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REVIEW

Potential T regulatory cell therapy in transplantation: how far have we come and how far can we go?

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Summary

Graft survival has been lately improved by the introduction of efficient immunosuppressive drugs. However, late graft loss caused by chronic rejection and the side effects of long-term immunosuppression remain major obstacles for successful transplantation. Operational tolerance, which is defined by the lack of acute and chronic rejection and indefinite graft survival with normal graft function in the absence of continuous immunosuppression, represents an attractive alternative. Nevertheless, tolerance after allogeneic transplantation is commonly considered the 'mission impossible' for both immunologists and clinicians. One of the mechanisms involved in tolerance is the suppression of graft-specific alloreactive T cells, which largely mediate graft rejection, by regulatory T cells (Tregs) or by soluble factors produced by Treg cells. With this review, I will make an effort to collect and describe the significant studies performed in transplanted patients, and not in animal models or in *in vitro* systems, with the attempt to: (i) understand how tolerance is achieved, (ii) define whether and how Treg cells influence transplant tolerance, (iii) describe the first clinical trials with Treg cells in humans (i.e. how far have we come) and (iv) predict the future of Treg cell-based therapy in humans (i.e. how far can we go).

Introduction

'Tolerance is the future of transplantation, and always will be (Norman Shumway)'. Shumway undoubtedly was a stubborn and talented surgeon who continued performing heart transplants after others had given up because of poor results. He pioneered the use of cyclosporin, which made the operation much safer and successful. He certainly was not a loser or someone who would concede defeat easily. He is remembered as a person who could predict whether something would work or not. He had quite a precise and clear idea about transplantation tolerance, but that was years ago. Would Shumway change his mind today if he were still alive? Are we now in a position to say that tolerance after transplantation is possible? What do we really need to achieve transplantation tolerance?

This review will attempt to analyse critically some of the more significant studies performed in transplanted patients. Studies performed in preclinical models (rodents and others) will not be included in this review, not because they are considered inappropriate. Studies in animals are clean and can provide sophisticated answers to crucial questions, but their translation into the human disease is not an easy task. Conversely, studies in humans are often masked by many other different factors and are limited to peripheral tissues, but are not, with no doubt, 'lost in translation'.

Human Treg cells

The immune system is a rather complicated network of many different players who interact with each other and cooperate to protect against diseases. The immune system is finely tuned to distinguish antigens (Ag) that belong to the body from those that do not, allowing it to deploy swiftly a potent array of defence mechanisms, whenever evidence of a foreign invasion is found. Among the many immune-system players, there are the regulatory T (Treg)

lymphocytes which act to suppress immune activation and thereby maintain immune homoeostasis and tolerance to self-Ag. This is an important 'self-check' built into the immune system to prevent excessive reactions, which would lead to autoimmunity. There are, however, situations in which the immune system should not recognize non-self Ag as dangerous and there should be no active immune responses. Allograft is such a situation and it is now clear that Treg cells can efficiently control undesired anti-allogeneic immune responses [1].

The 'Treg-cell team' comprises many players (reviewed in [2]), but this review, for simplicity and clarity, will focus only on two distinct human Treg-cell subsets: the naturally occurring $CD4^+$ $CD25^+$ $FOXP3^+$ Treg (nTreg) and the inducible T regulatory type 1 (Tr1) cells.

Human nTreg cells

The nTreg cells are selected in the thymus and represent about 5–10% of the total $CD4^+$ T cells in the periphery. They are crucial for maintaining tolerance by down-regulating undesired immune responses to self and nonself Ag. The nTreg cells are defined on the basis of the constitutive expression of high levels of CD25 and the transcription factor FOXP3 (forkhead box P3), and the inability to produce interleukin-2 (IL-2) and to proliferate *in vitro* (reviewed in [3]). The nTreg cells potently suppress activation, proliferation, and/or effector functions of $CD4^+$ and $CD8^+$ T cells and possibly natural killer, natural killer T, B and dendritic cells. They can presumably control such a variety of target cells in different phases of the immune response by implementing multiple modes of suppression in a multi-step manner. This includes cell-contact dependent suppression, functional modification or killing of antigen-presenting cells (APC) and secretion of immunosuppressive cytokines (reviewed in [4]). *In vivo* experiments supporting the role of IL-10 in nTreg cell-mediated suppression have been reported. However, the IL-10 role in human nTreg cells is still uncertain [5].

Human $CD4^+$ Tr1 cells

$CD4^+$ Tr1 cells arise in the periphery after encounter with the Ag in the presence of IL-10. The unique cytokine production profile ($IL\text{-}10^{++}IL\text{-}4^-TGF\beta^+IFN\text{-}\gamma^+IL\text{-}2^-$) distinguishes Tr1 cells from T helper 0 (Th0), Th1, Th2 and Th17 cells [6]. To date, no specific marker for Tr1 cells has been identified, but this cell subset has been recently classified in the peripheral blood as $CD4^+$ $CD45RA^-CD25^-CD127^-$ T cells [7]. Tr1 cells have a very low proliferative capacity following T-cell-receptor (TCR) activation *in vitro*, in part because of autocrine production of IL-10. Tr1 cells regulate immune responses through secretion of the immunosuppressive cytokines IL-10 and TGFβ, and they suppress both naïve and memory T-cell responses. In addition, they down-regulate the expression of co-stimulatory molecules and production of pro-inflammatory cytokines by APC. Importantly, Tr1 cells need to be activated through their TCR to exert their suppressive functions. However, once activated, they mediate suppression in an Ag-non-specific manner [8]. Many different approaches have been explored to induce Tr1 cells both *ex vivo* and *in vivo*, but IL-10 remains one of the indispensable factors (extensively reviewed in [8]).

Each of the above-mentioned Treg cell subsets has been shown to be required for tolerance induction, depending on the disease model. nTreg cells seem predominantly involved in controlling responses to self-Ag, whereas Tr1 cells may be important for controlling immune responses to non self-Ag, including alloantigens, allergens, or food antigens [2]. However, the respective role of these two Treg cell subsets in regulating immune responses to self- versus non self-Ag has not been completely elucidated.

Tolerance after solid organ transplantation

Although transplanted patients who tolerate the allograft in the absence of immunosuppressive therapy (defined as operational tolerance) are rare and it is of question whether the available tolerance-inducing protocols can be implemented on a large scale, there is no doubt that an in-depth analysis of such patients is fundamental to comprehending how tolerance can be achieved after solid organ transplantation.

Advances in our understanding of the induction and maintenance of tolerance have confirmed at least three major mechanisms to be active: clonal deletion, clonal anergy and regulation [9]. Most experts in the field agree that any durable tolerogenic therapy will involve manipulation of more than one mechanism, with the goal of profound reduction in clonal T-cell expansion accompanied by active immune regulation. In humans, this can be achieved by the co-transfer of donor cells with solid graft. Three manuscripts published at the same time reported patients tolerant to HLA-matched kidney [10], HLA-single haploidentical kidney [11] and completely mismatched liver grafts [12]. In all three cases, patients were exposed early after transplantation to donor cells either by a planned donor stem cell transfer [10,11] or by an unintended migration of donor passenger leucocyte from the graft into the host immune system [12]. Again in all three cases, the recipients' immune system has been weakened enough to reduce host-versus-graft disease, but not to the extent of completely eliminating anti-donor responses. This was achieved by a designed treatment with anti-T-cell antibodies prior to transplantation and

with just the right immunosuppressive therapy [10,11] or fortuitously by lymphopenia caused by a viral infection [12]. This has led to a 'pacific co-existence' of donor and host leucocytes either for not more than 21 days post-transplantation [11] or for a sustained period of time [10,12] leading to central clonal deletion and/or anergy of anti-donor responses, followed by peripheral tolerance, which, in one occurrence, was associated with high $FOXP3^+$ T cells within the graft without concomitant inflammatory response [11]. If this process does not occur in the first few weeks after transplantation, the outcome is rejection (i.e. recipient cells 'win over' donor cells) or graft-versus-host disease (i.e. donor cells 'win over' recipient cells).

These studies suggest that to achieve transplantation tolerance, the right dose and timing of immunosuppression with or without the aid of adjunct haematopoietic stem cells are mandatory. Should this hypothesis be correct, the reciprocal clonal exhaustion-deletion phase between donor and host cells does not have to be inhibited by either excessive immunosuppressive treatment or forced peripheral tolerance. Conversely, long-term tolerance seems to be more dependent on Treg cells.

Clinical evidence that Treg cells after transplantation are important

Preclinical studies clearly demonstrate that Treg cells are associated with transplantation tolerance and Treg cell therapy efficiently controls graft rejection (reviewed in [13]). Nonetheless, what clinical evidence do we have that Treg cells play a fundamental role in inducing and/or maintaining long-term tolerance after solid organ transplantation in humans?

In principle, it seems quite an easy task to identify Treg cells in the periphery of transplanted patients and to associate the presence or absence of Treg cells with a given clinical condition (e.g. engraftment versus rejection versus tolerance). Unfortunately, this is not the case. For instance, FOXP3 expression, which is constitutively high in nTreg cells, can be up-regulated in non-Treg cells upon activation [14,15]. Thus, functional *in vitro* assays performed with purified nTreg lymphocytes are fundamental to discriminate precisely between cells with regulatory and those with nonregulatory activity. However, cell-sorting strategies are often subjective and they might lead to isolation of cells with various degrees of 'purity'. Without mentioning that, suppressive assays are time-consuming experiments unlikely to be used routinely. Recent studies provide demonstration that glycoprotein-A repetitions predominant (GARP), an orphan toll-like receptor composed of leucine-rich repeats, is selectively expressed only in activated human nTreg cells and clones, but not in activated effector T cells [16]. GARP is required for latent TGF-β [contained in latency-associated peptide (LAP)] expression and these two molecules directly bind to each other on activated nTreg cells. However, GARP overexpression is insufficient to induce modification of LAP into active TGF-β [17] indicating that GARP on nTreg cells simply functions as a LAP 'transporter'. GARP is therefore one of the molecules that seems to be a bona fide nTreg-cell marker [18]. Thus far, no data associating $GARP^+$ nTreg cells and tolerance after transplantation have been produced. Recently, there has been a growing interest on the characterization of bona fide nTreg cells by the analysis of the Treg-specific demethylated region (TSDR) within the FOXP3 locus. This region is constantly demethylated in bona fide nTreg cells and not in inducible Treg cells or activated $FOXP3^+$ non-Treg cells [19]. Therefore, the TSDR analysis represents the ideal tool to discriminate among nTreg and non-nTreg cells and so far it is considered a reliable and environmentally uninfluenced marker for bona fide nTreg cells. Unfortunately, this assay is based on DNA analysis and thus does not allow for isolation of viable pure cells.

The identification and quantification of Tr1 cells have been even more complex because of the lack of any specific surface marker that could exclusively associate a tolerogenic state with the Tr1-cell presence and abundance. IL-10 production is a hallmark of Tr1 cells, but the very same cell has to produce very low levels of IFN-γ and IL-2 in the absence of IL-4 [8]. Intracytoplasmic staining is the only *in vitro* read-out which provides such information, but it requires TCR-mediated activation which has to be quite powerful (i.e. nonphysiological) to detect appreciable numbers of circulating Tr1 cells. T-cell cloning is the generally accepted technique that can give a rough idea of Tr1-cell frequency *in vivo* [20]. This is, however, extremely time-consuming and cannot be proposed as routine screening for transplanted individuals. Serum IL-10 levels and/or IL-10 release by T cells upon *in vitro* activation are the accepted techniques to date [20]. Given the drawbacks of these approaches, it is very likely that the data generated so far in transplanted patients may not be comprehensive.

We are therefore facing important technical restraints that could possibly justify some contradictory results generated in the last few years and might have led to our partial view of the role played by Treg cells in promoting and maintaining transplantation tolerance in humans. Keeping this in mind, in case the reader craves for an overview of all the papers that positively correlate FOXP3 expression with graft tolerance and all the papers that negatively correlate graft outcome with FOXP3 expression, excellent investigators have already done a tremendous job (reviewed in [21]) which I shall not attempt to replicate. Considering the lack

of a bona fide nTreg cell marker, it is my opinion that it is almost impossible to provide a unique interpretation of all the published data in an attempt to correlate FOXP3 expression with operational tolerance. Similarly, Tr1 cell detection in transplanted patients is often limited to 'IL-10 yes'/'IL-10 no', which leads to an inevitably limited view. Should humans be treated like guinea-pigs, one could deplete/block Treg cells in tolerant patients and see whether the graft is rejected. As patients are definitely not guinea-pigs, we have to provide alternative convincing ways to prove that Treg cells are crucial for tolerance maintenance after solid organ transplantation.

The field has been refreshed by a more 'holistic' approach, which has led to the identification of biomarkers of operational tolerance in kidney-transplanted recipients [22]. Brouard *et al.* performed blood gene expression profiles from 75 renal-transplant patient cohorts (i.e. operational tolerance, acute and chronic rejection and stable graft function on immunosuppression) and 16 healthy individuals. The operational-tolerance gene-expression signature obtained in this study demonstrated a minimal set of 49 genes differently expressed in tolerant patients when compared with other patients and suggested that tolerance is not just a paradigm shift towards a normal resting state. Co-stimulatory signals and Th1/Th2-related cytokines (e.g., TNF-α, IL-4, and IL-10) were down-regulated implying that tolerant patients have a normal immune system with donor-specific hyporesponsiveness. FOXP3, GITR and neurophilin, all nTreg-cell related molecules [4], were elevated in tolerant and normal controls as compared with chronic rejected patients, demonstrating a role of intact T-cell regulation in tolerance and, conversely, its loss during rejection. Interestingly, TGF-β, a key tolerogenic molecule [23], was not differently expressed between tolerant and rejected individuals, but 27% of the peripheral blood genes that distinguish between tolerance and rejection were regulated by TGF-β. These TGF-β-regulated genes include latent TGF-β binding protein (LTBP4), which functions to convert latent TGF-β into the active form and may represent the 'missing link' between TGF-β-shipping by nTreg cells (through GARP) and TGF-β nTreg cell-mediated suppression. Overall, it is clear that operational tolerance goes far beyond 'Treg cells yes'/'Treg cells no'. A complex interplay between effector and regulatory mechanisms occurs and the central question now is whether Treg cells can by themselves be the leading cause of operational tolerance induction.

Treg cell trials in humans: how far we have got

The first animal model used to test the *in vivo* regulatory activity of Treg cells was the allogeneic stem cell transfer in mice. It was shown that donor-derived nTreg cells do not induce graft-versus-host disease (GvHD). In contrast, nTreg cells prevent GvHD when co-transplanted with effector T cells [24,25]. Similarly, Tr1 cells generated *ex vivo* upon stimulation with alloantigens in the presence of IL-10 and TGFβ have been shown to be potent regulators of GvH responses after allogeneic bone marrow transplant. Infusion of unmanipulated cultured T cells induced lethal GvHD in all transplant recipients, whereas 75% of mice receiving *ex vivo* generated Tr1 cells survived [26]. Experimental GvHD represents the ideal model for the examination of Treg-cell-mediated suppression *in vivo* as: (i) the time of disease onset is known and thus Treg-cell administration can be performed either prophylactically or therapeutically; (ii) the lymphopenia in conditioned recipients supports the activation and *in vivo* expansion of the transferred Treg cells and (iii) it is clinically relevant. As a matter of fact, the first in-man clinical trials with Treg cells have been performed in stem cell transplanted patients with the aim of preventing/curing GvHD. The results of these trials have been published either as original papers or more recently as abstracts at the American Society of Hematology (ASH) meeting and they are briefly described below.

The group led by M. Martelli reported that transfer of freshly isolated $CD4^+$ $CD25^+$ T cells (consisting of $CD25^{high}$ 25.6% ± 11.2 and $FOXP3^+$ cells 64% ± 1 mean ± SD) 3 days prior to transplantation of haploidentical $CD34^+$ stem cells favours immune reconstitution (Blood, ASH Annual Meeting Abstracts, Nov 2009; **114**: 4). No GvHD was observed in 17 of 20 valuable patients. Two patients developed grade I cutaneous self-limited untreated GvHD and one developed grade III GvHD (this patient had received the lowest Treg cell doses). This study suggests that in the setting of haploidentical stem cell transplantation, the infusion of freshly purified Treg cells prior to transplant provides long-term protection from GvHD and robust immune reconstitution.

Wagner and colleagues performed adoptive transfer of umbilical cord blood (UCB)-derived Treg cells to recipients of non-myeloablative unrelated UCB transplantation (Blood, ASH Annual Meeting Abstracts, Nov 2009; **114**: 513). $CD25^+$ cells were obtained from a 3rd UCB unit and expanded in the presence of anti-CD3/CD28 mAb coated beads and IL-2 for an average of 18 days. Expanded Treg-cell dose escalation levels (from 1 to 30×10^5/kg) were transferred on day +1, and 30×10^5/kg on days +1 and +15 after UCBT. After infusion, an increase in the proportion of peripheral blood comprising $CD4^+$ $FOXP3^+$ $CD127^-$ cells was observed. Donor Treg cells were clearly detected in all patients receiving Treg cells that were HLA-disparate. The co-infusion of *ex vivo* expanded and activated UCB-derived Treg cells to

recipients of nonmyeloablative UCBT: (i) was safe at the tested dose levels, (ii) led to a detectable increase in donor-derived circulating nTreg cells and (iii) resulted in an increased proportion of mixed chimerism.

An even bolder study was conducted by Trzonkowski and colleagues who performed a clinical trial with *ex vivo* expanded $CD4^+$ $CD25^+$ $CD127^-$ nTreg cells for the treatment of acute or chronic GvHD [27]. The therapy gave significant alleviation of the symptoms and reduction in pharmacological immunosuppression in the case of chronic GvHD. However, in the case of grade IV acute GvHD, it improved the clinical condition only temporarily.

The group of M.G. Roncarolo is currently conducting a phase I/II non-randomized study to establish the safety and efficacy of a cellular therapy with alloantigen specific donor-derived Tr1 cells in patients transplanted with $CD34^+$ stem cells from haploidentical donors. Peripheral blood mononuclear cells (PBMC) were collected from both the donor prior to mobilization and the host prior to conditioning. Subsequently, a mega-dose of T-cell depleted $CD34^+$ stem cells was infused in the myeloablated host. Once there were signs of neutrophyl engraftment, the donor anti-host Tr1 cells were infused in the host in the absence of immunosuppression for GvHD prophylaxis, with the ultimate goal of providing immune reconstitution without severe GvHD [28]. Sixteen patients received $CD34^+$ selected stem cells and 12 patients were treated with IL-10 anergized cell therapy at day +30 post-transplant, at the dose of 10^5 $CD3^+$ cells/kg with the exception of two patients who received 3×10^5 $CD3^+$ cells/kg. Five patients died from infections by day +30 after Treg-cell infusion and two patients dropped out because of graft rejection. Five patients achieved immune reconstitution followed by progressive normalization of the TCR repertoire, memory/naïve phenotype, and T-cell functions *in vitro* and *in vivo*. Acute GvHD grade III was observed in one patient who received 3×10^5 $CD3^+$ cells/kg; GvHD grade II was observed in four patients who received 10^5 $CD3^+$ cells/kg and were successfully immune-reconstituted. Four patients are alive and disease-free and they do not require immunosuppressive treatment (Blood, ASH Annual Meeting Abstracts, Nov 2009; **114**: 45). Overall, cellular therapy with cells comprising donor anti-host Tr1 cell-precursors is safe and feasible. In addition, based on the available informative patients, this trial demonstrates to sustain immune reconstitution associated with a reduced severity of GvHD and no occurrence of disease relapse.

After waiting years for some 'real data' in patients treated with Treg cell-based therapy, the time is now ripe and final definitive publications are expected in the next few years. The above-mentioned clinical trials, albeit preliminary, demonstrate the feasibility and safety of such an approach. Importantly, if confirmed, they will set the stage for phase III studies that will establish the true impact of Treg cells on engraftment and GvHD. In addition, those studies will pave the way for future studies in the treatment of T-cell mediated diseases, solid organ transplantation included. How far we can go now and which problems should be first overcome are described below.

Treg cell trials in humans: how far we can go

The success of Treg-cell therapy in solid organ transplantation is subject not only to confirmation of the above-mentioned results in preventing/curing GvHD but also to definition of the effects of various immunosuppressive (IS) therapies on Treg-cell survival, proliferation and function. Today, no clinician would dare perform an allotransplant in the absence of IS treatment. T effector and Treg cells, by having a different phenotype and also using different signalling pathways, are likely to be differently targeted by the same IS drug. There is therefore a compelling need to determine the exact role of each of the currently used IS compounds on Treg cells.

In an attempt to do this, I will report the effects of IS drugs on Treg cells described in transplanted patients and also in autoimmune individuals who often undergo treatment with a single drug unlike the former who are commonly treated with multiple drug combinations. I will try not to include studies carried out on animals and on *in vitro* cultured cells since, as already mentioned, it is difficult to translate these data into a clinical situation.

Standard IS therapy targeting T-lymphocytes can be classified into four different groups based on the T-cell activation level at which they operate. Namely: (i) depleting agents, (ii) inhibitors of early T-cell activation, (iii) inhibitors of late T-cell activation and (iv) inhibitors of T-cell proliferation.

Depleting agents (ATG)

The most commonly used depleting agent is the anti-thymocyte globulins (ATG) made of horse (hATG) or rabbit (rATG)-derived antibodies against human T-cells which is used in the prevention and treatment of acute rejection in organ and bone marrow transplantation to prevent GvHD. ATG works through depletion of immunocompetent cells trough complement-dependent lysis or activation induced apoptosis and modulation of several molecules on residual circulating leucocytes that are involved in regulating the leucocyte-endothelium adhesion and leucocyte migration (e.g. chemokine receptors CXCR4, CCR5, and CCR7) [29]. *In vitro* evidence first

suggested that rATG causes rapid and sustained *in vitro* expansion of $CD4^+$ $CD25^+$ T cells from human PBMC. This was due mainly to conversion of $CD4^+$ $CD25^-$ into $CD4^+$ $CD25^+$ T cells [30]. These findings were confirmed by other investigators [31,32] and recently questioned by Broady *et al.* [33]. *In vivo* data demonstrated that rATG in kidney transplanted patients have the same depleting effects on both nTreg and T effector cells. However, while rATG affects the function of recovered T effector cells, the suppressive activity of newly generated Treg cells remains proportionally unaltered leading to an effective capacity to control allogeneic immune responses (albeit numerically reduced) [34]. When the TSDR analysis prior to and after rATG treatment was performed in PBMC of three kidney transplanted patients, nTreg-cell levels decreased in all patients tested [19].

Peripheral blood mononuclear cell treated with rATG display increased IL-10 release in culture supernatants as compared with those treated with hATG [31]. In addition, Th2 cytokines (IL-10 included) are significantly up-regulated in PBMC exposed to rATG *in vitro* [30].

Overall, rATG seems not to have a different effect on the ability to deplete T effector versus nTreg cells, but it might spare the nTreg-cell suppressive ability while impairing their effector activity. In addition, rATG does not inhibit the ability of T cells to produce IL-10.

Inhibitors of early T-cell activation (Cyclosporin A, tacrolimus)

Cyclosporin A and tacrolimus are calcineurin inhibitors (CNI) licensed for use in organ transplantation. TCR engagement with donor MHC/peptide normally triggers calcium-dependent intracellular signalling resulting in activation of the calcium/calmodulin-dependent phosphatase calcineurin. This leads to the de-phosphorylation of NF-AT allowing translocation into the nucleus where it enhances the binding of transcription factors to genes encoding for pro-inflammatory cytokines (e.g. IL-2, IL-3, IL-4, IFN-γ and TNF-α). After entering the cytoplasm, CNI form complexes with their immunophilins. Cyclosporin binds to cyclophilin and tacrolimus binds to the 12 kDa FK506-binding protein (FKBP12). The CNI–immunophilin complexes inhibit calcineurin activity, and hence prevent nuclear translocation of NF-AT and cytokine gene transcriptions. The net result is that CNI block the production of cytokines such as IL-2 and inhibit T-cell activation and proliferation. Considering the importance of IL-2 for Treg-cell function and homoeostasis, it is likely that CNI have a detrimental effect on Treg cells *in vivo*. As a matter of fact, several reports suggest that CNI are not beneficial for nTreg cells but rather detrimental to their generation, survival and function (reviewed in [35]). However, recent data from Wang and colleagues demonstrated that low doses of tacrolimus in solid organ transplantation may favour the induction of donor-specific Treg cells maintaining transplantation tolerance to alloantigens [36], thus leaving open the final definitive answer.

Interleukin-10 production does not seem inhibited by the presence of CNI drugs. In fact, increased IL-10 production was detected in a group of stable renal allograft recipients maintained on CNI-based therapy [37]. In addition, gene expression profile of total PBMC isolated from solid organ transplanted patients demonstrated that IL-10 levels are higher in PBMC of individuals under CNI therapy as compared with those in PBMC of individuals under non-CNI treatments [38]. One could therefore envisage that CNI drugs do not interfere with the generation and/or function of Tr1 cells.

Inhibitors of late T-cell activation (daclizumab, rapamycin)

Daclizumab is a non-depleting humanized anti-CD25 (i.e. IL-2R alpha) mAb that disrupts IL-2 signalling by binding to CD25 and by preventing the assembly of the high affinity IL-2R. Clearly, anti-CD25 mAb therapy can be expected to affect not only activated alloreactive T cells but also nTreg cells. A clean study where multiple sclerosis patients were treated with long-term maintenance (i.e. one infusion every 4 weeks for 54 weeks) of daclizumab monotherapy [39] showed a 44% reduction in $CD4^+$ $FOXP3^+$ T cells after 7.5 months of therapy and Treg-cell frequency recovered after treatment withdrawn to near baseline levels. Impaired homoeostatic proliferation of nTreg cells during anti-CD25 treatment has also been reported [38]. Daclizumab in transplanted patients is, however, used at reduced extent and frequency but rarely as monotherapy; it is therefore difficult to extrapolate clean data. One infusion of daclizumab at the time of transplant leads to loss of CD25 expression (because of internalization or receptor shedding) in 75% of the cells at 1 month after infusion. $CD25^+$ cells return to baseline levels at 6 months after liver transplant, but $CD4^+$ $FOXP3^+$ T cells are not reduced [40]. Functional nTreg cells are present in kidney transplanted recipients upon mAb clearance [41] thus suggesting that daclizumab leads to transient IL-2 insensitivity in T cells (because of lack of CD25 expression), but $FOXP3^+$ T cells do not die in this time frame and they return to express CD25 and to be functional upon mAb clearance. Surprisingly, analysis of the TSDR very close after daclizumab therapy (1–2 days after treatment) demonstrated a reduced frequency of nTreg cells upon anti-CD25 therapy thus leaving open the possibility that daclizumab might indeed deplete nTreg cells [19].

Daclizumab leads also to contraction of activated effector T cells while expanding $CD56^{bright}$ NK cells as shown both in multiple sclerosis [39] and uveitis patients [42]. The induced $CD56^{brigt}$ NK cells secrete high amounts of IL-10. In addition, daclizumab does not hamper IL-10 release by pancreatic-islet reactive T cells [43].

Overall, short-term treatment with daclizumab transiently 'freezes' nTreg cells to IL-2 sensitivity, but not to the extent that they die and disappear. Fully expressing CD25 nTreg cells, which recover after daclizumab therapy, are perfectly functioning *in vitro*. It therefore seems that a short peri-transplant treatment with anti-CD25 mAb is not nTreg-cell detrimental, but further studies are needed. Similarly, IL-10 seems not to be negatively impaired by the administration of daclizumab.

Rapamycin belongs to the group of immunosuppressive agents called mammalian target of rapamycin (mTOR) inhibitors. It was originally identified as a potent antifungal metabolite. This macrolide, which is produced by *Streptomyces hygroscopicus*, was found to inhibit cell proliferation and to have potent immunosuppressive activity. It is currently used for the prevention of transplant rejection, but rapamycin and its derivatives are also undergoing clinical testing for prophylaxis of graft rejection and GvHD (reviewed in [44]). Rapamycin binds to FKBP12 but, unlike FK506, does not inhibit calcineurin activity. The rapamycin/FKBP12 complex is an highly specific inhibitors of mTOR complex 1 (mTORC1) [45] which is a serine/threonine kinase involved in the phosphatidylinositol 3-kinase (P12K)/AKT (protein kinase B) signalling pathway. We were the first to demonstrate that rapamycin selectively allows proliferation and expansion of mouse and human nTreg cells while sparing proliferation of effector T cells [46,47]. This selective activity of rapamycin seems to be connected to two effects. First, nTreg cells do not down-regulate phosphatase and tensin homologue (PTEN) expression after TCR engagement, which impedes the activation of the rapamycin-susceptible PI3K–AKT–mTOR pathway. Second, FOXP3 drives the expression of PIM2, reinforced by IL-2- and TCR-mediated activation of signal transducer and activator of transcription 5 (STAT5); PIM2 compensates for AKT inactivity and promotes cell cycle progression [48]. *In vivo* data are in accordance with such a view. Kidney transplanted patients treated with rapamycin have a markedly increased frequency of nTreg cells compared with total $CD4^+$ T-cell numbers. This effect is reversed in patients treated with CNI [49,50]. Exactly the same data were generated in lung transplant recipients treated with rapamycin as compared with those treated with cyclosporin [51]. We also showed that type 1 diabetic patients undergoing rapamycin monotherapy prior to islet transplantation have circulating nTreg cells with an improved *in vitro* suppressive function as compared with those isolated prior to rapamcyin therapy [52].

Collectively, these data provide strong evidence that the differential effects of rapamycin on effector T cells and nTreg cells favour its ability to promote tolerance and support its use in tolerance-promoting protocols.

Mammalian target of rapamycin complex 1 stimulates activation of the signal transducer and activator of transcription 3 (STAT3) to promote expression of IL-10; therefore, its inhibition by rapamycin leads to a drastic decrease in IL-10 production by myeloid phagocytes [53,54]. It is possible that rapamycin inhibits Tr1 cell generation *in vivo* while its role on Tr1 cell function is still unknown.

Inhibitors of cell-proliferation (MMF)

Mycophenolate mofetil (MMF) is rapidly converted in the liver into mycophenolic acid which is the active compound. The target of mycophenolic acid is inosine monophosphate dehydrogenase (IMPDH), the rate-limiting enzyme in the *de novo* synthesis of guanosine nucleotides, themselves essential for DNA synthesis. Most cell types can generate guanosine nucleotides by two pathways, the IMPDH pathway and a salvage pathway. Lymphocytes do

Table 1. The effect of the commonly used immunosuppressive drugs on human nTreg and Tr1 cells.

IS drug	nTreg cells	Reference	Tr1 cells/IL-10 production	Reference
Depleting agents (rATG)	Numerically ☹	[19,33]	😐	[29,30]
	Functionally 😐	[33]		
Inhibitors of early activation (cyclosporin A, tacrolimus)	☹/?	[34,35]	😐	[36,37]
Inhibitors of late activation				
(Daclizumab)	😐/?	[19,39,40]	😐	[41,42]
(Rapamycin)	😊	[48–51]	☹/?	[52,53]
Inhibitors of cell proliferation (MMF)	😐	[39]	?	

IS, immunosuppressive; MMF, mycophenolate mofetil.

not possess such a salvage pathway and hence blockade of the IMPDH pathway results in relatively selective blockade of lymphocyte proliferation. There are two isoforms of the IMPDH enzyme, the type I isoform being found predominantly on resting cells, and the type II isoform being induced and expressed on activated lymphocytes. Mycophenolic acid preferentially inhibits the type II isoform of IMPDH, expressed on the activated lymphocyte population [55]. MMF is currently used to prevent allograft rejection. There is now increasing evidence that MMF has similar effects on Treg cells to rapamycin. In several experimental models, MMF does not interfere with nTreg cell function and positively affects tolerance induction [56–58]. In humans, MMF therapy can overturn the suppressive effect of CNI on circulating nTreg cells in liver transplanted patients [40]. Overall, MMF does not appear to suppress survival and suppressive function of human nTreg cells.

In-depth studies analysing the effect of MMF on IL-10 producing Tr1 cells are still lacking.

Conclusions

The evaluation of the impact of therapeutic agents on Treg cells *in vivo* is rapidly improving, but it is clearly far from complete (Table 1). As anticipated earlier, the reciprocal exhaustion-deletion phase between donor and host cells just after transplantation should not be inhibited by either excessive immunosuppressive treatments or forced peripheral tolerance. Therefore, the perfect balance between donor leucocytes infiltrating the graft, recipient alloreactive T effector cells and Treg cells should always be the target of current IS treatment. Unfortunately, we still lack the tools to define such a 'perfect balance'. Once determined, the important and numerous studies aimed at determining the molecular mechanisms of each IS compound will be of key importance for the resolution of the 'mission impossible' of transplantation, hopefully leading to stable and easily achievable tolerance.

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References

1. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* 2008; **133**: 775.
2. Curotto de Lafaille MA, Lafaille JJ. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity* 2009; **30**: 626.
3. Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat Immunol* 2010; **11**: 7.
4. Shevach EM. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 2009; **30**: 636.
5. Miyara M, Sakaguchi S. Natural regulatory T cells: mechanisms of suppression. *Trends Mol Med* 2007; **13**: 108.
6. Groux H, O'Garra A, Bigler M, *et al.* A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997; **389**: 737.
7. Haringer B, Lozza L, Steckel B, Geginat J. Identification and characterization of IL-10/IFN-gamma-producing effector-like T cells with regulatory function in human blood. *J Exp Med* 2009; **206**: 1009.
8. 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.
9. Li XC, Strom TB, Turka LA, Wells AD. T cell death and transplantation tolerance. *Immunity* 2001; **14**: 407.
10. Scandling JD, Busque S, Dejbakhsh-Jones S, *et al.* Tolerance and chimerism after renal and hematopoietic-cell transplantation. *N Engl J Med* 2008; **358**: 362.
11. Kawai T, Cosimi AB, Spitzer TR, *et al.* HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med* 2008; **358**: 353.
12. Alexander SI, Smith N, Hu M, *et al.* Chimerism and tolerance in a recipient of a deceased-donor liver transplant. *N Engl J Med* 2008; **358**: 369.
13. Long E, Wood KJ. Regulatory T cells in transplantation: transferring mouse studies to the clinic. *Transplantation* 2009; **88**: 1050.
14. Roncarolo MG, Gregori S. Is FOXP3 a bona fide marker for human regulatory T cells? *Eur J Immunol* 2008; **38**: 925.
15. Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol* 2007; **37**: 129.
16. Wang R, Kozhaya L, Mercer F, Khaitan A, Fujii H, Unutmaz D. Expression of GARP selectively identifies activated human FOXP3+ regulatory T cells. *Proc Natl Acad Sci USA* 2009; **106**: 13439.
17. Stockis J, Colau D, Coulie PG, Lucas S. Membrane protein GARP is a receptor for latent TGF-beta on the surface of activated human Treg. *Eur J Immunol* 2009; **39**: 3315.
18. Battaglia M, Roncarolo MG. The Tregs' world according to GARP. *Eur J Immunol* 2009; **39**: 3296.
19. Wieczorek G, Asemissen 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.
20. Serafini G, Andreani M, Testi M, *et al.* Type 1 regulatory T cells are associated with persistent split erythroid/lymphoid chimerism after allogeneic hematopoietic stem cell transplantation for thalassemia. *Haematologica* 2009; **94**: 1415.

21. Brown K, Wong W. Diagnostic value of regulatory T cells: a new facet of a much studied cell population. *Transplantation* 2008; **86**: 1485.
22. Brouard S, Mansfield E, Braud C, *et al.* Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. *Proc Natl Acad Sci USA* 2007; **104**: 15448.
23. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol* 2006; **24**: 99.
24. Cohen JL, Trenado A, Vasey D, Klatzmann D, Salomon BL. CD4(+)CD25(+) immunoregulatory T Cells: new therapeutics for graft-versus-host disease. *J Exp Med* 2002; **196**: 401.
25. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med* 2002; **196**: 389.
26. Zeller JC, Panoskaltsis-Mortari A, Murphy WJ, *et al.* Induction of CD4+ T cell alloantigen-specific hyporesponsiveness by IL-10 and TGF-beta. *J Immunol* 1999; **163**: 3684.
27. 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.
28. Battaglia M, Gregori S, Bacchetta R, Roncarolo MG. Tr1 cells: from discovery to their clinical application. *Semin Immunol* 2006; **18**: 120.
29. Mohty M. Mechanisms of action of antithymocyte globulin: T-cell depletion and beyond. *Leukemia* 2007; **21**: 1387.
30. 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.
31. Feng X, Kajigaya S, Solomou EE, *et al.* Rabbit ATG but not horse ATG promotes expansion of functional CD4+ CD25highFOXP3+ regulatory T cells *in vitro*. *Blood* 2008; **111**: 3675.
32. LaCorcia G, Swistak M, Lawendowski C, *et al.* Polyclonal rabbit antithymocyte globulin exhibits consistent immunosuppressive capabilities beyond cell depletion. *Transplantation* 2009; **87**: 966.
33. Broady R, Yu J, Levings MK. ATG-induced expression of FOXP3 in human CD4+ T cells *in vitro* is associated with T cell activation and not the induction of FOXP3+ T regulatory cells. *Blood* 2009; **114**: 5003.
34. Sewgobind VD, Kho MM, van der Laan LJ, *et al.* The effect of rabbit anti-thymocyte globulin induction therapy on regulatory T cells in kidney transplant patients. *Nephrol Dial Transplant* 2009; **24**: 1635.
35. De Serres SA, Sayegh MH, Najafian N. Immunosuppressive drugs and Tregs: a critical evaluation! *Clin J Am Soc Nephrol* 2009; **4**: 1661.
36. Wang Z, Shi B, Jin H, Xiao L, Chen Y, Qian Y. Low-dose of tacrolimus favors the induction of functional CD4(+)CD25(+)FoxP3(+) regulatory T cells in solid-organ transplantation. *Int Immunopharmacol* 2009; **9**: 564.
37. Mittal SK, Sharma RK, Gupta A, Naik S. Increased interleukin-10 production without expansion of CD4+ CD25+ T-regulatory cells in early stable renal transplant patients on calcineurin inhibitors. *Transplantation* 2009; **88**: 435.
38. Niemczyk M, Zegarska J, Pawlowska M, Wyzgal J, Ciszek M, Paczek L. Different profile of gene expression of cytokines in peripheral blood mononuclear cells of transplant recipients treated with m-TOR inhibitor and calcineurin inhibitor. *Transpl Immunol* 2009; **20**: 139.
39. Oh U, Blevins G, Griffith C, *et al.* Regulatory T cells are reduced during anti-CD25 antibody treatment of multiple sclerosis. *Arch Neurol* 2009; **66**: 471.
40. Demirkiran A, Sewgobind VD, van der Weijde J, *et al.* Conversion from calcineurin inhibitor to mycophenolate mofetil-based immunosuppression changes the frequency and phenotype of CD4+ FOXP3+ regulatory T cells. *Transplantation* 2009; **87**: 1062.
41. Kreijveld E, Koenen HJ, Klasen IS, Hilbrands LB, Joosten I. Following anti-CD25 treatment, a functional CD4+ CD25+ regulatory T-cell pool is present in renal transplant recipients. *Am J Transplant* 2007; **7**: 249.
42. Li Z, Lim WK, Mahesh SP, Liu B, Nussenblatt RB. Cutting edge: *in vivo* blockade of human IL-2 receptor induces expansion of CD56(bright) regulatory NK cells in patients with active uveitis. *J Immunol* 2005; **174**: 5187.
43. van de Linde P, Tysma OM, Medema JP, *et al.* Mechanisms of antibody immunotherapy on clonal islet reactive T cells. *Hum Immunol* 2006; **67**: 264.
44. Thomson AW, Turnquist HR, Raimondi G. Immunoregulatory functions of mTOR inhibition. *Nat Rev Immunol* 2009; **9**: 324.
45. Mita MM, Mita A, Rowinsky EK. The molecular target of rapamycin (mTOR) as a therapeutic target against cancer. *Cancer Biol Ther* 2003; **2**: S169.
46. Battaglia M, Stabilini A, Migliavacca B, Horejs-Hoeck J, Kaupper T, Roncarolo MG. 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. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+ CD25+ FoxP3+ regulatory T cells. *Blood* 2005; **105**: 4743.
48. Fox CJ, Hammerman PS, Thompson CB. The Pim kinases control rapamycin-resistant T cell survival and activation. *J Exp Med* 2005; **201**: 259.
49. Noris M, Casiraghi F, Todeschini M, *et al.* Regulatory T cells and T cell depletion: role of immunosuppressive drugs. *J Am Soc Nephrol* 2007; **18**: 1007.
50. Segundo DS, Ruiz JC, Izquierdo M, *et al.* Calcineurin inhibitors, but not rapamycin, reduce percentages of

CD4+ CD25+ FOXP3+ regulatory T cells in renal transplant recipients. *Transplantation* 2006; **82**: 550.
51. Lange CM, Tran TY, Farnik H, *et al.* Increased frequency of regulatory T Cells and selection of highly potent CD62L+ cells during treatment of human lung transplant recipients with rapamycin. *Transpl Int* 2010; **23**: 266.
52. Monti P, Scirpoli M, Maffi P, *et al.* Rapamycin monotherapy in patients with type 1 diabetes modifies CD4+ CD25+ FOXP3+ regulatory T-cells. *Diabetes* 2008; **57**: 2341.
53. Baker AK, Wang R, Mackman N, Luyendyk JP. Rapamycin enhances LPS induction of tissue factor and tumor necrosis factor-alpha expression in macrophages by reducing IL-10 expression. *Mol Immunol* 2009; **46**: 2249.
54. Saemann MD, Haidinger M, Hecking M, Horl WH, Weichhart T. The multifunctional role of mTOR in innate immunity: implications for transplant immunity. *Am J Transplant* 2009; **9**: 2655.
55. Allison AC, Eugui EM. Purine metabolism and immunosuppressive effects of mycophenolate mofetil (MMF). *Clin Transplant* 1996; **10**: 77.
56. Blaha P, Bigenzahn S, Koporc Z, *et al.* The influence of immunosuppressive drugs on tolerance induction through bone marrow transplantation with costimulation blockade. *Blood* 2003; **101**: 2886.
57. Gregori S, Casorati M, Amuchastegui S, Smiroldo S, Davalli AM, Adorini L. Regulatory T cells induced by 1 alpha,25-dihydroxyvitamin D3 and mycophenolate mofetil treatment mediate transplantation tolerance. *J Immunol* 2001; **167**: 1945.
58. Zeiser R, Nguyen VH, Beilhack A, *et al.* Inhibition of CD4+ CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood* 2006; **108**: 390.

