies was prematurely suspended since these agents were found to precipitate arterial and venous thromboembolism, likely due to interactions with CD40L-expressing activated platelets and endothelial cells.

On the other hand, activation of inhibitory molecules (such as PD1) may also result in anergization of alloreactive T cells. Moreover, PD1 signaling could also convert Th1 cells into a regulatory phenotype.[103] However, major limitation of such approaches would be the probable simultaneous abrogation of graft-versus-tumor effects.

4.2.1.3. Inhibition of IL-2- and other cytokine-induced signal transduction pathways. Inhibition of T-cell proliferation in response to IL-2 by interfering with the mTOR pathway (with mTOR inhibitors, such as sirolimus (siro) or everolimus) has been explored as GVHD prevention for several years. Impact of mTOR inhibitors on cell-cycle progression predominates in Tconvs while preserving Treg that depend on the STAT-5 pathway for IL-2 signaling.[29,104] A recent large randomized phase III trial compared postgrafting immunosuppression with tacro/siro to tacro/MTX in patients transplanted from HLA-identical siblings after TBI-based myeloablative conditioning.[92] The authors observed similar aGVHD incidence and aGVHD-free survival (primary endpoint) but faster engraftment, and less mucosal toxicity with siro/tacro. In contrast, use of sirolimus in patients given myeloablative doses of busulfan is contraindicated because it is associated with high incidence of sinusoidal obstruction syndrome.[105] The use of siro for GVHD prophylaxis is also being explored in a randomized study in the setting of RIC-alloHSCT (ClinicalTrials.gov#NCT01428973). Blocking cytokine-induced signal transduction in T cells by interfering with JAK/STAT pathways is another emerging approach to attenuate GVHD (Figure 1). In preclinical models, inhibition of JAK3 (i.e. with tofacitinib [106]) or JAK1/2 (i.e. with ruxolitinib [107,108]) reduced aGVHD by impairing Th1 and Th17 differentiation, and by increasing Tregs. A recent retrospective multicenter study of 54 patients treated with ruxolitinib for cortico-refractory aGVHD reported encouraging results (81.5% overall response, including 46.3% complete responses).[93] Based on these data, ruxolitinib is currently being investigated for aGVHD prevention in a clinical trial (#NCT02528877).

4.2.2. Gene expression modulation in T cells and other immune cells (epigenetic modulators)

Gene expression is modulated through several epigenetic pathways. For example, DNA methylation of gene promoters can result in gene silencing. On the other hand, histone octamers are major structural protein complexes that package DNA into chromatin. Hence, covalent modification on the amino terminal of the core histones (i.e. through acetylation, methylation, ADP-ribosylation, phosphorylation, ubiquitylation) can also affect chromatin assembly and therefore gene accessibility for transcription. Epigenetic regulation of gene expression in immune cells plays major role in orchestrating their fate. Hence, DNA demethylation and histone deacetylation recently emerged as new promising strategies for GVHD prevention (Figure 1).

Demethylating agents, such as 5-azacytidine (5-aza) and 5-aza-2′-deoxycytidine (decitabine) can be incorporated into DNA and act as DNA methyltransferase (DNMT) inhibitors. They are currently largely used for the treatment of myelodysplastic syndromes and acute myeloid leukemia. Further, murine studies have demonstrated that demethylating agents prevented aGVHD by inhibiting the proliferation of Tconvs and by inducing Treg (through the demethylation of the FoxP3 gene/promoter).[109] De Lima et al. investigated low-dose 5-aza in the post-transplant setting in patients with acute myeloid leukemia or myelodysplastic syndrome, initially in an attempt to prevent relapse.[110] They reported encouraging results in terms of disease control, but also observed a low incidence of aGVHD. Recently, in a phase I–II study, Goodyear et al. reported that post-transplant 5-aza significantly increased Treg numbers but also induced cytotoxic $CD8^+$ T-cell response against several tumor antigens, thus potentially preventing GVHD without compromising graft-versus-tumor effects.[94] These promising results emphasize the need to continue exploring demethylating agents for GVHD prophylaxis in further prospective studies (Table 2).

Histone acetylation regulates gene expression by modulating DNA accessibility. Lysine residues at the amino-terminus of histones are deacetylated by histone deacetylase enzymes (HDACs). Hence, HDAC inhibition results in accumulation of hyperacetylated histones and thus decreases gene accessibility for transcription. Moreover, emerging data also demonstrate that HDACs can additionally target nonhistone cellular proteins (such as STAT3), thereby modulating their function and stability. Recently, HDAC inhibition with SAHA (vorinostat) and ITF2357 (givinostat), that both mostly target class I and II HDACs, have been shown to prevent experimental GVHD.[111] On this basis, Choi et al. performed a phase I–II study investigating the use of vorinostat (from day −10 to day +100) along with tacro/MMF for aGVHD prevention after related donor

alloHSCT, and reported a lower incidence of grade II–IV aGVHD than expected (22% grade II–IV).[95] In agreement with preclinical observations, treated patients had low serum levels of inflammatory cytokines, high levels of IDO and high Treg counts.[112] Phase II studies are currently exploring the addition of vorinostat to standard tacro-MTX in the unrelated donor alloHSCT setting (#NCT01789255, #NCT01790568). Importantly, it should be stressed that not all HDAC inhibitors have similar effects on GVHD and, for example, the potent HDAC inhibitor (LBH589, panobinostat) accelerated experimental GVHD.[113]

4.2.3. Targeting extracellular mediators

4.2.3.1. Targeting cytokines and cytokine receptors.

As mentioned above, IL-2 is an essential cytokine for T-cell homeostasis. Blockade of IL-2 signaling with anti-IL-2 receptor monoclonal antibodies (basiliximab and daclizumab) has been investigated in several studies for controlling GVHD. Unfortunately, IL-2 blockade increased GVHD-mortality in a phase III study,[114] perhaps because of the negative impact of IL-2 blockade on Tregs who depend on IL-2 for their homeostasis. In the opposite, a recent study has investigated the administration of ultralow dose IL-2 (100,000–200,000 IU/m^2 × 3 per week) after transplantation with the aim of promoting Tregs and preventing GVHD.[115] Authors observed that IL-2 was well tolerated, expanded Tregs, and was associated with a very low incidence of aGVHD (0/16 patients experienced grade II–IV aGVHD).

Murine models have demonstrated an important role of IL-6 in the pathophysiology of aGVHD.[116] Based on these data, IL-6 inhibition with tocilizumab, an anti-IL-6 receptor monoclonal antibody, has recently been assessed in a phase I–II clinical trial.[96] Adding tocilizumab to standard GVHD prophylaxis after HLA-matched donor alloHSCT resulted in low rate of grade II–IV aGVHD (12%) compared to historical controls.

TNF-α is another important cytokine in aGVHD pathogenesis. Previous clinical reports have shown that a large increase TNF-receptor-1 levels (a good surrogate for TNF-α levels) early after the conditioning regimen predicted aGVHD.[117,118] This evidence prompted investigations on the efficacy of TNF-α inhibition for GVHD prevention. Unfortunately, prospective studies showed that addition of TNF-α inhibitors (infliximab or etanercept) to standard GVHD failed to prevent aGVHD.[119] Similarly, a large phase III trial showed that blockade of the proinflammatory cytokine IL-1 failed to prevent GVHD.[120] Finally, IL-21 has recently emerged as a major inducer of Th1 and Th17 differentiation and while administration of IL-21 inhibitors attenuated GVHD in mice.[121] Several other cytokines (such as IL-17, IL-12, IL-23, IL-22, IL-27, etc.) have also been implicated in GVHD pathogenesis and their inhibition should also be evaluated in clinical trials.[27]

4.2.3.2. Targeting chemokine and chemokine receptors.

T-cell chemotaxis into target organs is mediated by chemokine- and homing-receptors. A recent phase I-II study investigated the addition of a CCR5 inhibitor (maraviroc) to tacro/MTX for GVHD prophylaxis and demonstrated low incidence of grade II–IV aGVHD (14.7%).[97] Additional phase II trials are currently ongoing (Table 2). Other molecules, such as integrins, also play pivotal roles in the homing of alloreactive cells to target organs. Tissue specificity shown by integrins make them interesting targets for future GVHD therapy.

4.2.3.3. Blocking "danger" signals.

Administration of alpha 1-antitrypsin was shown to reduce heparan sulfate levels and to prevent experimental GVHD.[122] Based on these observations, a pilot clinical trial of exogenous alpha 1-antitrypsin supplementation in steroid-refractory GVHD is underway (NCT01523821). However, to our knowledge, no study assessing this strategy for GVHD prevention is currently ongoing. Uric acid was also described as a potential DAMP in aGVHD. Rasburicase is a recombinant urate-oxidase enzyme that catalyzes the oxidation of uric acid into an inactive soluble metabolite. In a phase I study of myeloablative alloHSCT, Yeh et al. administrated rasburicase during the conditioning regimen (0.2 mg/kg for five consecutive days) and observed a low incidence of aGVHD (24% grade II–IV).[98]

In a similar concept, manipulation of gut microbiota to reduce PAMP production has also been explored as a way to attenuate GVHD. Gut decontamination with metronidazole or ciprofloxacin during the peritransplant period seemed to confer protection from aGVHD in patients given grafts from HLA-identical siblings.[123] However, recent data suggesting a link between loss of microbial diversity and intestinal aGVHD [23–25] are now challenging the concept of gut antibiotic decontaminating strategies. Indeed, increased abundance of gut commensal flora belonging to the Clostridiales and Blautia species has been reported to be associated with reduced lethal aGVHD and improved overall survival.[23–25] Hence, novel strategies aimed at preserving such a "protective flora" might help preventing the development of aGVHD.

4.2.4. Targeting B cells

As mentioned above, B-cell involvement in the pathogenesis of aGVHD has remained controversial. However, administration of the B-cell depleting agent rituximab before and after alloHSCT is currently being explored in clinical trials (#NCT01044745, #NCT01810926).

4.3. Cellular therapies to promote immune tolerance

Several cell subtypes are able to mediate immune tolerance. Preclinical and early clinical data suggest that infusion or modulation of immune regulatory cells will be important tools for preventing GVHD in the future (see Table 3).

4.3.1. Regulatory T cells (Tregs)

Experimental studies have clearly demonstrated that infusion of high doses of Tregs (one Treg for 1–2 CD4$^+$ Tconv infused) prevented murine and xenogeneic GVHD.[37,39] Based on these observations, Treg infusion has been evaluated in the clinical setting. Specifically, safety and efficacy of Treg infusions were evaluated in a phase I clinical trial including 23 patients given double UCB-alloHSCT.[124] In that study, post-transplant administration of escalated dose of *in vitro* expanded Tregs (0.1–30 × 10^5 Tregs/kg, on day +1, ± day +15) from a third-party UCB unit was safe and resulted in a

Table 3. Cellular approaches.

Drug or pathway	Main mechanism of action	Level of clinical evidence	Ongoing clinical studies (ClinicalTrials.gov)
Regulatory T cells (Tregs)			
Treg infusion	Promotion of immune tolerance	Phase I–II [75,124]	Phase I: #NCT01795573, #NCT01937468 Phase II: #NCT02118311
Low dose IL-2	Promotion of Treg expansion and immune tolerance	Phase I [115]	Phase I: #NCT01937468 Phase II: #NCT01927120
Mesenchymal stem cells (MSCs)			
MSC coinfusion	Promotion of Tregs	Phase I-II [125,126]	Phase II: #NCT01045382
Invariant natural killer T (iNKT) cells			
iNKT cell content in the graft	Promotion of immune tolerance	Retrospective study [127]	Phase 0: # NCT02194868
Conditioning regimen involving total lymphoid irradiation and ATG	Sparing of iNKT cells	Phase II [128,129]	Phase II: # NCT01566656, #NCT00896493
Myeloid-derived suppressor cells (MDSCs)			
MDSC infusion	Promotion of immune tolerance	Animal models [47,48]	–

decreased incidence of aGVHD as compared to historical controls (43% versus 61%, $P = 0.05$).[124] Another pilot study of 28 patients transplanted from HLA-haploidentical donors analyzed whether pretransplant Treg infusions could allow coinfusion of $CD34^+$ selected stem cells with Tconvs (to enhance engraftment and immune recovery) at a dose that otherwise causes fatal aGVHD.[130] In that study, Tregs were freshly isolated from donors and infused at a dose of 2×10^6/kg 4 days before alloHSCT. Only two patients developed grade II–IV aGVHD despite the absence of any postgrafting immunosuppressive therapy. More recently, the same group reported the results of the first 43 patients with acute leukemia given HLA-haploidentical Tregs as described earlier.[75] Only 6/41 patients (15%) developed grade II–IV aGVHD. At a median follow-up of 46 months, only 2/41 evaluable patients had relapsed, suggesting that this strategy of Treg + Tconv infusion in the HLA-haploidentical setting increased graft-versus-tumor effects without increasing GVHD.

Further efforts are directed at improving Treg manufacturing and at determining which are the best Treg subsets for Treg infusion.

4.3.2. MSCs

MSC may harbor a wide range of immunosuppressive properties. Moreover, due to the absence of HLA class I molecules on their surface, MSC are hypoimmunogenic and can therefore be transferred from third-party HLA-mismatched donors. A number of preclinical studies using various animal models have assessed the efficacy of MSC to mitigate GVHD.[42] Results were variable, with some studies having reported benefits [131] while others having not.[132] Various factors, including cell dose, timing of infusion, and pre-activated status of MSCs might have added heterogeneity between studies and influenced results. Pilot clinical studies have also suggested a potential role for MSCs as GVHD prevention.[125,126] Prospective randomized studies are currently underway in order to more definitely assess the impact of MSC co-transplantation on GVHD (Table 3).

4.3.3. Invariant natural killer T cells (iNKTs)

iNKT is another rare immunoregulatory T cell population, with restricted T-cell receptors. Recent mouse experiments have shown that infusion of *in vitro* expanded donor iNKT cells attenuated experimental GVHD.[133] In patients, early post-transplant recovery of donor-derived iNKT cells after alloHSCT has been associated with reduced risk of aGVHD.[45] Accordingly, high content of iNKT cells in the stem cell graft has also been associated with a reduced risk of aGVHD.[127] Thus, expanding iNKT (by *in vivo* or *ex vivo* manipulations) might provide a new potential approach for controlling GVHD.

4.3.4. MDSCs

MDSCs are a heterogeneous population of immature myeloid progenitors that have the ability to suppress effector T cell responses and promote the development of Tregs. Several mouse experiments have reported that adding functional MDSCs in donor graft could alleviate aGVHD.[47,48] These observations, however, remain to be confirmed in clinical trials.

4.4. Risk stratification directed strategy for aGVHD prevention

Alternative approaches to decrease aGVHD-related morbidity and mortality are focusing on the ability to predict aGVHD occurrence, in an effort to provide an opportunity to abort aGVHD development in patients at high risk for aGVHD by intensifying aGVHD prophylaxis without exposing patients at low risk to undue toxicity from excessive immunosuppressive therapy. Studies have identified clinical factors of aGVHD (i.e. age, type of donor, HLA-disparities, type of conditioning, pretransplant comorbidities), genetic variants (i.e. NOD2 polymorphisms), and biomarkers (i.e. TNF receptor-1 [TNFR1], IL-2 receptor [IL-2R], IL-8, hepatocyte growth factor as well as, elafin regenerating islet-derived protein 3α [REG3α] and suppressor of tumorigenicity 2 [ST2]) to correlate with risks for aGVHD (nicely reviewed in Paczesny [134]) Hence, it is possible to envision the future development of algorithms that would predict risk of aGVHD for individual patients and would further guide risk-adapted aGVHD prophylaxis.

5. Impact on cGVHD

Interestingly, some approaches for aGVHD prophylaxis also result in mitigating cGVHD while others do not.[9] Current

Table 4. Impact of aGVHD prophylactic strategies on cGVHD.

Strategy	Results on GVHD	Reference
Current strategies		
Calcineurin inhibitor ± methotrexate (MTX)	Little/no impact	[9]
Calcineurin inhibitor + mycophenolate mofetil (MMF)	Little/no impact	[9]
Anti-T cell globulin (ATG)	Decreased incidence	[63,64]
Alemtuzumab	Decreased incidence	[67]
Ex vivo $CD34^+$ cell selection	Decreased incidence	[74]
Novel strategies		
Pentostatin	Unknown	–
Suicide gene therapy in T cells	Decreased incidence[a]	[77]
Post-transplant cyclophosphamide (Cy)	Decreased incidence	[79]
Ex vivo photodepletion of anti-host reactive donor T cells (TH9402, Kiadis)	Unknown	–
Ex vivo depletion of $TCR\alpha\beta^+$ donor T cells	Decreased incidence[a]	[81]
Ex vivo depletion of $CD45RA^+$ naive T cells	Decreased incidence[a]	[82]
Proteasome inhibitors (bortezomib)	Decreased incidence[a]	[90]
CTLA-4 immunoglobulin	Unknown	–
mTOR inhibitors (sirolimus)	Little/no impact	[92]
JAKs inhibitors	Unknown	–
Demethylating agents (5-azacytidine)	Decreased incidence[a]	[94]
Histone deacetylase inhibitors	Unknown	–
Anti-IL-6 receptor antibody (tocilizumab)	Unknown	–
CCR5 inhibitor (maraviroc)	Unknown	–
Recombinant urate-oxidase (rasburicase)	Unknown	–
Anti-CD20 antibody (rituximab)	Decreased incidence	[9]
Treg infusion	Unknown	–
Low dose IL-2	Unknown	–
MSC coinfusion	Unknown	–

[a]These results have to be validated in further studies.

knowledge concerning the impact of aGVHD prophylactic strategies on cGVHD are summarized in Table 4.

6. Conclusion

Numerous novel approaches for aGVHD prevention are undergoing preclinical development or clinical testing based upon the deeper understanding of the complex immunologic processes involved in aGVHD. As Tconvs have been demonstrated to play major role in aGVHD, donor T-cell depletion strategies (e.g. with ATG, alemtuzumab, *ex vivo* $CD34^+$ cell positive selection of the graft) have been investigated during the last decades and have demonstrated potent efficacy for aGVHD prevention. Thereafter, refined T-cell depleting approaches progressively developed and still continue to emerge, aimed at specifically targeting activated alloreactive T cells (e.g. post-transplant Cy, suicide gene therapies, *ex vivo* photodepletion of anti-host reactive donor T cells) or specific T cell subpopulations (e.g. $TCR\alpha\beta^+$, naive cells) while preserving immune recovery and anti-tumor T cells. Some of them (e.g. post-transplant Cy) already demonstrated potent efficacy in large prospective trials. Other novel strategies aimed at functionally impairing T-cell activation (e.g. proteasome inhibitors, costimulation blockers, mTOR inhibitors), modulating T-cell fate (e.g. JAKs inhibitors, demethylating agents, histone deacetylase inhibitors) and interfering with homing properties (e.g. CCR5 inhibitors) have also shown encouraging results for aGVHD prevention. Approaches aimed at controlling the milieu to make it less ``GVHD favorable'' are also emerging, targeting pro-inflammatory cytokines (e.g. anti-IL6 receptor antibody) and danger signals (e.g. recombinant urate-oxidase and modulation of microbiota). In addition, Treg-based therapies to promote immune tolerance have also demonstrated efficacy to mitigate aGVHD in preclinical experiments as well as in initial clinical studies. Other cell subsets such as iNKTs, MSCs, and MDSCs were also recently reported to mediate immune tolerance after alloHSCT and are currently under investigation for aGVHD prevention. The ultimate step would likely be to provide a risk-stratified strategy for aGVHD prevention, with intensified prophylactic regimens (e.g. by using and/or combining novel approaches) for patients at high risk for aGVHD and alleviated prophylaxis for patients at low risk in a way to avoid them undue toxicity from excessive immunosuppressive therapy.

7. Expert opinion

New understanding in aGVHD pathogenesis will likely lead to new standards for aGVHD prophylaxis in the future. According to our opinion, the most promising novel pharmacological approaches for aGVHD prophylaxis include post-transplant Cy administration, HDAC inhibitors (such as vorinostat), proteasome inhibitors (such as bortezomib) as well as IL-6 blockade (as example with tocilizumab). Further, although they have not been assessed in phase II studies for GVHD prevention yet, JAKs inhibitors (such as the JAK 1–2 inhibitor ruxolutinib) and hypomethylating agents (such as azacitidine) are also promising. In addition, the blocking of other cytokines such as IL-21 deserves also to be investigated in appropriate phase I–II studies.

The most promising novel cellular approach for aGVHD prevention in our view consists of Treg co-transplantation. Importantly, this approach seems to prevent aGVHD without affecting graft-versus-tumor effects, both in murine models of GVHD and in humans.[38,75] Current challenges for Treg therapy include the difficulty to infuse a sufficient number of Tregs, with a good purity, and to ensure that infused cells persist and keep an immunoregulatory profile in the host. The

utility of adding low-doses of IL-2 to donor Treg infusion remains to be investigated, especially in patients on calcineurin inhibitors.

Besides pharmacological and cellular therapies, modulation of the microbiome will also likely play an important role in the prevention of gut GVHD in the future.

An important limitation in the field has been the difficulty to translate discoveries made in mouse-to-mouse models of GVHD directly into clinical practice. This is partly due to differences between human and murine immune systems, and more importantly, to the large genetic diversity within the human species in contrast to what is present in inbred mice. Recent developments in humanized models of GVHD may provide additional tool for testing new immunoregulatory approaches on human immune cells *in vivo* and offers the possibility to assess various donors with different genetic background,[39,40,49] while the canine model of unrelated dog-leukocyte antigen-mismatched transplantation has remained the only animal model that allows direct translation to the clinic.[55]

The holy grail of GVHD prophylaxis is to efficiently prevent grade >1 aGVHD without compromising post-transplant immune recovery and graft-versus-tumor effects. Unfortunately, besides possibly the use of low-doses of ATG on the one hand, and of donor Treg infusion in the other hand, the vast majority of approaches that decrease GVHD in humans also impair graft-versus-tumor effects. This is in contrast to what has been observed in mouse-to-mouse models of GVHD where a number of approaches are able to differentiate GVHD and graft-versus-tumor effects. This can be probably explained by the fact that many successful therapeutic interventions in mouse-to-mouse models of aGVHD are efficient in CD4-dependent GVHD models, while the graft-versus-tumor effect systems used are largely mediated by CD8+ T cells.[18]

In the future, we can envision that the ultimate step for ideal aGVHD prophylaxis would be an individualized approach, in which the type and intensity of the immune suppression would be dictated by algorithms evaluating the type of transplantation and the risks for aGVHD determined by both pre-transplant genetic testing and early post-transplant biomarkers.

Declaration of interests

S Servais is Postdoctoral Researcher, F Baron Senior Research Associate, and L Delens, G Ehx, G Francolet are Televie PhD students at the National Fund for Scientific Research (FNRS) Belgium. S Humblet-Baron is postdoctoral researcher at the Fonds Wetenschappelijk Onderzoek - Vlaanderen (FWO). The review was in part supported by funds from the FNRS, the Belgian Foundation against Cancer (FBC), the Anti-Cancer Center and the Leon Fredericq Foundation from the University of Liege, the Terry Fox Foundation, and Plan Cancer from the Belgian Government. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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•• of considerable interest

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