Tolerogenic therapies in transplantation

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Since the concept of immunologic tolerance was discovered in the 1940s, the pursuit of tolerance induction in human transplantation has led to a rapid development of pharmacologic and biologic agents. Short-term graft survival remains an all-time high, but successful withdrawal of immunosuppression to achieve operational tolerance rarely occurs outside of liver transplantation. Collaborative efforts through the NIH sponsored Immune Tolerance Network and the European Commission sponsored Reprogramming the Immune System for Establishment of Tolerance consortia have afforded researchers opportunity to evaluate the safety and efficacy of tolerogenic strategies, investigate mechanisms of tolerance, and identify molecular and genetic markers that distinguish the tolerance phenotype. In this article, we review traditional and novel approaches to inducing tolerance for organ transplantation, with an emphasis on their translation into clinical trials.

Keywords: B cell therapeutics, cellular therapies, costimulation blockade, mixed chimerism, regulatory T cells, T cell depletion, tolerance, transplantation

INTRODUCTION

Immunologic tolerance was first introduced in 1945 when Ray Owen observed that placental interchange resulted in red cell chimerism between dizygotic bovine twins (Owen, 1945). In the ensuing decade, Peter Medawar, McFarlane Burnet, and colleagues elaborated upon this phenomenon of acquired immunologic tolerance with experimental models of transplantation, which awarded them the Nobel Prize in Physiology or Medicine in 1960. Most of the work at the time involved non-self antigen exposure in immunologically immature hosts, until 1959 when Schwartz and Dameshek demonstrated a marked delay in the adult rabbit immune response to iodine-labeled injections of human serum albumin when treated with 6-mercaptopurine (Schwartz and Dameshek, 1959). Their descriptions of the inhibition of immune pathways in this “drug-induced immunological tolerance” notably foreshadowed the era of pharmacologic development for tolerance induction.

The next 50 years heralded a boom in drug development and subsequent improvements in graft survival. In contrast to 1-year graft survival in 1977 of 53 and 78% for deceased and living-related donors, respectively (Lemons, 1988), modern immunosuppression has enabled transplant recipients to enjoy very favorable graft survival. One-year rates having asymptotically approached 93–96%; therefore, short-term graft survival alone can no longer be held as the metric of success for new immunosuppressants. Instead, as 10-year graft survival rates still trail at 47–61%, new agents must address factors leading to chronic rejection as well as the comorbidities associated with chronic immunosuppression. The decisive measure of success is for a therapy to demonstrate allospecific immunotolerance while minimizing side effects and preserving immune competence to infectious pathogens and cancer during drug administration, and permanent graft survival after its withdrawal.

While transplant tolerance has been largely elusive in human organ transplantation, it has been an achievable feat in animal – particularly murine – models. Non-human primate studies have identified successful preclinical tolerogenic approaches, from T cell depletion and mixed chimerism to costimulation blockade and cellular therapies (Hamawy and Knechtle, 1998; Kawai et al., 2011). Our experience with FN18-CRM9 CD3 immunotoxin in rhesus macaques showed that T cell depletion led to graft survival over 600 days, with five of six long-term survivors demonstrating donor-specific tolerance by skin grafting (Knechtle et al., 1997; Torrealba et al., 2003). Kawai et al. (1995) reported tolerance induction in four cynomolgus macaques that developed multilineage mixed chimerism. Costimulation (CD154) blockade enhanced mixed chimerism and tolerance induction when added to their chimerism-inducing non-myeloablative regimen (Kawai et al., 2004). In the above studies, however, a considerable number of animals developed chronic rejection, sometimes even years before their grafts were terminally rejected. This underscores the metastable nature of tolerance, at least in non-human primates, which is likely mediated by donor-specific regulatory T cells expressing TGFβ (Knechtle and Burlingham, 2004; Torrealba et al., 2004; Ashton-Chess et al., 2007).

Tolerance is inextinguishable achieved outside of liver transplantation in humans and is often encountered serendipitously due to non-compliance or physician-driven immunosuppression withdrawal for severe adverse effects or malignancy. In clinical practice, operational tolerance is defined as “a well-functioning graft lacking pathological signs of rejection, in the absence of any immunosuppressive drugs (for at least 1 year), in an immunocompetent host” (Ashton-Chess et al., 2007; Orlando et al., 2009). Orlando et al. (2009) provided a comprehensive review of all successful and unsuccessful cases of clinical operational tolerance after liver or kidney transplantation. One hundred of 461 liver recipients (22%) remained immunosuppression free 1 year after withdrawal, a total of 163 cases of successful withdrawal were reported (Orlando et al., 2009). In kidney transplantation, over 200 claimed cases of operational tolerance of over 1 year were reviewed (Orlando et al., 2010).
With approximately 28,000 patients undergoing organ transplantation each year, clinicians face a daunting statistic stacked against them.

In pursuit of tolerance, a concerted international effort was made to translate promising basic science findings into clinical practice in transplantation. The US National Institute of Allergy and Infectious Diseases (NIAID) recruited partnerships through tolerance experts in academia, industry, and foundations, and established the US National Institutes of Health sponsored Immune Tolerance Network (ITN) in 1999 (Bicmont et al., 2010). Similarly, the European Commission funded the multinational consortium Reprogramming the Immune System for Establishment of Tolerance (RRISET) in 2003. These consortia afforded researchers to evaluate the safety and efficacy of tolerogenic strategies, investigate mechanisms of tolerance, and identify molecular and genetic markers that distinguish the tolerance phenotype. Here, we review traditional and novel approaches to inducing tolerance for organ transplantation (Figure 1, Table 1). We will discuss within each topic the pre-clinical studies that have or may lead to clinical trials, to focus this topic on the translation of these therapies.

**MOLECULE-BASED APPROACHES**

**T CELL THERAPIES – DEPLETION**

Early attempts at transplantation in humans were fraught with early graft failure due to a robust alloimmune response mediated by activated T cells. We have since learned that the suppression of these alloreactive T cells permits long-term graft survival and, at times, operational tolerance (Starzl et al., 1963; Meier-Kriesche et al., 2004; Womer and Kaplan, 2009). In the 1980s, Strober et al. (1989) observed that some renal transplant patients undergoing total lymphoid irradiation acquired tolerance to their allografts after withdrawal of immunosuppression and demonstrated donor-specific unresponsiveness in vitro. Over 30 years later, the concept of eliminating alloreactive T cells upon induction continues to prevail, as T cell depletion remains the most common induction therapy in the US (IHHS/HRSA/HSB/DOT, 2009). While steroids, calcineurin inhibitors, rapamycin, and mycophenolate mofetil comprise essential components of most immunosuppressive regimens, we will focus our discussion on induction strategies.

Anti-thymocyte globulin (ATG), the oldest depleting agent dating back to the late 1890s, has been a mainstay in induction therapy since the 1960s (Gaber et al., 2010). Due to its potency and markedly heterogeneous target antigen specificities, ATG is particularly useful in high-risk recipients as well as in preventing ischemia-reperfusion injury (Cocka et al., 1993; Shield et al., 1997; Michallet et al., 2003; Bunnapradist and Yekta, 2003; Chappell et al., 2006; Bettsa-Fernandez et al., 2009). ATG has been found to promote regulatory T cells in vitro and in murine studies (Lopez et al., 2006; Shumoni et al., 2012). The NIAID and ITN are currently conducting a phase II clinical trial using rabbit ATG and rituximab (plus tacrolimus and sirolimus) for tolerance induction (Bluestone et al., 2010). Similarly, the European Commission funded the multinational consortium Reprogramming the Immune System for Establishment of Tolerance (RRISET) in 2003. These consortia afforded researchers to evaluate the safety and efficacy of tolerogenic strategies, investigate mechanisms of tolerance, and identify molecular and genetic markers that distinguish the tolerance phenotype. Here, we review traditional and novel approaches to inducing tolerance for organ transplantation (Figure 1, Table 1). We will discuss within each topic the pre-clinical studies that have or may lead to clinical trials, to focus this topic on the translation of these therapies.

**T CELL THERAPIES – COSTIMULATION BLOCKADE**

Alloreactive T cell activation requires antigen-specific engagement of the T cell receptor with major histocompatibility complex molecules (signal 1), followed by antigen non-specific ligation of a variety of receptor–ligand combinations, or costimulation (signal 2; Jenkins and Schwartz, 1987). Blockade of costimulation effectively prevents T cell activation and allograft rejection (Kirk et al., 1997; Li et al., 1999). While costimulation blockade renders the T cell anergic (Schwartz, 1990), these anergic T cells may express inducible costimulator (ICOS) and play a regulatory role (Vermeiren et al., 2004). In addition, costimulation blockade does not require radical ablation of the immune system by lymphocyte depletion or irradiation, thus shifting the emphasis from induction to maintenance immunosuppression (Larsen et al., 2006).

Costimulatory signals of the CD28/CTLA-4 (CD80/86) and CD40/CD154 (CD40L) tumor necrosis factor (TNF) family are the most studied and potentially most important activating costimulation pathways. Cytotoxic lymphocyte antigen-4 (CTLA-4) shares about 30% homology with CD28, and binds with 10–20-fold higher affinity than CD28 to B7 molecules on the antigen presenting cell (APC). Not only does this potently inhibit the T cell, but also its ligation with APC B7 molecules induces indoleamine 2,3-dioxygenase expression, promoting the suppressive functions of CD8+ T cells (Maini et al., 2004). Abatacept (Orencia, Bristol-Myers Squibb) and belatacept (Nulojix, Bristol-Myers Squibb), fusion proteins composed of CTLA-4 and immunoglobulin IgG1, have utilized this mechanism to confer potent inhibition of alloreactive T cell responses. Belatacept was developed to increase affinity for CD86, with an increase in affinity by fourfold for CD86 and by twofold for CD80. Belatacept more effectively inhibited T cell activation in vitro compared to its predecessor CTLA-4lg (Larsen et al., 2005). Preclinical studies
FIGURE 1 | Approaches to transplant tolerance induction. (Top left) Mixed chimerism is achieved by infusing donor bone marrow into myelo-conditioned recipients, to establish co-existence of donor and recipient cells in the setting of organ transplantation. The dotted arrows indicate cell types originating from the bone marrow, unrelated to mixed chimerism. (Top right) Allospecific T cell responses can be abrogated through a number of mechanisms, including irradiation, pharmacologic lymphodepletion by ATG or alemtuzumab, suppression of activation by costimulation blockade or IL-2 receptor blockade. (Bottom right) Tolerogenic cell types, including regulatory T cells, macrophages, and mesenchymal stromal cells, can inhibit effector T cells through direct ligation or inhibitory cytokine production. (Bottom left) The humoral response can be suppressed through B cell depletion, and blockade of survival factors (BAFF), plasma cells, and complement.

using CD28:B7 blockade were able to demonstrate prolonged graft survival in non-human primate models of islet transplantation (Adams et al., 2002).

In a randomized, phase III human clinical trial called Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression Trial (BENEFIT), recipients of living or standard criteria deceased donors underwent basiliximab induction with mycophenolate mofetil and a steroid taper. Belatacept maintenance, compared to cyclosporine, resulted in superior renal function, cardiovascular and metabolic profiles in the first 2 years (Larsen et al., 2010; Vanrenterghem et al., 2011; Pestana et al., 2012); extension of the trial to recipients of extended criteria
Table 1 | Strategies for tolerance induction. This table outlines the pharmacologic, biologic, and cellular therapies discussed in this article, categorized by T cell agents, B cell agents, and cellular therapies (including mixed chimerism).

| Category | Therapeutic | Mechanism |
|----------|-------------|-----------|
| T cell depletion | Anti-thymocyte globulin (ATG) | Depleting polyclonal antibodies to thymocytes that express multiple target antigens; possible induction of regulatory T cells |
| | Alemtuzumab | Depleting mAb to CD52, on T, B, NK cells, some monocytes |
| Costimulation blockade | Abatacept | CTLA-4 Ig, blockade of CD28:CD80/86 costimulatory pathway |
| | Belatacept | CTLA-4 Ig, blockade of CD28:CD80/86 costimulatory pathway |
| | Efalizumab | Blockade of LFA-1:ICAM-1 costimulatory pathway |
| | Basiliximab | Blockade of CD25 (Interleukin 2 receptor α chain) |
| | Basiliximab + rapamycin | Interleukin 2 + rapamycin, to increase regulatory T cell proliferation and survival, and stabilize the expression of Forkhead box P3 (FoxP3) |
| B cell therapeutics | Rituximab | Depleting mAb to CD20 |
| | Belimumab | Blockade of B cell activating factor (BAFF), causing depletion of follicular and alloreactive B cells, decrease in alloantibody response, and promotion of immature/transitional B cell phenotype and a regulatory cytokine environment |
| | Atacicept | Blockade of BAFF and APRIL |
| | BR3-Fc | Blockade of BAFF, causing decrease in peripheral, marginal zone, and follicular B cells |
| | Bortezomib | Proteosome inhibitor, causing apoptosis of mature plasma cells |
| | Eculizumab | Blockade of complement protein C5, to prevent complement mediated injury due to circulating alloantibody |
| Cellular therapy | Mixed chimerism | Infusion of donor bone marrow into myeloablated/immune-conditioned recipient, to produce coexistence of donor and recipient cells |
| Regulatory T cells | Regulatory T cells | Infusion of expanded regulatory T cells, to inhibit inflammatory cytokine production, downregulate costimulatory and adhesion molecules, promote anergy and cell death, convert effector T cells to a regulatory phenotype, and produce suppressive cytokine IL-10, TGF-β, and IL-35 |
| Regulatory T cells + IL-2 | Regulatory T cells + IL-2 | As above, plus the addition of IL-2 to promote Treg survival, development, and expansion |
| Dendritic cells | Dendritic cells | Immunomodulatory effects include their ability to acquire and present antigen, expand and respond to antigen-specific Tregs, constitutively express low levels of MHC and costimulatory molecules, produce high IL-10 and TGF-β and low IL-12, resist activation by danger signals and CD40 ligation, resist killing by natural killer or T cells, and promote apoptosis of effector T cells |
| Macrophages | Macrophages | Immune suppression mediated through the enroachment of CD4+ CD25+ Foxp3 cells and cell contact- and caspase-dependent depletion of activated T cells |
| Mesenchymal stromal cells | Mesenchymal stromal cells | Inhibition of T cell activation and proliferation, potentially due to production of IL-10, NO, and IDO, and suppression of INFγ and IL-17 |

Donors found similar protective effects on graft function as measured by mean calculated glomerular filtration rate (Pestana et al., 2012). All studies, however, documented increased risk of post-transplant lymphoproliferative disorder in the belatacept-treated arm, compared to the cyclosporine-treated arm.

Activated T cells rapidly upregulate CD154 (CD40L) expression and can bind to CD40, which is constitutively expressed on B cells, dendritic cells (su), and macrophages (van Kooten and Banchereau, 1997; Ju). Blockade of this pathway significantly prolongs allograft survival in non-human primate kidney, heart, skin, peripheral nerve, allotest, and xenoislet transplantation (Kirk et al., 1997, 1999; Pearson et al., 2002; Xu et al., 2002, 2003; Brenner et al., 2004; Kawai et al., 2004; Adams et al., 2005; Arima et al., 2006; Hering et al., 2006; Pearl et al., 2007, Aoyagi et al., 2009; Thompson et al., 2011; Badell et al., 2012). Newer antibodies targeting this pathway have avoided platelet activation-induced thromboembolic complications observed with older anti-CD154 mAbs (Koyama et al., 2004). Newer CD40/CD40L blocking agents have yet to be translated to clinical trials.

The lymphocyte function-associated antigen (LFA-1): intracellular adhesion molecule (ICAM) costimulation pathway has also been studied through therapeutic blockade in transplantation. Badell et al. (2010) reported that short-term treatment with LFA-1 prolonged islet allograft in rhesus macaques, and
suggested its utility in treating CD28-costimulation blockade-resistant T cell populations. Turgeon et al. (2010) observed that efalizumab (Raptiva, Genentech/Merck Serono) treated patients experienced fewer immunosuppression-related events compared to the standard Edmonton protocol, and also required no additional iatrogenic insults to achieve insulin independence. Efalizumab was withdrawn from the market in 2009 due to a reported increased risk of progressive multifocal leukoencephalopathy (Carson et al., 2009).

OTHER T CELL THERAPIES

While numerous other surface molecules such as ICOS and very late antigen 4 (VLA-4) have been targeted (Matthews et al., 2003), we will limit discussion here to two trials sponsored by the ITN. In 1999, Shapiro and colleagues presented results from a multicenter, international clinical trial evaluating the Edmonton protocol for islet transplantation, which used interleukin-2 receptor α chain (CD25) blockade for induction (Shapira et al., 2006). Fifty-eight percent of patients achieved insulin independence, although only 31% of them remained independent after 2 years. While dazaximab (Zenapax, Hoffmann-La Roche), used in the trial, was discontinued in 2009, basiliximab (Simulect, Novartis) remains a popular induction agent. The Kidney Disease: Improving Global Outcomes (KDIGO) group and European Renal Best Practice Advisory Board recommended for all non-high risk kidney transplant recipients to receive IL2R blockade as first line induction therapy (Kasiske et al., 2010).

The ITN is also sponsoring a phase I trial in type 1 diabetes, using a combination of IL-2 adseleukin (Proleukin, Prometheus) and rapamycin to arrest islet cell destruction. Animal studies have shown that treatment with IL-2 increases regulatory T cell proliferation and survival (Rabinovitch et al., 2002; Tang et al., 2008). Combination with rapamycin, which stabilizes the expression of Forkhead box P3 (FoxP3) and enhances suppression (Battaglia et al., 2006; Singh et al., 2012), may promote tolerance in these autoimmune and potentially alloimmune settings.

B CELL THERAPIES

The role of B cells in operational tolerance has yet to be defined. On one hand, an ITN-sponsored collaboration identified a unique B cell signature associated with 25 operationally tolerant renal transplant candidates, but recently terminated the study for not reaching efficacy in its primary goals (Zarkhin et al., 2009). Its blockade using human recombinant mAb belimumab (Benlysta, Human Genome Sciences/GlaxoSmithKline) promotes tolerance in murine cardiac and islet allograft models by (1) depleting follicular and alloreactive B cells, (2) promoting an immature/transitional B cell phenotype, (3) abrogating the alloantibody response, and (4) sustaining a regulatory cytokine environment (Zarkhin et al., 2009; Vivek et al., 2011). The same group evaluated belimumab in a phase II clinical trial for the desensitization of kidney transplant candidates, but recently terminated the study for not reaching efficacy in its primary goals (clinicaltrials.gov ID: NCT01025193). Atacicept (ZymoGenetics/Merck Serono) and BR3–Fc (Briobacept, Genentech/Biogen Idec, discontinued in 2011) are two other BAFI pathway-targeting agents that have demonstrated reduction of alloantibodies and peripheral B cells in non-human primates (Vugmeyster et al., 2010).
AMR in ABO blood group incompatible living donor kidney transplants and the clinical implementation of his and his colleagues’ findings in solid organ transplant recipients suggests a number of barriers needed to be overcome before clinical application of chimerism could be successful (Jankowski and Ildstad, 1997). The most significant of those barriers is the conditioning of donor’s and recipients to produce an environment where both donor and host hematopoietic cells can co-exist (Jankowski and Ildstad, 1997; Sachs et al., 2011). In somewhat simplistic terms, a mature host immune system has had time to develop and produce a presumably robust and crowded repertoire of immune cell populations. In order to produce a mixed population of cells, that crowded repertoire must be reduced in size to allow donor hematopoietic cells to exist. Furthermore, recipients must be conditioned to accept these donor cells. Finally, donor cells that could attack the host and cause graft-versus-host disease (GVHD) also need to be eliminated while at the same time preserving the recipient’s ability to produce immune populations that can defend against infections (Jankowski and Ildstad, 1997; Sachs et al., 2011).

These barriers favored a strategy of pursuing mixed chimerism in solid organ transplant recipients, as total marrow ablation associated with full chimerism was thought to be too risky in patients undergoing a semi-elective procedure who would otherwise do well with standard immunosuppression regimens (Sachs et al., 2011). Numerous groups but particularly those of Ildstad and Sachs demonstrated in animal and non-human primate studies that partial irradiation of the recipient bone marrow with peripheral deletion of recipient T cells allowed for the development of both donor and recipient hematopoietic cells and induction of tolerance to donor tissue without the need for full myeloablation (Ildstad and Sachs, 1984; Sharabi and Sachs, 1989; Kaufman and Ildstad, 1994; Colson et al., 1995). Mixed chimerism was also found to be beneficial over full chimerism from an infectious risk standpoint both in Ildstad and Sachs’ work as well as in humans undergoing bone marrow transplantation for hematopoietic malignancies (Rayfield and Brent, 1983; Ruedi et al., 1989). While non-myeloablative conditioning only promoted transient mixed chimerism in the HLA-mismatched setting, long-term renal allograft survival was achieved in most patients (Kawai et al., 2011). Sachs and colleagues took their experimental findings and then went on to implement these strategies in the clinic (Kawai et al., 2008; Spitzer et al., 2011). To date, their group has published two series on induction of mixed chimerism in kidney transplant recipients and subsequent induction of tolerance. Having found that tolerance in chimerism has both a central and peripheral component, their induction strategy now includes thymic irradiation to allow for development of a donor T cell reservoir in these solid organ recipients (Kawai et al., 2008; Sachs et al., 2011; Spitzer et al., 2011).

The results from the aforementioned studies indicate that in both HLA-matched and -mismatched recipients induction of mixed chimerism may be a viable strategy for inducing tolerance in solid organ recipients. To date, of the HLA-matched recipients, seven of eight experienced no episodes of rejection with the single patient with rejection being treated and back on standard immunosuppression. All of these patients also had multiple myeloma so they underwent concomitant bone marrow transplantation. Unfortunately, despite the success of their solid organ transplants, three of the recipients have had recurrence of
their multiple myeloma (Sachs et al., 2011; Spitzer et al., 2011). Among the HLA-mismatched patients, one of nine experienced acute rejection, which was effectively treated, and one of nine currently has chronic allograft injury (Kawai et al., 2008; Sachs et al., 2011). The Stanford group recently published their experience of sixteen patients undergoing HLA-matched kidney and hematopoietic cell transplants (Scandling et al., 2012). Conditioning with total lymphoid irradiation and ATG promoted increased proportions of CD4^+ CD25^+ regulatory T cells (compared to naive CD4 T cells) and chimerism in 15 patients. Eight patients had successful withdrawal of immunosuppression for 1–3 years, and only four were unable to withdraw due to recurrent disease or rejection. These results, though limited, indicate an exciting future for chimerism as a strategy for inducing tolerance in solid organ transplant recipients. They serve as evidence that observations in basic science serve as the basis for new discovery of effective clinical immunosuppressive therapies in the field of transplant surgery.

OTHER CELL-BASED APPROACHES

REGULATORY T CELLS

The immune repertoire of experimental animal models and operationally tolerant patients strongly suggests a major role of regulatory T cells (Tregs) in inducing and maintaining tolerance (Graca et al., 2002; Levitsky, 2011). The mechanisms by which these CD4^+ CD25^+ T cells exert regulatory control of immune responses are diverse. Upon allorecognition via direct or indirect pathways, Tregs can suppress other T cells through inhibition of cytokine production, down-regulation of costimulatory and adhesion molecules, promotion of anergy and cell death, and conversion of effector T cells to a regulatory phenotype (Wood and Sakaguchi, 2003). O’Garra and Vieira, 2004). A key transcription factor in Treg development and function, Forkhead box protein 3 (Foxp3) has been commonly used to distinguish this population (Hori et al., 2003; Collison et al., 2007), although Foxp3− T cells producing suppressive cytokines IL10 (type I), TGFβ (type 3), and IL35 (type 35) have been identified (Nakamura et al., 2004; Vieira et al., 2004; Collison et al., 2007).

In vitro expansion of Tregs has been shown to preserve suppressive function (Levings et al., 2001; Godfrey et al., 2004), thus making it an attractive tolerogenic therapy. Polyclonal expansion using magnetic beads coated with CD3 and CD28 antibodies may yield a several hundred-fold expansion of antigen non-specific Tregs that maintain classic surface and intracellular Treg markers and more importantly their regulatory function (Bluestone, 2005). Hoffmann et al. (2004) documented up to 40,000-fold expansion in vitro by repeatedly stimulating with CD3 and CD28 and high dose interleukin 2. While using this technique significantly inhibits graft-versus-host disease (GVHD) as well as allo- and auto-immunity (Taylor et al., 2002; Xia et al., 2006), the inhibitory effect is more pronounced when antigen-specific Tregs are administered (Masteller et al., 2003; Trenado et al., 2006; Nagahama et al., 2007; Zenk et al., 2009; Brennan et al., 2011).

Antigen-specific Tregs can be generated in several ways. Cohen et al. (2002) co-cultured purified CD4^+ CD25^+ CD62L^− T cells with irradiated splenocytes and observed a significant delay in GVHD development in a murine model. Interestingly, the treated mice later developed severe GVHD, suggesting a limited half-life of these ex vivo expanded Tregs. Joffre et al. (2008) observed long-term tolerance in irradiated mice were treated with allograft-specific Tregs in bone marrow, and subsequent skin and cardiac allograft models. In a rat liver transplant model, Pu et al. (2007) found that donor-specific splenocyte-stimulated Tregs prolonged graft survival when compared to third party splenocyte stimulated Tregs and freshly isolated syngeneic Tregs. Short-term tacrolimus administration with donor-specific Tregs further enhanced long-term graft acceptance. Yamazaki et al. (2006) observed that dendritic cells were more effective than splenocytes at expanding Tregs and sustaining their Foxp3 expression. Godshalk et al. (2007) used autologous dendritic cells pulsed with an allogeneic peptide to promote skin graft tolerance; this approach was later implemented on murine cardiac allografts and paired with short-term rapamycin treatment to achieve indefinite graft survival in three of four mice (Tsang et al., 2009). Peptide-MHC multimers can also be used to create antigen-specific Tregs. Masteller et al. (2005) employed beads coated with recombinant islet peptide mimic-MHC class II plus CD28 antibodies and IL-2; expanded islet peptide mimic-specific Tregs were more efficiently able to suppress autoimmune diabetes in non-obese diabetic mice than polyclonally activated Tregs. Antigen-specific Tregs have also been generated using lentiviral T cell receptor gene transfer into polyclonally expanded cells (Brusko et al., 2010). Finally, Tregs expanded up to 50 million fold by artificial APCs have been shown to maintain suppressor function and reduce GVHD lethality (Hippen et al., 2011). The ability to massively expand functional Tregs in such ways may overcome the challenge of extracting enough circulating Tregs for therapeutic preparation.

In vivo expansion of antigen-specific Tregs has also been described in a mouse model (Nishimura et al., 2004). Yamazaki et al. (2003) described the use of antigen-loaded dendritic cells to stimulate CD4^+ CD25^+ T cell proliferation in vivo, and induce expansion of adoptively transferred CD4^+ CD25^+ T cells as well. Walker et al. (2003) found that Tregs deemed anergic based on in vitro stimulation assays were capable of proliferating in vivo in response to immunization. These studies suggest that therapeutically administered antigen-specific Tregs can continue to be expanded in vivo.

The initial clinical trials utilizing Treg immunotherapy for hematopoietic stem cell transplantation (HSCT) have shown promising results (Edinger and Hoffmann, 2011). Brunstein et al. (2011) recently published the University of Minnesota experience, where umbilical cord blood (UCB) derived Tregs were CD3/CD28/IL2 expanded and infused after double UCB transplantation. UCB Tregs were detectable for 14 days, were free of infusion toxicities, and reduced the incidence of severe GVHD. In Janini et al. (2011) from the University of Perga, Italy, observed that co-infusion of Tregs with conventional T cells in the absence of concurrent immunosuppression prevented lethal GVHD and promoted immune reconstitution and protective immunity in 28 patients undergoing HLA-haploidentical HSCT. As interleukin-2 has been found to be critical for Treg survival, development, and expansion (Nelson, 2004; Malik, 2006), it has been administered in clinical trials of autoimmunity and refractory chronic GVHD to augment Treg numbers (Koreth et al., 2011; Saadoun et al. 2011).
CD4 described as a non-dendritic cell and more mature form of rest-reduced inflammation from chronic colitis. These IFN-MdC, procured from mouse spleen, blood, and bone marrow of interferon gamma-stimulated monocyte-derived cells (IFN-γ-MdC), have been shown to significantly suppress allogeneic lymphocyte proliferation in mixed lymphocyte reactions, by suppressing IFN-γ, IL-12, and TNF-α production by mature DCs, TH1 cells, and NK cells, and increasing IL-10, IL-4, and PGE2. Co-infusion of MSCs with donor bone marrow has been shown to enhance mixed chimerism, reverse GVHD, and improve vascularized skin grafts in rats (Akou et al., 2008).

In a rat islet transplantation model, Solar et al. (2009) demonstrated long-term islet allograft survival, normal serum insulin levels, and normoglycemia when autologous MSCs were co-transplanted with marginal islet masses. Promising results from a phase II clinical trial showed that 39 of 55 patients with steroid-resistant, severe acute GVHD responded to MSC therapy and experienced a significant survival benefit (Le Blanc et al., 2008). Phase III randomized, placebo-controlled clinical trials, however, failed to show benefit in the setting of refractory GVHD (Allison, 2009; Askew and Karp, 2010).

Recently, MSCs harvested from term fetal membranes have been shown to significantly suppress allogeneic lymphocyte proliferation in mixed lymphocyte reactions, by suppressing IFN-γ, IL-12, and TNF-α production by mature DCs, TH1 cells, and NK cells, and increasing IL-10, IL-4, and PGE2. Co-infusion of MSCs with donor bone marrow has been shown to enhance mixed chimerism, reverse GVHD, and improve vascularized skin grafts in rats (Akou et al., 2008).

CONCLUSION
Operational tolerance in organ transplant patients continues to be an elusive clinical goal but has stimulated a broad variety of approaches. Research in tolerance has elucidated mechanistic pathways of rejection, T cell regulation, and T cell activation previously unknown. In concert with therapeutic approaches to tolerance, diagnostic assays to identify tolerance and distinguish it from “non-tolerance” are needed, and progress continues in this area relying in part on microarray analysis of tolerant patients. For instance, Li et al. (2012) have identified a small set of
13 genes common to both adult and pediatric liver transplant patients demonstrating operational tolerance. The work by the group of Sanchez-Fueyo continues to demonstrate that operational tolerance can be achieved in pediatric patients and that the mechanisms involved may differ significantly from those seen in adults. The results suggest that both blood and liver tissue gene expression can predict the outcome of immunosuppression withdrawal in children. The clinical significance of tol-
erance in liver transplantation may differ significantly from that of kidney transplantation for reasons that are unknown at this time (Sagoo et al., 2010). While most clinical work on tolerance focuses on liver transplantation since this organ lends itself best to transplant tolerance, only a miniscule fraction of liver transplant patients appear to have achieved stable tolerance, and efforts in this area need to be conducted under strict clin-
cical guidance in protocols designed to protect the patients’ best interests (Levitsky, 2011). Nevertheless, it would appear likely that as immunologic monitoring evolves into a clinical reality, ongoing efforts in this arena need to be conducted under strict clinical guidance in protocols designed to protect the patients’ best interests. (Levitsky, 2011). Nevertheless, it would appear likely that as immunologic monitoring evolves into a clinical reality, ongoing efforts in this arena need to be conducted under strict clinical guidance in protocols designed to protect the patients’ best interests.
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