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Treg Therapy in Transplantation: How and When Will We Do It?

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Abstract Adoptive therapy with regulatory T cells (Tregs) has shown great promises in many experimental models to induce permanent graft acceptance and tolerance to alloantigens. However, although their tolerogenic therapeutic potential has been demonstrated in clinical hematopoietic stem cell transplantation and partially in patients with early onset type I diabetes, translation into clinical testing for solid organ transplantation is still lacking or only slowly starting. This is in part due to the fact that many questions regarding not only the optimal type of Tregs but also the best-suited clinical trial design are unsolved and highly debated within the scientific community. With facilities for purifying and expanding Tregs and the clinical trials being very expensive, these points should be carefully discussed. Here, we summarized recent results regarding adoptive Treg therapy in solid organ transplantation additionally addressing the above-mentioned questions.

Keywords Transplantation · Cell therapy · Regulatory T cells

Introduction

Since the first discovery by Gershon and Kondo [1], the importance of regulatory T cells (Tregs) for installing and controlling immune homeostasis has become widely accepted [2–4]. With only 5–10 % of circulating $CD4^+$ T cells being Tregs, they represent only a small fraction of our immune cells. Nevertheless, they are instrumental in controlling self and oral tolerance, and thus preventing occurrence of autoimmune diseases and undesired immune reactions against harmless antigens in the majority of people [4–8]. They have shown therapeutic effects in treating autoimmune disease such as type 1 diabetes (T1D) [9, 10•, 11••, 12], GvHD after hematopoietic stem cell transplantation (HSC) [8, 13•, 14••, 15] and also preventing rejection of solid organ transplants [3, 7, 16]. In some studies, higher proportions of Tregs or certain subsets were detected in the peripheral blood of "operationally tolerant" patients [17, 18] with their frequencies or numbers being reduced in some patients experiencing rejection [18, 19]. In addition, Tregs can impose regulatory function onto conventional alloreactive T cells in a process now being known as infectious tolerance [16, 20]. These properties make Tregs an attractive therapeutic alternative to conventional lifelong immunosuppressive drugs. The hope is to utilize the immunoprotective function of Tregs to prolong graft survival or induce tolerance while preserving the general immunocompetence of the patient. In this review, we outline the current status in research developing applicable strategies for adoptive therapy with Tregs in solid organ transplantation.

Type of Regulatory T Cells

nTregs or Tr1

Among the various T cell subsets with regulatory potential are two that show the greatest potential for cell therapy. On the one hand, there are the thymus-derived regulatory Tregs (tTregs) [21], and on the other hand, peripherally or ex vivo-

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induced IL-10 producing type 1 regulatory T cells (Tr1) [22–24]. Thymus-derived Tregs are defined as CD4+CD25highCD127low cells with stable high Foxp3 expression [25, 26]. Utilizing tTregs as a bulk population based on their high expression of CD25 and low expression of CD127 offers the advantage of isolation and application without the need of donor material. As such, these cells were already applied in T1D patients [9, 10•, 11••] as well as prophylaxis and treatment of GvHD in the setting of HSC transplantation [14••, 15, 27••, 28]. On the contrary, Tr1 cells in the thymus are equivalent to naive T cells and initially get transformed into regulatory cells in the presence of interleukin 10 (IL-10) in the periphery [29, 30]. Tr1 cells mediate locally suppression by generating IL-10 themselves [31]. However, Tr1 do not express Foxp3 why they are described as CD4+CD25−Foxp3−IL10+IL4− cells [31]. Tr1 cells were first applied in studies of GvHD and showed promising results [13•]. It is difficult to purify these cells from peripheral blood, which is why naive recipient T cells are taken and incubated with donor antigen-presenting cells (APCs) in the presence of IL-10 to generate them [32]. Therefore, generation of Tr1 cells is dependent on donor cells making clinical translation more challenging [30]. The recent identification of Tr1 specific surface markers LAG-3 and CD49b can help to improve purity of generated Tr1 cells, as now isolation/enrichment using beads or flow sorting is possible [31]. However, in many studies, tTregs are preferentially used because of established isolation protocols and intracellular Foxp3 expression or Foxp3 locus demethylation being widely accepted quality control markers [7, 33–36, 37•]. Additionally, they take part in the in vivo induction of Tr1 cells [17, 23]. Nevertheless, both cell types were effective in vivo in mice [18] and are attractive candidates for future studies.

Isolation and Expansion of Tregs for Adoptive Therapy

There is no specific single marker for identifying and isolating tTregs. That is why a combination of many different markers has to be used (CD4, CD25, CD127, ±RA) to enrich tTregs with a high purity [11••, 26, 38]. So far, only with flow cytometry-based enrichment multiple phenotypic characteristics can be considered. Thereby, a purity of over 90 % CD4+CD25highCD127lowFoxp3+ cells is achievable [11••, 20, 39, 40]. However, flow cytometry-based enrichment is only possible or allowed in certain labs and countries so far. That is why most laboratories are applying the CliniMACS-System (Miltenyi) instead. However, so far only enrichment for parallel expression of CD4 and CD25 is possible. As there is a large overlap between Tregs and CD25dim effector T cells in humans, only 60–80 % of the CD4 and CD25 positive cells express Foxp3 and show demethylation of the Treg-specific demethylation region (TSDR) within the Foxp3 locus [36, 37•, 41, 42]. Additionally, CD4+CD25highCD127low cells

have been shown to be more effective as compared to Tregs isolated according to CD4+CD25+ expression only [3, 26, 39, 41, 42]. Importantly, both tTregs isolated by bead-based and flow cytometry-based methods can keep their suppressive function in vivo [30]. So each procedure is potentially applicable.

As explained later in some circumstances to gain sufficiently high cell numbers expansion of Tregs is required. The ex vivo expansion of CD4+CD25+ tTregs is well established [43]. Interestingly, it has been noticed that fresh isolated Tregs were less effective in preventing GvHD than in vitro-expanded cells [8]. The cells can either be stimulated with antigen or unspecifically with anti-CD3/CD28 beads [36, 37•, 40, 42, 44, 45]. Usually interleukin 2 (IL-2) is added to stimulate Treg expansion. The issue on advantages or disadvantages of selecting and expanding antigen-reactive Tregs will be discussed in the following text in greater detail.

Also, for tTregs, it is possible to distinguish between naïve (CD45RA+) and memory cells (CD45RA−) [38, 46]. Several studies have revealed that naïve tTregs are the more stable and especially suppressive cells [46–48]. In contrast, memory tTregs show a higher potential of transforming into IL-17 producing inflammatory cells. With the frequencies and numbers of CD45RA+ naïve tTregs decreasing with age, it might be impossible in some patients to use them as starting population (Lei et al. American Journal of Transplantation, in press). Therefore, alternative strategies for preventing phenotypic alterations and outgrowth of inflammatory T cells had to be defined. Interestingly, with addition of rapamycin to tTreg cultures, this can be easily achieved [48, 49]. It selectively enforces proliferation of CD4+CD25highFoxp3+ cells, and prevents IL-17 production and outgrowth of contaminating effector T cells. Additionally, tTregs cultured in the presence of rapamycin show increased suppressive function [48]. In all expansion protocols, a frequent stimulation was associated with loss of suppressive function and increased cell death [44].

Polyclonal vs. Alloantigen-Reactive Tregs

Anti-CD3/CD28 bead-stimulated polyclonal tTregs have broad antigen reactivity, and potentially, a state of general suppression could be acquired [40]. If that would be the case, polyclonal Tregs would cause similar side effects in vivo as conventional IS. Indeed, although the first study on polyclonal Treg therapy in conjunction with umbilical cord blood transplantation (UCB) reported no deleterious effects on risks of infections in comparison to historical controls [14••], later analysis revealed a higher risk of opportunistic infections within the first 30 days following UCB infusion [50•]. However, this was only the case for the first 30 days, the time period in which the infused Tregs were still present and these early events had no impact on long-term outcome. In addition,

conditioning in conjunction with HSC transplantation is creating a post-transplant nadir phase, in which the patients are highly vulnerable for opportunistic infections. It is still unanswered and need to be investigated whether adoptive transfer of polyclonal tTregs in solid organ transplantation will increase the risk of infectious complications. Another issue is their disposition to lose their suppressive function and subsequently develop into proinflammatory cells especially when restimulated too often [51].

In contrast, alloantigen-reactive Tregs stimulated and expanded by e.g., use of donor, APCs would selectively react to donor antigen, and thus not induce a state of general immunosuppression [51]. In addition, upon antigen-specific stimulation, reactive tTregs can be discriminated from reactive effector T cells by a selective expression pattern of certain activation markers [20, 41, 52•, 53, 54].

The group of Andreas Thiel has identified a selective expression of CD137 (4-1BB) on antigen-reactive Tregs shortly upon antigen restimulation, whereas antigen-reactive conventional helper T cells were characterized by CD154 (CD40L) expression [52•].

Sagoo and colleagues screened tTregs for the expression of activation markers upon stimulated with allogeneic PBMCs or dermal dendritic cells [54]. Expression of CD69 and CD71 peaked between day 3 and 5 in alloantigen-reactive tTregs. In addition, $CD69^+CD71^+$ tTregs more potently inhibited donor but not third party effector responses.

Also, other groups did show a higher and also more specific suppressive capacity of antigen-reactive vs. polyclonal tTregs [51, 54]. They can cause an inhibition of effector responses at ratios of up to 1:2000 Tregs to Teff [51]. This indicates again that antigen-reactive Tregs could have fewer side effects. In addition, no loss of Foxp3 expression was detected during expansion or after extensive stimulation [37•, 51]. In fact, the expansion rate was even higher using an antigen-stimulation-based approach [37•, 55]. The best results were reached using B cells as antigen-presenting cells [37•, 45]. In animal studies alloantigen-reactive Tregs appeared to be significantly more suppressive than polyclonal cells [20, 45, 55, 56]. They are 5 to 25 times more potent than polyclonal Tregs and suppress 9 to 27 times more effective when stimulated with their cognate antigen as compared to other stimuli [45]. In addition, it was shown that only antigen-reactive Tregs are able to efficiently inhibit IFN-y production by memory T cells [55].

Therefore, it is believed that with antigen-reactive Tregs, one can decrease the total cells needed about 10 times [37•, 55–57]. This makes them even more attractive. Nevertheless, the stimulation with alloantigen might not always be possible. Since in case of deceased donation, the donor is not known before transplantation and some time is needed for isolation and expansion, adoptive transfer of polyclonal Tregs might be the better option. In addition, recognition of donor alloantigen

occurs at least by direct and indirect presentation. Direct alloantigen presentation is easy to achieve, whereas reproducible indirect presentation is difficult to achieve.

Number of Tregs Needed

Tregs represent less than 10 % of $CD4^+$ T cells, which therefore make up about 13×10^9 cells in the whole body [56]. A ratio of Tregs to effector T cells of 1:3 showed a positive outcome in biopsies of rats [57]. Lower ratios were more connected to rejection. Other studies revealed that ratios of 1:2 up to 1:1 are necessary to inhibit rejection [30, 56, 58]. To reach a sufficient cell number in a non-immune-compromised patient with a body weight of 70 kg, a dose of $5–8 \times 10^{10}$ Tregs is needed [56, 59]. By leukophoresis, a maximum of 8×10^9 lymphocytes with a fraction of 2×10^8 Tregs can be isolated [30, 56]. Thus, the required cell number cannot be reached without stimulation and expansion for several rounds [60]. Taylor et al. even showed that expanded cells were more effective than freshly isolated [8]. Expanded Tregs suppressed effector response by 50 % at a ratio of 1:243 in contrast to freshly isolated Tregs at a ratio of 1:1 [51].

From the few performed clinical trials in HSC transplantation and diabetes, it is difficult to estimate how many Tregs are really needed to inhibit GVHD and induce long-term remission but are also safe. In the first clinical trial in stem cell transplantation, a dose escalation was performed starting from 0.1 up to 30×10^5/kg polyclonal Tregs [14••]. The majority of patients received 30×10^5/kg Tregs with either concomitant CsA/MMF or sirolimus/MMF therapy. This maximum dose seemed to be efficient to reduce the incidence of severe GVHDs in comparison to historical controls. However, as pointed out already earlier, this is a special situation following conditioning of patients.

In early onset, T1D administration of two cycles of 3×10^7 polyclonally expanded Tregs lead to remission at 1 year in 8 out of 12 patients [10•].

It is postulated that using antigen-reactive Tregs, e.g., donor-reactive Tregs, with direct specificity in case of allogeneic transplantation, the required cell dose can be reduced at least up to 10 times [61]. However, large clinical trials in solid organ transplantation testing polyclonal vs. antigen-reactive Tregs in conjunction with Treg favoring, IS such as rapamycin or reduced CNI dosage are needed to clarify this issue.

Assessment of Their Functional Capacity and Stability

It is crucial to perform quality measurements on the isolated and expanded Tregs prior to in vivo transfer. The biggest fears are (1) concomitant transfer of contaminating effector T cells

and (2) in vivo conversion of Tregs into pathogenic helper T cells. Especially the later fact has been frequently observed in animal models or in vitro upon addition of inflammatory stimuli and in the absence of stabilizing agents such as rapamycin. As mentioned already earlier, the epigenetic status of the Foxp3 locus especially demethylation of the TSDR region is deeply connected with stability and thus function of Tregs [17, 41, 52•]. Braza et al. have reported that the amount of CD4^{+} T cells with a TSDR demethylation is significantly higher in tolerant patients compared to controls [17]. Foxp3 is known to inhibit RORγt, the master switch transcription factor of IL-17 producing Th17 cells [40]. A loss of TSDR demethylation is associated with increased IL-17 production [33, 40]. According to this, a high stable Foxp3 expression in vitro could suggest a small risk for an IL-17 production in vivo under inflammatory conditions. Collectively, those data point to the assessment of TSDR demethylation as being an important quality control. However, current methods applied to assess TSDR demethylation take more than a day hampering its use as a release criterion.

In addition to assessing the stability of infused Tregs validation of their suppressive function is important. This is often done analyzing their capacity to inhibit proliferation or cytokine production of co-cultured conventional T cells. Typically, CSFE-labeled effector T cells are incubated at various ratios with Tregs and one without [27••, 48]. Although widely used, the relevance is highly questioned within the community. It is known that activated conventional T cells are also able to suppress proliferation of co-cultured naïve T cells. Performing this test under inflammatory conditions, which is more physiological, might generate more convincing results. With lack of alternative assays, testing the potential of Tregs to inhibit proliferation is widely performed. An attractive addition to proliferation-based functional assays would be the analysis to inhibit expression of activation markers on co-cultured conventional T cells. Here again, the expression analysis of CD154 seems to be a good candidate [62].

Clinical Indications for Adoptive Treg Therapy

From studies in animal models, we know that the best effect is observed when Tregs are given in a prophylactic manner prior to or at the time of transplantation [5, 7, 57, 63]. Here, the aim is to prevent acute rejection of allogeneic transplants and to induce tolerance very often without concomitant use of other immunomodulatory agents. In the clinical situation, this early transfer of Tregs is hard to achieve especially in the case of deceased donation. Also, the intensive IS regimen, ethically so far unavoidable, early after transplantation might interfere with the regulatory function of the transferred Tregs. However, in more advanced experimental animal models, mimicking clinical challenges also delayed application of regulatory cells

to revert developing signs of chronic rejection was successful [64].

In the next paragraphs, we will therefore discuss the options we have to utilize he immunoregulatory potential of Tregs in clinical trials.

Boosting Operational Tolerance for Drug Minimization or Withdrawal

Ideally adoptive transfer of Tregs would result in induction of long-term graft acceptance or tolerance, and thus one could avoid permanent use of IS with all the known side effects. Thus, similar to studies performed in animal models, Tregs would be transferred at the time or early after transplantation to efficiently prevent differentiation of alloreactive pathogenic conventional T cells and B cells. This, however, might be a challenging task and require large numbers of Tregs to be infused in a tolerance permissive IS environment, which as mentioned above from an ethical standpoint not feasible at least within the first clinical trials. In addition, it would require tools or biomarkers by which we can identify the successfully treated and thus tolerant patients. Although gene markers or immune cell subpopulations being more abundant in "operationally" tolerant patients have been identified [17, 65, 66], they were never validated in prospective trials, a prerequisite before using them as endpoints in Treg trials.

In the clinical trials on an early adoptive therapy of Tregs in conjunction with HSC immunosuppression could be successfully tapered in patients not experiencing GVHD with the overall incidences of GVHD being lower as compared to historical controls [14••, 27••]. Thus, Treg therapy seemed to favor tolerance induction. Whether this is also possible in unconditioned patients, receiving "just" a solid organ graft remains to be investigated.

First dose escalation trials have to be run to prove safety of Treg application in solid organ transplantation. Thus, it will take a few more years before efficacy results on the tolerance promoting capacity of polyclonal or antigen-reactive Tregs will become publically available. "The ONE Study" is a consortium evaluating the safety of Treg transfer shortly after transplantation in living kidney donation [67]. Within that project safety of various regulatory cell populations will be tested and also their impact on abundance of tolerance biomarkers will be tested [68]. In future studies, efficacy of Treg therapy can be assessed. However, currently there is a big discussion within the scientific community whether one should really aim for complete withdrawal of IS by tolerance promoting Tregs therapy or whether drug minimization to very low dose monotherapy would be safer in the long term [67]. It would be already a success to achieve at a relatively early time point a low dose CNI monotherapy, thereby minimizing the side effects but avoiding unnoticed inflammatory

processes due to e.g., bystander activation of cross-reactive T cells. Regardless whether aiming for complete or partial IS withdrawal, one should carefully consider the variable effect of concomitantly applied drugs on Treg in vivo survival, expansion, and function. From studies in animal models, we know that a high dose of CNIs hampers Treg proliferation, whereas rapamycin seems to selectively promote Treg expansion [49]. At a low dose, CNIs can be combined with a Treg therapy [64, 69]. As rapamycin cannot be given to every patient especially early post transplant, a possible combination with low dose CNIs sounds appealing. In addition it is highly debated whether Tregs can or should be given in conjunction with an induction agent such as ATG. Depletion-induced lymphopenia might give the transferred Tregs an advantage or even induce their maturation and expansion [70, 71]. However, it might also lead to expansion of depletion-refractory preexisting memory T cells, which are more difficult to regulate by Tregs [63, 72, 73]. Along the same line, it is frequently discussed whether a delay of Treg transfer by a few days following induction therapy or application of a second batch should be taken into consideration.

Treatment of Chronic Inflammation and/or Rejection

In addition to the induction of tolerance, the immunoregulatory potential of Tregs could be also used to treat developing chronic rejection, which would also lead to improved long-term graft survival, and thus be of immense clinical benefit [74]. Development of chronic rejection is the most feared event following organ transplantation, because it is difficult to recognize early and hardly any treatment options that exist. Current IS except for mycophenolate mofetil (MMF) even favors development of chronic rejection [75]. Notably, it has been recognized that peripheral Treg levels are extremely low in patients with chronic rejection [76–78]. Thus, it seems appealing that adoptive transfer of Tregs can halt or reverse inflammatory processes leading to chronic rejection and graft loss. Indeed, also in experimental models, it was shown that Treg transfer can prevent antibody-mediated and T cell-mediated chronic rejection [64, 79]. For the prevention of antibody-mediated chronic rejection, early recognition by e.g., slowly increasing levels of anti-donor antibodies and treatment is instrumental for the success. In addition, it has been reported that for successful prevention of chronic rejection transfer of Tregs with indirect specificity is needed [79]. Thus, to ensure success of a Treg-based prevention or treatment of chronic rejection, we should use cells with mixed, direct and indirect, specificities, and install extensive immune monitoring of our patients.

Boosting Regeneration—Treatment of Delayed Graft Function (DGF) or Acute Kidney Injury

With the increasing demand for organs, there is an urge to transplant organs of poorer quality. Especially organs of deceased donors can cause delayed graft function [80–82]. In fact, up to 50 % of the transplanted kidney grafts can get affected. This is linked with a significant reduction of long-term graft survival making any approach leading to the prevention or treatment of DGF important. Severe and prolonged ischemic reperfusion injury (IRI) is discussed as leading cause of DGF. An infiltration of CD8^{+} and CD4^{+} T cells [83] and a higher expression of MHC molecules were detected in grafts following IRI. Interestingly, an infiltration of Foxp3^{+} Tregs was observed a few days after reperfusion during graft recovery [84]. So they obviously modulate repair responses. Gandolfo et al. have observed that a decrease of Tregs in the healing process can delay repair and increase the infiltration rate of Teff cells which produced TNF-a and IFN-y [83]. Therefore, Tregs may downregulate cytokine production by effector T cells, and thus mediate faster recovery. Similar findings were made with the use of anti-CD25 antibodies as an induction agent [84]. Clearly more necrosis and reduced recovery were detected with the use of the antibody. Additionally, a decreased Treg function prior to transplantation was observed in patients later developing DGF [85]. Thus, Tregs may play a major role in preventing DGF. Treating the patients with Tregs before or during transplantation may ameliorate IRI and prevent DGF [86–88].

Thus, with an early transfer of Tregs, one not only would utilize their beneficial effects for prevention of DGF, but ultimately also acute and chronic rejection. However, this would limit it to the use of polyclonal Tregs.

Monitoring Safety and Efficacy of Treg Therapy

With any novel therapy, safety concerns do exist. In the case of adoptive Treg therapy, clinicians fear an increased risk of infectious complications caused by herpes virus reactivation or opportunistic pathogens [4]. As mentioned earlier, the first clinical trials applying Treg therapy did not, at first, observe an increased infection risk [14••, 15, 27••]. Although, a more detailed analysis by Brunstein and colleagues revealed critical 30-day post-infusion period in which the patients seemed to be more susceptible to infections as compared to historical controls [50•]. These trials were conducted on patients receiving HSC grafts for treatment of malignancies. Due to the conditioning, the patients are prone to develop infections, and it is as such not uncommon phenomenon. It is however reassuring that no serious infectious complications and normal post-vaccination response was observed in children with early onset T1D upon Treg transfer [10•]. But one should be aware of

the potential risk especially when transferring polyclonal Tregs, and thus accompany the treatment with a close screening of signs of e.g., viral replication (CMV, EBV, BKV). In addition, other biomarkers indicating a systematic state of immunodepression such as HLA-DR expression on monocytes or dendritic cells could be included within a post-infusion immune monitoring [89–91]. In addition, any cellular therapy can lead to a cytokine storm causing severe complications [92]. This is a bigger problem for tumor- or virus-specific cellular therapy; it cannot be excluded upon Treg transfer.

Although the first Treg trials are focusing on safety issues, we have to prepare ourselves for measuring efficacy or other parameters in future studies. Of course, efficacy will be first based on clinical outcome measures such as incidence of biopsy proven acute rejection or change in graft function over time. But as mentioned, it appears to be attractive to use additionally change in immune cell composition or function as additional indications for a therapy success or failure. As such, successful Treg therapy might inhibit development of anti-donor specific antibodies, and thereby prevent development of chronic antibody-mediated rejection [64]. In addition, it could lead to a reduction of pathogenic memory T cells as measured by IFN-γ Elispot or flow cytometry-based methods [52•, 93–96]. In contrast, Treg therapy could lead to increased numbers of transitional or regulatory B cells accompanied by a specific peripheral gene expression pattern as observed for "operationally" tolerant patients [65, 66]. However, to be able to report reproducible results and compare them across different clinical trials, we need to increase our efforts for method standardization as currently been shown for the IFN-γ Elispot and flow cytometry [97, 98, 99•].

Conclusion

Treg therapy is developing into a treatment alternative in solid organ transplantation. Animal studies and first human trials in HSC transplantation and T1D show promising results. The current trials in solid organ transplantation will give some insights into safety and partially efficacy. As a community, we will need to discuss issues on best-suited type of Tregs, time point, and clinical indication. In addition, efforts to standardize immune monitoring accompanying Treg trials will be of importance.

Compliance with Ethics Guidelines

Conflict of Interest Nadja Niemann and Birgit Sawitzki declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Papers of particular interest, published recently, have been highlighted as:
• Of importance
•• Of major importance

1. Gershon RK, Kondo K. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. Immunology. 1970;18:723–37.
2. Maloy KJ, Powrie F. Regulatory T cells in the control of immune pathology. Nat Immunol. 2001;2:816–22.
3. Schliesser U, Streitz M, Sawitzki B. Tregs: application for solid-organ transplantation. Curr Opin Organ Transplant. 2012;17:34–41. doi:10.1097/MOT.1090b1013e32834ee32869f.
4. Smigiel KS, Srivastava S, Stolley JM, Campbell DJ. Regulatory T-cell homeostasis: steady-state maintenance and modulation during inflammation. Immunol Rev. 2014;259:40–59. doi:10.1111/imr.12170.
5. Oliveira V, Sawitzki B, Chapman S, Appelt C, Gebuhr I, Wieckiewicz J, 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.
6. Sakaguchi S, Fukuma K, Kuribayashi K, Masuda T. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. J Exp Med. 1985;161:72–87.
7. Schliesser U, Chopra M, Beilhack A, Appelt C, Vogel S, Schumann J, et al. Generation of highly effective and stable murine alloreactive Treg cells by combined anti-CD4 mAb, TGF-beta, and RA treatment. Eur J Immunol. 2013;43:3291–305.
8. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. Blood. 2002;99:3493–9.
9. Marek-Trzonkowska N, Mysliwec M, Siebert J, Trzonkowski P. Clinical application of regulatory T cells in type 1 diabetes. Pediatr Diabetes. 2013;14:322–32.
10.• Marek-Trzonkowska N, Mysliwiec M, Dobyszuk A, Grabowska M, Derkowska I, Juscinska J, et al. Therapy of type 1 diabetes with CD4(+)CD25(high)CD127-regulatory T cells prolongs survival of pancreatic islets—results of one year follow-up. Clin Immunol. 2014;153:23–30. doi:10.1016/j.clim.2014.1003.1016. **First study indicating efficacy of Treg in patients receiving pancreatic islets**.
11.•• Marek-Trzonkowska N, Mysliwiec M, Dobyszuk A, Grabowska M, Techmanska I, Juscinska J, et al. Administration of CD4+ CD25highCD127− regulatory T cells preserves beta-cell function in type 1 diabetes in children. Diabetes Care. 2012;35:1817–20. **First study indicating efficacy of Treg in early onset type 1 diabetes patients**.
12. Zhang Y, Bandala-Sanchez E, Harrison LC. Revisiting regulatory T cells in type 1 diabetes. Curr Opin Endocrinol Diabetes Obes. 2012;19:271–8.
13.• Bacchetta R, Lucarelli B, Sartirana C, Gregori S, Lupo Stanghellini MT, Miqueu P, et al. Immunological outcome in haploidentical-HSC transplanted patients treated with IL-10-anergized donor T cells. Front Immunol. 2014;5:16. doi:10.3389/fimmu.2014.00016. eCollection 02014. **Results of the first clinical trial with Tr1 cells in patients receiving hematopoietic stem cells**.
14.•• Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. Blood. 2011;117:1061–70. **One of the first two**

studies investigating safety and partially efficacy of Tregs in patients receiving hematopoietic stem cell grafts.

15. Trzonkowski P, Dukat-Mazurek A, Bieniaszewska M, Marek-Trzonkowska N, Dobyszuk A, Juscinska J, et al. Treatment of graft-versus-host disease with naturally occurring T regulatory cells. BioDrugs. 2013;27:605–14.

16. Waldmann H, Adams E, Fairchild P, Cobbold S. Infectious tolerance and the long-term acceptance of transplanted tissue. Immunol Rev. 2006;212:301–13.

17. Braza F, Dugast E, Panov I, Paul C, Vogt K, Pallier A, et al. Central role of CD45RA-Foxp3hi memory regulatory T cells in clinical kidney transplantation tolerance. J Am Soc Nephrol:. 2015;2014050480.

18. Sawitzki B, Reinke P, Pascher A, Volk HD. State of the art on the research for biomarkers allowing individual, tailor-made minimization of immunosuppression. Curr Opin Organ Transplant. 2010;15:691–6.

19. Iwase H, Kobayashi T, Kodera Y, Miwa Y, Kuzuya T, Iwasaki K, et al. Clinical significance of regulatory T-cell-related gene expression in peripheral blood after renal transplantation. Transplantation. 2011;91:191–8.

20. Juvet SC, Whatcott AG, Bushell AR, Wood KJ. Harnessing regulatory T cells for clinical use in transplantation: the end of the beginning. Am J Transplant. 2014;14:750–63.

21. Abbas AK, Benoist C, Bluestone JA, Campbell DJ, Ghosh S, Hori S, et al. Regulatory T cells: recommendations to simplify the nomenclature. Nat Immunol. 2013;14:307–8.

22. Battaglia M, Gregori S, Bacchetta R, Roncarolo MG. Tr1 cells: from discovery to their clinical application. Semin Immunol. 2006;18:120–7.

23. Levings MK, Roncarolo MG. T-regulatory 1 cells: a novel subset of CD4 T cells with immunoregulatory properties. J Allergy Clin Immunol. 2000;106:S109–12.

24. Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol Rev. 2006;212:28–50.

25. Edozie FC, Nova-Lamperti EA, Povoleri GA, Scotta C, John S, Lombardi G, et al. Regulatory T-cell therapy in the induction of transplant tolerance: the issue of subpopulations. Transplantation. 2014;98:370–9.

26. Hartigan-O'Connor DJ, Poon C, Sinclair E, McCune JM. Human CD4+ regulatory T cells express lower levels of the IL-7 receptor alpha chain (CD127) allowing consistent identification and sorting of live cells. J Immunol Methods. 2007;319:41–52.

27.•• Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. Blood. 2011;117:3921–8. **One of the first two studies investigating safety and partially efficacy of Tregs in patients receiving hematopoetic stem cell grafts.**

28. Martelli MF, Di Ianni M, Ruggeri L, Falzetti F, Carotti A, Terenzi A, et al. HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse. Blood. 2014;124:638–44.

29. Bacchetta R, Gregori S, Serafini G, Sartirana C, Schulz U, Zino E, et al. Molecular and functional characterization of alloantigen-specific anergic T cells suitable for cell therapy. Haematologica. 2010;95:2134–43.

30. Hippen KL, Riley JL, June CH, Blazar BR. Clinical perspectives for regulatory T cells in transplantation tolerance. Semin Immunol. 2011;23:462–8.

31. Gagliani N, Magnani CF, Huber S, Gianolini ME, Pala M, Licona-Limon P, et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. Nat Med. 2013;19:739–46.

32. Gregori S, Roncarolo MG, Bacchetta R. Methods for in vitro generation of human type 1 regulatory T cells. Methods Mol Biol. 2011;677:31–46. doi:10.1007/1978-1001-60761-60869-60760_60763.

33. Canavan JB, Afzali B, Lord GM, Lombardi G. Assessment of regulatory T-cell function in forthcoming clinical trials of cell therapy. Expert Rev Mol Diagn. 2013;13:5–7. doi:10.1586/erm.1512.1133.

34. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Del Papa B, Perruccio K, et al. Immunoselection and clinical use of T regulatory cells in HLA-haploidentical stem cell transplantation. Best Pract Res Clin Haematol. 2011;24:459–66.

35. Kendal AR, Chen Y, Regateiro FS, Ma J, Adams E, Cobbold SP, et al. Sustained suppression by Foxp3+ regulatory T cells is vital for infectious transplantation tolerance. J Exp Med. 2011;208:2043–53.

36. Peters JH, Preijers FW, Woestenenk R, Hilbrands LB, Koenen HJ, Joosten I. Clinical grade Treg: GMP isolation, improvement of purity by CD127 depletion, Treg expansion, and Treg cryopreservation. PLoS One. 2008;3:e3161.

37.• Putnam AL, Safinia N, Medvec A, Laszkowska M, Wray M, Mintz MA, et al. Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation. Am J Transplant. 2013;13:3010–20. **Very straight forward description of clinical grade manufacturing of Tregs.**

38. Canavan JB, Scotta C, Vossenkamper A, Goldberg R, Elder MJ, Shoval I, et al. Developing in vitro expanded CD45RA+ regulatory T cells as an adoptive cell therapy for Crohn's disease. Gut. 2015;2014:306919.

39. Nadig SN, Wieckiewicz J, Wu DC, Warnecke G, Zhang W, Luo S, et al. In vivo prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells. Nat Med. 2010;16:809–13.

40. Tang Q, Bluestone JA, Kang SM. CD4(+)Foxp3(+) regulatory T cell therapy in transplantation. J Mol Cell Biol. 2012;4:11–21. doi:10.1093/jmcb/mjr1047. Epub 2011 Dec 1014.

41. Bacher P, Kniemeyer O, Schonbrunn A, Sawitzki B, Assenmacher M, Rietschel E, et al. Antigen-specific expansion of human regulatory T cells as a major tolerance mechanism against mucosal fungi. Mucosal Immunol. 2014;7:916–28.

42. Scheffold A. How can the latest technologies advance cell therapy manufacturing? Curr Opin Organ Transplant. 2014;19:621–6.

43. Hoffmann P, Eder R, Kunz-Schughart LA, Andreesen R, Edinger M. Large-scale in vitro expansion of polyclonal human CD4(+)CD25high regulatory T cells. Blood. 2004;104:895–903.

44. 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.

45. Chen LC, Delgado JC, Jensen PE, Chen X. Direct expansion of human allospecific FoxP3+ CD4+ regulatory T cells with allogeneic B cells for therapeutic application. J Immunol. 2009;183:4094–102.

46. Hoffmann P, Eder R, Boeld TJ, Doser K, Piseshka B, Andreesen R, et al. Only the CD45RA+ subpopulation of CD4+ CD25high T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion. Blood. 2006;108:4260–7.

47. Safinia N, Leech J, Hernandez-Fuentes M, Lechler R, Lombardi G. Promoting transplantation tolerance; adoptive regulatory T cell therapy. Clin Exp Immunol. 2013;172:158–68.

48. Scotta C, Esposito M, Fazekasova H, Fanelli G, Edozie FC, Ali N, et al. Differential effects of rapamycin and retinoic acid on expansion stability and suppressive qualities of human CD4(+)CD25(+)FOXP3(+) T regulatory cell subpopulations. Haematologica. 2013;98:1291–9.

49. Hester J, Schiopu A, Nadig SN, Wood KJ. Low-dose rapamycin treatment increases the ability of human

regulatory T cells to inhibit transplant arteriosclerosis in vivo. Am J Transplant. 2012;12:2008–16.

50.• Brunstein CG, Blazar BR, Miller JS, Cao Q, Hippen KL, McKenna DH, et al. Adoptive transfer of umbilical cord blood-derived regulatory T cells and early viral reactivation. Biol Blood Marrow Transplant. 2013;19:1271–3. **Study reports on in vivo effects of Treg treatment in HSC patients**.

51. Veerapathran A, Pidala J, Beato F, Yu XZ, Anasetti C. Ex vivo expansion of human Tregs specific for alloantigens presented directly or indirectly. Blood. 2011;118:5671–80.

52.• Schoenbrunn A, Frentsch M, Kohler S, Keye J, Dooms H, Moewes B, et al. A converse 4-1BB and CD40 ligand expression pattern delineates activated regulatory T cells (Treg) and conventional T cells enabling direct isolation of alloantigen-reactive natural Foxp3+ Treg. J Immunol. 2012;189:5985–94. **Study describes an elegant method to distinguish simultaneously between antigen-reactive effector and regulatory T cells**.

53. Canavan JB, Afzali B, Scotta C, Fazekasova H, Edozie FC, Macdonald TT, et al. A rapid diagnostic test for human regulatory T-cell function to enable regulatory T-cell therapy. Blood. 2012;119:e57–66.

54. Sagoo P, Ali N, Garg G, Nestle FO, Lechler RI, Lombardi G. Human regulatory T cells with alloantigen specificity are more potent inhibitors of alloimmune skin graft damage than polyclonal regulatory T cells. Sci Transl Med. 2011;3:83ra42. doi:10.1126/scitranslmed.3002076.

55. Koenen HJ, Fasse E, Joosten I. CD27/CFSE-based ex vivo selection of highly suppressive alloantigen-specific human regulatory T cells. J Immunol. 2005;174:7573–83.

56. Tang Q, Lee K. Regulatory T-cell therapy for transplantation: how many cells do we need? Curr Opin Organ Transplant. 2012;17:349–54.

57. Tang Q, Kang SM. Interpretation of transplant biopsies and immune responses following Treg cell therapy. Curr Opin Organ Transplant. 2014;19:616–20.

58. 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.

59. McMurchy AN, Bushell A, Levings MK, Wood KJ. Moving to tolerance: clinical application of T regulatory cells. Semin Immunol. 2011;23:304–13.

60. Hippen KL, Merkel SC, Schirm DK, Sieben CM, Sumstad D, Kadidlo DM, et al. Massive ex vivo expansion of human natural regulatory T cells (T(regs)) with minimal loss of in vivo functional activity. Sci Transl Med. 2011;3:83ra41. doi:10.1126/scitranslmed.3001809.

61. Tang Q, Bluestone JA. Regulatory T-cell therapy in transplantation: moving to the clinic. Cold Spring Harb Perspect Med. 2013;3(11):a015552.

62. Landwehr-Kenzel S, Issa F, Luu SH, Schmuck M, Lei H, Zobel A, et al. Novel GMP-compatible protocol employing an allogeneic B cell bank for clonal expansion of allospecific natural regulatory T cells. Am J Transplant. 2014;14:594–606.

63. Siepert A, Ahrlich S, Vogt K, Appelt C, Stanko K, Kuhl A, et al. Permanent CNI treatment for prevention of renal allograft rejection in sensitized hosts can be replaced by regulatory T cells. Am J Transplant. 2012;12:2384–94.

64. Siepert A, Brosel S, Vogt K, Ahrlich S, Schmitt-Knosalla I, Loddenkemper C, et al. Mechanisms and rescue strategies of calcineurin inhibitor mediated tolerance abrogation induced by anti-CD4 mAb treatment. Am J Transplant. 2013;13:2308–21.

65. Sagoo P, Perucha E, Sawitzki B, Tomiuk S, Stephens DA, Miqueu P, et al. Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans. J Clin Invest. 2010;120:1848–61.

66. Newell KA, Asare A, Kirk AD, Gisler TD, Bourcier K, Suthanthiran M, et al. Identification of a B cell signature associated with renal transplant tolerance in humans. J Clin Invest. 2010;120:1836–47.

67. Geissler EK, Hutchinson JA. Cell therapy as a strategy to minimize maintenance immunosuppression in solid organ transplant recipients. Curr Opin Organ Transplant. 2013;18:408–15.

68. Schlickeiser S, Boes D, Streitz M, Sawitzki B. The use of novel diagnostics to individualize immunosuppression following transplantation. Transpl Int. 2015;12527.

69. Wang XJ, Leveson-Gower D, Golab K, Wang LJ, Marek-Trzonkowska N, Krzystyniak A, et al. Influence of pharmacological immunomodulatory agents on CD4(+)CD25(high)FoxP3(+) T regulatory cells in humans. Int Immunopharmacol. 2013;16:364–70.

70. Bloom DD, Chang Z, Fechner JH, Dar W, Polster SP, Pascual J, 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.

71. Lopez M, Clarkson MR, Albin M, Sayegh MH, Najafian N. A novel mechanism of action for anti-thymocyte globulin: induction of CD4+ CD25+ Foxp3+ regulatory T cells. J Am Soc Nephrol. 2006;17:2844–53.

72. Pearl JP, Parris J, Hale DA, Hoffmann SC, Bernstein WB, McCoy KL, et al. Immunocompetent T-cells with a memory-like phenotype are the dominant cell type following antibody-mediated T-cell depletion. Am J Transplant. 2005;5:465–74.

73. Piotti G, Ma J, Adams E, Cobbold S, Waldmann H. Guiding post-ablative lymphocyte reconstitution as a route toward transplantation tolerance. Am J Transplant. 2014;14:1678–89.

74. Issa F, Chandrasekharan D, Wood KJ. Regulatory T cells as modulators of chronic allograft dysfunction. Curr Opin Immunol. 2011;23:648–54.

75. Joosten SA, Sijpkens YW, van Kooten C, Paul LC. Chronic renal allograft rejection: pathophysiologic considerations. Kidney Int. 2005;68:1–13.

76. Ballet C, Giral M, Ashton-Chess J, Renaudin K, Brouard S, Soulillou JP. Chronic rejection of human kidney allografts. Expert Rev Clin Immunol. 2006;2:393–402.

77. Louis S, Braudeau C, Giral M, Dupont A, Moizant F, Robillard N, et al. Contrasting CD25hiCD4+ T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance. Transplantation. 2006;81:398–407.

78. Braudeau C, Racape M, Giral M, Louis S, Moreau A, Berthelot L, et al. Variation in numbers of CD4+ CD25highFOXP3+ T cells with normal immuno-regulatory properties in long-term graft outcome. Transpl Int. 2007;20:845–55.

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

80. Cooper JE, Wiseman AC. Acute kidney injury in kidney transplantation. Curr Opin Nephrol Hypertens. 2013;22:698–703.

81. Legendre C, Canaud G, Martinez F. Factors influencing long-term outcome after kidney transplantation. Transpl Int. 2014;27:19–27. doi:10.1111/tri.12217. Epub 12013 Nov 12218.

82. Sharif A, Borrows R. Delayed graft function after kidney transplantation: the clinical perspective. Am J Kidney Dis. 2013;62:150–8.

83. Gandolfo MT, Jang HR, Bagnasco SM, Ko GJ, Agreda P, Satpute SR, et al. Foxp3+ regulatory T cells participate in repair of ischemic acute kidney injury. Kidney Int. 2009;76:717–29.

84. Monteiro RM, Camara NO, Rodrigues MM, Tzelepis F, Damiao MJ, Cenedeze MA, et al. A role for regulatory T cells in renal acute kidney injury. Transpl Immunol. 2009;21:50–5.

85. Nguyen MT, Fryml E, Sahakian SK, Liu S, Michel RP, Lipman ML, et al. Pre-transplantation recipient regulatory T cell suppressive

function predicts delayed and slow graft function after kidney transplantation. Transplantation. 2014;98:745–53.

86. Chan L. Harnessing regulatory T cells for therapeutic purposes. Kidney Int. 2012;81:935–6.

87. Kinsey GR, Huang L, Vergis AL, Li L, Okusa MD. Regulatory T cells contribute to the protective effect of ischemic preconditioning in the kidney. Kidney Int. 2010;77:771–80.

88. Kinsey GR, Sharma R, Okusa MD. Regulatory T cells in AKI. J Am Soc Nephrol. 2013;24:1720–6.

89. Monneret G, Venet F, Meisel C, Schefold JC. Assessment of monocytic HLA-DR expression in ICU patients: analytical issues for multicentric flow cytometry studies. Crit Care. 2010;14:432.

90. Poehlmann H, Schefold JC, Zuckermann-Becker H, Volk HD, Meisel C. Phenotype changes and impaired function of dendritic cell subsets in patients with sepsis: a prospective observational analysis. Crit Care. 2009;13:R119.

91. Schefold JC, Porz L, Uebe B, Poehlmann H, von Haehling S, Jung A, et al. Diminished HLA-DR expression on monocyte and dendritic cell subsets indicating impairment of cellular immunity in preterm neonates: a prospective observational analysis. J Perinat Med. 2014;2014:0226.

92. DeFrancesco L. CAR-T cell therapy seeks strategies to harness cytokine storm. Nat Biotechnol. 2014;32:604.

93. Bestard O, Cruzado JM, Lucia M, Crespo E, Casis L, Sawitzki B, et al. Prospective assessment of antidonor cellular alloreactivity is a tool for guidance of immunosuppression in kidney transplantation. Kidney Int. 2013;84:1226–36.

94. Dinavahi R, Heeger PS. T-cell immune monitoring in organ transplantation. Curr Opin Organ Transplant. 2008;13:419–24.

95. Gerlach UA, Vogt K, Schlickeiser S, Meisel C, Streitz M, Kunkel D, et al. Elevation of CD4+ differentiated memory T cells is associated with acute cellular and antibody-mediated rejection after liver transplantation. Transplantation. 2013;95:1512–20.

96. Hricik DE, Rodriguez V, Riley J, Bryan K, Tary-Lehmann M, Greenspan N, et al. Enzyme linked immunosorbent spot (ELISPOT) assay for interferon-gamma independently predicts renal function in kidney transplant recipients. Am J Transplant. 2003;3:878–84.

97. Ashoor I, Najafian N, Korin Y, Reed EF, Mohanakumar T, Ikle D, et al. Standardization and cross validation of alloreactive IFNgamma ELISPOT assays within the clinical trials in organ transplantation consortium. Am J Transplant. 2013;13:1871–9.

98. Bestard O, Crespo E, Stein M, Lucia M, Roelen DL, de Vaal YJ, et al. Cross-validation of IFN-gamma Elispot assay for measuring alloreactive memory/effector T cell responses in renal transplant recipients. Am J Transplant. 2013;13:1880–90.

99.• Streitz M, Miloud T, Kapinsky M, Reed MR, Magari R, Geissler EK, et al. Standardization of whole blood immune phenotype monitoring for clinical trials: panels and methods from the ONE study. Transplant Res. 2013;2:17. doi:10.1186/2047-1440-1182-1117. **The studies describes approaches for a standardization of low cytometry-based immune monitoring for clinical trials**.