

TRANSPLANTATION

Advances and challenges in immunotherapy for solid organ and hematopoietic stem cell transplantation

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Although major advances have been made in solid organ and hematopoietic stem cell transplantation in the last 50 years, big challenges remain. This review outlines the current immunological limitations for hematopoietic stem cell and solid organ transplantation and discusses new immune-modulating therapies in preclinical development and in clinical trials that may allow these obstacles to be overcome.

INTRODUCTION

Since the first successful allogeneic bone marrow and deceased-donor kidney transplants in the 1960s, the fields of hematopoietic stem cell transplantation (HSCT) and solid organ transplantation have grown immensely. In the United States, more than 21,000 patients per year receive solid organ transplants (Organ Procurement and Transplantation Network, <http://optn.transplant.hrsa.gov>), and another 7000 patients undergo allogeneic HSCT (1). HSCT is a life-saving, curative treatment for a range of diseases, including hematological disorders and malignancies, immunodeficiencies, metabolic storage diseases, and certain extracellular matrix disorders such as the debilitating skin disease epidermolysis bullosa. Solid organ transplantation not only extends life in patients with organ failure, but it can also improve quality of life, a feat difficult to achieve with other therapies. Unfortunately, serious immune reactions complicate both HSCT [graft-versus-host disease (GVHD)] and solid organ transplantation (graft rejection). Although broadly immunosuppressive agents can help to control these events, immunosuppression confers additional complications, such as opportunistic infections and an increased incidence of a variety of conditions, including malignancy, cardiovascular disease, and diabetes. Thus, big challenges remain in the field of transplantation. Here, we outline the current immunological limitations for both HSCT and solid organ transplantation and discuss new immune-modulating therapies that may enable these barriers to be overcome.

CURRENT IMMUNOLOGICAL CHALLENGES

The primary immunological barrier to allogeneic HSCT efficacy is GVHD. With a fatality rate of nearly 20%, GVHD is the second leading cause of death in patients undergoing allogeneic HSCT, behind only mortality from primary disease (1). Acute GVHD occurs in 20 to 70% of patients (2), and chronic GVHD, the primary long-term cause of morbidity after allogeneic HSCT, can affect >50% of patients (3). Both acute and chronic GVHD result from the transfer of alloreactive donor T cells within the stem cell graft, but their pathogenesis (Fig. 1, A and B) and clinical features are distinct. Acute GVHD has a strong inflammatory component, with robust T cell activation

and proliferation causing immune-mediated destruction of recipient organs, in particular, the skin, gastrointestinal (GI) tract, and liver (4). Chronic GVHD displays more autoimmune and fibrotic features, with donor T cells interacting with bone marrow-derived B cells along with recipient macrophages and fibroblasts to cause widespread antibody deposition and tissue fibrosis (5). Yet, despite our increased understanding of GVHD pathogenesis, current GVHD prophylaxis and treatment approaches are primarily based on the use of nonspecific immunosuppressive drugs such as calcineurin inhibitors, rapamycin, mycophenolate mofetil, steroids, and anti-T cell antibodies (6). Additionally, whereas rigorous donor T cell depletion can avert GVHD, the immediate consequences of pan-T cell removal are similar to global immune suppression, that is, increased risk of infection and tumor recurrence.

With improvements in surgical techniques and ancillary care over the past several decades, graft rejection is now the primary limitation for solid organ transplantation. Although the incidence varies by type of graft, rejection plays a major role in the loss of graft function over time. Rejection has three forms: hyperacute, acute, and chronic. Hyperacute rejection occurs within hours after transplantation and is mediated by preformed complement-fixing antibodies (typically directed against donor MHC class I antigens) that activate complement and coagulation cascades and mediate rapid destruction of the graft (7). The discovery of the basis for hyperacute rejection and the introduction of crossmatching to detect anti-donor antibodies before transplant have virtually eliminated the occurrence of hyperacute rejection in solid organ transplantation (8). Acute rejection typically manifests weeks to months after transplantation and is mediated by recipient alloreactive T cells, which destroy the donor graft through both direct cytotoxicity and activation of innate immune cells (9) (Fig. 2A). In addition, a subset of activated T cells can provide help for B cell antibody class switching, affinity maturation, and ultimately the production of donor-specific antibodies. Development of donor-specific antibodies after transplantation plays a limited role in most instances of acute rejection, with the exception of some of the most severe cases. However, these antibodies have been implicated in chronic rejection (10) (Fig. 2B), which manifests months to years after transplantation. Chronic rejection likely involves both non-immune-mediated and immune-mediated processes (11), in particular, donor-specific antibody deposition that directs innate immune cell activation and graft injury. The primary prophylaxis and treatment strategies for addressing solid organ transplant rejection are similar to those for GVHD, comprising nonspecific immunosuppressant drugs.

Although GVHD and solid organ transplant rejection are distinct clinical entities, they share many features, including key steps in pathogenesis and limitations of current therapies. In this light, the following

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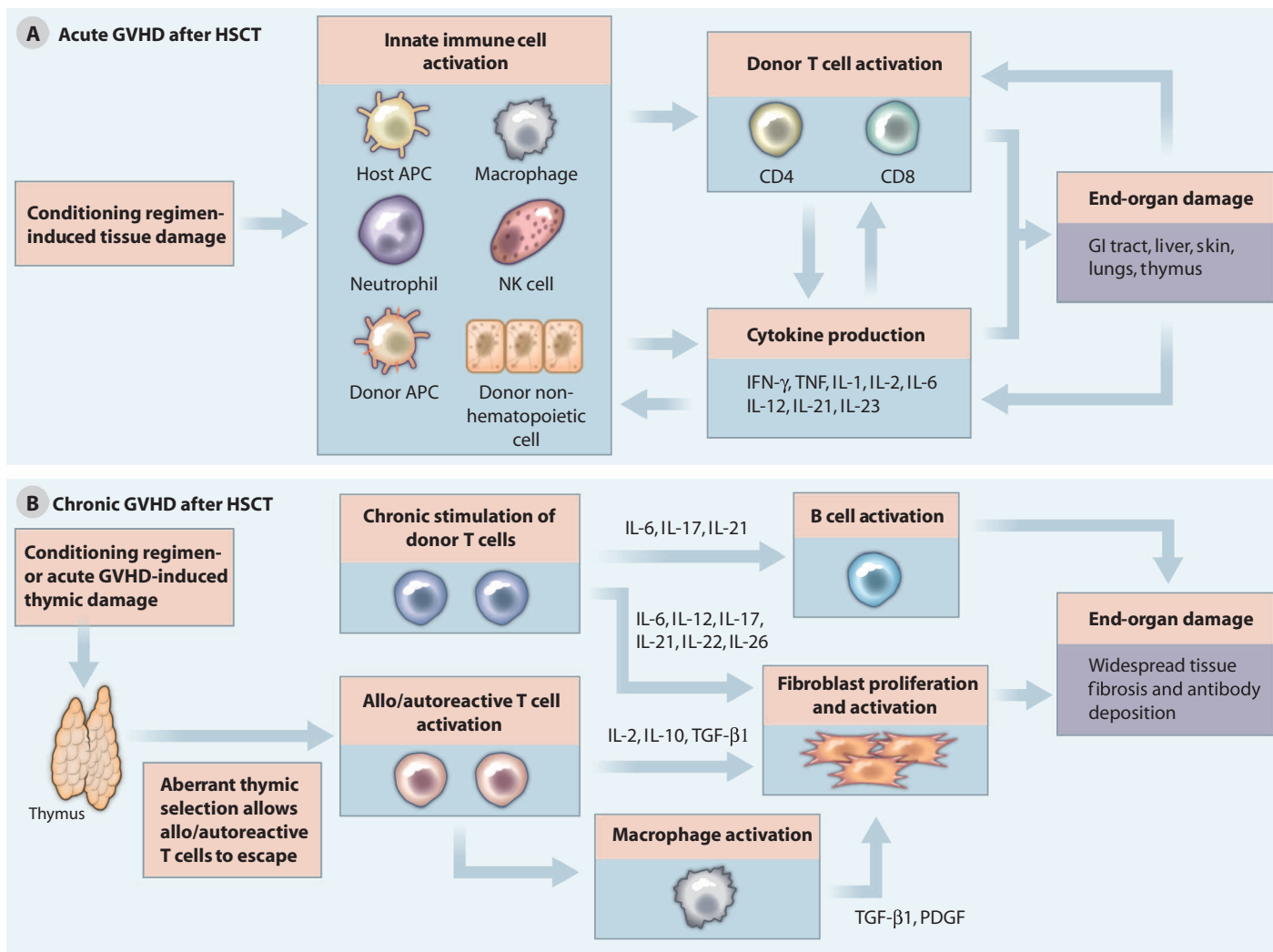


Fig. 1. The pathophysiology of and initiating factors involved in GVHD after HSCT. (A and B) Shown are the immune processes and molecules involved in the development of acute (A) or chronic (B) GVHD after HSCT. (A) Acute GVHD begins with a conditioning regimen such as chemotherapy combined with total body irradiation, which induces tissue damage. This tissue damage causes the release of danger signals, such as cytokines and chemokines, that activate recipient innate immune cells, including antigen-presenting cells (APCs). Donor APCs, which are a component of the stem cell graft, are also activated by this highly inflammatory milieu. A combination of donor and recipient APCs then activates donor CD4 and CD8 T cells. Cytokine production and direct cytotoxicity of host cells by these T cells, as well as by host macrophages, neutrophils, and natural killer (NK) cells, cause end-organ damage. The resulting tissue destruction further amplifies acute GVHD, creating a positive feedback loop that can

be difficult to stop even with immunosuppressive drug treatment. IFN- γ , interferon- γ ; TNF, tumor necrosis factor; IL-1, interleukin-1. (B) Thymic destruction, either from pretransplant conditioning or acute GVHD, and chronic stimulation of donor T cells contribute to chronic GVHD after HSCT. Thymic damage alters the selection of T cells, which can result in the release of lymphocytes that react to host tissues. Depending on the antigen, this reaction to the host can be considered allo- or autoreactive. Once activated, these T cells stimulate fibroblast proliferation and macrophage activation, both of which result in tissue fibrosis. Donor T cells also contribute to fibroblast activation and play a key role in activating B cells, which produce antibodies with specificities for host tissues. All of these events contribute to the highly fibrotic syndrome of chronic GVHD. TGF- β 1, transforming growth factor- β 1; PDGF, platelet-derived growth factor.

sections highlight promising new targeted immunotherapies specific for each condition, as well as treatments that may be efficacious in both GVHD and solid organ transplant rejection (Fig. 3 and Table 1). Whereas we present primarily positive data for the therapies discussed below, many have significant risks or adverse effects. We have highlighted some of these risks, but a comprehensive discussion of each therapy is beyond the scope of this review. In addition, all of these

therapeutics will need rigorous large-scale clinical trial testing to assess fully their potential benefits, side effects, and drawbacks. When discussing solid organ transplantation research and clinical trials, we primarily focus on kidney transplantation because in the United States >50% of patients undergoing solid organ transplantation receive kidney allografts (<http://optn.transplant.hrsa.gov>), and most of new translational therapies first enter the clinic in this area.

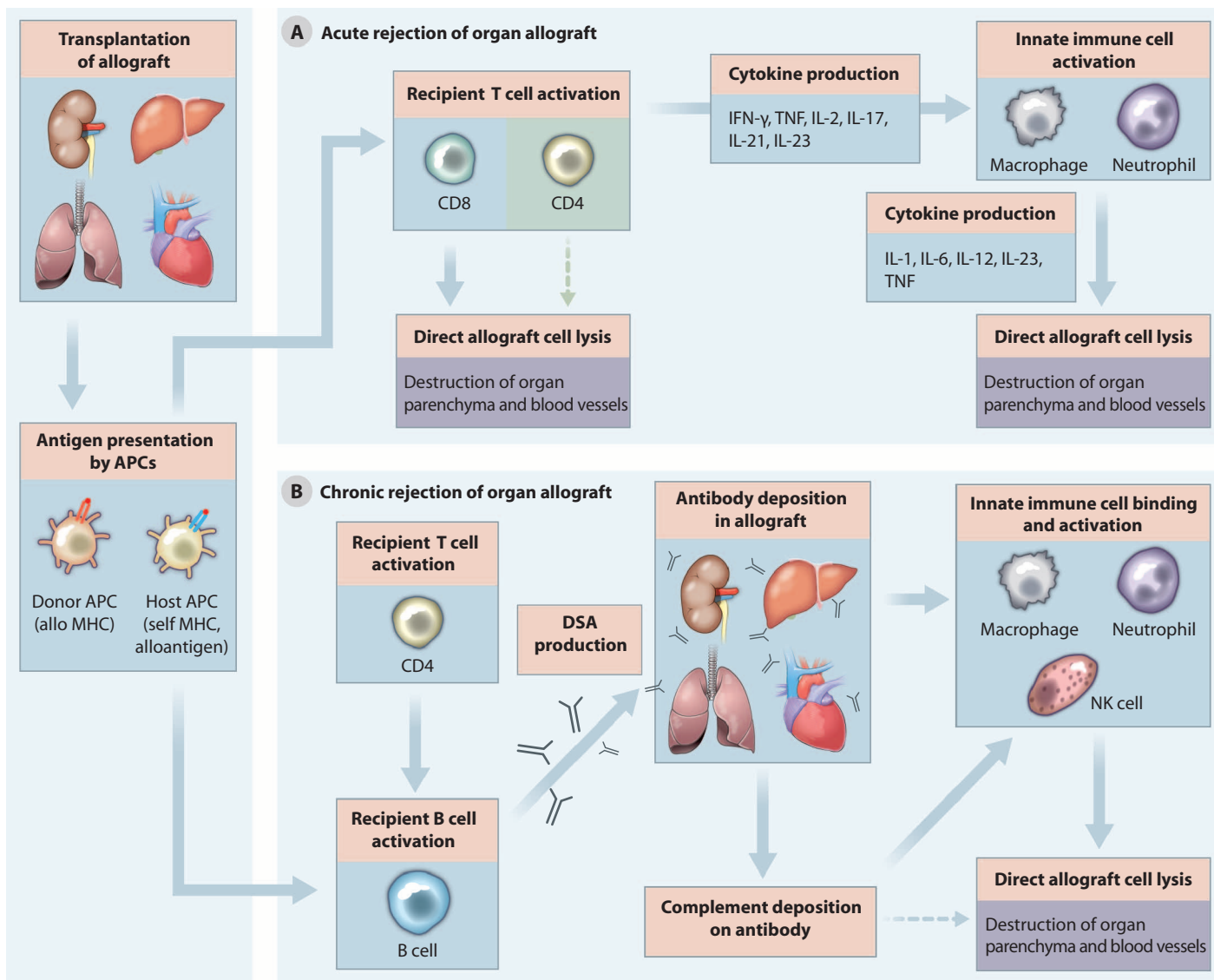


Fig. 2. The pathophysiology of and initiating factors involved in rejection of solid organ transplants. (A and B) Shown are the factors involved in the development of acute (A) and chronic (B) rejection of solid organ transplants. (A) The process of acute allograft rejection begins with recipient CD4 and CD8 T cells becoming activated through interactions with donor and recipient APCs (respectively termed direct and indirect allorecognition). After activation, CD8 T cells and, to a lesser extent, CD4 T cells directly destroy both graft blood vessels and parenchyma. Recipient CD4 T cells primarily contribute to acute rejection by producing a variety of cytokines that activate macrophages and neutrophils. These innate cells then attack and lyse graft cells. The combination of lymphocyte- and innate cell-directed

graft destruction results in allograft dysfunction and acute rejection. MHC, major histocompatibility complex. (B) In chronic allograft rejection, CD4 T cells help to induce antibody class switching, affinity maturation, and ultimately the production of donor-specific antibodies (DSA) by recipient B cells. Binding of DSA to graft cells enhances neutrophil-, macrophage-, and NK cell-mediated destruction of the graft (through Fc receptor binding) and results in complement deposition. Subsequent activation of the complement cascade results in direct lysis of graft cells through the complement membrane attack complex and further augments innate cell recognition and destruction of the graft. Although this process evolves over months to years, it results in chronic allograft dysfunction and eventual complete rejection.

NEW THERAPIES IN CLINICAL TRIALS

Advances in our understanding of the pathogenesis of GVHD and solid organ transplant rejection has led to the development of new immunomodulatory approaches, and many of these have been translated from preclinical models into clinical trials. A number of these therapies are approved by the U.S. Food and Drug Administration (FDA) for other conditions, enhancing their translational potential.

Below we identify crucial mechanisms involved in the pathogenesis of both GVHD and solid organ transplant rejection and discuss some of the most interesting and promising treatment approaches that are being tested in the clinic.

Reducing inflammatory cytokines

The cytokine interleukin-6 (IL-6) plays a role in the early inflammatory reactions of both acute GVHD and solid organ transplant rejection (12–14)

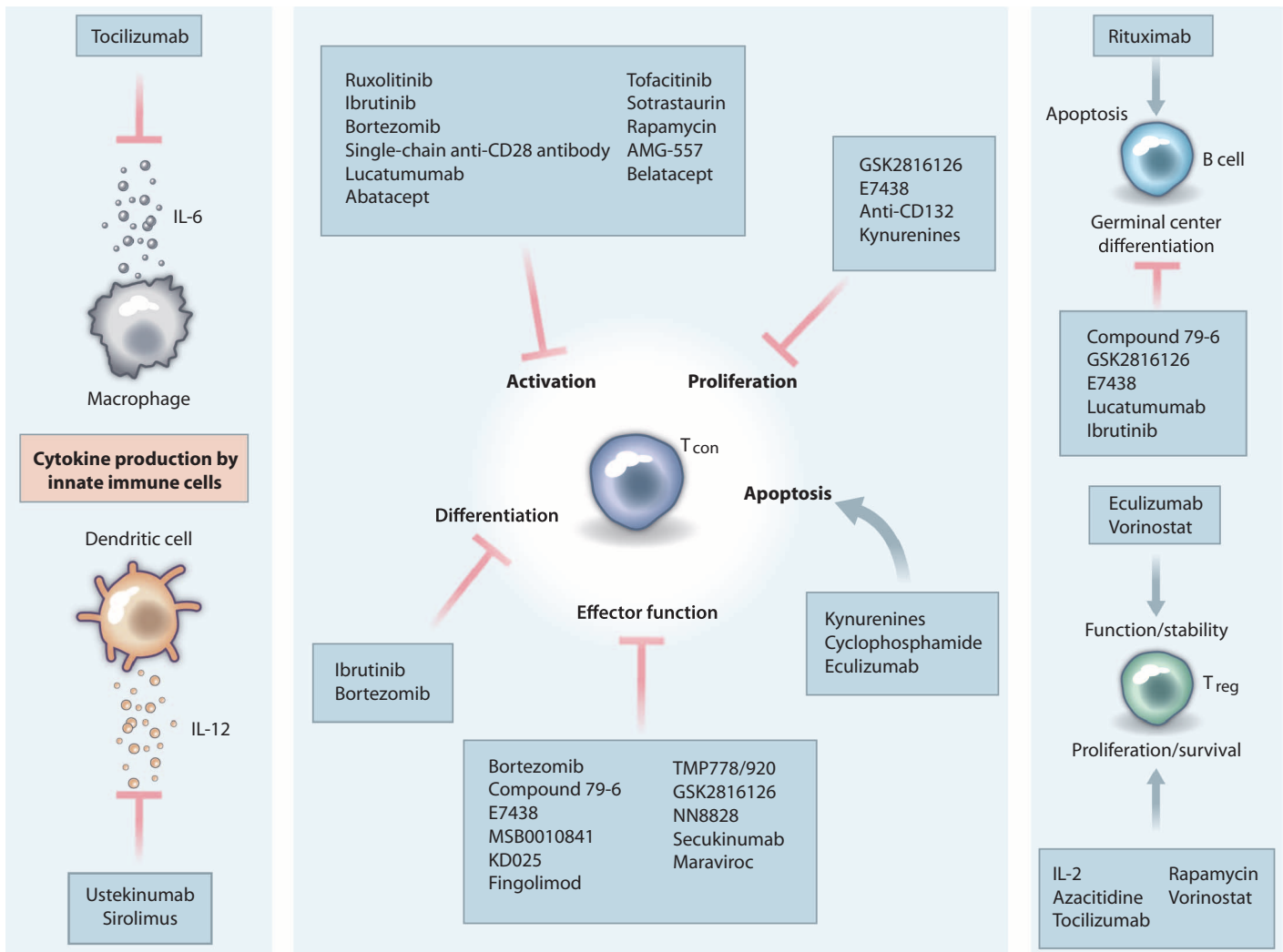


Fig. 3. Mechanisms of action for promising immunomodulatory therapies. Shown are known mechanisms by which new agents alter critical aspects of the pathogenesis of GVHD and solid organ transplant rejection. Most of these therapies focus on inhibiting various functions of conventional T cells (T_{con}), which are the primary drivers of many aspects of GVHD and allograft rejection. Abatacept and belatacept, CTLA4-Ig fusion protein inhibitors of CD80/86; azacitidine, DNA-hypomethylating agent; sotrastaurin, pan-PKC inhibitor with preferential selectivity for PKC- θ ; anti-CD132, anti-IL-2 common γ -chain (CD132) monoclonal antibody (mAb); AMG 557, anti-ICOS/B7RP1 mAb; bortezomib, proteasome inhibitor; compound 79-6, Bcl-6 inhibitor; cyclophosphamide, DNA-alkylating agent; E7438, EZH2

methyltransferase inhibitor; eculizumab, complement inhibitor; fingolimod, sphingosine-1-phosphate receptor inhibitor; GSK2816126, EZH2 methyltransferase inhibitor; ibrutinib, BTK/ITK inhibitor; KD025, ROCK2 inhibitor; kynurenes, products of L-tryptophan catabolism; lucatumumab, anti-CD40 mAb; maraviroc, CCR5 antagonist; MSB0010841, anti-IL-17A/F nanobody; NN8828, anti-IL-21 mAb; rapamycin, mTOR inhibitor; rituximab, anti-CD20 mAb; ruxolitinib, JAK1/2 inhibitor; secukinumab, anti-IL-17A mAb; single-chain anti-CD28 antibody, anti-CD28 mAb; TMP778, retinoic acid receptor-related orphan receptor γ (ROR γ t) antagonist; TMP920, ROR γ t antagonist; tocilizumab, anti-IL-6R mAb; tofacitinib, JAK3 inhibitor; ustekinumab, anti-IL-12/23 mAb; vorinostat/suberanilohydroxamic acid, HDAC inhibitor; T_{reg} , regulatory T cell.

and, as such, represents a common target for both diseases. In mouse studies, inhibition of IL-6 signaling with an anti-IL-6 receptor (IL-6R) monoclonal antibody (mAb) reduced GVHD (15). Additionally, testing of tocilizumab—a humanized anti-IL-6R mAb approved by the FDA for rheumatoid arthritis—in addition to a standard GVHD prophylactic regimen in a cohort of HSCT patients demonstrated a reduction in acute GVHD incidence compared to patients given standard GVHD prophylaxis alone, without affecting immune reconstitution, infectious immunity,

or tumor recurrence (16). However, it should be noted that rheumatoid arthritis patients treated with tocilizumab have increased susceptibility to infections, as well as some instances of neutropenia (17), meaning that careful monitoring for both of these potential side effects will be required in further clinical trials. In solid organ transplantation, IL-6 modulation also represents a potential therapeutic strategy and could have additional benefits beyond reducing inflammation because IL-6 plays a role in ischemia-reperfusion injury and antibody-mediated

Table 1. New approaches for preventing or treating GVHD and solid organ transplant rejection.

Category	Therapy	Description	Category	Therapy	Description
Reducing inflammatory cytokines	Tocilizumab	Anti-IL-6R mAb	Cell therapies	T _{regs}	CD4 ⁺ CD25 ⁺ Foxp3 ⁺ suppressive T cell
	Ustekinumab	Anti-IL-12/23 mAb		Type 1 T regulatory (Tr1) cells	CD4 ⁺ Lag3 ⁺ CD49b ⁺ suppressive T cell
	NN8828	Anti-IL-21 mAb		Mesenchymal stem cells	Suppressive stem cell population
	MSB0010841	Anti-IL-17A/F nanobody		Regulatory macrophages	Suppressive macrophages
Altering immune cell trafficking	Secukinumab	Anti-IL-17A mAb	Regulatory dendritic cells (DCs)	Suppressive DCs	
	Maraviroc	CCR5 small-molecule inhibitor	Stem cell transplant with solid organ transplant	Chimerism to induce tolerance	
Inhibition of T and B cell signaling	Fingolimod	Sphingosine-1-phosphate receptor modulator	Inhibition of T cell costimulation	Abatacept	CTLA4-Ig fusion protein
	Ruxolitinib	JAK1/2 small-molecule inhibitor		Belatacept	CTLA4-Ig fusion protein
	Tofacitinib	JAK3 small-molecule inhibitor		Single-chain CD28 antibody	Anti-CD28 mAb
	Ibrutinib	ITK/BTK small-molecule inhibitor		Lucatumumab	Anti-CD40 mAb
	Sotrastaurin	PKC- θ small-molecule inhibitor		AMG-557	Anti-ICOS-L mAb
	KD025	ROCK2 small-molecule inhibitor		Complement inhibition	Ecuzumab
B cell depletion	TMP778, TMP920	ROR γ small-molecule inhibitors	Targeting metabolic pathways	Kynurenine infusion	Tryptophan metabolite
	Rituximab	Anti-CD20 mAb	Blocking germinal center formation	TLR7/8 Agonist	Enhances kynurenine production by APCs
Preferential in vivo expansion of T _{regs}	Rapamycin (+IL-2)	mTOR small-molecule inhibitor		Compound 79-6	Bcl-6 small-molecule inhibitor
	Azacitidine (+IL-2)	DNA-hypomethylating agent	GSK2816126	EZH2 small-molecule inhibitor	
	IL-2	Antiapoptotic, proliferative cytokine	E7438	EZH2 small-molecule inhibitor	
	Vorinostat	HDAC small-molecule inhibitor	Other	Bortezomib	Proteasome small-molecule inhibitor
	Cyclophosphamide	DNA-alkylating agent			

injury (18). Tocilizumab is now being investigated in two clinical trials for kidney transplant (NCT01594424 and NCT02108600).

Critical to the pathogenesis of both acute and chronic GVHD is the production of proinflammatory cytokines. In particular, two members of the IL-12 cytokine family, IL-12 and IL-23, have been shown to play key roles in GVHD initiation. In mouse studies, blockade of IL-12 and IL-23 reduced acute GVHD while still maintaining the graft-versus-tumor (GVT) response of donor T cells (19, 20). Ustekinumab, a humanized mAb approved by the FDA for treating plaque psoriasis, inhibits both IL-12 and IL-23. As a result of the success with IL-12 and IL-23 blockade in preclinical and phase 1 studies, ustekinumab is now in phase 2 clinical testing for GVHD prophylaxis (NCT01713400). Although results have not been reported for the clinical trial, ustekinumab did have efficacy in a cohort of patients with steroid-refractory GVHD (21). Whereas psoriasis patients taking ustekinumab have an increased risk of minor infections (22), there did not appear to be a higher incidence of infections in these steroid-refractory patients.

Altering immune cell trafficking

One of the most critical steps in GVHD pathogenesis is the recruitment of activated T cells to target organs. Chemokine-chemokine receptor interactions mediate much of this migration (5), and CCR5 has been shown to be a key mediator of T cell trafficking to GVHD target organs, especially the GI tract (23, 24). CCR5 expression on donor graft dendritic cells (DCs) also correlates with greater incidence and higher-grade GVHD in patients after allogeneic HSCT (25), indicating that CCR5 may also regulate donor DC activation and migration in GVHD. A recently completed phase 1/2 clinical trial with the CCR5 inhibitor maraviroc (NCT00948753), which is FDA-approved as an HIV antiretroviral, demonstrated a reduction in GVHD, primarily as a result of reduced GI tract involvement (26). However, different conditioning regimens in a mouse model altered the efficacy of CCR5 inhibition (27), indicating that further assessment of maraviroc will need to take into account the type of conditioning a patient receives. Additional insight about the efficacy of this drug will be gleaned from the three ongoing clinical trials using

maraviroc to prevent acute GVHD (NCT01785810, NCT02208037, and NCT02167451).

Inhibition of T and B cell signaling

Activated T cells play a major role in the pathogenesis of both GVHD and solid organ transplant rejection, with B cells critically involved in chronic GVHD progression (5) and chronic allograft rejection (28). Inhibition of T cell function has long been a strategy for treating GVHD and preventing solid organ transplant rejection, but several new agents that target either T cells or both T and B cells are currently being tested.

Janus-activated kinases (JAKs) are required for T cell activation and differentiation in response to inflammatory cytokines. In particular, JAK1 and JAK2 are critical in GVHD initiation (29), making them attractive therapeutic targets for early abrogation of GVHD. Ruxolitinib, a selective JAK1 and JAK2 inhibitor approved by the FDA for treating myelofibrosis, was recently shown to be efficacious in treating GVHD in mice (30). In addition, a subset of patients with steroid-refractory GVHD responded well to ruxolitinib and had no adverse events as a result of therapy (30). These clinical data, along with our unpublished results of ongoing studies, suggest that ruxolitinib may be useful in GVHD. Although worsening anemia and thrombocytopenia have been seen in myelofibrosis patients on ruxolitinib (31), the early results from treating steroid-refractory GVHD patients would suggest that these effects may not be as prominent in the HSCT setting. Further assessment of these potential side effects in HSCT patients is required to understand fully the potential drawbacks of this therapy.

Inhibiting JAK3 signaling reduces mouse T cell proliferation and GVHD lethality in a mouse model (32), suggesting that JAK3 may be a target for GVHD prevention or therapy in the clinic. JAK3 has also been shown to be important for rejection of solid organ transplants. Tofacitinib, a relatively selective JAK3 inhibitor currently FDA-approved for rheumatoid arthritis, was found to inhibit renal transplant rejection in cynomolgus monkeys (33) and to have additive effects with mycophenolate mofetil (34), which is commonly used for prophylaxis of transplant rejection. In phase 1/2 clinical trials using tofacitinib for renal transplantation (35, 36), a high incidence of infectious complications has been observed. This suggests that whereas this drug holds promise for preventing rejection of solid organ allografts, the ideal patient profiles and concurrent immunosuppressive regimens have not yet been elucidated.

Two members of the tyrosine kinase expressed in hepatocellular carcinoma (TEC) family of kinases, IL-2-inducible kinase (ITK) and Bruton's tyrosine kinase (BTK), share close homology and play critical roles in both T and B cell function. ITK helps to drive T cell activation as well as the differentiation of CD4 T helper cell subsets, and BTK is essential for B cell receptor signaling (37, 38). In mouse studies, treatment with ibrutinib, an ITK and BTK inhibitor that is FDA-approved for the treatment of chronic lymphocytic leukemia (CLL), reversed lung pathology and pulmonary dysfunction in mice with established chronic GVHD in a model dependent on cooperation between T follicular helper cells (T_{FH}) and germinal center B cells; additionally, ibrutinib reduced the progression of sclerodermatous chronic GVHD in mice (39). Further testing in mice established that ibrutinib does not adversely affect clearance of intracellular pathogens, suggesting that ibrutinib may be efficacious in GVHD without increasing infection risk (40). However, there is some evidence in CLL patients that ibrutinib may cause anemia, thrombocytopenia, and neutropenia (41);

assessment of these cytopenias will be critical in HSCT patients. An ongoing phase 1/2 clinical trial (NCT02195869) is evaluating ibrutinib in patients with steroid-dependent or steroid-refractory chronic GVHD.

Inhibiting protein kinase C

Activation of phospholipase C after T cell receptor (TCR) and CD28 ligation results in the generation of the second messenger diacylglycerol, which in turn activates the θ isoform of protein kinase C (PKC- θ). In a randomized phase 2 trial, the PKC- θ inhibitor sotrastaurin was shown to be comparable to mycophenolate mofetil when used as part of a standard tacrolimus (calcineurin inhibitor)-based regimen for immunosuppression in renal transplantation (42). Further results from completed studies are not yet available. Sotrastaurin is currently being explored in GVHD as well. Preclinical results with mice suggest that administration of sotrastaurin prevents GVHD while maintaining GVT (43). In combination with the results with sotrastaurin for kidney transplant, there may be translational potential for this small molecule in GVHD.

B cell depletion

Given the critical role that antibodies play in chronic GVHD (44), depletion of B cells using the FDA-approved anti-CD20 mAb rituximab is a potentially attractive therapeutic option. In mice, a highly depletory anti-CD20 mAb was effective in preventing chronic GVHD when used as a prophylactic agent, but showed less than optimal therapeutic benefit in reversing established chronic GVHD (45). Two phase 1/2 clinical trials have reported that rituximab has efficacy in preventing chronic GVHD (46, 47), although there was an increased susceptibility to infections. Nevertheless, these preclinical and early clinical trial data indicate that rituximab may be an effective prophylactic agent, and a variety of additional clinical trials are assessing the long-term efficacy of rituximab in chronic GVHD.

Rituximab also has been shown to be effective as part of a desensitization protocol in renal transplantation where recipients have preformed anti-human leukocyte antigen (HLA) antibodies (48). Smaller studies have used rituximab as prophylaxis or treatment for acute graft rejection or as treatment for chronic antibody-mediated rejection, but because of their size, these studies are not conclusive.

Other strategies

The proteasome, the cellular organelle that disposes of misfolded proteins, is required for the maintenance and regulation of basic cellular processes, including proliferation, differentiation, and apoptosis (49). Preclinical mouse work with the FDA-approved proteasome inhibitor bortezomib demonstrated that it is a potent inhibitor of alloreactive T cells, and that bortezomib treatment reduced acute GVHD without affecting GVT (50, 51). In addition, one of the mechanisms by which bortezomib reduced GVHD severity was by diminishing the production of the inflammatory cytokine IL-6 (discussed above). In chronic GVHD, preclinical mouse studies and early clinical trial results show clinical efficacy with bortezomib treatment (52, 53). Some patients treated with bortezomib were able to taper steroid doses, a significant achievement for patients who were steroid-dependent. Clinical trials are evaluating the long-term efficacy of bortezomib for treating GVHD.

Bortezomib has been considered in solid organ transplantation as well, with many single patient reports or small scale trials in literature, although a study of four treated patients showed that bortezomib was not effective as a single agent in reducing the production of donor-specific antibodies

(54). The BORTEJECT (bortezomib in late antibody-mediated kidney transplant rejection) study (NCT01873157), the only trial currently recruiting patients to test bortezomib in solid organ transplantation, is designed to determine if this modality can be used to treat chronic antibody-mediated renal transplant rejection.

Preferential in vivo expansion of regulatory T cells

Regulatory T cells (T_{regs}) are $CD4^{+}25^{+}FoxP3^{+}$ suppressor cells that are critical mediators of peripheral tolerance (55). A reduced ratio of T_{regs} to conventional T cells (T_{con}) ($T_{reg} < T_{con}$) is associated with the development of chronic GVHD in allogeneic HSCT patients; low-dose IL-2 therapy reduces chronic GVHD by preferentially expanding T_{regs} during the IL-2 administration period (56). For chronic GVHD, such in vivo T_{reg} expansion is an attractive means to tip the T_{reg}/T_{con} ratio in favor of tolerance. Several ongoing clinical trials are building on the success with low-dose IL-2 treatment for chronic GVHD. In particular, two trials are combining drugs that favor T_{regs} versus T_{con} with low-dose IL-2 to further enhance T_{reg} expansion. The first trial (NCT01927120) pairs rapamycin (sirolimus) with IL-2 and tacrolimus to prevent acute GVHD. Sirolimus is an FDA-approved mammalian target of rapamycin (mTOR) inhibitor that has been shown to prevent GVHD by restricting T_{con} proliferation while also permitting preferential expansion of T_{regs} (57). The addition of sirolimus to IL-2 and tacrolimus has the potential to limit T_{con} responses while supporting IL-2-driven T_{reg} proliferation, which could further enhance T_{reg} -mediated GVHD prevention. A second trial (NCT01453140) combines IL-2 and sirolimus with the DNA-hypomethylating agent azacitidine for treating steroid-refractory acute GVHD, with or without cyclophosphamide, which is used to deplete alloreactive T_{con} but not T_{regs} (see below). Azacitidine favors T_{regs} versus T_{con} , which could be beneficial for GVHD prevention, and in one phase 1 trial, it also was shown to enhance GVT (58, 59). Given its potential to augment GVT while expanding T_{regs} , azacitidine may prove to be an effective GVHD therapeutic. Although it appears that the addition of sirolimus \pm azacitidine to low-dose IL-2 (and other agents) could further augment T_{reg} numbers in vivo, it remains to be seen whether this T_{reg} expansion will reduce GVHD without sacrificing long-term infectious immunity or GVT. Similar concepts regarding in vivo expansion of endogenous T_{regs} using IL-2 alone or in combination with sirolimus have been proposed for solid organ transplantation. A single clinical trial of IL-2 in patients receiving tissue allografts consisting of skin, subcutaneous, neuromuscular, and vascular tissue (vascularized composite tissue allografts) is currently under way (NCT01853111).

Epigenetic modification of the *Foxp3* locus and other loci is critically important in the development and maintenance of T_{regs} (60). In keeping with this, histone deacetylase (HDAC) inhibitors are effective therapeutics in animal models of solid organ and tissue transplantation (61). In a phase 1/2 study, the HDAC inhibitor vorinostat reduced acute GVHD incidence when paired with mycophenolate mofetil and tacrolimus (62) by increasing the number and suppressive function of T_{regs} (63).

Cyclophosphamide, an alkylating agent FDA-approved for a variety of malignancies and autoimmune diseases, has been shown to preferentially deplete alloreactive T_{con} while sparing T_{regs} (64). As a result, several phase 1/2 studies have examined posttransplant cyclophosphamide as a means to reduce GVHD. These trials revealed that even short courses of cyclophosphamide after HSCT can reduce GVHD, with particular efficacy in preventing chronic GVHD (65, 66). This indicates that this strategy may merit further clinical testing in GVHD and potentially solid organ transplantation as well.

Cell-based therapies

In addition to targeted drugs, cell-based therapies represent another potentially successful intervention for preventing GVHD and solid organ transplant rejection. Several distinct types of immunosuppressive cells are currently under investigation in clinical trials.

A large body of preclinical data has demonstrated that the adoptive transfer of T_{regs} can prevent solid organ transplant rejection and acute GVHD (67–69), and this has provided the impetus for ongoing and planned clinical trials. In solid organ transplantation, some studies use ex vivo unselected T_{regs} (NCT02129881 and NCT02088931), whereas others focus on recipient T_{regs} that have been expanded using donor-derived antigen (NCT02091232, NCT02188719, and NCT02244801). The approach to develop the antigen-specific T_{regs} used in the NCT02091232 trial, that is, culturing recipient T cells with donor antigen in the presence of CTLA4-Ig (cytotoxic T lymphocyte-associated antigen 4-immunoglobulin) fusion protein (to block CD28-B7 costimulation), is derived from a series of early-phase trials for GVHD prophylaxis using donor bone marrow precultured with recipient antigen-presenting cells (APCs) plus CTLA4-Ig (70, 71). T_{reg} studies in solid organ transplantation are still focusing on safety; whether T_{regs} may be most effective in preventing or treating rejection or whether they allow for a reduction in conventional immunosuppression awaits further determination.

In GVHD, a recent first-in-human phase 1 clinical trial using umbilical cord blood-derived T_{regs} showed that third-party ex vivo expanded T_{reg} infusions were well tolerated and reduced grade II to IV acute GVHD (72). A phase 2 trial using freshly purified T_{regs} also showed that T_{reg} infusions were efficacious in preventing GVHD in a haploidentical HSCT setting (73). As a result of these phase 1 and 2 successes, a variety of trials are now investigating ex vivo expanded or freshly purified T_{reg} infusions as a means to prevent both acute and chronic GVHD. These new trials are also assessing the efficacy of T_{regs} derived from multiple sources, with the goal of understanding which populations are most efficacious and easier to generate in large numbers.

Type 1 T regulatory (Tr1) cells are a suppressive subset of CD4 T cells that express CD49b and Lag3 (lymphocyte activation gene 3) and can be generated by and produce IL-10 (74). Tr1 cells play a role in peripheral tolerance but are distinct from $Foxp3^{+}$ T_{regs} . Tr1 cells have only recently been investigated as a therapeutic: in four haploidentical HSCT patients treated with infusions of Tr1 cells shortly after transplant, immune reconstitution was enhanced compared to historical controls (who had undergone haploidentical HSCT) with no GVHD and no long-term immunosuppression required (75). Studies to examine these cells in solid organ transplantation are just being initiated.

In addition to T_{regs} and Tr1 cells, other cell types with immunomodulatory properties are being studied. Mesenchymal stem cells (MSCs) are being explored as a therapeutic for treating both HSCT and solid organ transplant rejection. MSCs are multipotent adult stem cells that can suppress immune responses, repair tissues, and stimulate hematopoiesis (76). Because MSCs can be derived from a variety of tissues, they represent a potentially autologous or off-the-shelf third-party source of immunosuppressive cells that can be generated in large numbers and thus are an attractive cell-based therapeutic. To date, third-party MSCs have been used in tandem with front-line immunosuppressant drugs to treat GVHD with minimal side effects and some encouraging clinical results (77–79). However, primary endpoint reporting from a phase 3 trial (NCT00366145) using an industry-produced MSC product showed that these MSCs failed to increase complete responses in patients with steroid-resistant

GVHD (80). Whereas full results from this trial have yet to be published, the mix of positive and negative results with MSCs in GVHD underscores the potential need to identify specific subsets of patients that may benefit from this therapy. In solid organ transplantation, both live autologous and irradiated donor MSCs are being investigated. In the latter instance, such cells may be immunomodulatory and may also provide an ongoing source (in the case of repeated administration) of systemic donor antigen. Further testing of MSCs from a variety of sources is currently ongoing.

Regulatory macrophages have been used in renal transplantation (81) and are the focus of further ongoing clinical studies (NCT02085629). Autologous regulatory DCs, typically immature DCs or cells cultured in the presence of rapamycin, have been tested in a variety of preclinical HSCT and solid organ transplant mouse models (82–84) and are just now being introduced into the clinic for solid organ transplantation (NCT02252055).

Stem cell transplantation

More than three decades ago, hematopoietic chimerism was shown to convey donor-specific organ and tissue transplantation tolerance (85). Since then, extensive preclinical studies have led to the development of several clinical trials to refine this approach. Two current protocols use nonmyeloablative HSCT to create transient mixed hematopoietic chimerism. In the most extensive data set with patients given HLA-mismatched kidneys, 7 of 10 patients achieved excellent stable renal function and were able to discontinue immunosuppressive drugs for at least 5 years (86). Another approach using an infusion of a proprietary product composed of CD8⁺, TCR⁻ cells (termed graft facilitating cells), along with nonmyeloablative conditioning and HSCT, successfully achieved durable chimerism in some patients after HLA-mismatched kidney transplantation (87). Whereas follow-up is still limited in many patients, positive results have been achieved, and GVHD has not been problematic, perhaps because of the use of a cell product in the infusion or because the high-dose cyclophosphamide regimen spared T_{regs} (64). At present, these conceptually attractive strategies still await large-scale clinical trials to delineate the optimal approaches and to determine whether they produce superior results compared with other regimens that achieve minimization (although not discontinuation) of immunosuppressive drug treatment.

ON THE HORIZON

In this section, we identify new therapeutics that are showing promise in preclinical testing but have yet to be translated into the clinic.

Reducing inflammatory cytokines

The proinflammatory cytokine IL-21, a member of the IL-2 common γ -chain family of cytokines, has been shown to promote both acute and chronic GVHD (5). In mouse studies, inhibition of IL-21 reduced acute and chronic GVHD mortality and severity (88, 89). Although IL-21 inhibition has yet to be brought to the clinic for GVHD, a humanized anti-IL-21 mAb (NN8828, Novo Nordisk) was recently tested in several phase 1/2 clinical trials as a therapeutic for autoimmune inflammatory conditions (NCT01208506, NCT01565408, NCT01751152, and NCT01647451), enhancing the translational potential of anti-IL-21 therapy for GVHD.

In addition to IL-21, several other common γ -chain cytokines (IL-2, IL-7, and IL-15) play critical roles in acute and chronic GVHD patho-

genesis. Using a mAb against the IL-2 common γ -chain (CD132) in mice reduced acute GVHD and reversed established chronic GVHD (32). These initial preclinical data indicate that CD132 may be a useful therapeutic target in GVHD. However, it is as yet unclear how inhibiting such a critical cytokine pathway would affect infectious immunity or GVT, although mouse studies with anti-CD132 mAb in acute GVHD showed retention of GVT in that model system (32).

T helper 17 (T_H17)-skewed CD4 T cells produce both IL-21 and IL-17, a proinflammatory cytokine that works in tandem with IL-21 to mediate acute and chronic GVHD. The production of both cytokines by T_H17 cells requires rho-associated kinase 2 (ROCK2), a molecule linked to a variety of fibrotic diseases, including some with features similar to highly fibrotic chronic GVHD (90, 91). The selective ROCK2 inhibitor KD025 was successful in treating mouse collagen-induced arthritis (92) and is currently in clinical trials for psoriasis (NCT02106195). Given that both IL-17 and IL-21 play critical roles in acute and chronic GVHD pathogenesis, ROCK2 inhibition may prove to be a successful, translatable strategy for GVHD prevention. Alternatively, strategies that seek to neutralize IL-17A [currently being tested in autoimmune diseases such as psoriasis (MSB0010841, anti-IL-17A/F nanobody) and inflammatory bowel disease (secukinumab, anti-IL-17A mAb)] or that use ROR γ inhibitors (93) to target T_H17 cells may be useful in GVHD and solid organ transplantation settings.

Altering immune cell trafficking

Migration of T cells from secondary lymphoid organs to inflamed tissues is critical for nearly all immune responses. The interaction between sphingosine-1-phosphate and its receptor helps to mediate T cell emigration from secondary lymphoid organs, a step required for activated T cells to migrate to inflamed tissues. Fingolimod, which is FDA-approved for multiple sclerosis, is a sphingosine-1-phosphate receptor modulator that prevents T cell emigration from secondary lymphoid organs, resulting in reduced numbers of activated T cells in inflamed tissues (94). In mouse GVHD testing, fingolimod decreased acute GVHD mortality without affecting the GVT response (95), but somewhat unexpectedly, this reduced mortality was not mediated by a reduction in activated T cells in target tissues (96). Rather, fingolimod reduced the number of splenic DCs and activated T cells early after transplant, indicating that fingolimod may have additional mechanisms of action. Regardless of its exact effect, fingolimod remains a candidate for GVHD prevention or treatment. In contrast to its potential for future development in GVHD, in renal transplantation, fingolimod did not display superior efficacy to mycophenolate mofetil in a phase 3 clinical trial (97), and thus its developmental path in solid organ transplantation remains uncertain.

Inhibition of T cell costimulation

Early mouse studies demonstrated the efficacy of a soluble form of CTLA-4 (the inhibitory homolog of CD28) linked to an IgG1 fusion partner (CTLA4-Ig) to block CD28/CTLA4–B7 ligand interactions, prolong solid organ graft acceptance (98), and reduce GVHD (99). The likely mechanism of action of CTLA4-Ig is to bind to CD80 and CD86 with a higher affinity than CD28, thus preventing CD28-mediated costimulation. Abatacept, a humanized CTLA4-Ig fusion protein, is FDA-approved for treating autoimmune arthritis. A trial of abatacept for GVHD prevention demonstrated a low incidence of acute GVHD (100), and as a result, abatacept is being tested in two additional phase 1 and 2 trials (NCT01954979 and NCT01743131). Belatacept, a modified version of CTLA4-Ig, is FDA-approved for use as prophylaxis for

rejection in renal transplantation. However, both abatacept and belatacept have the undesired effect of inhibiting endogenous CTLA-4 from binding to CD80 and CD86, thus interrupting a normal negative immunoregulatory signal. One means to circumvent this would be using agents that directly block CD28. Single-chain antibodies that block CD28 without binding to CTLA-4 were first described more than a decade ago, and their subsequent development has led to a better understanding of how to develop highly specific antibodies (101). These antibodies have been used successfully in a number of nonhuman primate models of renal transplantation (102) and seem likely to be developed for clinical use in solid organ transplantation in the near future. An inhibitory anti-CD28 antibody also was recently shown to reduce human-to-mouse xenogeneic GVHD (102), indicating that CD28 blockade could be beneficial in this setting as well.

Blockade of CD40-CD154 interactions has been shown to prevent T and B cell-mediated alloimmune responses in many animal models. Anti-CD154 mAb showed great promise in nonhuman primate studies of renal transplantation and mouse GVHD models, but clinical trials of the initial anti-CD154 mAb were halted because of thrombotic complications (103). Given these complications, new anti-CD154 therapies, as well as anti-CD40 mAbs, are currently being explored in nonhuman primate and mouse models (104, 105). Notably, translation of anti-CD40 therapies may be bolstered by lucatumumab, an anti-CD40 mAb currently in phase I testing for follicular lymphoma (NCT01275209). However, it remains to be seen whether these new therapies are devoid of the thrombotic complications in humans that hindered the initial anti-CD154 mAb.

Inhibition of the inducible costimulator (ICOS) pathway with anti-ICOS antibodies reduced germinal center formation (where T and B cells cooperate to generate class-switched antibodies and induce affinity maturation) and chronic GVHD in mouse studies using models in which disease induction is dependent on antibody secretion and tissue deposition (89). The humanized anti-ICOS ligand (ICOS-L) mAb AMG-557 is currently in clinical trial testing for lupus (NCT01683695 and NCT00774943), enhancing the translational potential of anti-ICOS/ICOS-L therapeutics for potential treatment of both chronic GVHD and chronic solid organ allograft rejection.

Complement inhibition

Complement proteins represent the humoral arm of the innate immune system and play a role in modulating innate and adaptive immunity. Complement proteins C3a and C5a diminish T_{reg} function (106) and reduce T cell apoptosis (107). In mouse GVHD studies, pharmacological blockade of C3a/C5a signaling limited acute GVHD by promoting T_{reg} stability (106). In solid organ transplant rejection, C5a seems to play a role in both T cell activation and ischemia-reperfusion injury. In a preliminary report, the anti-C5a mAb eculizumab, FDA-approved for use in paroxysmal nocturnal hemoglobinuria, was shown to be effective in preventing antibody-mediated damage in renal transplantation (108). It is being studied further in solid organ transplantation to prevent ischemia-reperfusion injury through a number of ongoing clinical trials.

Targeting metabolic pathways

Indoleamine 2,3-dioxygenase (IDO) is the rate-limiting enzyme in the conversion of tryptophan to kynurenines, metabolites that inhibit the proliferation of alloreactive T cells. Mouse studies showed that IDO expression and kynurenine production by APCs play a critical role in dampening GVHD (109). Both infusions of exogenous kynurenines

and stimulation of kynurenine production by APCs through agonism of toll-like receptors 7 and 8 (TLR7/8) reduced acute GVHD (109). In addition, IDO expression in DCs can induce a highly suppressive subset of T_{regs} , further potentiating the suppressive effect of IDO expression (110). Together, these data indicate that augmented IDO and kynurenine signaling, either through TLR7/8 agonists or kynurenine infusions, may be a therapeutic option for GVHD or solid organ transplantation.

Blocking germinal center formation by transcriptional and epigenetic modulation

In addition to blockade of costimulatory signals, inhibition of key transcriptional modulators involved in germinal center development (which is critical for the production of potentially pathogenic antibodies in chronic allograft rejection and chronic GVHD) is also under investigation. Given that expression of the transcription factor B cell lymphoma-6 (Bcl-6) is critical for B cells and T_{FH} in germinal centers, directly inhibiting Bcl-6 interactions may be an ideal way to limit germinal center formation. One highly specific small-molecule inhibitor, compound 79-6, has been investigated as a therapeutic for Bcl-6-dependent B cell lymphomas. In preclinical mouse studies, compound 79-6 was shown to kill both mouse and human cancer cells, with no in vivo toxicity and favorable pharmacokinetic properties (111). Given these results in B cell lymphoma models, compound 79-6 is under preclinical investigation in mice as a treatment option for chronic allograft rejection and chronic GVHD.

The histone methyltransferase EZH2 helps to maintain the gene expression profile of B cells in the germinal center through epigenetic modifications. Deactivating mutations and direct inhibition of EZH2 block germinal center formation and antibody production (112), indicating that this may be a useful target for treating chronic GVHD and chronic solid organ transplant rejection. In addition, EZH2 plays a role in the proliferation of alloreactive T cells (113), further enhancing interest in EZH2 inhibition for GVHD and solid organ transplant rejection. Investigation of EZH2 inhibitors in GVHD are under way, and two different EZH2 inhibitors are in clinical trials as therapeutics for B cell lymphomas (NCT02082977 and NCT01897571), enhancing the translational potential of EZH2 inhibition. Given that EZH2 contributes to T_{reg} stability, which may be important in chronic GVHD and tolerance to solid organ allografts, it will be important to see the extent to which prolonged blockade in the clinic will affect long-term tolerance induction (114).

IMMUNE MONITORING

The many new therapies discussed above represent some of the leading candidates for more specific immunosuppression. By focusing more precisely on the initiating pathophysiology of solid organ allograft rejection and GVHD, these new therapies also present the possibility of minimizing or even discontinuing immunosuppressive regimens. For this to be done safely, appropriate and simple peripheral blood-based immune monitoring tests must exist, both to identify “tolerance” and to detect early signs of graft rejection or GVHD.

Efforts to develop immune monitoring tests in solid organ transplantation have focused on kidney and liver transplant recipients. Whereas tolerant renal transplant patients are relatively rare, two series of patients ($n = \sim 20$ to 25) have been described with spontaneous tolerance to their grafts (115, 116). Surprisingly, both studies identified a strong

B cell “signature” of tolerance, with elevated expression of B cell–derived genes and increased numbers of naïve and transitional B cells in the peripheral blood of tolerant recipients compared with stable patients on immunosuppressive drugs. Tolerant patients closely resembled healthy controls, and thus, because of the nature of these studies, it remains possible that the B cell findings are merely a marker of the absence of immunosuppressive drug use. Thus, the ability to use these findings to direct immunosuppression management depends on further confirmation in other studies, determination of the prevalence of the signature among patients taking standard immunosuppression, and the development of assays that can be used to detect incipient rejection.

Parallel efforts have been under way in liver transplantation where, because the regenerative/repair capacity of the liver is high, immunosuppression withdrawal can be more safely studied. Here, natural killer (NK) cell markers have been most prominent, and there has been little overlap between gene signatures seen in liver and renal transplantation (117), highlighting the fact that all tolerance is not the same and that organ-specific differences exist.

In addition to the “tolerance signatures” identified in kidney and liver transplant patients, a new method of directly tracking donor-reactive T cells in solid organ transplant recipients was recently reported. By isolating recipient cells that proliferate in response to donor antigens and then performing deep TCR sequencing, donor-reactive T cells were identified (118). Using blood samples from patients who had undergone HLA-mismatched kidney grafts after nonmyeloablative HSCT (86), as well as patients who received conventional kidney allografts with long-term immunosuppression, donor-reactive T cells were tracked over time by comparing pretransplant blood samples with samples obtained sequentially after transplant. In tolerant patients who had undergone combined HSCT and kidney transplant, there was notable deletion of donor-reactive clones, whereas in non-tolerant patients, as well those who received conventional grafts with immunosuppression, deletion of donor-reactive clones was not observed. This suggests that one of the potential mechanisms behind tolerance in solid organ transplantation is deletion of donor-reactive T cells and that this method of tracking donor-reactive T cells can discriminate between tolerant and nontolerant patients. Whereas further testing is required to validate this technique and also to determine which subsets of donor-reactive cells are not deleted (for example, possibly T_{reg} s), the increasing availability of sequencing resources may allow this type of identification and analysis to become useful for predicting outcomes after solid organ transplantation.

For HSCT patients, a variety of biomarkers have recently been shown to be useful for predicting GVHD severity and treatment outcomes. In particular, a six-biomarker panel looking at plasma concentrations of IL-2 receptor α , TNF receptor 1 (TNFR1), hepatocyte growth factor, IL-8, elafin (a skin-specific marker), and regenerating islet-derived 3- α (Reg3 α ; a GI tract-specific marker) demonstrated that concentrations of these biomarkers correlated with responsiveness to treatment and 180-day mortality (119). Plasma concentrations of the soluble IL-33 receptor (termed ST2) have been useful for stratifying patients in terms of their risk for developing treatment-resistant GVHD, with higher ST2 concentrations correlating with increased risk of developing this severe form of GVHD (120). In addition, a step forward was made recently in predicting GVHD onset: elevated plasma concentrations of the chemokine CXCL9 were shown to correlate with an increased risk of developing chronic GVHD (121). Such studies have

led to a prognostic score for acute GVHD based on biomarkers using algorithms centered around measuring TNFR1, ST2, and Reg3 α (122). As these and additional biomarkers are validated, our ability to predict GVHD development and response to treatment will increase, and this will be critical for augmenting our ability to elucidate the efficacy of our therapies.

CONCLUSIONS AND OUTLOOK

The past decade has seen substantial advances in our understanding of the mechanisms of tolerance induction and allograft responses. Concurrent with these discoveries is a new armamentarium of drugs (both small molecules and biologics) and cell-based therapies that have been approved or are available for clinical testing, making this an exciting time for both the HSCT and solid organ transplant fields. Among the strategies discussed above, a few in particular are generating noteworthy attention in their fields. In solid organ transplantation, blocking CD40-CD154 interactions and developing tolerance with combined stem cell and renal transplantation are drawing particular interest. In acute GVHD, T_{reg} infusions, modification of IL-2 common γ -chain cytokines, and JAK1/2 inhibition with ruxolitinib are garnering substantial attention. In chronic GVHD, T_{reg} promotion with IL-2 combination therapies, as well as bortezomib and ibrutinib, has garnered much interest. Only further testing will reveal whether these potentially important new therapies merit the excitement they have created. Overall, the development of biomarker panels and repurposing of approaches designed for GVHD, solid organ transplant, and autoimmune disorders offer new and potentially more selective approaches for achieving the durable tolerance induction needed for successful transplantation and at the same time may obviate the need for global immune suppression.

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"Indoleamine 2,3-dioxygenase and PD-1/PD-L pathways in the activation of regulatory T cells"; #61/132,601 "Inducible regulatory T cell generation for hematopoietic cell transplants" [University of Minnesota (UMN) no. Z09026]; "Large-scale expansion approaches for regulatory T cells" (UMN no. 20100109); "Methods to expand a T regulatory cell master cell bank" (University of Pennsylvania/UMN no. 61322186); #61/910,944 "Methods of treating and preventing alloantibody driven chronic graft-versus-host diseases"; #61/977,564 "Role of ROCK2 inhibitors in chronic GVHD"; and #62/076,358 "Use of compositions modulating chromatin structure for graft versus host disease (GVHD)." C.M.-H. declares that he has no competing interests.

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