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Regulatory T-Cell Therapy in Transplantation: Moving to the Clinic

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Regulatory T cells (Tregs) are essential to transplantation tolerance and their therapeutic efficacy is well documented in animal models. Moreover, human Tregs can be identified, isolated, and expanded in short-term ex vivo cultures so that a therapeutic product can be manufactured at relevant doses. Treg therapy is being planned at multiple transplant centers around the world. In this article, we review topics critical to effective implementation of Treg therapy in transplantation. We will address issues such as Treg dose, antigen specificity, and adjunct therapies required for transplant tolerance induction. We will summarize technical advances in Treg manufacturing and provide guidelines for identity and purity assurance of Treg products. Clinical trial designs and Treg manufacturing plans that incorporate the most up-to-date scientific understanding in Treg biology will be essential for harnessing the tolerogenic potential of Treg therapy in transplantation.

One of the major challenges facing the field of transplantation is the management of immunosuppression. Although immunosuppression is necessary to prevent immune attacks of the transplanted organ, it also imposes substantial morbidity and mortality risks for transplant recipients. Chronic global immunosuppression impairs immune responses to microbial pathogens and hinders tumor immunosurveillance. Often, infections and posttransplant cancers, rather than allograft rejection, are the major contributors of transplant-related mortality, especially beyond the first year after transplant (Penn 1990; Euvrard et al. 2003; Soltys et al. 2007). In addition to these immunological complications, immunosuppressive drugs are often causes of morbidity owing to their off-target effects such as nephrotoxicity, diabetes, hyperlipidemia, hypertension, cardiovascular diseases, and obesity (Berenson et al. 1992; Textor et al. 2000; Nair et al. 2002; Ojo et al. 2003). All these complications may necessitate reduction or even withdrawal of immunosuppression that leads to graft rejection and graft loss. Therefore, the key to improving immunosuppression after transplantation is to selectively block the immune responses against the graft without impeding other protective immune functions or causing nonspecific toxicities.

In this article, we propose that such goal can be accomplished by harnessing the natural immune regulatory mechanisms using cell-

based therapies. Various types of T cells have been shown to contribute to transplant tolerance. These include the CD4$^+$CD25$^+$ regulatory T cells (Tregs) that express the transcription factor FOXP3, IL-10-producing Tr1 cells, CD8$^+$28$^-$ T cells, and anergic T cells. In this article, we will focus on the FOXP3-expressing Tregs. We summarize parameters that are important for effective application of Treg therapy to prevent graft rejection in experimental models and review advances in translating these preclinical experiences to the clinic.

ADVANTAGES OF Treg THERAPY IN TRANSPLANTATION

None of the current immunosuppressive drugs can suppress immune responses to transplant antigens without potentially altering immune surveillance toward tumor antigens and microbial pathogens. This is because immunosuppressive drugs target common pathways of immune activation, such as calcium signaling (cyclosporin A, FK506), purine biosynthesis (Mycophenolate), and T-cell costimulation (CTLA4Ig). Other immunosuppressive drugs such as antithymocyte globulin, Campath-1, and anti-CD20, massively and nonspecifically delete immune cells. In contrast, T cells have an extraordinary ability to distinguish minute differences among different antigens. Aside from the specificity conferred by the T-cell receptors, specificity of therapeutic T cells is also amplified by their ability to seek their targets throughout the body and deliver effector functions locally where they are most effective and specific. These properties underlie the remarkable efficacy of T-cell therapy in treating drug-resistant recurrent cancers (Restifo et al. 2012; Scholler et al. 2012). For example, using autologous cytotoxic T cells engineered to express a chimeric receptor that recognizes CD19, 19 out 20 patients with end-stage therapy-refractory B-cell lymphoma have been successfully treated (Porter et al. 2011; Scholler et al. 2012). Thus, T cells can be used to deliver highly specific and targeted therapies.

Similarly, immune tolerance mediated by Tregs is also highly antigen specific. Immunosuppressive functions of Tregs are activated by the engagement of their T-cell receptors locally at the site of antigen deposition. In a mouse model of autoimmune diabetes and autoimmune pancreatitis, we have observed complete protection against islet destruction using islet antigen-specific Tregs, whereas autoimmune attack of the surrounding exocrine pancreas progressed without hindrance (Meagher et al. 2008). In the transplant setting, tolerance maintained by Tregs is specific to the graft donor, whereas unrelated grafts are rejected (Joffre et al. 2008). Tolerance can spread to a new transplant antigen, and this "linked suppression" is mediated by Tregs in the graft. The establishment of linked suppression requires that the new antigen and the antigen the host is already tolerant to are present in the same graft. Thus, immune tolerance to transplant antigens mediated by Tregs is highly specific and highly localized. Despite their high antigen specificity, Tregs are highly versatile and can control responses of various immune cells including conventional CD4$^+$ T cells, CD8$^+$ T cells, natural killer (NK) cells, natural killer T (NKT) cells, B cells, and various antigen-presenting cells. Moreover, Tregs have a collection of more than a dozen different immunosuppressive mechanisms and can deploy different strategies depending on the tissue microenvironment (Tang and Bluestone 2008; Yamaguchi et al. 2011). In comparison to standard immunosuppressive drugs used in transplantation today, Tregs are "smart" therapeutic agents that are highly antigen specific and highly adaptable, capable of selectively targeting graft tissues by tuning their activities in response to the tissue microenvironment.

Besides the problems with long-term global immunosuppression and off-target toxicities, most of the current immunosuppressive drugs prevent tolerance induction and create a dependence on continuous immunosuppression. Immunosuppressive drugs used in the clinic today are selected based on their ability to prevent immune activation. Research from the past three decades shows that acquisition of immune tolerance to self-antigens and transplantation antigens is an active process that requires antigen exposure. A tolerogenic antigen exposure leads to inactivation of antigen-reactive T

cells through apoptosis, anergy, and induction and expansion of immune regulatory mechanisms that maintain tolerance. The current approach of immunosuppressing transplant recipients blindfolds the immune system to prevent rejection, but also impedes tolerance induction. This may explain why spontaneous transplant tolerance is rare and the best predictor of tolerance is time after transplant (Sanchez-Fueyo 2011), likely through the cumulative effects of a low level of donor antigen exposure over a long period of time. To induce tolerance and freedom from chronic immunosuppression, transplant recipients should receive immunoregulatory regimens that block rejection while permitting donor antigen recognition. In this regard, Tregs can induce other T cells to acquire regulatory functions in vitro (Dieckmann et al. 2002; Jonuleit et al. 2002; Oliveira et al. 2011). In a mouse model of transplantation, Treg therapy not only prevents rejection, but also allows the induction of new Tregs with broader specificities through a process of infectious tolerance (Waldmann 2008). Therefore, Treg therapy can potentially turn the graft tissue from a target of immune attack into a tolerogenic organ that promotes its own long-term survival.

CRITICAL PARAMETERS FOR EFFECTIVE Treg THERAPY IN TRANSPLANTATION
Dosing

Tregs ensure normal immune homeostasis by providing a counterbalance for the effector arm of the immune system. In an immunologically quiescent state, Tregs represent $\sim 5\% - 10\%$ of $CD4^+$ T cells in lymphoid tissues. At this ratio, Tregs prevents unwanted immune activation by reducing expression of costimulatory molecules CD80 and CD86 via CTLA-4-mediated trogocytosis (Muthukumar et al. 2005; Dijke et al. 2007) and by sopping up IL-2 and other common γ-chain-binding cytokines (Pandiyan et al. 2007; O'Gorman et al. 2009). During an active immune response, Tregs proliferate, traffic, and accumulate at the site of inflammation, particularly at the later phase of the response, to restore normal immune homeostasis using a wider array of effector mechanisms including immunosup-

pressive cytokines IL-10, IL-35, TGF-β, cell-surface ATPases, granzyme-dependent killing of antigen-presenting cells, IL-9-mediated recruitment of mast cell, etc. (Tang and Bluestone 2008; Yamaguchi et al. 2011). Consequently, the number of Tregs often increases with inflammation and graft rejection (Muthukumar et al. 2005; Dijke et al. 2007). In the transplant setting, such increases are usually not sufficient and too late to prevent graft damage; therefore, effective Treg therapy should be given at a dose sufficient to tip the balance in favor of Tregs before the rise of effector responses.

Two approaches can be applied to estimate the effective dose of Tregs in humans to prevent graft rejection: one based on achieving a percentage of Tregs required to tip the balance to tolerance and the other based on allometric scaling from mouse transplant models. Early proof-of-principle experiments in mouse models relied on the use of adoptive transfer of a mixture of effector T cells and Tregs to lymphopenic hosts to precisely control and balance between the two populations (Hara et al. 2001; Graca et al. 2002). In these settings, a high ratio of at least one Treg per two effector T cells, and sometimes as high as five Tregs per one effector cell, is needed to prevent rejection (Nishimura et al. 2004; Golshayan et al. 2007). This suggests that a minimum of 33% Tregs is required to prevent rejection. Experiments in lymphoreplete mice also find that 30% Tregs in the grafts is associated with prevention of graft rejection (Fan et al. 2010). Interestingly, Tregs are also found to accumulate to 30% in tumor tissues and are thought to contribute to the suppression of antitumor immunity. Thus, 30% Tregs is likely the tipping point between productive immunity and tolerance. We have estimated that an average adult human has 166×10^9 $CD4^+$ T cells in the body, including lymphoid and non-lymphoid organs, and among which 13×10^9 are Tregs (Tang and Lee 2012). To increase the percentage of Tregs to 30% by using Treg therapy alone, 53×10^9 Treg would be needed. Aside from the technical challenge of producing such a high dose of Tregs (see below), it is not clear if Tregs can engraft when infused at such a high bolus dose. However, 10 times less (5×10^9)

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Treg would be sufficient if Treg therapy is combined with prior depletion of 90% of the T cells using an immune T-cell-depleting agent such as antithymocyte immunoglobulin.

Scaling for immune cell therapy may be less complicated and unpredictable than that for small molecule drugs because pharmacokinetic and pharmacodynamic properties of immune cells (i.e., rules for cell trafficking, turnover, and dose response) are relatively insensitive to the differences in body mass and metabolic rate of different species (Wiegel and Perelson 2004; Perelson and Wiegel 2009). For example, clinical data obtained from hematopoietic stem cell transplant shows that the minimal effective dose for neutrophil and platelet reconstitution in humans is consistent with that predicted by allometric scaling of data from animal models. Thus, the number of Tregs needed to achieve efficacy in humans may be extrapolated using numbers from mouse experiments multiplied by the ratio between the sizes of the human and the mouse lymphoid compartments. One way to approximate the relative size of the mouse and human lymphoid compartments is by comparing the lymphocyte cellularity of the spleens. Adult human spleens contain 70×10^9 lymphocytes (Westermann and Pabst 1992; Ganusov and De Boer 2007; Nylen et al. 2007), 1000 times that in adult mouse spleens. Therefore effective Treg dose in humans is likely 1000 times that found in mouse models. There is very little experimental evidence for effective Treg dose in lymphoreplete mouse models. In a mouse islet transplant model, 1×10^6 unmanipulated polyclonal Tregs were able to prolong graft survival by 2 wk (Zhang et al. 2009). Others used 2 to 5×10^6 Tregs enriched for donor antigen reactivity and also observed limited prolongation of graft survival unless combined with other treatments (Golshayan et al. 2007; Joffre et al. 2008; Tsang et al. 2008). We have found that 30×10^6 polyclonal Tregs was able to induce long-term graft survival only when combined with substantial deletion of donor-reactive T cells from the hosts (K Lee and Q Tang, unpubl.). Thus, using allomeric scaling, effective dose of polyclonal Tregs for preventing rejection in humans is estimated to be $>30 \times 10^9$.

The total number of Tregs in an adult human is estimated to be 13×10^9 and most of the Tregs reside in lymphoid organs (Tang and Lee 2012). The number of Tregs circulating in the blood is 0.25×10^9, which is the most that can be isolated from an autologous source for therapeutic use. It is important to point out that there is limited information about the percentage of Tregs in the body that actually circulates. Tregs in the skin and gut have been suggested to be largely resident with little ability to circulate. This is especially true of memory Tregs (Rosenblum et al. 2011). In a recent clinical trial in type 1 diabetes conducted at UCSF, deuterium-labeled ex vivo expanded Tregs were tracked in vivo. Our preliminary results showed that a dose of $\sim 350 \times 10^6$ led to a 3%–5% increase of the circulating Tregs. It is clear that ex vivo expansion is needed to dramatically increase the number of Tregs (JA Bluestone, MK Hellerstein, SE Gitelman et al., unpubl.).

Specificity

In mouse models of Treg therapy in transplantation, a consistent feature is that Tregs purified from tolerant hosts are more effective in transferring tolerance to new hosts than Tregs from naïve hosts. The improved efficacy is likely owing to an increased frequency of donor alloantigen-reactive Tregs induced by various tolerance-inducing protocols (Bushell et al. 1995; Cobbold et al. 2004; Karim et al. 2004; Ochando et al. 2006; Yates et al. 2007; Verginis et al. 2008; Francis et al. 2011). There are two types of alloantigen-reactive T cells. The "direct" alloantigen-reactive T cells recognize intact alloantigen expressed on donor cells, and the "indirect" alloantigen-reactive T cells recognize processed donor alloantigens presented by host antigen-presenting cells (Gould and Auchincloss 1999; Rogers and Lechler 2001). Interestingly, allograft tolerance is primarily mediated through the indirect pathway (Hara et al. 2001; Yamada et al. 2001; Callaghan et al. 2007; Sanchez-Fueyo et al. 2007; Gokmen et al. 2008), and many tolerance-inducing protocols expand Tregs with the indirect specificity (Wise et al. 1998; Ochando et al. 2006; Verginis et al. 2008). Two independent studies compared

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the efficacy of direct Tregs with Tregs of mixed direct and indirect specificities, and both found that additional indirect specificity improved efficacy (Joffre et al. 2008; Tsang et al. 2008). Indirect Tregs alone confer some protection against rejection, but the protection is very limited (Golshayan et al. 2009; Tsang et al. 2009), not much better than protection conferred by direct Tregs with a single specificity from T-cell receptor (TCR) transgenic mice (Brennan et al. 2011). Because current Treg manufacturing technology is only capable of large-scale production of polyclonal and direct alloreactive Tregs (see below for more details), we have compared the relative potency of direct and polyclonal Tregs. We found that when combined with deletion of 80% donor-reactive T cells, Tregs of direct alloreactivity could induce long-term islet allograft survival. Polyclonal Tregs were also capable of inducing long-term graft acceptance, but five times more cells were needed (K Lee and Q Tang, unpubl.). It has been thought that the impact of direct Tregs would be short lived because their activation and function depends on short-lived donor-derived antigen-presenting cells. However, there is some evidence that host antigen-presenting cells can acquire intact donor human leukocyte antigen (HLA) antigens expressed by the grafts and present alloantigens in a "semidirect" fashion, thus professional direct alloantigen presentation, and hence direct Treg function, may persist long after transplantation (Sagoo et al. 2012). Alternatively, direct Treg may create a tolerogenic milieu in the graft tissue to promote indirect Tregs through infectious tolerance, thus achieving long-term protection. Taken together, we believe combining direct and indirect alloreactive Tregs may be optimal in inducing graft survival when limited Treg numbers and/or minimal immunosuppression are used. When combined with adequate preconditioning and given in sufficient numbers, both direct and polyclonal Tregs may be able to induce long-term graft survival.

Adjunct Immunosuppression

Experimental data show that Tregs cannot prevent rejection as a stand-alone therapy. Treg in-

duction of long-term graft survival requires short-term adjunct immunosuppression to create a therapeutic window. Most immunosuppressive drugs used in the transplant clinics today were selected based on their ability to block immune responses before the existence of Tregs and their role in transplant tolerance was established. Some of these immunosuppressive drugs antagonize Treg function and survival, whereas others are less harmful to Tregs or even beneficial (Table 1). Therefore, selection of adjunct immunosuppression will have a significant impact on the efficacy of Treg therapy. Currently planned clinical trials have to rely on available drugs to combine with Treg therapy, and developing new "Treg-supportive" immune-modulatory drugs will be instrumental for improving efficacy of Treg therapy. For example, activation of antigen-presenting cells via CD40 can abrogate Treg-mediated suppression (Serra et al. 2003), and blocking CD40 and CD40L interaction in mouse and nonhuman primate models of transplantation has shown promising results (Li et al. 2008). However, anti-CD40L antibodies had unexpected thrombotic complications in humans owing to platelet activation. Using nonthrombogenic alternatives or targeting CD40 may solve the problem. Given the differential impact of PI3 kinase-AKT-mTOR signaling in Tregs and conventional T cells, it is also possible to preferentially inhibit conventional T-cell activation by antagonizing the PI3 kinase-signaling pathway (Han et al. 2012). In addition, directly boosting Tregs may be an effective strategy to combine with Treg therapy. IL-2 therapy induces significant expansion of Tregs, but can also expand $CD8^+$ T cells and NK cells, particularly when higher doses of IL-2 are used (Bluestone 2011; Koreth et al. 2011; Saadoun et al. 2011; Long et al. 2012). In mouse models, it was possible to preferentially deliver IL-2 to Tregs using anti-IL-2 antibodies and repair defects of Tregs in a mouse model of autoimmune diabetes (Boyman et al. 2006; Tang et al. 2008). The efficacy of such strategy in humans remains to be determined. Last, histone deacetylases destabilize Tregs by promoting FOXP3 degradation, and histone deacetylase inhibitors have been shown to improve Treg homeostasis and

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Table 1. Impact of immunosuppressive drugs on Tregs

Drug	Mechanism of immunosuppression	Impact on Tregs	References
Corticosteroids	Binds to nuclear receptor to inhibit AP1 and NF-κB and expression of proinflammatory cytokines	May support Tregs by reducing inflammation	Karagiannidis et al. 2004; Xu et al. 2009
CNI	Inhibits calcineurin, calcium signaling pathway, NFAT activation, IL-2 production	Detrimental to Treg function and survival	Baan et al. 2005; Pascual et al. 2008; Zeiser et al. 2008; Demirkiran et al. 2009
Rapamycin	Inhibits mTOR, protein synthesis, proliferation	Spares Tregs, thus increases percentage of Tregs	Baan et al. 2005; Pascual et al. 2008; Zeiser et al. 2008; Demirkiran et al. 2009
MMF	Inhibits purine biosynthesis, T- and B-cell proliferation	Likely neutral	Baan et al. 2005; Pascual et al. 2008; Zeiser et al. 2008; Demirkiran et al. 2009
ATG	Deletes T cells, NK cells, B cells	Deletes Tregs less efficiently, thus increases percentage of Tregs	Lopez et al. 2006; Morelon et al. 2010
Anti-CD25	Deletes CD25-expressing cells	Deletes Tregs	Bluestone et al. 2008; Toso et al. 2009
CTLA4-Ig	Blocks CD80 and CD86 costimulation of CD28 and T-cell clonal expansion	Spares Tregs when used at subsaturating dose	Bluestone et al. 2008
Anti-LFA-1	Blocks LFA-1 and ICAM-1 interaction and T-cell activation, blocks leukocyte trafficking	Dramatically increases circulating Treg percentage	Posselt et al. 2010

CNI, calcineurin inhibitor; MMF, mycophenolate mofetil; ATG, antithymocyte globulin.

induce allograft tolerance (Tao and Hancock 2007; Tao et al. 2007). New discoveries and understanding in Treg biology and transplantation tolerance have led to ever expanding therapeutic opportunities. However, it is extremely unlikely that any single agent will be effective against transplant rejection or capable of inducing tolerance. The challenge is to design an optimal combinational regimen and delivery program to steer the immune system away from rejection and into tolerance.

Treg MANUFACTURING

Treg Isolation

The first step in Treg expansion is the isolation of Tregs. A two-step magnetic activated cell sorting (MACS) protocol has been proposed for good manufacturing practice (GMP)-compliant isolation of human Tregs (Hoffmann et al. 2006; Peters et al. 2008b). However, Tregs isolated using this protocol are often contaminated with conventional T cells. This is because CD25 expression on human $CD4^+$ cells is not restricted to Tregs and there is no clear separation between Tregs and non-Tregs based on the level of CD25 expression. Selecting only the top 2% $CD25^{hi}$ Tregs increases purity at the expense of low yield. The additional use of CD127 allowed high-yield and high-purity recovery human Tregs based on the cell-surface phenotype of $CD4^+CD25^+$ $CD127^{lo/-}$ (Liu et al. 2006), and on average 1×10^6 Tregs can be isolated from 100 mL of blood using this approach. Tregs selected based on $CD4^+CD25^+CD127^{lo/-}$ markers have been

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found to be more effective than $CD4^+CD25^+$ Tregs in controlling alloimmune-mediated arterial atherosclerosis in a humanized mouse model (Nadig et al. 2010). It has also been reported that human Tregs can be identified on the basis of CD4, CD25, and CD45RA expression with naïve Tregs having the $CD4^+CD25^{mid}CD45RA^+$ phenotype and antigen-experienced effector Tregs having a $CD4^+CD25^{hi}CD45RA^-$ phenotype (Miyara et al. 2009). It has been shown that $CD45RA^+$ Tregs are more stable during repeated in vitro stimulation with anti-CD3 and anti-CD28, whereas $CD45RA^-$ Tregs progressively lost FOXP3 expression after each round of stimulation (Hoffmann et al. 2009). We have found that $CD4^+CD25^+CD127^{lo/-}$ Tregs contain both $CD45RA^+$ and $CD45RA^-$ populations and the $CD45RA^+$ subset preferentially expands in culture (Putnam et al. 2009), as others have reported (Miyara et al. 2009), and the presence of the $CD45RA^-$ subset does not negatively impact the purity of the Tregs at the end of the expansion. Selecting Tregs with three or more cell-surface markers improves purity and yield, but makes it challenging to isolate the cells using MACS. Fluorescence-activated cell sorting (FACS) is capable of identifying cell subsets based a panel of three or more markers, however, currently, there is no GMP-compliant FACS and the process is slow, which poses a limit on the number of Tregs that can be purified and manufactured. An instrument that combines the bulk processing capability of MACS with the high precision multi-parameter-based FACS would be ideal for fast isolation of highly pure Tregs.

Polyclonal Treg Expansion

Tregs can be readily expanded using anti-CD3 and anti-CD28-coated beads supplemented with IL-2 (Levings et al. 2001; Herold et al. 2002; Hoffmann et al. 2004; Earle et al. 2005; Putnam et al. 2009). Conventional $CD4^+$ T cells and $CD8^+$ T cells expand better than Tregs using this protocol, therefore high purity of the starting population is essential for producing highly pure expanded Tregs. This is particularly problematic for MACS-purified $CD4^+CD25^+$ Tregs, as they are often contaminated with $CD25^+$

$FOXP3^-$ conventional cells. The addition of rapamycin improves the purity of the culture because rapamycin suppresses the proliferation of conventional T cells and Treg growth is less affected (Battaglia et al. 2005). Despite these advances, large-scale manufacturing of Tregs remains challenging because even highly pure Tregs lose FOXP3 expression with repeated stimulation even in the presence of rapamycin (Hoffmann et al. 2009; Hippen et al. 2011b). The loss of FOXP3 is likely owing to destabilization of FOXP3 expression in Tregs instead of outgrowth of a few contaminating conventional T cells (Hoffmann et al. 2009). The cellular and molecular basis for Treg destabilization during in vitro stimulation is presently unclear. Nonetheless, it will be important to determine an optimized expansion protocol to maximize yield without compromising purity.

Alloantigen-Reactive Treg Expansion

The frequency of direct alloreactive Tregs has been estimated to be between 1% and 10% (Lin et al. 2008; Veerapathran et al. 2011). Proof-of-principle experiments have shown that alloantigen-reactive Tregs can be expanded using donor antigen-presenting cells such as dendritic cells, B cells, and unfractionated peripheral blood mononuclear cells (Peters et al. 2008a; Chen et al. 2009; Sagoo et al. 2011; Veerapathran et al. 2011; Tran et al. 2012). Collectively, these studies reported that alloantigen-expanded Tregs were 5–32 times more potent at suppressing alloantigen-stimulated proliferation in vitro than polyclonal Tregs. These results suggest that 5–32 times less alloantigen-expanded Tregs may be sufficient to achieve the same therapeutic efficacy as polyclonal Tregs. Based on our estimate that 5×10^9 polyclonal Tregs would be sufficient to induce tolerance when combined with 90% deletion of endogenous T cells, 150×10^6 to 1×10^9 alloantigen-reactive Tregs would be needed to achieve similar efficacy. Thus, clinical translation of alloantigen-reactive Treg therapy will require reliable manufacturing of more than hundreds of millions cells under GMP conditions. In this regard, we have developed a process using CD40L-ac-

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tivated allogeneic B cells to selectively stimulate the expansion of alloantigen-reactive Tregs (Putnam et al. 2013). After primary expansion, the cells are restimulated with anti-CD3 and anti-CD28-coated beads to increase cell yield. Using this approach, we are able to achieve 200- to 4000-fold expansion in 16 d. The cells are highly donor reactive and have demethylated the Treg-specific demethylation region (TSDR). Clinical trials applying Tregs manufactured with this protocol in kidney and liver transplantations are planned to start in 2014.

Although most successes in expanding human alloantigen-reactive Tregs have been in generating direct Tregs, efforts have been made to expand human indirect Tregs with less spectacular results (Jiang et al. 2003; Veerapathran et al. 2011). This is likely because of the 100 times lower frequency of indirect alloreactive Tregs in the Treg pool (Veerapathran et al. 2011). Given the experimental evidence of improved efficacy in combining direct and indirect Tregs to induce tolerance, developing protocols to manufacture indirect alloreactive Tregs is an important future direction. Alternative to selective expansion of indirect Tregs from the existing repertoire, forced expression of TCRs with indirect alloreactivity during expansion of direct alloreactive Tregs can generate dual specificity Tregs with improved ability to protect grafts in mouse models (Tsang et al. 2008). Genetic engineering Treg specificity has been successfully shown with human Tregs (Brusko et al. 2010). In addition to TCRs, other desirable features can be introduced into the Tregs such as traceable markers, tunable TCRs, chemotactic receptors to synthetic ligands, and drug-inducible suicidal enzymes (Lim 2010). These "designer" features would allow monitoring of the infused Tregs, controlling their activities and trafficking patterns, and eliminating them when needed.

Ex Vivo Induction of Tregs

Alternative to isolating preexisting Tregs for ex vivo expansion, it has been proposed that conventional $CD4^+$ T cells can be converted to Tregs during ex vivo expansion with the addition of TGF-β together with rapamycin or all-*trans* ret-

inoic acid (Lu et al. 2010; Hippen et al. 2011a). These in vitro-induced Tregs acquire some features of Tregs such as expression of CD25 and FOXP3, reduced expression of effector cytokines, and ability to suppress in vitro and in vivo in a humanized mouse model of xenogeneic graft-versus-host disease (GvHD). Activated human T cells transiently express FOXP3; therefore FOXP3 expression alone does not distinguish activated T cells from Tregs. One distinction between ex vivo-isolated Tregs and in vitro induced Tregs is the DNA methylation status of the TSDR, which is important for Treg commitment and stability (more details in the paragraph below). Ex vivo-isolated Tregs have a demethylated TSDR, whereas in vitro-induced Tregs have fully methylated TSDR suggesting that they are not committed Tregs (Baron et al. 2007; Hippen et al. 2011a). Inhibiting or knocking down the DNA methyltransferase promotes demethylation of the FOXP3 locus and may drive commitment and stabilization of induced Tregs (Kim and Leonard 2007; Lal et al. 2009; Sanchez-Abarca et al. 2010). Further experimental evidence on the commitment and stability of ex vivo induced Tregs is needed before they can be considered as a viable source of therapeutic Tregs for humans.

Assessing Treg Identity and Purity after Expansion

Tregs are typically $CD3^+CD4^+$ and express the transcription factor FOXP3. Although CD3 and CD4 are clearly not unique for Tregs, conventional T cells transiently express FOXP3 after activation; therefore FOXP3 expression does not distinguish Tregs from conventional T cells either (Allan et al. 2007). It has been proposed that Tregs are best defined by their ability to suppress conventional T-cell proliferation in the "classical" in vitro suppression assay. It is important to remember that activated human conventional $CD4^+$ T cells show dose-dependent inhibition of T-cell proliferation similar to the suppression curve obtained using Tregs (Walker et al. 2003, 2005). On the other hand, there are numerous examples in mouse models that cells with normal suppressive activities

measured in the in vitro suppression assay fail to function in vivo (Zhou et al. 2008; Wohlfert et al. 2011; Ouyang et al. 2012). In addition, in vitro suppression assays cannot detect contaminating Tconv cells. Typically, Tregs suppress maximally when used at 1:2 to 1:4 Treg to conventional T-cell ratios, which means this assay cannot detect the presence of as many as 50% contaminating T cells. Collectively, the in vitro suppression assay can misidentify cells as Tregs and is not an appropriate approach for assessing the identity or the purity of a Treg product.

To properly identify Tregs, it is helpful to know how Tregs develop and what defines their lineage. Treg development in the thymus is a multistep process controlled by cytokines and signaling through T-cell receptor and CD28, which culminates in FOXP3 expression and epigenetic modification of the FOXP3 locus (Burchill et al. 2008; Lio and Hsieh 2008; Long et al. 2009; Lio et al. 2010; Ohkura et al. 2012). The TSDR of the FOXP3 locus is demethylated in cells that have committed to the Treg lineage to ensure stable inheritance of FOXP3 expression in dividing cells and the lineage stability of Tregs (Floess et al. 2007; Huehn et al. 2009; Josefowicz and Rudensky 2009; Zheng et al. 2010; Ohkura et al. 2012). Although FOXP3 expression is necessary for Treg development and function, Treg lineage specification and stabilization also depends on coordinated activities of up to 300 proteins to ensure persistent high expression of FOXP3 (Hill et al. 2007; Fu et al. 2012; Rudra et al. 2012; Samstein et al. 2012). This suggests that coexpression of FOXP3 and a collection of proteins essential for Treg lineage specification may define a Treg. However, the list of these essential proteins is not defined currently and there is evidence that many of these proteins have redundant roles in defining the Treg lineage (Fu et al. 2012) and there may never be an unambiguous definition for Tregs based on patterns of protein expression. Because the function of these proteins is reflected in the epigenome of a Treg to ensure inheritable high expression of FOXP3 and simultaneous repression of IL-2 locus and other genes characteristic of effector cells (Ohkura et al. 2012), an epigenetic profile most accurately defines a bona fide

Treg (Hori 2008). Until a comprehensive epigenomic fingerprint of a Treg is defined, demethylated TSDR can be used currently to determine the purity and identity of Treg products (Wieczorek et al. 2009).

EARLY EXPERIENCE OF REGULATORY T-CELL THERAPY IN HUMANS

As of April 2013, there have been four reported clinical trials of Treg therapy in humans, three in GvHD and one in type 1 diabetes (Trzonkowski et al. 2009; Brunstein et al. 2010; Di Ianni et al. 2011; Marek-Trzonkowska et al. 2012). In addition, there are seven Treg trials registered in www.clinicaltrials.org, four in GvHD, one in type 1 diabetes, one in liver transplantation (NCT01624077), and one in kidney transplantation (NCT01446484). Some features of the published trials are summarized in Table 2 with emphasis on the Treg product infused. All four reported trials showed acceptable safety and promising efficacy of the treatment. Although none of these trials are in solid organ transplantation, information from these trials is instrumental for the design of future solid organ trials. For example, in the Brunstein et al. (2010) trial, because of the mismatch of HLA of the Treg cord blood donors from the host and the conventional T-cell donors, persistence of Tregs can be tracked. The infused Tregs could be detected between 4 hr and 7 d after infusion. The increase in infused Tregs in circulation was dramatically less after a second infusion of cryopreserved Treg products, suggesting cryopreservation may compromise the viability or stability of the Tregs, although other factors may also contribute. In addition, a clear trend of more consistent overall increase in Tregs was observed in rapamycin-treated patients when compared with cyclosporine-treated patients. In the Marek-Trzonkowska et al. trial, we learn that infusion of 20×10^6/kg body weight polyclonally expanded Tregs in type 1 diabetic children between the ages of 8 and 16 is safe and the treatment is associated with immediate doubling of the percentage of circulating Tregs and a trend of increase at 2 wk. In the Di Ianni trial, therapeutic Tregs enabled the infusion of a higher dose of conventional T cells and

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Table 2. Summary of published Treg trials in humans

References	Indication (No. of patients)	Tregs			
		Isolation	Expansion	% FOXP3$^+$	Dosing/kg
Trzonkowski et al. 2009	GvHD ($n = 2$)	FACS	2–4 Wk, weekly stimulations	40%–90%	$0.1–3 \times 10^6$
Brunstein et al. 2010	GvHD ($n = 23$)	MACS	18 ± 1 d, Anti-CD3/ CD28 on day 0	31%–96%	$0.1–6 \times 10^6$
Di Ianni et al. 2011	GvHD ($n = 28$)	MACS	None	$69\% \pm 14\%$	$2–4 \times 10^6$
Marek-Trzonkowska et al. 2012	Type 1 diabetes in children ($n = 10$)	FACS	14 d, Anti-CD3/CD28 on days 0 and 7	90%–97%	$10–20 \times 10^6$

GvHD, graft-versus-host disease; FACS, fluorescence-activated cell sorting; MACS, magnetic-activated cell sorting.

better immunity against opportunistic infections. Particularly, the investigators observed markedly improved prevention against cytomegalovirus (CMV) disease. Five out of seven patients who received influenza vaccine after Treg infusion achieved protective antibody titers. All these results suggest that Treg infusion did not impede protective immunity against infections or lead to global immunosuppression. Overall, experience in clinical application of Treg therapy thus far shows that it is feasible, safe, and potentially effective.

It is worthwhile to review the quality of the Treg products in these trials closely, so that lessons learned from these early experiences in humans can be applied to the planned trials in solid organ transplantations. The investigator reported a wide range of Treg purity ranging from 31% to 97% FOXP3$^+$. Two factors contributed to the variability of Treg purity. In the Di Ianni trial when no ex vivo expansion was used, purity below 50% was reported. In the Brunstein trial, the percentage of FOXP3$^+$ cells after MACS isolation varied between 20% and 80% despite the use of cord blood as source materials. These results illustrate the problem with the current MACS technology. Additionally, loss of FOXP3 during repeated stimulation of Tregs was evident in the Trzonkowski trial that reported the percentage of FOXP3$^+$ decreasing from 90% to 70% and 40% after successive weekly stimulations. It is encouraging that by combining FACS purification and limiting to two rounds of stimulations in the Marek-Trzonkowska trial, the puri-

ty of the expanded Tregs was consistently above 90%, however, the authors noted an insufficient Treg yield in four out of 10 patients. We have completed infusion of 10 type 1 diabetic patients of up to 320×10^6 expanded Tregs at UCSF. By using FACS purification, and two rounds of anti-CD3 and anti-CD28 stimulations, we are able to produce on average 2×10^9 Tregs (range $0.2–3.1 \times 10^9$) with an average of 92% FOXP3$^+$ (range 76%–97%) from 1 unit of blood donation (J Bluestone, unpubl.). In the GvHD setting, up to 70% of FOXP3$^-$ conventional T cells are tolerated because conventional T cells are needed for improving hematopoietic stem cell engraftment, restoring immunity, and mediating graft-versus-leukemia effect. In the solid organ transplant setting, high-purity Tregs will be needed to ensure safety, potency, and consistency of results.

CONCLUDING REMARKS

Taming a powerful multifaceted immune response against transplanted organ requires equally potent and versatile therapy without compromising the overall immune competence of the patient. Tregs have the desired specificity, versatility, and adaptability and decades of research has shown their therapeutic efficacy in transplantation. However, Tregs do not have sufficient potency as a stand-alone therapy for transplantation, and factors critical to the efficacy of Treg therapy in transplantation are dose, specificity, and adjunct immunosuppression.

Although early clinical trials will be mainly focused on safety, it is important to design these trials with efficacy end points in mind so that the safety of the right dose with the desired specificity administered with optimized adjunct therapy can be evaluated. Central to the successful implementation of Treg therapy is the reliable production of consistently high-quality Tregs and the challenge is in balancing the yield and the purity of the products. The future of Treg therapy depends on effective and informative clinical trial designs, technological advancement in Treg manufacture, and better mechanistic understanding of Treg biology and transplantation tolerance in humans.

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REFERENCES

Allan SE, Crome SQ, Crellin NK, Passerini L, Steiner TS, Bacchetta R, Roncarolo MG, Levings MK. 2007. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol* **19:** 345–354.

Baan CC, van der Mast BJ, Klepper M, Mol WM, Peeters AM, Korevaar SS, Balk AH, Weimar W. 2005. Differential effect of calcineurin inhibitors, anti-CD25 antibodies and rapamycin on the induction of FOXP3 in human T cells. *Transplantation* **80:** 110–117.

Baron U, Floess S, Wieczorek G, Baumann K, Grutzkau A, Dong J, Thiel A, Boeld TJ, Hoffmann P, Edinger M, et al. 2007. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3⁺ conventional T cells. *Eur J Immunol* **37:** 2378–2389.

Battaglia M, Stabilini A, Roncarolo MG. 2005. Rapamycin selectively expands CD4⁺CD25⁺FoxP3⁺ regulatory T cells. *Blood* **105:** 4743–4748.

Berenson GS, Wattigney WA, Tracy RE, Newman WP 3rd, Srinivasan SR, Webber LS, Dalferes ER Jr, Strong JP. 1992. Atherosclerosis of the aorta and coronary arteries and cardiovascular risk factors in persons aged 6 to 30 years and studied at necropsy (The Bogalusa Heart Study). *Am J Cardiol* **70:** 851–858.

Bluestone JA. 2011. The yin and yang of interleukin-2-mediated immunotherapy. *N Engl J Med* **365:** 2129–2131.

Bluestone JA, Liu W, Yabu JM, Laszik ZG, Putnam A, Belingheri M, Gross DM, Townsend RM, Vincenti F. 2008. The effect of costimulatory and interleukin 2 receptor blockade on regulatory T cells in renal transplantation. *Am J Transplant* **8:** 2086–2096.

Boyman O, Kovar M, Rubinstein MP, Surh CD, Sprent J. 2006. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. *Science* **311:** 1924–1927.

Brennan TV, Tang Q, Liu FC, Hoang V, Bi M, Bluestone JA, Kang SM. 2011. Requirements for prolongation of allograft survival with regulatory T cell infusion in lymphosufficient hosts. *J Surg Res* **169:** e69–e75.

Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, Defor T, Levine BL, June CH, Rubinstein P, et al. 2010. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: Safety profile and detection kinetics. *Blood* **117:** 1061–1070.

Brusko TM, Koya RC, Zhu S, Lee MR, Putnam AL, McClymont SA, Nishimura MI, Han S, Chang LJ, Atkinson MA, et al. 2010. Human antigen-specific regulatory T cells generated by T cell receptor gene transfer. *PLoS ONE* **5:** e11726.

Burchill MA, Yang J, Vang KB, Moon JJ, Chu HH, Lio CW, Vegoe AL, Hsieh CS, Jenkins MK, Farrar MA. 2008. Linked T cell receptor and cytokine signaling govern the development of the regulatory T cell repertoire. *Immunity* **28:** 112–121.

Bushell A, Morris PJ, Wood KJ. 1995. Transplantation tolerance induced by antigen pretreatment and depleting anti-CD4 antibody depends on CD4⁺ T cell regulation during the induction phase of the response. *Eur J Immunol* **25:** 2643–2649.

Callaghan CJ, Rouhani FJ, Negus MC, Curry AJ, Bolton EM, Bradley JA, Pettigrew GJ. 2007. Abrogation of antibody-mediated allograft rejection by regulatory CD4 T cells with indirect allospecificity. *J Immunol* **178:** 2221–2228.

Chen LC, Delgado JC, Jensen PE, Chen X. 2009. Direct expansion of human allospecific FoxP3⁺CD4⁺ regulatory T cells with allogeneic B cells for therapeutic application. *J Immunol* **183:** 4094–4102.

Cobbold SP, Castejon R, Adams E, Zelenika D, Graca L, Humm S, Waldmann H. 2004. Induction of *foxP3*⁺ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. *J Immunol* **172:** 6003–6010.

Demirkiran A, Sewgobind VD, van der Weijde J, Kok A, Baan CC, Kwekkeboom J, Tilanus HW, Metselaar HJ, van der Laan LJ. 2009. Conversion from calcineurin inhibitor to mycophenolate mofetil-based immunosuppression changes the frequency and phenotype of CD4⁺FOXP3⁺ regulatory T cells. *Transplantation* **87:** 1062–1068.

Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, Del Papa B, Zei T, Ostini RI, Cecchini D, et al. 2011. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* **117:** 3921–3928.

Dieckmann D, Bruett CH, Ploettner H, Lutz MB, Schuler G. 2002. Human CD4+CD25+ regulatory, contact-dependent T cells induce interleukin 10-producing, contact-independent type 1-like regulatory T cells [corrected]. *J Exp Med* **196:** 247–253.

Dijke IE, Velthuis JH, Caliskan K, Korevaar SS, Maat AP, Zondervan PE, Balk AH, Weimar W, Baan CC. 2007. Intragraft FOXP3 mRNA expression reflects antidonor immune reactivity in cardiac allograft patients. *Transplantation* **83:** 1477–1484.

Earle KE, Tang Q, Zhou X, Liu W, Zhu S, Bonyhadi ML, Bluestone JA. 2005. In vitro expanded human CD4+ CD25+ regulatory T cells suppress effector T cell proliferation. *Clin Immunol* **115:** 3–9.

Euvrard S, Kanitakis J, Claudy A. 2003. Skin cancers after organ transplantation. *N Engl J Med* **348:** 1681–1691.

Fan Z, Spencer JA, Lu Y, Pitsillides CM, Singh G, Kim P, Yun SH, Toxavidis V, Strom TB, Lin CP, et al. 2010. In vivo tracking of "color-coded" effector, natural and induced regulatory T cells in the allograft response. *Nat Med* **16:** 718–722.

Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, Schlawe K, Chang HD, Bopp T, Schmitt E, et al. 2007. Epigenetic control of the *foxp3* locus in regulatory T cells. *PLoS Biol* **5:** e38.

Francis RS, Feng G, Tha-In T, Lyons IS, Wood KJ, Bushell A. 2011. Induction of transplantation tolerance converts potential effector T cells into graft-protective regulatory T cells. *Eur J Immunol* **41:** 726–738.

Fu W, Ergun A, Lu T, Hill JA, Haxhinasto S, Fassett MS, Gazit R, Adoro S, Glimcher L, Chan S, et al. 2012. A multiply redundant genetic switch "locks in" the transcriptional signature of regulatory T cells. *Nat Immunol* **13:** 972–980.

Ganusov VV, De Boer RJ. 2007. Do most lymphocytes in humans really reside in the gut? *Trends Immunol* **28:** 514–518.

Gokmen MR, Lombardi G, Lechler RI. 2008. The importance of the indirect pathway of allorecognition in clinical transplantation. *Curr Opin Immunol* **20:** 568–574.

Golshayan D, Jiang S, Tsang J, Garin MI, Mottet C, Lechler RI. 2007. In vitro-expanded donor alloantigen-specific CD4+CD25+ regulatory T cells promote experimental transplantation tolerance. *Blood* **109:** 827–835.

Golshayan D, Wyss JC, Abulker CW, Schaefer SC, Lechler RI, Lehr HA, Pascual M. 2009. Transplantation tolerance induced by regulatory T cells: In vivo mechanisms and sites of action. *Int Immunopharmacol* **9:** 683–688.

Gould DS, Auchincloss H Jr, 1999. Direct and indirect recognition: The role of MHC antigens in graft rejection. *Immunol Today* **20:** 77–82.

Graca L, Thompson S, Lin CY, Adams E, Cobbold SP, Waldmann H. 2002. Both CD4+CD25+ and CD4+CD25− regulatory cells mediate dominant transplantation tolerance. *J Immunol* **168:** 5558–5565.

Han JM, Patterson SJ, Levings MK. 2012. The role of the PI3K signaling pathway in CD4+ T cell differentiation and function. *Front Immunol* **3:** 245.

Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, Morris PJ, Powrie F, Wood KJ. 2001. IL-10 is required

for regulatory T cells to mediate tolerance to alloantigens in vivo. *J Immunol* **166:** 3789–3796.

Herold KC, Hagopian W, Auger JA, Poumian-Ruiz E, Taylor L, Donaldson D, Gitelman SE, Harlan DM, Xu D, Zivin RA, et al. 2002. Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. *N Engl J Med* **346:** 1692–1698.

Hill JA, Feuerer M, Tash K, Haxhinasto S, Perez J, Melamed R, Mathis D, Benoist C. 2007. Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* **27:** 786–800.

Hippen KL, Merkel SC, Schirm DK, Nelson C, Tennis NC, Riley JL, June CH, Miller JS, Wagner JE, Blazar BR. 2011a. Generation and large-scale expansion of human inducible regulatory T cells that suppress graft-versus-host disease. *Am J Transplant* **11:** 1148–1157.

Hippen KL, Merkel SC, Schirm DK, Sieben CM, Sumstad D, Kadidlo DM, McKenna DH, Bromberg JS, Levine BL, Riley JL, et al. 2011b. Massive ex vivo expansion of human natural regulatory T cells (T$_{regs}$) with minimal loss of in vivo functional activity. *Sci Transl Med* **3:** 83ra41.

Hoffmann P, Eder R, Kunz-Schughart LA, Andreesen R, Edinger M. 2004. Large-scale in vitro expansion of polyclonal human CD4+CD25^{high} regulatory T cells. *Blood* **104:** 895–903.

Hoffmann P, Boeld TJ, Eder R, Albrecht J, Doser K, Piseshka B, Dada A, Niemand C, Assenmacher M, Orso E, et al. 2006. Isolation of CD4+CD25+ regulatory T cells for clinical trials. *Biol Blood Marrow Transplant* **12:** 267–274.

Hoffmann P, Boeld TJ, Eder R, Huehn J, Floess S, Wieczorek G, Olek S, Dietmaier W, Andreesen R, Edinger M. 2009. Loss of FOXP3 expression in natural human CD4+ CD25+ regulatory T cells upon repetitive in vitro stimulation. *Eur J Immunol* **39:** 1088–1097.

Hori S. 2008. Rethinking the molecular definition of regulatory T cells. *Eur J Immunol* **38:** 928–930.

Huehn J, Polansky JK, Hamann A. 2009. Epigenetic control of FOXP3 expression: The key to a stable regulatory T-cell lineage? *Nat Rev Immunol* **9:** 83–89.

Jiang S, Camara N, Lombardi G, Lechler RI. 2003. Induction of allopeptide-specific human CD4+CD25+ regulatory T cells ex vivo. *Blood* **102:** 2180–2186.

Joffre O, Santolaria T, Calise D, Al Saati T, Hudrisier D, Romagnoli P, van Meerwijk JP. 2008. Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes. *Nat Med* **14:** 88–92.

Jonuleit H, Schmitt E, Kakirman H, Stassen M, Knop J, Enk AH. 2002. Infectious tolerance: Human CD25+ regulatory T cells convey suppressor activity to conventional CD4+ T helper cells. *J Exp Med* **196:** 255–260.

Josefowicz SZ, Rudensky A. 2009. Control of regulatory T cell lineage commitment and maintenance. *Immunity* **30:** 616–625.

Karagiannidis C, Akdis M, Holopainen P, Woolley NJ, Hense G, Ruckert B, Mantel PY, Menz G, Akdis CA, Blaser K, et al. 2004. Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma. *J Allergy Clin Immunol* **114:** 1425–1433.

Karim M, Kingsley CI, Bushell AR, Sawitzki BS, Wood KJ. 2004. Alloantigen-induced CD25+CD4+ regulatory T cells can develop in vivo from CD25−CD4+ precursors

in a thymus-independent process. *J Immunol* **172:** 923–928.

Kim HP, Leonard WJ. 2007. CREB/ATF-dependent T cell receptor-induced *FoxP3* gene expression: A role for DNA methylation. *J Exp Med* **204:** 1543–1551.

Koreth J, Matsuoka K, Kim HT, McDonough SM, Bindra B, Alyea EP 3rd, Armand P, Cutler C, Ho VT, Treister NS, et al. 2011. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med* **365:** 2055–2066.

Lal G, Zhang N, van der Touw W, Ding Y, Ju W, Bottinger EP, Reid SP, Levy DE, Bromberg JS. 2009. Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. *J Immunol* **182:** 259–273.

Levings MK, Sangregorio R, Roncarolo MG. 2001. Human CD25$^+$CD4$^+$ T regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* **193:** 1295–1302.

Li XL, Menoret S, Le Mauff B, Angin M, Anegon I. 2008. Promises and obstacles for the blockade of CD40-CD40L interactions in allotransplantation. *Transplantation* **86:** 10–15.

Lim WA. 2010. Designing customized cell signalling circuits. *Nat Rev Mol Cell Biol* **11:** 393–403.

Lin YJ, Hara H, Tai HC, Long C, Tokita D, Yeh P, Ayares D, Morelli AE, Cooper DK. 2008. Suppressive efficacy and proliferative capacity of human regulatory T cells in allogeneic and xenogeneic responses. *Transplantation* **86:** 1452–1462.

Lio CW, Hsieh CS. 2008. A two-step process for thymic regulatory T cell development. *Immunity* **28:** 100–111.

Lio CW, Dodson LF, Deppong CM, Hsieh CS, Green JM. 2010. CD28 facilitates the generation of Foxp3$^-$ cytokine responsive regulatory T cell precursors. *J Immunol* **184:** 6007–6013.

Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B, et al. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4$^+$ T reg cells. *J Exp Med* **203:** 1701–1711.

Long M, Park SG, Strickland I, Hayden MS, Ghosh S. 2009. Nuclear factor-κB modulates regulatory T cell development by directly regulating expression of Foxp3 transcription factor. *Immunity* **31:** 921–931.

Long SA, Rieck M, Sanda S, Bollyky JB, Samuels PL, Goland R, Ahmann A, Rabinovitch A, Aggarwal S, Phippard D, et al. 2012. Rapamycin/IL-2 combination therapy in patients with type 1 diabetes augments Tregs yet transiently impairs β-cell function. *Diabetes* **61:** 2340–2348.

Lopez M, Clarkson MR, Albin M, Sayegh MH, Najafian N. 2006. A novel mechanism of action for anti-thymocyte globulin: Induction of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells. *J Am Soc Nephrol* **17:** 2844–2853.

Lu L, Zhou X, Wang J, Zheng SG, Horwitz DA. 2010. Characterization of protective human CD4CD25 FOXP3 regulatory T cells generated with IL-2, TGF-β and retinoic acid. *PLoS ONE* **5:** e15150.

Marek-Trzonkowska N, Mysliwiec M, Dobyszuk A, Grabowska M, Techmanska I, Juscinska J, Wujtewicz MA, Witkowski P, Mlynarski W, Balcerska A, et al. 2012. Administration of CD4$^+$CD25^{high}CD127$^-$ regulatory T cells

preserves β-cell function in type 1 diabetes in children. *Diabetes Care* **35:** 1817–1820.

Meagher C, Tang Q, Fife BT, Bour-Jordan H, Wu J, Pardoux C, Bi M, Melli K, Bluestone JA. 2008. Spontaneous development of a pancreatic exocrine disease in CD28-deficient NOD mice. *J Immunol* **180:** 7793–7803.

Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C, Heike T, Valeyre D, et al. 2009. Functional delineation and differentiation dynamics of human CD4$^+$ T cells expressing the FoxP3 transcription factor. *Immunity* **30:** 899–911.

Morelon E, Lefrancois N, Besson C, Prevautel J, Brunet M, Touraine JL, Badet L, Touraine-Moulin F, Thaunat O, Malcus C. 2010. Preferential increase in memory and regulatory subsets during T-lymphocyte immune reconstitution after Thymoglobulin induction therapy with maintenance sirolimus vs cyclosporine. *Transpl Immunol* **23:** 53–58.

Muthukumar T, Dadhania D, Ding R, Snopkowski C, Naqvi R, Lee JB, Hartono C, Li B, Sharma VK, Seshan SV, et al. 2005. Messenger RNA for FOXP3 in the urine of renal-allograft recipients. *N Engl J Med* **353:** 2342–2351.

Nadig SN, Wieckiewicz J, Wu DC, Warnecke G, Zhang W, Luo S, Schiopu A, Taggart DP, Wood KJ. 2010. In vivo prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells. *Nat Med* **16:** 809–813.

Nair S, Verma S, Thuluvath PJ. 2002. Obesity and its effect on survival in patients undergoing orthotopic liver transplantation in the United States. *Hepatology* **35:** 105–109.

Nishimura E, Sakihama T, Setoguchi R, Tanaka K, Sakaguchi S. 2004. Induction of antigen-specific immunologic tolerance by in vivo and in vitro antigen-specific expansion of naturally arising Foxp3$^+$CD25$^+$CD4$^+$ regulatory T cells. *Int Immunol* **16:** 1189–1201.

Nylen S, Maurya R, Eidsmo L, Manandhar KD, Sundar S, Sacks D. 2007. Splenic accumulation of IL-10 mRNA in T cells distinct from CD4$^+$CD25$^+$ (Foxp3) regulatory T cells in human visceral leishmaniasis. *J Exp Med* **204:** 805–817.

O'Gorman WE, Dooms H, Thorne SH, Kuswanto WF, Simonds EF, Krutzik PO, Nolan GP, Abbas AK. 2009. The initial phase of an immune response functions to activate regulatory T cells. *J Immunol* **183:** 332–339.

Ochando JC, Homma C, Yang Y, Hidalgo A, Garin A, Tacke F, Angeli V, Li Y, Boros P, Ding Y, et al. 2006. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol* **7:** 652–662.

Ohkura N, Hamaguchi M, Morikawa H, Sugimura K, Tanaka A, Ito Y, Osaki M, Tanaka Y, Yamashita R, Nakano N, et al. 2012. T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. *Immunity* **37:** 785–799.

Ojo AO, Held PJ, Port FK, Wolfe RA, Leichtman AB, Young EW, Arndorfer J, Christensen L, Merion RM. 2003. Chronic renal failure after transplantation of a nonrenal organ. *N Engl J Med* **349:** 931–940.

Oliveira VG, Caridade M, Paiva RS, Demengeot J, Graca L. 2011. Sub-optimal CD4$^+$ T-cell activation triggers autonomous TGF-β-dependent conversion to Foxp3$^+$ regulatory T cells. *Eur J Immunol* **41:** 1249–1255.

Q. Tang and J.A. Bluestone

Ouyang W, Liao W, Luo CT, Yin N, Huse M, Kim MV, Peng M, Chan P, Ma Q, Mo Y, et al. 2012. Novel Foxo1-dependent transcriptional programs control T_{reg} cell function. *Nature* **491:** 554–559.

Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. 2007. $CD4^+CD25^+Foxp3^+$ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector $CD4^+$ T cells. *Nat Immunol* **8:** 1353–1362.

Pascual J, Bloom D, Torrealba J, Brahmbhatt R, Chang Z, Sollinger HW, Knechtle SJ. 2008. Calcineurin inhibitor withdrawal after renal transplantation with alemtuzumab: Clinical outcomes and effect on T-regulatory cells. *Am J Transplant* **8:** 1529–1536.

Penn I. 1990. Cancers complicating organ transplantation. *N Engl J Med* **323:** 1767–1769.

Perelson AS, Wiegel FW. 2009. Scaling aspects of lymphocyte trafficking. *J Theoret Biol* **257:** 9–16.

Peters JH, Hilbrands LB, Koenen HJ, Joosten I. 2008a. Ex vivo generation of human alloantigen-specific regulatory T cells from $CD4^{pos}CD25^{high}$ T cells for immunotherapy. *PLoS ONE* **3:** e2233.

Peters JH, Preijers FW, Woestenenk R, Hilbrands LB, Koenen HJ, Joosten I. 2008b. Clinical grade Treg: GMP isolation, improvement of purity by $CD127^{pos}$ depletion, Treg expansion, and Treg cryopreservation. *PLoS ONE* **3:** e3161.

Porter DL, Levine BL, Kalos M, Bagg A, June CH. 2011. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med* **365:** 725–733.

Posselt AM, Szot GL, Frassetto LA, Masharani U, Tavakol M, Amin R, McElroy J, Ramos MD, Kerlan RK, Fong L, et al. 2010. Islet transplantation in type 1 diabetic patients using calcineurin inhibitor-free immunosuppressive protocols based on T-cell adhesion or costimulation blockade. *Transplantation* **90:** 1595–1601.

Putnam AL, Brusko TM, Lee MR, Liu W, Szot GL, Ghosh T, Atkinson MA, Bluestone JA. 2009. Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes* **58:** 652–662.

Putnam A, Safinia N, Medvec A, Laszkowska M, Wray M, Mintz M, Trotta E, Szot G, Liu W, Lares A, et al. 2013. Clinical grade manufacturing and therapeutic advantage of human alloantigen-reactive regulatory T cells in transplantation. *Am J Transplant* (to be published).

Restifo NP, Dudley ME, Rosenberg SA. 2012. Adoptive immunotherapy for cancer: Harnessing the T cell response. *Nat Rev Immunol* **12:** 269–281.

Rogers NJ, Lechler RI. 2001. Allorecognition. *Am J Transplant* **1:** 97–102.

Rosenblum MD, Gratz IK, Paw JS, Lee K, Marshak-Rothstein A, Abbas AK. 2011. Response to self antigen imprints regulatory memory in tissues. *Nature* **480:** 538–542.

Rudra D, deRoos P, Chaudhry A, Niec RE, Arvey A, Samstein RM, Leslie C, Shaffer SA, Goodlett DR, Rudensky AY. 2012. Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nat Immunol* **13:** 1010–1019.

Saadoun D, Rosenzwajg M, Joly F, Six A, Carrat F, Thibault V, Sene D, Cacoub P, Klatzmann D. 2011. Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. *N Engl J Med* **365:** 2067–2077.

Sagoo P, Ali N, Garg G, Nestle FO, Lechler RI, Lombardi G. 2011. Human regulatory T cells with alloantigen specificity are more potent inhibitors of alloimmune skin graft damage than polyclonal regulatory T cells. *Sci Transl Med* **3:** 83ra42.

Sagoo P, Lombardi G, Lechler RI. 2012. Relevance of regulatory T cell promotion of donor-specific tolerance in solid organ transplantation. *Front Immunol* **3:** 184.

Samstein RM, Arvey A, Josefowicz SZ, Peng X, Reynolds A, Sandstrom R, Neph S, Sabo P, Kim JM, Liao W, et al. 2012. Foxp3 exploits a pre-existent enhancer landscape for regulatory T cell lineage specification. *Cell* **151:** 153–166.

Sanchez-Abarca LI, Gutierrez-Cosio S, Santamaria C, Caballero-Velazquez T, Blanco B, Herrero-Sanchez C, Garcia JL, Carrancio S, Hernandez-Campo P, Gonzalez FJ, et al. 2010. Immunomodulatory effect of 5-azacytidine (5-azaC): Potential role in the transplantation setting. *Blood* **115:** 107–121.

Sanchez-Fueyo A. 2011. Hot-topic debate on tolerance: Immunosuppression withdrawal. *Liver Transpl* **17:** S69–S73.

Sanchez-Fueyo A, Domenig CM, Mariat C, Alexopoulos S, Zheng XX, Strom TB. 2007. Influence of direct and indirect allorecognition pathways on $CD4^+CD25^+$ regulatory T-cell function in transplantation. *Transpl Int* **20:** 534–541.

Scholler J, Brady TL, Binder-Scholl G, Hwang WT, Plesa G, Hege KM, Vogel AN, Kalos M, Riley JL, Deeks SG, et al. 2012. Decade-long safety and function of retroviral-modified chimeric antigen receptor T cells. *Sci Transl Med* **4:** 132ra153.

Serra P, Amrani A, Yamanouchi J, Han B, Thiessen S, Utsugi T, Verdaguer J, Santamaria P. 2003. CD40 ligation releases immature dendritic cells from the control of regulatory $CD4^+CD25^+$ T cells. *Immunity* **19:** 877–889.

Soltys KA, Mazariegos GV, Squires RH, Sindhi RK, Anand R. 2007. Late graft loss or death in pediatric liver transplantation: An analysis of the SPLIT database. *Am J Transplant* **7:** 2165–2171.

Tang Q, Bluestone JA. 2008. The $Foxp3^+$ regulatory T cell: A jack of all trades, master of regulation. *Nat Immunol* **9:** 239–244.

Tang Q, Lee K. 2012. Regulatory T-cell therapy for transplantation: How many cells do we need? *Curr Opin Organ Transplant* **17:** 349–354.

Tang Q, Adams JY, Penaranda C, Melli K, Piaggio E, Sgouroudis E, Piccirillo CA, Salomon BL, Bluestone JA. 2008. Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity* **28:** 687–697.

Tao R, Hancock WW. 2007. Regulating regulatory T cells to achieve transplant tolerance. *Hepatobiliary Pancreat Dis Int* **6:** 348–357.

Tao R, de Zoeten EF, Ozkaynak E, Chen C, Wang L, Porrett PM, Li B, Turka LA, Olson EN, Greene MI, et al. 2007. Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nat Med* **13:** 1299–1307.

Textor SC, Taler SJ, Canzanello VJ, Schwartz L, Augustine JE. 2000. Posttransplantation hypertension related to calcineurin inhibitors. *Liver Transpl* **6:** 521–530.

Toso C, Edgar R, Pawlick R, Emamaullee J, Merani S, Dinyari P, Mueller TF, Shapiro AM, Anderson CC. 2009. Effect of different induction strategies on effector, regulatory and memory lymphocyte sub-populations in clinical islet transplantation. *Transpl Int* **22:** 182–191.

Tran GT, Hodgkinson SJ, Carter NM, Verma ND, Plain KM, Boyd R, Robinson CM, Nomura M, Killingsworth M, Hall BM. 2012. Interleukin-5 (IL-5) promotes induction of antigen specific CD4$^+$CD25$^+$ T regulatory cells that suppress autoimmunity. *Blood* **119:** 4441–4450.

Trzonkowski P, Bieniaszewska M, Juscinska J, Dobyszuk A, Krzystyniak A, Marek N, Mysliwska J, Hellmann A. 2009. First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4$^+$CD25$^+$CD127$^-$ T regulatory cells. *Clin Immunol* **133:** 22–26.

Tsang JY, Tanriver Y, Jiang S, Xue SA, Ratnasothy K, Chen D, Stauss HJ, Bucy RP, Lombardi G, Lechler R. 2008. Conferring indirect allospecificity on CD4$^+$CD25$^+$ Tregs by TCR gene transfer favors transplantation tolerance in mice. *J Clin Invest* **118:** 3619–3628.

Tsang JY, Tanriver Y, Jiang S, Leung E, Ratnasothy K, Lombardi G, Lechler R. 2009. Indefinite mouse heart allograft survival in recipient treated with CD4$^+$CD25$^+$ regulatory T cells with indirect allospecificity and short term immunosuppression. *Transpl Immunol* **21:** 203–209.

Veerapathran A, Pidala J, Beato F, Yu XZ, Anasetti C. 2011. Ex vivo expansion of human Tregs specific for alloantigens presented directly or indirectly. *Blood* **118:** 5671–5680.

Verginis P, McLaughlin KA, Wucherpfennig KW, von Boehmer H, Apostolou I. 2008. Induction of antigen-specific regulatory T cells in wild-type mice: Visualization and targets of suppression. *Proc Natl Acad Sci* **105:** 3479–3484.

Waldmann H. 2008. Tolerance can be infectious. *Nat Immunol* **9:** 1001–1003.

Walker MR, Carson BD, Nepom GT, Ziegler SF, Buckner JH. 2005. De novo generation of antigen-specific CD4$^+$CD25$^+$ regulatory T cells from human CD4$^+$CD25$^-$ cells. *Proc Natl Acad Sci* **102:** 4103–4108.

Walker MR, Kasprowicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH, Ziegler SF. 2003. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4$^+$CD25$^-$ T cells. *J Clin Invest* **112:** 1437–1443.

Westermann J, Pabst R. 1992. Distribution of lymphocyte subsets and natural killer cells in the human body. *Clin Investig* **70:** 539–544.

Wieczorek G, Asemissen A, Model F, Turbachova I, Floess S, Liebenberg V, Baron U, Stauch D, Kotsch K, Pratschke J, et al. 2009. Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. *Cancer Res* **69:** 599–608.

Wiegel FW, Perelson AS. 2004. Some scaling principles for the immune system. *Immunol Cell Biol* **82:** 127–131.

Wise MP, Bemelman F, Cobbold SP, Waldmann H. 1998. Linked suppression of skin graft rejection can operate through indirect recognition. *J Immunol* **161:** 5813–5816.

Wohlfert EA, Grainger JR, Bouladoux N, Konkel JE, Oldenhove G, Ribeiro CH, Hall JA, Yagi R, Naik S, Bhairavabhotla R, et al. 2011. GATA3 controls Foxp3$^+$ regulatory T cell fate during inflammation in mice. *J Clin Invest* **121:** 4503–4515.

Xu L, Xu Z, Xu M. 2009. Glucocorticoid treatment restores the impaired suppressive function of regulatory T cells in patients with relapsing-remitting multiple sclerosis. *Clin Exp Immunol* **158:** 26–30.

Yamada A, Chandraker A, Laufer TM, Gerth AJ, Sayegh MH, Auchincloss H Jr, 2001. Recipient MHC class II expression is required to achieve long-term survival of murine cardiac allografts after costimulatory blockade. *J Immunol* **167:** 5522–5526.

Yamaguchi T, Wing JB, Sakaguchi S. 2011. Two modes of immune suppression by Foxp3$^+$ regulatory T cells under inflammatory or non-inflammatory conditions. *Semin Immunol* **23:** 424–430.

Yates SF, Paterson AM, Nolan KF, Cobbold SP, Saunders NJ, Waldmann H, Fairchild PJ. 2007. Induction of regulatory T cells and dominant tolerance by dendritic cells incapable of full activation. *J Immunol* **179:** 967–976.

Zeiser R, Leveson-Gower DB, Zambricki EA, Kambham N, Beilhack A, Loh J, Hou JZ, Negrin RS. 2008. Differential impact of mammalian target of rapamycin inhibition on CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells compared with conventional CD4$^+$ T cells. *Blood* **111:** 453–462.

Zhang N, Schroppel B, Lal G, Jakubzick C, Mao X, Chen D, Yin N, Jessberger R, Ochando JC, Ding Y, et al. 2009. Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. *Immunity* **30:** 458–469.

Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. 2010. Role of conserved non-coding DNA elements in the *Foxp3* gene in regulatory T-cell fate. *Nature* **463:** 808–812.

Zhou X, Jeker LT, Fife BT, Zhu S, Anderson MS, McManus MT, Bluestone JA. 2008. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. *J Exp Med* **205:** 1983–1991.