Published in final edited form as:

Curr Opin Organ Transplant. 2010 December ; 15(6): 757–764. doi:10.1097/MOT.0b013e32834017ae.

CD4+ regulatory T cells in solid organ transplantation

Fadi Issa and Kathryn J. Wood

Transplantation Research Immunology Group, Nuffield Department of Surgery, University of Oxford

Abstract

Purpose of review: Solid organ transplantation is the most effective treatment for end-stage organ failure, but the long-term outcomes remain suboptimal. CD4⁺ regulatory T cells (Treg) are emerging as a potential therapy to facilitate long-term allograft survival. This review provides a general overview of the biology of CD4⁺ Treg and then goes on to discuss the most relevant and recent experimental and clinical evidence for their therapeutic use in solid organ transplantation.

Recent findings: There have been major advances in our understanding of Treg, including improvements in methods for their isolation and expansion. Experimental models are providing very important data on the *in vitro* and *in vivo* behaviour of Treg in transplantation, while recent clinical trials of Treg cellular therapy in graft-versus-host disease are offering a valuable insight into the efficacy of Treg adoptive cellular therapy.

Summary: Data in favour of Treg cellular therapy in transplantation are mounting, and we predict that their use in clinical trials is on the horizon.

Keywords

Treg; tolerance; rejection; transplantation

INTRODUCTION

Solid organ transplantation (SOT) is one of the major medical success stories of the past 50 years. Although short-term graft and patient survival rates post-transplantation have improved dramatically over recent times, this has not been mirrored by similarly improved 10-year and long-term outcomes [1]. For the majority of cases, the immunosuppressive regimens used to prevent organ rejection are culprit. These drugs are nonspecific, require lifelong use, favor the development of opportunistic infections and tumors, directly damage transplanted organs and significantly increase cardiovascular morbidity and mortality [2, 3]. To address this, various therapies are being explored that may allow patients to retain a long-term functioning allograft under no immunosuppression, or minimal immunosuppression (termed tolerance [4] and prope tolerance [5], respectively). Within this field, regulatory T cells (Treg) have emerged as a promising candidate therapy [6]. Multiple

Corresponding author's contact details: Kathryn J. Wood, Transplantation Research Immunology Group, Nuffield Department of Surgery, University of Oxford, Level 6, John Radcliffe Hospital, Oxford OX3 9DU, Kathryn.Wood@nds.ox.ac.uk, +44-1865-222-508.

CONFLICTS OF INTEREST

None declared.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

heterogeneous populations of Treg exist, of which the CD4⁺ population has attracted the most interest [7, 8]. This review will summarise the state of the art of CD4⁺ Treg therapy in SOT, discussing recent advances both experimentally and clinically.

OVERVIEW OF REGULATORY T CELLS

Several regulatory mechanisms exist to avert excessive immune responses to pathogens and to prevent the development of autoimmune disease. CD4⁺ Tregs (termed Treg hereafter) are central to these mechanisms: mice lacking the Treg-specific transcription factor FoxP3 ('scurfy mice') develop a lymphoproliferative disorder [9], and humans with mutations in FoxP3 can develop IPEX (immunodysregulation, polyendocrinopathy and enteropathy, X-linked) syndrome [10]. FoxP3 is closely linked to suppressive activity and its sustained expression is required for the maintenance of regulatory activity [11].

Tregs can be divided into thymic-derived naturally occurring CD4⁺CD25^{hi}Foxp3⁺ Tregs (nTreg) [12] and induced or adaptive CD4⁺ Tregs (iTreg), which upregulate FoxP3 in the periphery under conditions of antigen-exposure and transforming growth factor β (TGF β) stimulation [13-15]. nTregs represent 5-10% of the peripheral CD4⁺ pool and constitutively express high levels of surface CD25 (the α -chain of the interleukin (IL)-2 receptor) although this is not a reliable marker due to its upregulation on activated non-regulatory T cells. It is unclear how much of the peripheral CD4⁺ population iTregs represent, but given that these cells are induced in specific inflammatory environments it is likely that the percentage is location and time-dependent.

Other described regulatory CD4⁺ T cells include Treg type 1 (Tr1) cells which employ a FoxP3-independent suppression mechanism mediated via IL-10 and TGF β [16]. Tr1 cells can be generated by stimulation with IL-10 or CD46-engagement and induce suppression by bystander regulation of effector T cells irrespective of their antigen specificity [16, 17].

In humans, FoxP3 is upregulated on non-suppressive recently-activated CD4⁺ T cells, making it a less useful marker than for mouse Tregs [18, 19]. Despite this, epigenetic studies have identified differences between the methylation status of the Treg-specific demethylated region (TSDR) site within the FoxP3 locus in different CD4⁺ T cells. In naïve and activated CD4⁺ T cells the TSDR is heavily methylated, whereas in iTregs it is partially demethylated and in nTregs is completely demethylated [20, 21**]. Analysis of Treg based on quantitative methylation of the TSDR site is therefore a useful adjunct to the phenotyping of Tregs for clinical use [22].

Differentiation of iTreg and nTreg may also be possible with the use of the transcription factor Helios, a member of the Ikaros transcription factor family. In a study by Thornton *et al* [23*], Helios was found to be expressed on all thymically-derived FoxP3⁺ cells, but not on iTregs. Interestingly, 70% of peripheral Tregs in this study were Helios positive, indicating that up to 30% of Tregs in the periphery may be iTregs.

Both nTregs and iTregs require T cell receptor (TCR) engagement for activation and induction of suppression *in vivo*. Once activated, Tregs suppress immune responses at multiple levels by directly inhibiting both CD4⁺ and CD8⁺ T cell activation and proliferation, as well as by preventing T cell activation through modulation of antigen presenting cell (APC) function [24]. Other described targets of Tregs include B cells, natural killer (NK) cells, natural killer T (NKT) cells and mast cells [24-27]. The molecular mechanisms involved in Treg suppressive activity are summarized in figure 1 [14, 28-41].

Treg suppressive activity may be inhibited by ligation of the glucocorticoid-induced tumour necrosis factor (TNF) receptor (GITR) on the surface of Treg [42]. Another member of the

TNF receptor superfamily, OX40 (CD134), which is constitutively expressed on the surface of Tregs, appears to have a more complex role. OX40 stimulation has been shown to reduce FoxP3 expression and Treg-mediated antitumour suppressive activity *in vivo* [43]. Furthermore, OX40- OX40 ligand interaction between Tregs and mast cells is important for suppression of degranulation and allergic responses [44]. However in the additional presence of IL-6, mast cells can force Tregs to differentiate into IL-17-producing proinflammatory cells [45]. OX40 is also required for the accumulation of Tregs in the colon to prevent T cell-induced colitis, and the lack of OX40 co-stimulation after Treg activation may lead to activation-induced cell death (AICD) [46*]. An insight into these distinct effects on Tregs is gained from a recent study of OX40 in experimental autoimmune encephalomyelitis (EAE), in which the local cytokine milieu at the time of ligation was important: OX40 ligation early in the response (during antigen priming) expanded Tregs and reduced the severity of EAE and OX40 ligation later (when effector T cells had already infiltrated the central nervous system) caused an enhancement of effector T cell responses and promoted the development of EAE [47].

Tregs may not remain suppressive throughout their lifetime. In a recent study it was demonstrated that some cells that lose FoxP3 develop an activated memory-type phenotype and are pathogenic *in vivo* [48**]. The loss of FoxP3 appears linked to a proinflammatory microenvironment. Under such conditions Tregs can acquire an effector T cell phenotype, secreting IL-17 and interferon γ (IFN γ) [49, 50], a phenomenon likely related to the ability of inflammatory cytokines such as IL-6 to methylate the FoxP3 TSDR site [21**]. Furthermore, transfer of Tregs into lymphopenic mice has been shown to result in the loss of FoxP3 in up to 50% of adoptively transferred cells [51], an important observation to consider if clinical Treg therapy is to be used in patients who are lymphopenic after immunosuppressive induction therapy.

In SOT there is a trend for an increase in the number of FoxP3⁺ cells in long-term operationally tolerant patients and, paradoxically, in patients undergoing acute rejection. For example, increased FOXP3 mRNA levels have been detected in rejecting cardiac [52] and renal allografts [53], as well as in the urine of patients with rejecting renal transplants [54]. Higher peripheral FoxP3⁺ cell numbers also predict a higher risk of development of cutaneous squamous cell carcinoma in renal transplant patients [55]. The increase in FoxP3⁺ cells in acute rejection may be related to its upregulation on activated effector T cells, or an increase in Tregs in response to high donor-activated effector T cell activity. Conversely, patients with renal transplants undergoing chronic rejection have lower numbers of peripheral FoxP3⁺ cells compared to those with either normal graft function under immunosuppression or those who are operationally tolerant. The increase in FoxP3⁺ cells in the graft and periphery is most striking in tolerant liver transplant recipients [56-59].

EXPERIMENTAL EVIDENCE FOR CD4⁺ REGULATORY T CELL USE IN SOLID ORGAN TRANSPLANTATION

Studies assessing the potential of nTreg, iTreg and Tr1 cells to promote allograft survival in experimental transplantation have yielded promising results to date. We will now discuss some of these important studies.

Mouse regulatory T cells

Tregs may be generated *in vivo* or *ex vivo* for later adoptive transfer. *In vivo* approaches are based on increasing the frequency or potency Tregs by exposure to antigen, inducing an expansion of nTregs or converting non-regulatory T cells to iTregs [60]. For example, Tregs may be generated *in vivo* by pre-treating mice with a donor alloantigen (in the form of a

donor-specific blood transfusion) along with a non-depleting anti-CD4 monoclonal antibody (mAb). Mice treated in this manner develop Tregs capable of preventing allograft rejection *in vivo* [61, 62]. Tregs isolated from these animals can also prevent allograft rejection in naïve mice by cellular adoptive transfer. These Tregs display an increase in alpha-1,2-mannosidase expression, an enzyme involved in the synthesis and processing of N-linked glycoproteins. The resultant altered N-glycosylation of Treg cell surface proteins has been shown to be necessary for Treg migration to lymph nodes where they can regulate priming [63]. Another method for *in vivo* generation involves injection of IL-2-IL-2 mAb complexes into mice, resulting in an over ten-fold expansion of Tregs *in vivo*. Animals treated by this method are resistant to EAE induction and display tolerance to islet allografts [64*].

Tregs may be generated by similar principles *in vitro*, by culturing CD4⁺ T cells with alloantigen and anti-CD4 antibody [65]. Tregs may also be generated *in vitro* by the conditioning of non-regulatory CD4⁺ cells with alloantigen in the presence of IFN γ , which helps convert these cells into Tregs and induces preferential AICD in the non-Treg population [66, 67]. IFN γ -conditioned Tregs have been shown to prevent the rejection of skin and islet allografts *in vivo* [67]. Furthermore, these cells can inhibit transplant-associated vasculopathy [68*], a phenomenon current immunosuppressive drugs have failed to influence significantly. Interestingly, Tregs isolated from tolerant animals produce IFN γ transiently and rapidly after activation, which acts in an autocrine manner to activate the signal transducer and activator of transcription (STAT)-1 intracellular signaling pathway necessary for regulation [69].

Human regulatory T cells

Human Tregs for therapy are generally produced by isolation of cells from peripheral or cord blood and subsequent *ex vivo* expansion or direct use. In order to isolate Treg efficiently and to a high purity, reliable markers of identification are required. Given the non-specificity of CD25 and FoxP3 expression, a number of other markers have been explored. Of these markers, CD127 (the IL7 receptor α -chain), CD49b (the α -chain of the integrin VLA-4 – α 4b1), CD45RA and the folate receptor 4 (FR4) are currently the most useful. Another surface marker, glycoprotein-A repetitions predominant (GARP or LRRC32), is linked to the expression of FoxP3 and associated with Treg suppressive activity but its role in efficiently distinguishing Tregs requires further investigation [70]. Other Treg markers include CTLA-4, GITR, CD69 and CD44 but these are less useful as they may also be expressed in almost identical patterns on non-regulatory activated T cells.

The low expression of CD127 (CD127^{lo}) correlates well with expression of FoxP3 and identifies Tregs that are highly suppressive *in vivo* [71-73]. In a recent study from our laboratory, CD25⁺CD127^{lo} Tregs were five-fold more effective at abrogating transplant arteriosclerosis in a humanised mouse model than Tregs selected on CD25 alone [74*]. The absence of CD49b is another good marker for Tregs [75]. The benefit of CD127 and CD49b is that a population of Tregs can be isolated by negative selection alone without the need for CD25 positive selection.

CD45RA allows cells to be divided into CD25⁺CD45RA⁺FoxP3^{lo} (resting naïve Tregs), CD25^{hi}CD45RA⁺FoxP3^{hi} (activated Tregs) and CD25⁺CD45RA⁺FoxP3^{lo} (non-suppressive T cells) populations [76*]. Resting naïve and activated Tregs are both suppressive *in vitro*, although only resting naïve Tregs proliferate *in vivo* and evolve into suppressive CD45RA⁺ Tregs. Umbilical cord blood (UCB) contains a high number of naïve CD45RA⁺ cells, and is therefore an attractive source of resting naïve Tregs [77]. However UCB Tregs are low in frequency and require either *in vitro* culture or pooling of multiple units. Furthermore, UCB Tregs are “third-party” (allogeneic to both the donor and recipient) - although given the increase in the frequency of UCB banking in recent years it may one day be possible to

infuse autologous UCB Treg into a patient. FR4 is constitutively expressed on the surface of Tregs and along with CD4 and CD25 identifies a population of Tregs that potentially suppress allograft rejection [78].

Ex vivo expansion of isolated Tregs is generally performed by TCR stimulation with anti-CD3/anti-CD28 microbeads in the presence of recombinant human IL-2 [79*, 80]. The non-specific TCR stimulation in this system leads to the production of a polyclonal population of Tregs. Another promising strategy for the production of human Tregs is based on lentivirus-mediated expression of FoxP3 in effector T cells, which results in stable and suppressive Treg populations [81]. Donor alloantigen-specific Tregs have been shown to be more potent suppressors *in vitro* and are theoretically safer for *in vivo* use. Current methods for the production of antigen-specific Tregs include culture with allogeneic stimulators rather than anti-CD3/anti-CD28 microbeads [82, 83] expansion of HLA-specific Tregs selected by tetramer [84], and retroviral transduction of Tregs with genes that encode for TCRs with known antigen specificities.

IMMUNOSUPPRESSION AND REGULATORY T CELLS

Treg adoptive cellular therapy in SOT is likely to be initially introduced under the cover of conventional immunosuppression. In light of this, it is important to delineate the effects of immunosuppression on Treg activity and proliferation [85]. Tregs and effector T cells preferentially employ different intracellular activation pathways: Tregs utilise IL-2-dependent STAT-5 [86, 87], whereas effector T cells utilise the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin (mTOR) pathway [88]. Immunosuppression with rapamycin (an mTOR inhibitor) takes advantage of this distinction. The beneficial effects of rapamycin on Treg survival and proliferation have been demonstrated *in vitro* and *in vivo* [89-94]. Alemtuzumab (a humanised anti-CD52 mAb used mainly for induction therapy) may also favour Treg survival, with evidence from one study demonstrating a higher proportional depletion of T effector cells than Tregs [95]. Data in this study may be confounded by the introduction of rapamycin in patients early after transplantation. Indeed, in a separate study Treg numbers in alemtuzumab-treated patients remained low until the late introduction of rapamycin [96].

There is no significant difference in circulating Treg numbers between renal transplant patients treated with belatacept (CTLA-4-Ig) in conjunction with basiliximab (chimaeric anti-CD25 mAb) compared with patients treated with calcineurin inhibitors (CNIs) [97]. Nevertheless, CNIs such as cyclosporine have a detrimental effect on Tregs [98, 99], complicating this observation. Interestingly, in this same study patients receiving belatacept displayed higher levels of intra-graft FoxP3⁺ T cells during acute rejection [97]. The effects of anti-CD25 mAb on Tregs are not entirely clear. In the former study, basiliximab depleted all CD25-bearing cells, including Tregs. However in another study examining daclizumab (a humanised anti-CD25 mAb) in cardiac transplant patients, Treg generation in the periphery was not affected [100]. Despite the detrimental effects of CNIs on Tregs, in a study examining topical CNIs for atopic dermatitis using tacrolimus, there was a significant reduction in intralesional CD25⁺ but not FoxP3⁺ cells [101].

In a recent study investigating a novel CD28 antagonist, there was an increase in the number and activity of Tregs in a non-human primate renal transplantation model [102*]. This co-stimulatory blockade, unlike CTLA-4-Ig, does not affect physiological CD80/86-CTLA-4 interaction, therefore allowing normal immune regulation through this important pathway to continue [37].

CLINICAL TRIALS

At the time of writing there are no ongoing clinical trials testing the efficacy of Tregs in SOT. There have, however, been several studies investigating Tregs for the treatment of graft-versus-host disease (GvHD) post-haematopoietic stem cell transplantation (HSCT). It is important to take note of these studies as they are paving the way for Treg therapy in SOT. Trzonkowski *et al* [79*] have reported the use of *ex vivo* expanded recipient-derived Tregs in two patients: in one case of chronic GvHD there was significant alleviation of the symptoms and a reduction of required immunosuppression, whereas in one case of severe grade IV acute GvHD there was only a transient improvement in symptoms and signs. Trials using Tr1 cells in GvHD are ongoing at the San Raffaele Hospital in Milan, Italy (M.G. Roncarolo, personal communication). Early results have been promising with no adverse side effects, although the beneficial effects on GvHD are not yet clear [103, 104].

Other ongoing trials include one conducted by Matthias Edinger at the University Hospital in Regensburg, Germany using CD25^{hi} magnetically-isolated non-expanded Treg infused into post-HSCT recipients [103] (M. Edinger, personal communication). At the time of writing nine patients have been treated with no adverse effects. Another trial at the University of Minnesota is exploring the use of UCB Tregs, which are expanded with anti-CD3/anti-CD28 beads and IL-2 before infusion into patients (B. Blazar) [77]. The largest trial to date is being conducted by Martelli *et al* [105] in Italy, where 28 patients receiving HSCT for high-risk acute leukemia have received magnetically-isolated unexpanded Tregs autologous to the HSC donor. Preliminary results are encouraging, with twenty-five out of twenty-nine patients not developing GvHD after transplantation. Patients in this trial are displaying overall faster post-transplant immune reconstitution as well as a reduction in cytomegalovirus (CMV) reactivation (Abstract 4, 51st meeting of the American Society for Haematology) [106].

CONCLUSION

Over the past decade there have been great advances in our overall understanding of Treg biology, and we are closer than ever to the first clinical trials in SOT. As a stepping-stone to these trials, humanised mouse *in vivo* models are proving invaluable. These models provide important efficacy data, as well as limited safety data. However, when considering Treg therapy in SOT it is important to keep in mind the excellent short-term outcomes with conventional immunosuppression. The beneficial effects of Treg therapy in the majority of cases are envisaged to be in the long-term. In order to evaluate Treg therapy in the short-term trials will need to be performed in situations in which acute rejection rates are high and conventional immunosuppression is risky, such as intestinal or composite tissue transplantation. In these cases, Treg cellular therapy may be given in the first instance as an adjunct together with reduced-dose immunosuppression, in an attempt to achieve proper tolerance. Whether Tregs will eventually emerge as a tolerance-inducing monotherapy is yet to be revealed.

Acknowledgments

Funding information: Work from the authors' own group referred to in this review was supported by grants from The Wellcome Trust, Medical Research Council UK, European Union – RISE framework 6 Integrated Project and British Heart Foundation.

REFERENCES

- [1]. Meier-Kriesche HU, Schold JD, Srinivas TR, Kaplan B. Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. *Am J Transplant.* 2004; 4:378–83. [PubMed: 14961990]
- [2]. Taylor AL, Watson CJ, Bradley JA. Immunosuppressive agents in solid organ transplantation: Mechanisms of action and therapeutic efficacy. *Crit Rev Oncol Hematol.* 2005; 56:23–46. [PubMed: 16039869]
- [3]. Marcen R. Immunosuppressive drugs in kidney transplantation: impact on patient survival, and incidence of cardiovascular disease, malignancy and infection. *Drugs.* 2009; 69:2227–43. [PubMed: 19852526]
- [4]. Wood KJ, Bushell AR, Jones ND. The discovery of immunological tolerance: now more than just a laboratory solution. *J Immunol.* 2010; 184:3–4. [PubMed: 20028657]
- [5]. Calne RY. Prope tolerance: the future of organ transplantation--from the laboratory to the clinic. *Transplantation.* 2004; 77:930–2. [PubMed: 15077040]
- [6]. Wood KJ, Sakaguchi S. Regulatory T cells in transplantation tolerance. *Nat Rev Immunol.* 2003; 3:199–210. [PubMed: 12658268]
- [7]. Shevach EM. From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity.* 2006; 25:195–201. [PubMed: 16920638]
- [8]. Issa F, Schiopu A, Wood KJ. Role of T cells in graft rejection and transplantation tolerance. *Expert Rev Clin Immunol.* 2010; 6:155–69. [PubMed: 20383898]
- [9]. Brunkow ME, Jeffery EW, Hjerrild KA, et al. Disruption of a new forkhead/winged-helix protein, scurfy, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet.* 2001; 27:68–73. [PubMed: 11138001]
- [10]. Bennett CL, Christie J, Ramsdell F, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet.* 2001; 27:20–1. [PubMed: 11137993]
- [11]. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. *Immunity.* 2009; 30:616–25. [PubMed: 19464984]
- [12]. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* 2003; 299:1057–61. [PubMed: 12522256]
- [13]. Karim M, Kingsley CI, Bushell AR, et al. Alloantigen-induced CD25+CD4+ regulatory T cells can develop in vivo from CD25–CD4+ precursors in a thymus-independent process. *J Immunol.* 2004; 172:923–928. [PubMed: 14707064]
- [14]. Kingsley CI, Karim M, Bushell AR, Wood KJ. CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. *J Immunol.* 2002; 168:1080–6. [PubMed: 11801641]
- [15]. Karim M, Feng G, Wood KJ, Bushell AR. CD25+CD4+ regulatory T cells generated by exposure to a model protein antigen prevent allograft rejection: antigen-specific reactivation in vivo is critical for bystander regulation. *Blood.* 2005; 105:4871–7. [PubMed: 15713793]
- [16]. Roncarolo MG, Gregori S, Battaglia M, et al. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev.* 2006; 212:28–50. [PubMed: 16903904]
- [17]. Kemper C, Chan AC, Green JM, et al. Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. *Nature.* 2003; 421:388–92. [PubMed: 12540904]
- [18]. Pillai V, Ortega SB, Wang CK, Karandikar NJ. Transient regulatory T-cells: a state attained by all activated human T-cells. *Clin Immunol.* 2007; 123:18–29. [PubMed: 17185041]
- [19]. Wang J, Ioan-Facsinay A, van der Voort EI, et al. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol.* 2007; 37:129–38. [PubMed: 17154262]
- [20]. Lal G, Bromberg JS. Epigenetic mechanisms of regulation of Foxp3 expression. *Blood.* 2009; 114:3727–35. [PubMed: 19641188]
- **[21]. Lal G, Zhang N, van der Touw W, et al. Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. *J Immunol.* 2009; 182:259–273. [PubMed: 19109157]
Study demonstrating the association between the methylation status of a CpG-rich island on the Foxp3 upstream enhancer and Treg subtype. This study also showed that demethylation of this

site with a DNA methyltransferase inhibitor can lead to stable induction of Foxp3. This is an interesting potential route for the generation of Treg for therapeutic use.

- [22]. Wieczorek G, Asemussen A, Model F, et al. Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. *Cancer Res.* 2009; 69:599–608. [PubMed: 19147574]
- *[23]. Thornton AM, Korty PE, Tran DQ, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol.* 2010; 184:3433–41. [PubMed: 20181882] An interesting study showing for the first time the use of the transcription factor Helios for the differentiation of naturally-occurring and peripherally-induced regulatory T cells.
- [24]. Shevach EM. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity.* 2009; 30:636–45. [PubMed: 19464986]
- [25]. Broere F, Wieten L, Klein Koerkamp EI, et al. Oral or nasal antigen induces regulatory T cells that suppress arthritis and proliferation of arthritogenic T cells in joint draining lymph nodes. *J Immunol.* 2008; 181:899–906. [PubMed: 18606641]
- [26]. Wang L, Toda M, Saito K, et al. Post-immune UV irradiation induces Tr1-like regulatory T cells that suppress humoral immune responses. *Int Immunol.* 2008; 20:57–70. [PubMed: 18006879]
- [27]. Ludwig-Portugall I, Hamilton-Williams EE, Gottschalk C, Kurts C. Cutting edge: CD25+ regulatory T cells prevent expansion and induce apoptosis of B cells specific for tissue autoantigens. *J Immunol.* 2008; 181:4447–51. [PubMed: 18802046]
- [28]. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol.* 2008; 8:523–32. [PubMed: 18566595]
- [29]. Gondek DC, Lu LF, Quezada SA, et al. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol.* 2005; 174:1783–6. [PubMed: 15699103]
- [30]. Grossman WJ, Verbsky JW, Barchet W, et al. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity.* 2004; 21:589–601. [PubMed: 15485635]
- [31]. Garin MI, Chu CC, Golshayan D, et al. Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood.* 2007; 109:2058–65. [PubMed: 17110462]
- [32]. Hara M, Kingsley CI, Niimi M, et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J Immunol.* 2001; 166:3789–96. [PubMed: 11238621]
- [33]. Collison LW, Workman CJ, Kuo TT, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature.* 2007; 450:566–9. [PubMed: 18033300]
- [34]. Tang Q, Boden EK, Henriksen KJ, et al. Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function. *Eur J Immunol.* 2004; 34:2996–3005. [PubMed: 15468055]
- [35]. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med.* 1998; 188:287–96. [PubMed: 9670041]
- [36]. Pandiyan P, Zheng L, Ishihara S, et al. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol.* 2007; 8:1353–62. [PubMed: 17982458]
- [37]. Wing K, Onishi Y, Prieto-Martin P, et al. CTLA-4 control over Foxp3+ regulatory T cell function. *Science.* 2008; 322:271–5. [PubMed: 18845758]
- [38]. Mellor AL, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol.* 2004; 4:762–74. [PubMed: 15459668]
- [39]. Liang B, Workman C, Lee J, et al. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J Immunol.* 2008; 180:5916–26. [PubMed: 18424711]
- [40]. Shalev I, Liu H, Kosciuk C, et al. Targeted deletion of fgl2 leads to impaired regulatory T cell activity and development of autoimmune glomerulonephritis. *J Immunol.* 2008; 180:249–60. [PubMed: 18097026]

- [41]. Borsellino G, Kleinewietfeld M, Di Mitri D, et al. Expression of ectonucleotidase CD39 by Foxp3⁺ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood*. 2007; 110:1225–32. [PubMed: 17449799]
- [42]. Bushell A, Wood K. GITR ligation blocks allograft protection by induced CD25⁺CD4⁺ regulatory T cells without enhancing effector T-cell function. *Am J Transplant*. 2007; 7:759–68. [PubMed: 17391121]
- [43]. Kitamura N, Murata S, Ueki T, et al. OX40 costimulation can abrogate Foxp3⁺ regulatory T cell-mediated suppression of antitumor immunity. *Int J Cancer*. 2009; 125:630–8. [PubMed: 19455675]
- [44]. Gri G, Piconese S, Frossi B, et al. CD4⁺CD25⁺ regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction. *Immunity*. 2008; 29:771–81. [PubMed: 18993084]
- [45]. Piconese S, Gri G, Tripodo C, et al. Mast cells counteract regulatory T-cell suppression through interleukin-6 and OX40/OX40L axis toward Th17-cell differentiation. *Blood*. 2009; 114:2639–48. [PubMed: 19643985]
- *[46]. Griseri T, Asquith M, Thompson C, Powrie F. OX40 is required for regulatory T cell-mediated control of colitis. *J Exp Med*. 2010; 207:699–709. [PubMed: 20368580] Study demonstrating a role for OX40 in the efficacy of Treg suppressive activity. In this study OX40 on the surface of Treg was required for their accumulation in the colon to prevent T cell-mediated colitis.
- [47]. Ruby CE, Yates MA, Hirschhorn-Cymerman D, et al. Cutting Edge: OX40 agonists can drive regulatory T cell expansion if the cytokine milieu is right. *J Immunol*. 2009; 183:4853–7. [PubMed: 19786544]
- **[48]. Zhou X, Bailey-Bucktrout SL, Jeker LT, et al. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol*. 2009; 10:1000–7. [PubMed: 19633673] Important study that demonstrating that Treg may not remain suppressive throughout their lifetime, with some cells losing FoxP3 and gaining a pathogenic effector memory T cell phenotype. An inflammatory milieu in the context of self-antigen contributes to this instability of phenotype. Cells with a high expression of CD25 were more resistant to the process of conversion. The possibility of Treg contributing to rather than alleviating disease is crucial to note when considering adoptive Treg cellular therapy.
- [49]. Yang XO, Nurieva R, Martinez GJ, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity*. 2008; 29:44–56. [PubMed: 18585065]
- [50]. Komatsu N, Mariotti-Ferrandiz ME, Wang Y, et al. Heterogeneity of natural Foxp3⁺ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc Natl Acad Sci U S A*. 2009; 106:1903–1908. [PubMed: 19174509]
- [51]. Duarte JH, Zelenay S, Bergman M-L, et al. Natural Treg cells spontaneously differentiate into pathogenic helper cells in lymphopaenic conditions. *Eur J Immunol*. 2009; 39:948–955. [PubMed: 19291701]
- [52]. Dijke IE, Velthuis JH, Caliskan K, et al. Intra-graft FOXP3 mRNA expression reflects antidonor immune reactivity in cardiac allograft patients. *Transplantation*. 2007; 83:1477–84. [PubMed: 17565321]
- [53]. Bunnag S, Allanach K, Jhangri GS, et al. FOXP3 expression in human kidney transplant biopsies is associated with rejection and time post transplant but not with favorable outcomes. *Am J Transplant*. 2008; 8:1423–33. [PubMed: 18510637]
- [54]. Muthukumar T, Dadhania D, Ding R, et al. Messenger RNA for FOXP3 in the urine of renal-allograft recipients. *N Engl J Med*. 2005; 353:2342–51. [PubMed: 16319383]
- [55]. Carroll RP, Segundo DS, Hollowood K, et al. Immune phenotype predicts risk for posttransplantation squamous cell carcinoma. *J Am Soc Nephrol*. 2010; 21:713–22. [PubMed: 20110382]
- [56]. Koshiba T, Li Y, Takemura M, et al. Clinical, immunological, and pathological aspects of operational tolerance after pediatric living-donor liver transplantation. *Transpl Immunol*. 2007; 17:94–7. [PubMed: 17306739]

- [57]. Li Y, Koshiba T, Yoshizawa A, et al. Analyses of peripheral blood mononuclear cells in operational tolerance after pediatric living donor liver transplantation. *Am J Transplant*. 2004; 4:2118–25. [PubMed: 15575917]
- [58]. Li Y, Zhao X, Cheng D, et al. The presence of Foxp3 expressing T cells within grafts of tolerance human liver transplant recipients. *Transplantation*. 2008; 86:1837–1843. [PubMed: 19104431]
- [59]. Martinez-Llordella M, Puig-Pey I, Orlando G, et al. Multiparameter immune profiling of operational tolerance in liver transplantation. *Am J Transplant*. 2007; 7:309–19. [PubMed: 17241111]
- [60]. Long E, Wood KJ. Regulatory T cells in transplantation: transferring mouse studies to the clinic. *Transplantation*. 2009; 88:1050–6. [PubMed: 19898199]
- [61]. Warnecke G, Bushell A, Nadig SN, Wood KJ. Regulation of transplant arteriosclerosis by CD25+CD4+ T cells generated to alloantigen *in vivo*. *Transplantation*. 2007; 83:1459–65. [PubMed: 17565319]
- [62]. Kingsley CI, Nadig SN, Wood KJ. Transplantation tolerance: lessons from experimental rodent models. *Transpl Int*. 2007; 20:828–41. [PubMed: 17711408]
- [63]. Long ET, Baker S, Oliveira V, et al. Alpha-1,2-mannosidase and hence N-glycosylation are required for regulatory T cell migration and allograft tolerance in mice. *PLoS One*. 2010; 5:e8894. [PubMed: 20126660]
- *[64]. Webster KE, Walters S, Kohler RE, et al. *In vivo* expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. *J Exp Med*. 2009; 206:751–60. [PubMed: 19332874] Study describing a method for the effective generation of Treg *in vivo* by the simple administration of an IL-2-IL-2 mAb complex. Treg in this model were very short lived (1-2 weeks) and highly activated. This method of Treg generation may prove useful for the treatment of acute pathology, although the potential for these activated Treg to convert into non-regulatory effector T cells must be fully evaluated.
- [65]. Oliveira V, Sawitzki B, Chapman S, et al. Anti-CD4-mediated selection of Treg *in vitro* - *in vitro* suppression does not predict *in vivo* capacity to prevent graft rejection. *Eur J Immunol*. 2008; 38:1677–88. [PubMed: 18465768]
- [66]. Feng G, Gao W, Strom T, et al. Exogenous IFN-gamma *ex vivo* shapes the alloreactive T-cell repertoire by inhibition of Th17 responses and generation of functional Foxp3 regulatory T cells. *Eur J Immunol*. 2008; 38:2512–2527. [PubMed: 18792404]
- [67]. Feng G, Wood KJ, Bushell A. Interferon-gamma conditioning *ex vivo* generates CD25+CD62L+Foxp3+ regulatory T cells that prevent allograft rejection: potential avenues for cellular therapy. *Transplantation*. 2008; 86:578–89. [PubMed: 18724229]
- *[68]. Warnecke G, Feng G, Goto R, et al. CD4+ Regulatory T Cells Generated *In Vitro* with IFN- γ and Allogeneic APC Inhibit Transplant Arteriosclerosis. *Am J Pathol*. 2010A study from our group showing that Treg produced *in vitro* through IFN γ -conditioning can prevent transplant arteriosclerosis *in vivo*. Transplant arteriosclerosis is the hallmark of chronic transplant dysfunction and an important cause of late graft loss.
- [69]. Wei B, Baker S, Wieckiewicz J, Wood KJ. IFN-gamma Triggered STAT1-PKB/AKT Signalling Pathway Influences the Function of Alloantigen Reactive Regulatory T Cells. *Am J Transplant*. 2009
- [70]. Wang R, Wan Q, Kozhaya L, et al. Identification of a regulatory T cell specific cell surface molecule that mediates suppressive signals and induces Foxp3 expression. *PLoS One*. 2008; 3:e2705. [PubMed: 18628982]
- [71]. Liu W, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med*. 2006; 203:1701–11. [PubMed: 16818678]
- [72]. Seddiki N, Santner-Nanan B, Martinson J, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med*. 2006; 203:1693–700. [PubMed: 16818676]
- [73]. Putnam AL, Brusko TM, Lee MR, et al. Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes*. 2009; 58:652–62. [PubMed: 19074986]

- *[74]. Nadig SN, Wieckiewicz J, Wu DC, et al. In vivo prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells. *Nat Med*. 2010. Another study from our group demonstrating the ability of *ex vivo* expanded Treg to control the difficult entity of transplant arteriosclerosis. This study showed a five-fold more effective control of the disease by CD127^{lo} Treg compared to CD25^{hi} Treg alone.
- [75]. Kleinewietfeld M, Starke M, Di Mitri D, et al. CD49d provides access to “untouched” human Foxp3+ Treg free of contaminating effector cells. *Blood*. 2009; 113:827–36. [PubMed: 18941119]
- *[76]. Miyara M, Yoshioka Y, Kitoh A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity*. 2009; 30:899–911. [PubMed: 19464196] Interesting study showing for the first time the ability of the surface marker CD45RA to distinguish between Treg of different potency and stability.
- [77]. Riley JL, June CH, Blazar BR. Human T regulatory cell therapy: take a billion or so and call me in the morning. *Immunity*. 2009; 30:656–65. [PubMed: 19464988]
- [78]. Yamaguchi T, Hirota K, Nagahama K, et al. Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor. *Immunity*. 2007; 27:145–59. [PubMed: 17613255]
- *[79]. Trzonkowski P, Bieniaszewska M, Juscinska J, et al. 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*. 2009; 133:22–6. [PubMed: 19559653] First phase 0/1 clinical trial of Treg cellular therapy. Although only two patients were included in this study the results are nonetheless very promising, with alleviation of symptoms in one patient.
- [80]. Sagoo P, Lombardi G, Lechler RI. Regulatory T cells as therapeutic cells. *Curr Opin Organ Transplant*. 2008; 13:645–53. [PubMed: 19060557]
- [81]. Allan SE, Alstad AN, Merindo N, et al. Generation of potent and stable human CD4+ T regulatory cells by activation-independent expression of FOXP3. *Mol Ther*. 2008; 16:194–202. [PubMed: 17984976]
- [82]. Peters JH, Hilbrands LB, Koenen HJ, Joosten I. Ex vivo generation of human alloantigen-specific regulatory T cells from CD4(pos)CD25(high) T cells for immunotherapy. *PLoS ONE*. 2008; 3:e2233. [PubMed: 18493605]
- [83]. Masteller EL, Warner MR, Tang Q, et al. Expansion of functional endogenous antigen-specific CD4+CD25+ regulatory T cells from nonobese diabetic mice. *J Immunol*. 2005; 175:3053–9. [PubMed: 16116193]
- [84]. Jiang S, Tsang J, Game DS, et al. Generation and expansion of human CD4+ CD25+ regulatory T cells with indirect allospecificity: Potential reagents to promote donor-specific transplantation tolerance. *Transplantation*. 2006; 82:1738–43. [PubMed: 17198269]
- [85]. Wekerle T. T-regulatory cells-what relationship with immunosuppressive agents? *Transplant Proc*. 2008; 40:S13–6. [PubMed: 19100898]
- [86]. Vogtenhuber C, Bucher C, Highfill SL, et al. Constitutively active Stat5b in CD4+ T-cells inhibits graft-versus-host disease (GVHD) lethality associated with increased regulatory T-cell (Treg) potency and decreased T effector cell (Teff) responses. *Blood*. 2010
- [87]. Burchill MA, Yang J, Vogtenhuber C, et al. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *J Immunol*. 2007; 178:280–90. [PubMed: 17182565]
- [88]. Delgoffe GM, Kole TP, Zheng Y, et al. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity*. 2009; 30:832–844. [PubMed: 19538929]
- [89]. Battaglia M, Stabilini A, Migliavacca B, et al. Rapamycin promotes expansion of functional CD4+CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J Immunol*. 2006; 177:8338–47. [PubMed: 17142730]
- [90]. Zeiser R, Leveson-Gower DB, Zambricki EA, et al. Differential impact of mammalian target of rapamycin inhibition on CD4+CD25+Foxp3+ regulatory T cells compared with conventional CD4+ T cells. *Blood*. 2008; 111:453–62. [PubMed: 17967941]

- [91]. Battaglia M, Stabilini A, Draghici E, et al. Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance. *Diabetes*. 2006; 55:40–9. [PubMed: 16380475]
- [92]. Gao W, Lu Y, El Essawy B, et al. Contrasting effects of cyclosporine and rapamycin in de novo generation of alloantigen-specific regulatory T cells. *Am J Transplant*. 2007; 7:1722–32. [PubMed: 17511761]
- [93]. Hendriks TK, Velthuis JH, Klepper M, et al. Monotherapy rapamycin allows an increase of CD4 CD25 FoxP3 T cells in renal recipients. *Transpl Int*. 2009; 22:884–91. [PubMed: 19453998]
- [94]. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood*. 2005; 105:4743–8. [PubMed: 15746082]
- [95]. Bloom DD, Chang Z, Fechner JH, et al. CD4+ CD25+ FOXP3+ regulatory T cells increase de novo in kidney transplant patients after immunodepletion with Campath-1H. *Am J Transplant*. 2008; 8:793–802. [PubMed: 18261176]
- [96]. Trzonkowski P, Zilvetti M, Chapman S, et al. Homeostatic repopulation by CD28–CD8+ T cells in alemtuzumab-depleted kidney transplant recipients treated with reduced immunosuppression. *Am J Transplant*. 2008; 8:338–47. [PubMed: 18211507]
- [97]. Bluestone JA, Liu W, Yabu JM, et al. The effect of costimulatory and interleukin 2 receptor blockade on regulatory T cells in renal transplantation. *Am J Transplant*. 2008; 8:2086–96. [PubMed: 18828769]
- [98]. Presser D, Sester U, Mohrbach J, et al. Differential kinetics of effector and regulatory T cells in patients on calcineurin inhibitor-based drug regimens. *Kidney Int*. 2009; 76:557–66. [PubMed: 19494797]
- [99]. Ma A, Qi S, Wang Z, et al. Combined therapy of CD4(+)CD25(+) regulatory T cells with low-dose sirolimus, but not calcineurin inhibitors, preserves suppressive function of regulatory T cells and prolongs allograft survival in mice. *Int Immunopharmacol*. 2009; 9:553–63. [PubMed: 19539558]
- [100]. Vlad G, Ho EK, Vasilescu ER, et al. Anti-CD25 treatment and FOXP3-positive regulatory T cells in heart transplantation. *Transpl Immunol*. 2007; 18:13–21. [PubMed: 17584597]
- [101]. Caproni M, Torchia D, Antiga E, et al. The effects of tacrolimus ointment on regulatory T lymphocytes in atopic dermatitis. *J Clin Immunol*. 2006; 26:370–5. [PubMed: 16773453]
- *[102]. Poirier N, Azimzadeh AM, Zhang T, et al. Inducing CTLA-4-dependent immune regulation by selective CD28 blockade promotes regulatory T cells in organ transplantation. *Sci Transl Med*. 2010; 2:17ra10. Study showing that blockade of CD28 rather than CD80/86 is effective at controlling graft rejection and vasculopathy in a non-human primate model. The study demonstrated an increase in intragraft and peripheral Treg. The blockade of CD28 rather than CD80/86 is an interesting alternative to CTLA-4-Ig as it leaves CTLA-4-dependent immunoregulation, as employed by Treg, intact.
- [103]. Roncarolo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nat Rev Immunol*. 2007; 7:585–98. [PubMed: 17653126]
- [104]. Allan SE, Broady R, Gregori S, et al. CD4+ T-regulatory cells: toward therapy for human diseases. *Immunol Rev*. 2008; 223:391–421. [PubMed: 18613849]
- [105]. Di Ianni, M.; Falzetti, F.; Carotti, A., et al. 51st ASH Annual Meeting AND Exposition Online Program and Abstracts; New Orleans. December 5-8, 2009; American Society of Hematology; <http://ash.confex.com/ash/2009/webprogram/Paper18555.html>
- [106]. Carlson RH. Adoptive Immunotherapy with ‘Tregs’ Prevents GvHD in Mismatched Transplants. *Oncology Times*. 2010; 32:19–20.

Figure 1. Molecular mechanisms of action of regulatory T cells.

Regulatory T cells (Treg) employ a multitude of redundant mechanisms to suppress immune responses [28]. 1) Tregs can cause direct cytotoxicity of target cells through perforin-dependent and -independent granzyme B mechanisms [29, 30]. 2) Galectin-1 expressed on the surface of Tregs can bind target cells to induce cell cycle arrest and apoptosis [31]. 3) Suppressive secreted cytokines such as interleukin (IL)-10, transforming growth factor β (TGF β) and IL-35 can induce bystander regulation of T cells [14, 32-34]. 4) Tregs inhibit the induction of IL-2 mRNA in target T cells [35]. 5) Surface CD25 on Tregs consumes IL-2 in the surrounding environment, depriving cells of this growth factor [36]. 6) Cytotoxic T Lymphocyte Antigen 4 (CTLA-4, CD152) interacts with CD80/CD86 on the surface of antigen presenting cells (APC) to prevent their binding to CD28, therefore indirectly inhibiting the activation of T cells [14, 37]. 7) The interaction of CTLA-4 and CD80/86 induces the expression of indoleamine 2,3-dioxygenase (IDO) from dendritic cells (DC), which inhibits T cell activation by depleting tryptophan from the extracellular milieu [38]. 8) Lymphocyte-activation gene 3 (Lag, CD223) on the surface of Treg binds MHC class II to inhibit DC maturation [39]. 9) Fibrinogen-like protein 2 (FGL2) on the surface of Tregs binds Fc γ RIIB on the surface of DC to prevent their maturation [40]. 10) CD39 on the surface of Tregs hydrolyses adenosine triphosphate (ATP) to adenosine monophosphate (AMP), reducing the availability of ATP-triggered proinflammatory signals [41].