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Review

Antibody-Based Preparative Regimens for Cell, Tissue and Organ Transplantation

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Abstract

The ability to successfully transplant cells and organs from a donor into an immunologically disparate recipient is one of the greatest treatment advances in the history of medicine. Nevertheless, acute and chronic rejection, graft versus host disease, and the inability to identify suitable donors continue to be challenges and limit broader application of cell and organ transplantation to the many patients that could benefit. Immunosuppression before and after allogeneic transplant has been found to dramatically improve allograft survival and, despite side effects, has been a mainstay of patient management. Inducing donor-specific tolerance is the holy grail in allotransplantation and is readily established in experimental animals but has been difficult to achieve in patients in settings apart from hematopoietic cell transplantation. Antibody-based conditioning to prepare the recipient is a promising approach

towards achieving transplant tolerance in both hematopoietic and solid organ transplant settings, and multiple targets are currently under consideration including those on circulating lymphocytes and hematopoietic stem cells. Here we review progress in the use of antibodies to support cell and tissue transplantation with a particular focus on induction of donor-specific tolerance for solid organ transplantation.

Keywords
Antibody-based conditioning; antibody-drug conjugates; immunotoxins; immune rejection; organ transplantation; organ tolerance

1. Introduction

Transplantation of cells and organs has evolved over the past century from compassionate use experimentation to lifesaving, standard-of-care treatments for a number of diverse conditions, making this one of the great achievements in the history of medicine [1, 2]. However, once major breakthroughs in donor tissue preservation and surgical technique were overcome, the prevention and treatment of graft rejection has remained as one of the greatest challenges to patient management and wider application. Graft rejection is the result of an alloimmune response by the recipient toward the donor graft and is predominantly mediated by T-cell recognition of recipient major histocompatibility complex (MHC) proteins [3]. As a result, immunosuppressive strategies have primarily focused on targeting host T-cells, either by blocking the production and release of cytokines from activated T-cells, downregulating and inhibiting T-cell receptors, inhibiting T-cell proliferation, or by depleting T-cells entirely [4]. A commonly accepted protocol for immunosuppressive therapy in both solid organ and hematopoietic cell transplantation (HCT) targets T-cells in 3 phases: induction, maintenance, and treatment of rejection episodes [5].

In the induction phase, high-intensity immunosuppression is usually given immediately before and/or immediately after the transplant, when the risk of rejection is the highest [4]. Immunosuppressive regimens used during this phase often include a T-cell-targeted antibody combined with a calcineurin inhibitor (CNI), typically cyclosporin A (CsA) or tacrolimus (FK 506). In addition to CNIs, a combination of corticosteroids and an antiproliferative agent, such as mycophenolate mofetil (MMF) or azathioprine, is also often given. In the setting of HCT this is usually combined with chemotherapy and/or irradiation-based conditioning to further enhance donor hematopoietic cell engraftment. In the maintenance phase, CNIs alone are usually sufficient to prevent rejection. Depending on the type of allograft and the side effect profile, MMF, azathioprine or mammalian-target-of-rapamycin (mTOR) inhibitors such as sirolimus and everolimus are also intermittently added. Patients are subsequently closely monitored for possible graft rejection and graft vs host disease. If rejection occurs, the graft can be damaged by the recipients' immune system or vice versa in graft vs host disease the host can be damaged by the transplanted hosts' immune system. In order to prolong allograft function and survival, corticosteroids and/or immunosuppressive antibodies are often given depending on the type of rejection [5].

Antibody therapy is considered a mainstay in assisting with stable engraftment, especially when there is a need to delay the introduction of calcineurin inhibitors or as steroid-sparing agents [4, 6]. Antibody therapy is generally well-tolerated, but use varies widely among transplant centers and organ types, ranging from a low use for liver transplants (31.1%) to a high use for pancreatic transplants (90.4%) [4]. Both depleting and non-depleting (inactivating) antibodies are used commonly, targeting antigens expressed on T-cells and B-cells. Both depleting polyclonal and monoclonal antibodies are used today.

The risk of acute rejection and early graft loss after solid organ allotransplantation has been dramatically reduced by the introduction of immunosuppressive therapies, beginning with cyclosporin in 1978 for kidney transplantation [7]. In data from 2016, one-year graft survival rates of >85% were reported for kidney, liver and heart allotransplantation [8-10]. Nevertheless, the 10-year survival rate for kidney transplants is only about 50%, and the five-year survival rate for lung transplants is less than 60% [11, 12], owing to chronic rejection and the deleterious side-effects of chronic immunosuppression which include major infections, malignancy, and cardiovascular disease [7, 11, 13, 14]. Moreover, broad application of solid organ transplantation remains severely constrained by the requirement for HLA-matching of donors and recipients, which has caused long, growing and inequitable waiting lists. Thus, there is a major unmet medical need to develop therapies for inducing allograft tolerance, which would allow transplants to be performed across HLA-mismatches without chronic immunosuppression.

The holy grail in allotransplantation is achieving donor-specific tolerance, which is defined as a recipient state in which the immune system is rendered unresponsive to antigens from the donor but not to other antigens, thereby avoiding the need for and complications of immunosuppressive agents [15]. While donor-specific tolerance is common in hematopoietic cell transplantation owing to the re-establishment of the donor immune system on the host's native architecture, establishing donor-specific tolerance in the solid organ transplant setting has been more challenging. There are several experimental protocols for tolerance induction, among them establishing stable multi-lineage mixed hematopoietic chimerism in the recipient by way of transplantation of donor hematopoietic cells. This approach has long been regarded as the most robust way to induce tolerance [16].

The history of transplantation tolerance began in 1945 when Ray David Owen observed a natural state of mixed chimerism in fraternal Freemantle bovine twins sharing a placental circulation [17]. Within a decade, Peter Medawar and colleagues demonstrated that these twins were tolerant to skin grafts from one another, launching a field of research into the underlying mechanisms and culminating in the use of hematopoietic cell transplantation (HCT) for the induction of immune tolerance [18]. Over the past 20 years, clinical trials with small numbers of patients have tested this approach, with groups such as those at Massachusetts General Hospital (MGH), Stanford University, and Northwestern University achieving success in weaning patients from immunosuppression following HLA-matched renal and hematopoietic cell transplantation [19-21]. In particular, in the MGH trial, immunosuppression was discontinued in 6 out of 10 patients for a duration ranging from 2-10 years [19]. The Stanford group was able to wean a total of 11 out of 16 patients off immunosuppression for 1-3 years, and none of the patients had rejection episodes [20]. Similarly, the Northwestern group demonstrated complete withdrawal of immunosuppression in 5 out of 10 patients for 16-36 months [22]. These efforts have since expanded to additional patients with

slightly modified regimens by these groups and others, including a recent study showcasing efficacy of a modified regimen in patients with Schimke Immuno-osseous Dysplasia (SIOD) [23].

Despite these promising, positive results, several challenges remain before this approach can be widely adopted in the clinical setting. HCT currently requires pre-transplant conditioning with non-selective genotoxic drugs and/or irradiation in order to eradicate endogenous hematopoietic stem cells (HSCs) and liberate space for donor HSCs to engraft. In addition, anti-thymocyte globulin (ATG), alemtuzumab, sirolimus and other cytotoxic immunosuppressive drugs are also critical in preventing the infused donor HSCs from being rejected, as observed in some haploidentical HCT trials [24]. This type of conditioning is highly toxic with both short-term and long-term side effects, and the destruction of many bystander cell types can result in serious complications such as pancytopenia, infections, enteritis and mucositis [25, 26]. An alternative approach to minimizing toxicity involves transplanting mega-doses of hematopoietic stem and progenitor cells, however this can be limited by the difficulty of collecting enough donor cells with megadose HCT additionally increasing the risk for graft-versus-host disease (GVHD). Although these complexities may be mitigated through robust HSC and progenitor collection via apheresis post mobilization and subsequent graft manipulation to remove alloreactive cells.

Despite many historic advances in the field, safer and more effective protocols for HCT are still needed not only for the treatment of blood and immune diseases but also for mixed chimerism-facilitated non-hematopoietic cell and solid organ allotransplantation. Numerous tissue-sparing agents have been studied recently, from the aforementioned conventional immunosuppressive drugs and antibodies to more recently developed HSC-depleting antibodies. This review will describe the state of the art in the principles and application of antibody-based therapies in the context of HCT and transplant tolerance, including the potential value of combining different antibody strategies. The basic properties of the antibodies we discuss can be found in Table 1.

Table 1 List of Antibodies discussed in this article.

Target antigens	Brand name (Trade name)	Company	Status	Approval date	Major side effects	References
CD25/IL-2R	Daclizumab (Zinbryta)	Biogen	Withdrawn from market (2018)	2016	Inflammatory encephalitis and meningoencephalitis, drug-induced liver injury	[27-32]
	Basiliximab (Simulect)	Novartis	Approved	1998		[33, 34]
CTLA-4Ig	Belatacept (Nulojix)	Bristol-Myers Squibb	Approved	2011	Increased risk of PTLD in EBV seronegative patients	[35-45]
C5a	Eculizumab (Soliris)	Alexion Pharmaceuticals	Approved	2007	Increased risk of invasive meningococcal disease	[46-53]
Polyclonal	ATG (Atgam, Thymoglobulin, Grafalon)	Pfizer, Sanofi, Neovii	Approved	1984 & 1998	Leukopenia, infection, cytokine release syndrome, PTLD	[54-69]
CD52	Alemtuzumab (Campath-1H/ Lemtrada)	Sanofi/Genzyme	Approved	2001	Leukopenia, infection, increased incidence of autoimmune disease	[70-82]
CD20	Rituximab (Rituxan)	Genentech Inc	Approved	1997		[83-91]
CD3	OKT3 (Muromonab)	Janssen-Cilag	Discontinued (2010)	1985	Flu-like cytokine release syndrome	[92-100]
	Visilizumab	Nuvion Plymouth	Phase 3 trial		Flu-like cytokine release syndrome	[101]
	Teplizumab	MacroGenics/Provention Bio	Phase 3 trial		Flu-like cytokine release syndrome	[101]
	Otelixizumab	Tolerx/GSK	Phase 3 trial		Flu-like cytokine release syndrome	[101]

	Foralumab	Tiziana Life Sciences	Phase 2 trial		Flu-like cytokine release syndrome	[101]
CD40L	Ruplizumab (Antova)	Biogen	Pre-clinical		Thromboembolic complications	[102-113]
	Toralizumab	IDEC Pharmaceuticals	Pre-clinical		Thromboembolic complications	[102-113]
	ABI 793	Novartis	Pre-clinical		Thromboembolic complications	[102-113]
	H106	Bristol Myers-Squibb	Pre-clinical		Thromboembolic complications	[102-113]
	BMS-986004	Bristol Myers-Squibb	Phase 1/2 trial			[114]
CD40	ch5D12		Pre-clinical			[112, 115]
	chi220		Pre-clinical			[112, 115]
	4D11		Pre-clinical			[116, 117]
	CFZ533	Novartis	Phase 1/2 trial			[118]
CD2	Alefacept (Amevive)	Astellas Pharma US	Withdrawn from market (2011)	2002	Malignancy	[119-127]
	OX34		Pre-clinical			[128, 129]
	BTI-332		Phase 2 trial		Malignancy	[130-133]
	Siplizumab	AstraZeneca	Phase 2 trial	2018	Lymphopenia	[134-139]
CD117	SR-1 (anti-mouse equivalent = ACK2)		Pre-clinical			[140-149]
	AMG 191/JSP 191	Jasper Therapeutics	Phase 1/2 trial			[150-153]
CD47	Magrolimab (and others)	Gilead Sciences	Phase 3 trial			[154-159]
Immunotoxins/Antibody-drug conjugates (ADCs)						
CD3	FN18-CRM9		Discontinued			[160-162]
	Resimmune	Angimmune	Phase 2 trial		Hypoalbuminemia, and hypophosphatemia	[162, 163]

CD3 / CD7	T-Guard	Xenikos	Phase 3 trial	Reversible hypoalbuminemia, microangiopathy, and thrombocytopenia	[164]
CD45	CD45-ADC	Magenta Therapeutics	Pre-clinical		[165-174]
CD117	MGTA-117	Magenta Therapeutics	Phase 1 trial		[175-181]

2. Antibodies Currently Used in Allotransplantation

2.1 Non-Depleting, Anti-T-Cell Monoclonal Antibodies

2.1.1 IL-2 Inhibitors–Daclizumab and Basiliximab

Interleukin-2 (IL-2) is produced by T-cells upon activation and binds to its receptor to trigger further T-cell activation and proliferation, or activation-induced T cell death. The IL-2 receptor is solely expressed on activated T-cells, including those mediating allograft rejection, and thus many attempts have been made to block the IL-2 receptor and its downstream signaling pathways in order to promote transplant tolerance [182, 183]. While CNIs inhibit the release of IL-2, they have significant toxicity and can dramatically impair renal function when administered chronically [184]. Daclizumab and basiliximab are antibodies that target CD25, the alpha chain of the heterotrimeric IL-2 receptor, [27] and were first introduced in the 1990s to serve as safer alternatives to CNIs [27].

Daclizumab is a humanized monoclonal antibody that reduces the incidence of acute rejection when administered with a CNI [28]. The first randomized controlled trials of daclizumab were conducted by Vincenti et al. in 1998, and Nashan et al. in 1999. In these trials, patients with low or standard immunological risk, meaning those who had not previously been sensitized or received transplants, were given daclizumab in addition to a standard immunosuppression regimen as conditioning for renal transplantation. Daclizumab reduced the rate of acute rejection and increased one-year survival rates, but did not appear to improve graft survival 3 years after transplantation [29, 30]. Later studies confirmed that daclizumab did not obviate CNIs [31]. Despite its efficacy in reducing acute rejection episodes, daclizumab was withdrawn from the market in 2018 due to reports of inflammatory encephalitis and meningoencephalitis in several patients [32].

Basiliximab is a chimeric monoclonal antibody that remains in use today. Basiliximab has been shown to reduce the rate of acute rejection in low to standard risk renal transplantation patients yet does not appear to improve one-year patient and graft survival as compared to controls. Given this, it is often combined with other agents. Importantly, basiliximab is well-tolerated in patients and does not increase the frequency of adverse events as compared to control groups [33, 34].

Thus, while anti-IL-2 receptor antibodies have been proven to improve transplant outcomes in the short-term, their use has thus far been unable to completely reduce dependency on CNIs. Additional studies must be conducted to understand the mechanistic differences between these types of drugs that lead to their varying efficacies in preventing allograft rejection.

2.1.2 Costimulation Blockade–Belatacept (CTLA-4Ig)

Belatacept is a fusion protein composed of the Fc fragment of human IgG1 linked to the extracellular domain of cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4, or CD152). Activation of antigen-specific T-cells requires two signals: an antigen-specific signal via the T-cell receptor (Signal 1) and a non-cognate costimulatory signal (Signal 2) that is provided by soluble factors or cell-surface molecules on the antigen presenting cell (APC) [35]. When both signals are provided, T-cells proliferate and secrete cytokines. Although multiple costimulatory pathways have been identified, including CD40 ligand (CD154)/CD40, CD2/CD58, LFA-1 (CD18)/ICAM-1 (CD54), and others, the CD28/B7 pathway is particularly important and is the best-characterized [36]. CD28 is a

44-kDa glycoprotein expressed on virtually all $CD4^+$ T-cells and the majority of $CD8^+$ T-cells. CTLA-4 is a T-cell-specific molecule with considerable homology to CD28 and competes with CD28 for binding to its ligands CD80 and CD86. Interactions between CD28 and CD80/86 promote T-cell activation; in contrast, engagement of CTLA-4 by CD80/86 inhibits T-cell activation and terminates the immune response [36]. As a fusion protein Belatacept is thought to bind to CD80/86 with greater affinity than CD28 [37-39] thus preventing CD28 engagement. In naïve T-cells, this binding would prevent T-cell co-stimulation and T-cell activation resulting in T-cell anergy or T-cell death [40]. In 1996, Larsen et al. observed that the addition of CTLA-4Ig to anti-CD40L antibody therapy leads to long-term acceptance of skin and cardiac allografts in mice [41]. Shortly thereafter, Kirk et al. noted a similar effect in renal transplants in MHC-mismatched rhesus macaques [42].

Belatacept was approved by the U.S. Food and Drug Administration and the European Medicines Agency in 2011, based on data from the phase 3 BENEFIT trial involving more than six hundred kidney transplant patients [43]. In this trial, the patients were given a belatacept-based regimen or a conventional CsA regimen and were followed for a full 84-month period. In patients receiving the belatacept-based regimen, there was a 43% reduction in the risk of death/graft loss (95% confidence interval: 0.35 to 0.95) 84 months after transplantation, as compared to patients receiving CsA. In addition, the mean estimated glomerular filtration rate (eGFR) increased over the observation period in patients receiving the belatacept regimen but declined in patients receiving the CsA regimen.

Despite this success, some limitations to belatacept-based immunosuppression in transplantation still exist, including an increased risk of post-transplant lymphoproliferative disease (PTLD) in Epstein-Barr virus (EBV)-seronegative recipients [44], and higher rates of early acute cell-mediated rejection. This suggests that not all T-cell subpopulations are equally responsive to belatacept-mediated costimulation blockade [44]. Moreover, the BENEFIT trial reported cumulative rates of biopsy-proven acute rejection of 24.4% with an intensive belatacept regimen, much higher compared to 11.4% with CsA at month 84. Medina Pestana et al. also reported a higher rejection rate of 18% with intensive belatacept compared to 16% with CsA by 3 years [43, 45]. These results have prompted further investigation targeting other co-stimulatory molecules, including CD40-CD40 ligand/CD154, inducible co-stimulator - inducible co-stimulator ligand (ICOS-ICOSL), and OX40-OX40L [44].

2.2 Non-Depleting, Anti-Complement Monoclonal Antibody

2.2.1 Eculizumab (Anti-C5a)

Complement activation has been shown to play a pivotal role in ischemia-reperfusion injury, which may lead to delayed allograft function, and the complement component C5a appears to play a prominent role in this process [46]. Eculizumab (Alexion) is a humanized monoclonal antibody directed against C5a, blocking its cleavage from C5 and its activity [47]. Eculizumab was approved for use in transplant patients in the US in 2011, as the mainstay protocol for preventing the recurrence of atypical hemolytic-uremic syndrome (aHUS) in kidney transplant patients whose primary underlying cause of renal failure was aHUS [47, 48].

The introduction of eculizumab is a therapeutic breakthrough in preventing aHUS recurrence which was prohibitive to successful transplantation, therefore its therapeutic applications have been extended to antibody-mediated rejection (AMR) and prevention of delayed graft function [48].

The efficacy of eculizumab in severe, progressive AMR has been reported, including cases in which the ongoing rejection episode was refractory to rituximab and intravenous immunoglobulin (IVIG)/plasmapheresis [49]. In a second prophylactic study, the rate of AMR decreased to 7.7% as compared to 41.2% in historical controls only receiving plasma exchange [50]. Several clinical trials have been conducted with the purpose to prevent delayed allograft function with eculizumab, but the initial results have not been promising [51, 52]. Although eculizumab is generally well tolerated, it significantly increases patient susceptibility to infections with encapsulated bacteria. The risk of invasive meningococcal disease is estimated at over 2000-fold increased compared with the normal population [53]. Given the efficacy of eculizumab in preventing and treating AMR, it might promote mixed chimerism and tolerance; however, this has not been tested yet.

2.3 Depleting Anti-T-Cell Polyclonal Antibodies

2.3.1 Anti-Thymocyte Globulin

Anti-thymocyte globulin (ATG) has been used for decades in high-risk transplantation patients as a means of inducing tolerance [54]. ATG is a polyclonal IgG produced by immunizing rabbits, horses, or goats with human lymphocytes and subsequently purifying the antibody from sera [54]. In 1963, M.F.A Woodruff first described the ability of ATG to deplete lymphocytes and prolong the survival of skin allografts in rats [55]. Shortly thereafter, Thomas Starzl began administering the drug to liver transplant patients as an additional immunosuppressive medication [56]. Rabbit ATG (rATG) was approved in 1984 in Europe and in 1999 in the United States, and is now the predominant form of ATG used clinically for the induction of immune tolerance and the treatment of acute rejection [27]. Several forms of rATG are in existence depending upon manufacturer and regulatory agency, with Thymoglobulin® being the most commonly used agent in the United States and Grafalon® being the most commonly used agent in Europe. Each has slightly different compositions and kinetics.

Due to the manner in which they are produced, ATG targets a wide variety of cell surface receptors on T-cells, B-cells and NK-cells, including CD2, CD3, CD4, CD8, CD11a, CD18, CD25 and CD45 [57, 58]. Upon administration, it is able to induce rapid and profound T-cell depletion. This is thought to be achieved largely through complement-dependent lysis but also through antibody-dependent cellular cytotoxicity and Fas-FasL-mediated apoptotic cell death [59]. ATG also triggers apoptosis in B-cells by binding to cell surface markers and interfering with T-cell-dependent activation [60]. NK-cells are also depleted due to the interaction of ATG with CD16 and CD56 [60].

rATG has been more successful in short-term management of transplantation than other immunosuppressive medications. In comparison studies with CNIs in low immunological risk renal transplant patients, rATG was found to have a lower incidence of acute rejection; however, it failed to improve one-year graft survival rates and dramatically increased the frequency of adverse events, most notably CMV and HSV infection, fever, leukopenia and thrombocytopenia [61, 62]. On the other hand, trials comparing rATG to basiliximab in high immunological risk patients have demonstrated that rATG confers a significant reduction in the rate and severity of acute rejection and improved outcomes over 5 years [63, 64]. Therefore, rATG is currently indicated for use by the 2009 Kidney Disease: Improving Global Outcomes (KDIGO) clinical practice guidelines in high immunological risk transplant patients [65]. Regardless of the benefits that rATG presents in the treatment of acute rejection, however, this antibody has not been shown to improve long-term graft or patient survival in this type of organ transplant setting [54].

rATG poses several significant challenges that limit its use in the clinic. Importantly following rapid and profound lymphocyte depletion, patients typically require several months to reconstitute the T-cell compartment to its original size and repertoire diversity, leaving them prone to developing leukopenia and infections during this period, particularly CMV, Epstein-Barr virus, and BK polyomavirus [61, 66, 67]. rATG also commonly triggers cytokine release syndrome as lymphocytes die, which can cause high fever, chills, tachycardia, hypotension, gastrointestinal disturbances, and bronchospasm [68]. It is therefore critical for patients to receive pre-treatment with corticosteroids, diphenhydramine, and acetaminophen and be carefully monitored during treatment [68].

In some but not all studies, rATG has also been reported to be a possible risk factor for post-transplant lymphoproliferative disease (PTLD) [69]. PTLD is a form of lymphoid hyperplasia that ranges from a benign condition to life-threatening disease that is associated with transplants from EBV-positive donors to EBV-negative recipients. Since EBV-specific $CD8^+$ T-cells are crucial in preventing this, agents that deplete T-cells such as rATG could increase the risk of PTLD. Although the literature is inconclusive, the plausibility that rATG may increase PTLD risk has emphasized the importance of treating patients with the lowest possible T-cell depleting dose and of giving anti-viral and anti-B-cell agents prophylactically.

Thus, while ATG holds great promise in the field of transplantation, determining the correct dosing regimen and its long-term effects will be essential in expanding its usage. Specifically, efforts to more carefully dose ATG based upon recipient lymphocyte count and time of ATG clearance are likely to improve use of these potent agents - such studies have been conducted and are underway by various groups.

2.4 Depleting Anti-Lymphocyte Monoclonal Antibody

2.4.1 Alemtuzumab (Anti-CD52)

Alemtuzumab (Sanofi), or Campath-1H, is a humanized monoclonal anti-CD52 antibody currently approved for the treatment of chronic lymphocytic leukemia although is also used off-label for inducing tolerance to solid organ transplants and for conditioning for allogeneic HCT. As CD52 is expressed on 95% of peripheral blood lymphocytes, monocytes and thymocytes, Alemtuzumab is able to dramatically deplete over 99% of lymphocytes with a single injection [70-72]. Alemtuzumab is thought to primarily induce cell death upon binding to CD52 through complement-mediated cytolysis, antibody-mediated cytotoxicity and apoptosis [73].

Alemtuzumab was first used for immune tolerance induction in 1998 by Calne et al., who administered the antibody to renal transplant patients in two 20 mg doses on days 0 and 1 post-transplant in combination with a low-dose, maintenance regimen of cyclosporine [74]. Twenty-nine of 31 recipients were able to retain functioning grafts over a year following the transplant and only 6 episodes of steroid-responsive rejection were observed. Although previous antibody-based therapies were shown to improve outcomes in solid organ transplantation, this pivotal study was the first evidence that these agents could also enable sparing of traditional immunosuppressive medications. Since then, several other groups have demonstrated the efficacy of Alemtuzumab in combination with reduced doses of immunosuppressive agents for renal and liver transplantation [72, 75-77].

Subsequent clinical trials have compared the safety and efficacy of Alemtuzumab to other antibody-based induction therapies, particularly basiliximab and rATG. In a 2011 study, Hanaway et

al. found that among low immunological risk renal transplant patients, those receiving Alemtuzumab had significantly lower rates of acute rejection at 6, 12 and 36 months following transplantation than those treated with basiliximab (3% vs. 15%, 5% vs. 17%, and 10% vs. 22%, respectively) [78]. Within the same study, the authors also compared Alemtuzumab to rATG in high immunological risk transplant patients. No significant differences were seen between the two treatment groups with regard to acute rejection or rates of adverse effects over 3 years following the transplant.

There are several notable considerations with the use of Alemtuzumab. In the Hanaway study, both low and high immunological risk patients receiving Alemtuzumab had increased rates of late rejection, defined as rejection first occurring between 12 and 36 months post-transplant, compared to those patients receiving basiliximab or rATG (8% with Alemtuzumab vs. 3% with conventional therapy). Alemtuzumab also had no benefit on long-term survival. Kirk et al. have also previously demonstrated that it is not able to induce tolerance on its own [79]. Furthermore, Alemtuzumab may also confer an increased risk of developing autoimmune disease or infection. In particular, two trials have observed an increased incidence of autoimmune disease following Alemtuzumab treatment (2/33 and 9/27 patients), although the mechanisms have not been defined [80, 81]. Like rATG, Alemtuzumab induces profound and long-lasting lymphocyte depletion, with T-cells only returning to one-third of their baseline levels after one year following injection [82]. Long-term studies of patients receiving Alemtuzumab have not demonstrated increased risk for infection or malignancy, yet this obviously remains a concern for long-term monitoring in this patient population [73].

While Alemtuzumab on its own is unable to induce complete tolerance and its impact on long-term outcomes has yet to be defined, its ability to reduce the dependency of transplant patients on immunosuppressive agents appears to be an advantage.

2.5 Depleting Anti-B-Cell Monoclonal Antibody

2.5.1 Rituximab (Anti-CD20)

Although the allograft immune response appears to be mainly driven by T-cells, B-cells are also likely to contribute. In addition to differentiating into antibody-secreting plasma cells, B-cells shape the T-cell response through a combination of antigen presentation, cytokine production and co-stimulation. Furthermore, B-cells have direct effects on the allograft that can be initiated by ischemic injury [185]. The first association of post-transplant HLA alloantibodies and graft failure was reported by Morris et al in 1969 [186]. Later, clinical data revealed that both pre-existing and de novo-produced donor-specific IgG antibodies (DSA) are strongly associated with acute and chronic antibody-mediated rejection (AMR) in kidney [187, 188], heart [189], lung [83], and liver allografts [84]. B-cell targeted therapies have been effective in treating and reducing the incidence of AMR and inducing tolerance [185].

Rituximab, an anti-CD20 monoclonal antibody, in particular has been used prominently in renal transplantation, often in conjunction with IVIG, plasmapheresis, or other agents, to prevent and reduce the incidence of AMR [85, 86]. An alternative approach is to desensitize patients who have preexisting DSA with rituximab. van den Hoogen et al. observed fewer rejection episodes in sensitized patients treated with rituximab compared to those treated with placebo [87]. In addition to preventing/alleviating rejections, rituximab treatment in kidney transplant patients caused

enrichment of transitional B-cells in the repopulating B-cells after memory B-cell depletion, suggesting a tolerogenic role of rituximab treatment, since transitional B-cells are believed to have immune regulatory functions by producing IL10 [88, 89]. However, there is controversy over the role of rituximab in tolerance induction. Clatworthy et al. observed increased incidence of acute cellular rejection in patients receiving rituximab as induction therapy for kidney transplantation [90]. A similar finding was reported in liver transplantation patients by Ide et al. [91]. Further investigation is necessary to understand how B-cells contribute to the humoral response against donor antigens, and how to selectively direct B-cells toward donor-specific tolerance.

3. Antibodies in Preclinical and Clinical Development

3.1 Lymphocyte-Modulating Antibodies

3.1.1 Anti-CD3

CD3 is a protein complex associated with the T-cell receptor (TCR) that is crucial for signal transduction upon TCR recognition of antigen. Since inhibition of CD3 blocks T-cell activation, many attempts have been made to develop antibodies against this complex for use in transplant tolerance. The first was OKT3 (Janssen-Cilag), also known as muromonab, approved in the United States in 1986 [92]. OKT3 is a mouse monoclonal IgG2a targeting the CD3 epsilon chain. OKT3 binding causes the CD3/TCR complex to disappear from the cell surface by shedding or internalization (antigenic modulation), rendering T-cells blind toward their cognate antigen. At the same time antibody-induced signaling through the CD3/TCR complex can render the T-cell anergic or trigger apoptosis. While antigenic modulation and anergy only render lymphocytes ignorant to antigen and lead to transient immunosuppression, instead anti-CD3 mAb-induced tolerance is dependent on apoptosis causing cell death [92].

OKT3 was used successfully for many years in the treatment of acute renal allograft rejection and later for refractory liver and heart rejection [93-97]. Since OKT3 is a product of murine origin, this agent was greatly immunogenic and patients rapidly cleared it from the circulation, neutralizing its effectiveness [98]. OKT3 is also a potent mitogen and causes a serious flu-like syndrome even following a single injection [92, 99]. The antibody was ultimately withdrawn from use due to low sales as other antibodies with fewer side effects were already on the market [100].

Although OKT3 is no longer clinically available, the drug development pipeline currently contains four other anti-CD3 monoclonal antibodies: three that are partially humanized (visilizumab [Nuvion Plymouth, MN], teplizumab [MacroGenics/Provention Bio], and otelixizumab [Tolerx, Inc., GlaxoSmithKline]), as well as the fully humanized foralumab (Tiziana Life Sciences) [101]. These antibodies have primarily been studied in the context of certain autoimmune diseases, including Type 1 diabetes, Crohn's disease, rheumatoid arthritis, psoriasis, and ulcerative colitis. Although early studies showed encouraging results for otelixizumab and teplizumab in the treatment of Type 1 diabetes, they noted similar flu-like syndromes as OKT3 upon injection, and larger randomized controlled trials also described concerns about the immunogenicity and diminished efficacy of the antibody [190-193]. Likewise, visilizumab and foralumab have yet to show favorable benefit-to-risk profiles for ulcerative colitis and Crohn's disease, respectively [194, 195]. Thus, the safety and efficacy of these antibodies must be improved before expanding their use to the field of transplantation.

3.1.2 Anti-CD40L/CD40

The interaction between CD40 and CD40L is another T-cell co-stimulatory pathway. CD40 is a member of the tumor necrosis factor receptor (TNFR) superfamily initially characterized on B-cells and is also expressed on various antigen-presenting cells (APCs), including dendritic cells (DCs), monocytes, platelets, and macrophages as well as by non-hematopoietic cells such as myofibroblasts, fibroblasts, epithelial, and endothelial cells [102, 103]. CD40 ligand (CD40L or CD154) is a type II transmembrane protein and a member of the TNF superfamily and is primarily found on activated T-cells, activated B-cells and platelets; under inflammatory conditions it is also induced on NK-cells, monocytic cells, mast cells, and basophils [102]. A shed, soluble form of CD40L has been reported that retains costimulatory activity. CD40-CD40L signaling in DCs promotes cytokine production, induces co-stimulatory molecule expression on the DC surface, and facilitates cross-presentation of antigen [102]. In B-cells CD40-CD40L signaling promotes germinal center (GC) formation, immunoglobulin (Ig) isotype switching, somatic hypermutation (SHM) of Ig to enhance affinity for antigen, as well as formation of long-lived plasma cells and memory B-cells [102]. The binding of CD40L to CD40 leads to the upregulation of CD80/86 on APCs. CD80/86, in turn, is able to bind CD28 on T-cells to induce full activation [104]. Inhibition of this pathway, chiefly accomplished through anti-CD40L antibodies, is thus able to prevent complete activation of T-cells, and may additionally cause T-cell depletion and apoptosis [105-107].

Several anti-CD40L antibodies have been developed for use in transplantation, such as ruplizumab (Biogen), toralizumab (IDEC Pharmaceuticals), ABI793 (Novartis), H106 and BMS-986004 (Bristol Myers-Squibb). Initially these antibodies showed great promise in their ability to reduce the rates of rejection and prolong allograft survival. Ruplizumab, or hu5c8, is a humanized IgG1 antibody that when used as monotherapy has been found to induce long-term, stable tolerance to MHC-mismatched allografts in rhesus macaques in both renal and islet transplant models [108, 109]. Toralizumab, known as IDEC-31, is also a humanized IgG1 that prolongs skin allograft survival in MHC-mismatched rhesus macaques when administered in combination with the conventional immunosuppressive agent rapamycin [110]. Though the rates of rejection appear to be higher with the fully humanized IgG1 antibody ABI793 than with ruplizumab and toralizumab, ABI793 prolongs renal allograft survival to a similar extent in macaques [111]. The combined use of H106 antibody and CTLA4-Ig was able to prevent donor specific antibody formation in a rhesus monkey kidney transplantation study [112]. However, translation of these antibodies to the clinic has been greatly limited by thromboembolic complications seen in early preclinical and clinical studies [103, 111, 113].

The mechanism underlying thrombosis associated with anti-CD40L mAb may involve activated platelet binding to the antibodies [196]. Abrogation of the thrombotic events was observed when prophylactic doses of heparin and NSAIDs were given before and during anti-CD40L mAb use [113, 197]. Moreover, there is evidence to suggest that high-order anti-CD40L immune complexes can bind to Fc-gamma receptor IIa and trigger signaling, thereby activating platelets [198]. Based on this finding, an anti-human CD40L dimeric blocking domain antibody was generated, named BMS-986004 [114]. The Ab was produced by fusing the anti-human CD40L Ab to the Fc portion of a modified IgG1 lacking effector function including Fc binding activity (Fc-silent). In a preclinical study using monkeys, the antibody significantly prolonged kidney allograft survival without any evidence of thromboembolism [114]. This gives hope for translation of this protocol into clinical practice.

As an alternative to anti-CD40L antibodies, there have been several attempts at developing antibodies to CD40. The chimeric antibodies ch5D12 and chi220 have been shown to be safe and effective as immunosuppressive agents in non-human primate kidney and islet transplantation but have yet to demonstrate the same degree of potency as anti-CD40L antibodies [112, 115]. Recently, Aoyagi et al. described the use of a novel human anti-CD40 antibody, 4D11, in suppressing T-cell-mediated immune responses and prolonging graft survival [116]. 4D11 may thus serve as a more promising costimulatory inhibitor than its predecessors [117]. CFZ533 is a fully human, Fc-silenced, non-depleting, IgG1 mAb that blocks CD40 pathway signaling and activation of $CD40^+$ cell types. It has shown promise in controlling acute rejection in a 12-month kidney transplant trial and may have potential to become an effective CNI-free treatment for kidney transplant patients [118].

3.1.3 Anti-CD2

CD2, or LFA-2, is an adhesion molecule and a member of the Ig superfamily. Expressed on T-cells, thymocytes, and NK-cells, CD2 binds to CD58 (originally known as LFA-3), on APCs and provides costimulatory signals [119]. In T-cells, this leads to the synthesis of IL-2, T-cell proliferation, and greater T-cell activation [120]. Disruption of CD2 not only inhibits cellular adhesion but also activates the cytolytic machinery of T-cells and induces apoptosis in those cells that are activated [120, 121]. Targeting CD2 to induce tolerance was first accomplished through the fusion protein alefacept (Astellas Pharma US), formed from CD58 and the constant portion of human IgG1 [122]. While alefacept was able to prolong survival when used in combination with CTLA4-Ig in a non-human primate renal transplantation model, it was unable to reduce the rates of acute rejection in Phase II clinical trials of kidney transplant recipients [123-125]. There are several reasons that may account for the lack of effect of Alefacept in this trial. Since the researchers followed the approved psoriasis dosing regimen, patients were likely under-dosed and only reached therapeutic levels about a month after initial administration, far too late for it to have sufficient coverage as an induction agent. Low doses of Alefacept early also caused slight immune activation, as measured by $CD69^+$, possibly contributing to the higher rates of acute rejection in patients receiving the drug as compared to the control group. This study may have seen more promising results had the dose and timing of Alefacept been optimal.

Two other trials have shown the promise of using Alefacept for tolerance induction in non-human primates with costimulatory blockade-resistant allograft rejection [126, 127]. Alefacept was able to deplete $CD8^+$ effector memory T cells, which express high levels of CD2 and low levels of CD28 making them otherwise resistant to Belatacept. Lo et al. demonstrated that the combination of Alefacept and Belatacept were able to almost entirely inhibit proliferation of alloreactive $CD8^+$ T cells in one-way mixed lymphocyte reactions. In mixed chimerism models of inducing tolerance, Lee et al. showed that Alefacept in combination with a conventional immunosuppression regimen of low dose total body irradiation, thymic irradiation, hATG, and anti-CD154 mAb led to prolonged renal allograft survival in non-human primates. Unfortunately, this study was halted when the sponsor decided to discontinue the production of Alefacept, citing business reasons.

Alefacept is no longer in production, but attempts at inhibiting CD2 have continued, primarily through the development of monoclonal antibodies. In the mid-1990s, the anti-CD2 IgG2a antibody OX34 was shown in two separate studies to induce indefinite allograft survival in a rat cardiac transplantation model [128, 129]. Around the same time, Latinne et al. demonstrated that the rat

anti-CD2 IgG2b monoclonal antibody, BTI-322, was capable of inducing alloantigen hyporesponsiveness and apoptosis in activated T-cells [130]. In a clinical trial, BTI-322 administration alongside conventional immunosuppression significantly reduced the incidence of rejection in renal transplant recipients without increasing the rate of adverse events as compared to controls (25% rejection rate with BTI-322 compared to 60% of controls at 6 months) [131]. Similar findings were observed 9 months following transplant [132]. Lerut et al. also demonstrated that the combination of BTI-322 and tacrolimus compared to tacrolimus alone reduced the rates of histological rejection and improved graft survival in patients undergoing liver transplant [133].

Furthermore, a humanized anti-CD2 antibody derived from BTI-322, siplizumab (MEDI-507), was later developed. Siplizumab has a unique three-in-one mode of action: selective T-cell depletion with relative sparing and upregulation of regulatory T cells (Tregs) [134-138]. Through these mechanisms, Siplizumab provided the first example of tolerance induction in HLA-mismatched renal transplantation in humans [139]. Despite these promising results, however, Siplizumab production was halted for business reasons. This program has since been revived and is currently being tested in several additional clinical settings in combination with other agents.

The use of anti-CD2 antibodies with other antibodies in mouse transplantation models has yielded especially encouraging results. Qin et al. demonstrated that the administration of anti-CD2 and an antibody targeting CD48, the murine analog of CD58, leads to better graft survival than either antibody alone (over 100 days with both antibodies compared to a median of 24.4 and 19.9 with anti-CD2 and anti-CD48 alone, respectively) [199]. Chavin et al. described a similar synergistic effect with anti-CD2 and anti-CD3 antibodies in a mouse cardiac transplantation model [200]. The combination of multiple immunosuppressive antibodies may thus be more commonly used in the future in clinical transplant and immune tolerance trials.

3.2 Hematopoietic Stem Cell-Depleting Antibodies

3.2.1 Anti-CD117

Over the last decade, we and our colleagues have pioneered new techniques for enabling hematopoietic chimerism through antibody-mediated depletion of host HSCs. Specifically, in collaboration with Kraft, Weissman and Bhattacharya, in mouse models, we initially showed that host HSCs compete with transplanted donor HSCs limiting their engraftment, and further showed that targeted depletion of host HSCs could be an effective strategy to enable increased donor HSC engraftment. Specifically we showcased this concept using an antagonistic antibody to mouse c-kit/CD117, ACK2, which we proved could deplete murine HSCs as a single agent in *in vitro* and *in vivo* studies and subsequently showed this could enable enhanced engraftment of donor hematopoietic stem cells in immunodeficient settings resulting in disease correction [140]. These studies have since paved the way for development of various anti-HSC-based conditioning regimens for both hematopoietic cell and solid organ transplantation.

Although the CD117 antigen is expressed on some downstream hematopoietic effector cells including mast cells and on rare cells of other tissues, within the hematopoietic compartment it is expressed primarily on HSCs and proximal multipotent and oligopotent progenitors making it an ideal anti-HSC-antigen to target [141]. Studies of enhancing donor hematopoietic cell engraftment by c-kit depletion started with syngeneic transplantation studies in mice. ACK2, an antagonistic naked anti-CD117 antibody [142], was first tested as a conditioning agent in immunocompetent

adult mice where it failed to alone enhance donor bone marrow cell engraftment [143]. However, when it was tested in immunocompromised $Rag2^{-/-}\gamma_c^{-/-}$ mice, 500 μg of ACK2 led to a ~99% decrease in the number of phenotypic and functional HSCs at 7 days after intravenous injection, with a mean donor granulocyte chimerism of 16.1% achieved post transplantation of 5000 wildtype syngeneic HSCs, a >10-fold increase over control unconditioned recipients, with even higher donor chimerism of >80% achieved post transplantation of higher HSC doses [140]. Although this agent was found to be ineffective as a single-conditioning agent in adult immunocompetent mice, subsequently meaningful chimerism was found to be achievable in this setting when sublethal irradiation, CD47 blockade or 5-azacytidine were added to the conditioning regimen [144-146].

Based on the promising results of ACK2 enhancing hematopoietic cell transplantation in immunocompromised animals and potential efficacy in other settings, we began searching for a parallel agent against human HSCs and identified the antagonistic anti-CD117 antibody to human c-kit/CD117, SR-1 [148]. We further showed that this antibody had a similar ability to deplete human HSCs as a single agent in both *in vitro* and *in vivo* xenograft studies [149]. From there we subsequently identified humanized monoclonal anti-CD117 antibody, AMG 191 (Amgen), as an agent with parallel properties that could potentially be used in both preclinical and clinical settings and established a collaboration with Amgen, Shizuru, Logan and colleagues to test this agent in this capacity. In subsequent studies, AMG 191, now renamed JSP 191, was found to have similar activity to SR-1 depleting human HSCs as a single agent in *in vitro* and *in vivo* xenograft studies [147, 150]. Furthermore, these agents have also been reported to have activity against HSCs from non-human primates although there is conflicting data on the full extent of HSC depletion with *in vivo* administration in this setting with lack of reported robust enhanced engraftment post transplantation of gene-marked cells [150, 201].

Based upon these various results and the high need for improved conditioning approaches, Agarwal, Shizuru and colleagues subsequently initiated a Phase I dose escalation clinical trial using the humanized monoclonal anti-CD117 antibody, AMG 191/JSP 191 in doses of 0.1 to 1.0 mg/kg, as the sole conditioning agent for HCT in patients with Severe Combined Immunodeficiency (SCID) [151]. Four of six initial patients included in the study demonstrated robust engraftment of donor hematopoietic grafts, defined by sustained donor myeloid engraftment and T and B-cell lymphopoiesis. Given these promising results, this study has now been expanded to more patients at the predicted optimal 0.6 mg/kg dose. This treatment has additionally been found to be safe and efficacious in newborn SCID patients further expanding the patient population that could benefit from such a treatment [152].

Further studies combining AMG 191/JSP 191 with immunosuppressive agents are now underway to determine whether similar results could be attainable in immunocompetent patients with various blood and immune diseases. Such a finding could ultimately allow AMG 191/JSP 191 to be used in conjunction with other conditioning regimens for HCT for the purpose of immune tolerance as well. Based upon preclinical studies showing anti-CD117 antibody efficacy against myelodysplastic syndrome (MDS) cells [147], a parallel clinical trial has been initiated using AMG 191/JSP 191 conditioning in MDS and acute myeloid leukemia (AML) patients [153]. The results of this study have been promising to date; however, these patients have additionally received significant chemotherapy and total body irradiation as a confounder. Subsequent studies with other immunosuppression backbones are underway to test this agent in this manner including in patients with Fanconi Anemia that have increased sensitivity to genotoxic agents.

While this agent is showing promise for use in several settings, alternative anti-CD117 mAbs for depletion of HSCs have also been reported to be in preclinical development by several other groups. These agents are likely to become available for clinical use in the future although their potency and efficacy across settings remains to be determined.

3.2.2 Anti-CD47

CD47 is a pentaspanin membrane glycoprotein that is expressed ubiquitously in all tissues and is the ligand of signal regulatory protein (SIRP)-alpha (CD172a, SHPS-1), an inhibitory receptor expressed mainly on the surface of myeloid cells. CD47-SIRP-alpha interaction provides a 'don't eat me' signal to macrophages and prevents phagocytosis of autologous hematopoietic cells. CD47-SIRP-alpha signaling also regulates dendritic cell (DC) endocytosis, activation, and maturation [154, 155]. High expression of CD47 by cancer cells confers resistance to macrophage elimination, and blockade of the CD47-SIRP-alpha pathway has also been reported to be effective in promoting tumor elimination by macrophages and in decreasing cancer cell dissemination [156]. Many clinical trials are simultaneously being conducted using various humanized anti-CD47 antibodies, including Magrolimab, for the treatment of diverse solid tumors and hematologic malignancies.

In the field of organ transplantation, the use of anti-CD47 blocking antibodies has been tested in various animal models. Specifically, treatment was found to significantly reduce ischemia-reperfusion injury (IRI) after rat kidney transplantation, possibly by preventing nitric oxide (NO) signaling pathway inhibition [157]. This agent could also potentially be used to enhance the efficacy of anti-HSC and immune depleting antibodies. In a mouse HCT study, Chhabra et al. demonstrated that blocking CD47 with a monoclonal antibody significantly enhanced recipient HSC depletion by anti-CD117 (c-kit) monoclonal antibody ACK2 in immunocompetent animals under syngeneic and allogeneic conditions in combination with antibody-based immunosuppression. Robust donor HSC engraftment and durable hematopoietic mixed chimerism were observed in these HSC-depleted animals [145]. Furthermore, George et al. have shown that when additional antibody-based immunosuppression was added to this regimen and combined with haplo-identical transplantation, stable mixed chimerism could be established that also contributed to allograft tolerance, demonstrating a promising approach that has the potential to induce donor-specific tolerance by mixed chimerism without significant long-term immunoablation [158]. Similar results showing combination anti-CD117 and anti-CD47 antibody mediated depletion of HSCs in non-human primates have also been presented [159], with subsequent clinical programs that are now in development by multiple groups.

3.3 Immunotoxins/Antibody-Drug Conjugates (ADC)

For conventional immunosuppressive agents, non-specific cytotoxicity and genotoxicity to normal proliferating cells lowers the therapeutic index and narrows the therapeutic window. Therefore, the next frontier in immune tolerance protocol development is specific cell subset targeting to minimize collateral damage. In the late 1970's Thorpe et al introduced the concept of using antibodies to direct toxins to specific cell types [202]. Since then, this has become a promising approach for specific cytoreductive therapy, especially in cancer therapy [203]. The experimental use of immunotoxins in organ transplantation was initially pioneered in the 1980's [204], using diphtheria toxin and plant toxins (ricin, gelonin and saporin) [204-206]. Two platforms now exist:

antibody-directed delivery of cytotoxic drugs (antibody drug conjugates: ADCs) and antibody-directed delivery of plant and bacterial toxins (immunotoxins) [207].

As an example, saporin is an *N*-glycosidase derived from the seeds of *Saponaria officinalis* (common name: soapwort) and a member of the ribosome inactivating protein (RIP) family that includes the plant toxins ricin and abrin. Saporin works by depurinating a specific nucleotide in 28S ribosomal RNA, thereby irreversibly blocking protein synthesis resulting in cell death. However, unlike other toxins, saporin lacks a cell entry domain and therefore on its own it is relatively non-toxic [208]. When conjugated to antibodies to cell surface antigens, saporin can be internalized at highly lethal concentrations. In an *in vitro* cytotoxicity assay of melanoma cells, a saporin-based immunotoxin efficiently killed antigen-expressing cells, with a half-maximal inhibitory concentration (IC_{50}) of 1×10^{-10} M; while the viability of antigen-negative melanoma cells was not affected at concentrations as high as 1×10^{-7} M, suggesting the immunotoxin has a high therapeutic index of more than 1000 [209].

3.3.1 Anti-CD3

As an alternative to an anti-CD3 antibody, several groups have developed CD3-targeted immunotoxins. In 1996, Neville et al. first developed FN18-CRM9, an immunotoxin made with an anti-CD3 antibody (FN18) and a binding site mutant of diphtheria toxin (CRM9), which they reported could profoundly yet transiently deplete T-cells in rhesus macaques [160]. Shortly thereafter, Knechtle et al. described the ability of FN18-CRM9 to prolong MHC-mismatched renal allograft survival in 14 of 14 recipient monkeys, with 5 of 6 monkeys who survived long-term exhibiting donor-specific tolerance to skin allografts [161]. FN18-CRM9 was also well-tolerated by the animals, with no evidence of cytokine release syndrome, fever, rash, diarrhea, or respiratory concerns. However, there is a potential problem using CRM9 or other diphtheria toxin-based immunotoxins in human, since most people have a pre-existing anti-diphtheria toxin antibody titers due to diphtheria vaccination which could potentially inhibit or alter the efficacy of these immunotoxins [162].

Resimmune is a recombinant immunotoxin composed of catalytic and translocation domains of diphtheria toxin (DT_{390}) fused to the extracellular domain of CD3 and has low sensitivity to *in vitro* inhibition with serum-containing anti-diphtheria toxin antibodies [162]. In a single-arm, multicenter inter-patient dose escalation Phase 1 trial study of 25 patients of cutaneous T-cell lymphoma, Resimmune achieved a response rate of 36% (95% CI: 18~57%) and complete remission in 4 patients (16%, 95% CI: 5~36%), with acceptable adverse effects, including fever, chills, hypotension, edema, hypoalbuminemia, and hypophosphatemia [163].

The only CD3-immunotoxin currently registered in a transplantation-related clinical trial is T-guard (Xenikos), a Ricin Toxin A-based immunotoxin against CD3 and CD7. In a phase I/II trial examining safety and efficacy in patients receiving allogeneic stem cell transplantation or post-transplantation donor lymphocyte infusion, 12 out of 20 (60%) patients showed responses in steroid-refractory acute graft versus host disease 28 days after the start of therapy, with 10 (50%) patients achieving a complete response; the toxin appeared to be safe and well tolerated with relatively low prevalence of manageable and reversible hypoalbuminemia, microangiopathy, and thrombocytopenia [164]. It is currently being tested in an open-label, single arm phase III multicenter trial.

3.3.2 Anti-HSC Immunotoxins/ADCs

Given the promise of antagonistic antibodies to HSCs in enabling hematopoietic chimerism, we and colleagues also began exploring HSC-targeted immunotoxins for this purpose [167, 175]. Several different candidate HSC antigens were tested, including anti-CD27, CD45, CD49d, CD84, CD90, CD110, CD117, CD133, CD135, and CD184, but only ckit/CD117 and CD45 have been selected for further study.

3.3.3 Anti-CD45

CD45 is expressed exclusively by all hematopoietic cells with the exception of platelets and erythrocytes, and CD45-targeting radioimmune depletion is currently under clinical investigation for hematologic malignancies [165]. Naked antibodies targeting CD45 depleted only lymphoid cells and additional genotoxic chemotherapy was required to deplete HSCs [166]. To enhance HSC depletion while avoiding bystander toxicity (neutropenia, lymphopenia, and thrombocytopenia) caused by CD45-radioimmunotherapy, Palchaudhuri et al. developed a saporin-based CD45 (CD45-SAP) immunotoxin using a biotin-streptavidin linker [167].

With this CD45-SAP immunotoxin they showed that a single intravenous dose (3 mg/kg) was not only able to deplete a majority of lymphocytes, but was also able to deplete almost all HSCs in the bone marrow of wildtype mice by 8 days after injection. Moreover, when syngeneic donor bone marrow cells were transplanted 8 days after CD45-SAP injection, donor cell engraftment was significantly enhanced. In particular, a dose of ten million donor bone marrow cells (~2% of total bone marrow in mice) was able to induce 75–90% donor cell chimerism among total white blood cells in peripheral blood at 4 months after bone marrow transplantation. CD45-SAP pre-treatment caused profound lymphodepletion but there was rapid recovery of adaptive and innate immunity post-transplantation. Further, correction of sickle cell disease using this approach has been demonstrated in a surrogate mouse model [167]. Given these studies, clinical development has begun to generate CD45-ADCs able to deplete human HSCs and additional work in mice has shown potential utility in immunodeficiency models [168, 169], autoimmune models [170, 171] and allogeneic settings [172-174, 178].

3.3.4 Anti-CD117

Due to the cross-species success of antagonistic anti-CD117 antibodies, this target has been further pursued in the development of HSC-directed immunotoxins and antibody-drug-conjugates. Specifically, we initially showed that a non-antagonistic anti-mouse CD117 antibody, 2B8, could be used to generate an effective HSC depleting/conditioning immunotoxin [175]. In its naked form, single dose 2B8 conditioning did not decrease phenotypic or functional HSC numbers *in vivo*, as compared to ACK2 conditioning of immunocompromised mice. Similarly, enhanced HSC engraftment was not observed in immunocompetent mice after naked 2B8 treatment and bone marrow transplantation. However, saporin-conjugated 2B8 antibody (CD117-SAP) was able to deplete >99% endogenous immunophenotypic and functional HSCs in immunocompetent mice, at a single dose of 1.5 mg/kg. Infusion of 10 million congenic donor bone marrow cells at 8-9 days after CD117-SAP conditioning resulted in >98% donor myeloid chimerism as early as 4 weeks after transplantation and persisted for > 20 weeks, suggesting the engraftment of donor bone marrow

cells was significantly enhanced. Importantly, CD117-SAP conditioning was not associated with significant anemia or profound lymphopenia, with just a minor decrease in platelets. Immunity to LCMV infection was also preserved in the treated animals, and toxicity was limited to transient elevations of liver function tests in the first week after treatment. Furthermore, we and colleagues have also shown efficacy of this agent to enable engraftment of gene modified mouse HSCs [176].

Based on these promising results, CD117-SAP-mediated HSC depletion was then investigated in the allogeneic setting using a stringent fully MHC-mismatched BALB/c to C57BL/6 mouse sequential bone marrow and skin allotransplantation model [177]. The extent of HSC depletion and donor bone marrow cell engraftment was assessed by flow cytometric analysis of donor mixed chimerism in the peripheral blood. In this model, the conditioning agents were given once, 6 days before bone marrow transplantation. Transient immunosuppression was applied in the first 30 days after bone marrow transplantation to prevent acute graft rejection (one dose each of depleting anti-CD8 mAb, and non-depleting anti-CD4 and anti-CD154 mAbs on days 0, +2, and +4, plus rapamycin on days +6 and +30). Sequential bone marrow donor-type (Balb/c) and third party (Cba/Ca) skin transplants were then applied on days 160 (primary skin allograft) and 240 (secondary skin allograft) after bone marrow transplantation, all on the same animal. At 100 days after allotransplantation of 20 million-total bone marrow cells, donor mixed chimerism was greatly enhanced in mice receiving CD117-SAP conditioning, ranging from 5-45% with an average of ~20%, as compared to 1-2% in mice receiving megadoses (50 million total bone marrow cells) or transplants conditioned with the naked anti-CD117 antibody. High level chimerism was fairly stable in most animals and durable out to >600 days. High level donor chimerism was also observed at sacrifice in all immune organs except thymus and for both mature myeloid and lymphoid cells as well as hematopoietic stem and progenitor cells.

Similar to the syngeneic study, no significant side effects of CD117-SAP were observed with the exception of a transient ALT/AST elevation. Importantly, skin transplantation experiments in these chimeric mice confirmed a strong association between donor-specific skin allograft tolerance and donor-type mixed hematopoietic chimerism, as evidenced by prompt rejection of skin allografts from the unrelated third-party donors and long-term survival (>411 days) of donor-type skin allografts. However, active immune cell infiltration of mostly B-cells and macrophages persisted in the viable skin allografts. Further studies are needed to determine whether this represents a slowly rejecting alloimmune effector response versus a tolerogenic response; however, importantly, it is clear that the skin was grossly and histologically intact more than one year after allotransplantation following CD117-SAP conditioning and without chronic immunosuppression. Subsequent efforts by Persaud et al. have shown a similar ability to achieve allotransplantation in mismatched settings with both CD117-SAP and CD45-SAP in combination with JAK1/2 inhibitors [178].

The idea of CD117 immunotoxin and ADC-based HSC depletion and conditioning has since been extended to human and rhesus macaque models. Specifically, Pearse et al. and Lanieri et al. have reported that an amanitin-based CD117-ADC effectively depleted normal and AML human HSCs and progenitors *in vitro* and *in vivo* in xenografts [179-181], in addition to showing safety and efficacy in non-human primates [210] with subsequent engraftment of autologous gene-modified hematopoietic stem cells in non-human primates as well [210]. Clinical testing of this agent recently began in a Phase 1 clinical trial with single-dose escalating cohorts in relapsed/refractory AML and MDS patients. Results of this clinical study will likely guide subsequent clinical development, with potential utility ranging from non-malignant diseases to use in establishing mixed chimerism for solid organ transplantation as was shown to be possible in proof-of-concept pre-clinical studies.

4. Conclusions

Immune modulation is required to enable allogeneic cell, tissue and organ graft survival and generate overall positive outcomes in hematopoietic and solid organ transplantation. Historically, this has been achieved using non-targeted or broadly immunosuppressive agents that dramatically affect the entire immune system, resulting in significant adverse effects and long-term complications for patients. As knowledge of the mechanisms underlying rejection expands, it has been possible to target specific components of the alloreactive immune response using naked and conjugated antibodies. Developing the appropriate antibodies with improved safety and efficacy profiles, as well as determining the optimal combinations of these agents may ultimately enable improved outcomes for allogeneic hematopoietic cell and organ transplantation, with the added potential utility in enabling induction of immune tolerance to enable transplantation without lifelong immune suppression thereby further improving outcomes and expanding patient/donor and disease target eligibility.

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Author Contributions

All authors contributed to the writing and editing of this review manuscript.

Competing Interests

Z.L., P.M.M., and A.C. are listed as inventors on a patent application disclosing CD117 antibody-drug-conjugates as a conditioning agent in allotransplantation filed with the US Patent and Trademark Office (16/498,572). Additional disclosures for A.C.: inventor, US patent applications (US 12/447,634; US 14/536,319; US 15/025,222; and US 15/148,837); A.C. also discloses financial interests in the following entities working in the rare genetic disease and transplantation space: Beam Therapeutics, Decibel Therapeutics, Editas Medicines, Global Blood Therapeutics, GV, Lyrik Therapeutics, Magenta Therapeutics, Prime Therapeutics and Spotlight Therapeutics.

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