Promoting transplantation tolerance; adoptive regulatory T cell therapy

Summary
Transplantation is a successful treatment for end-stage organ failure. Despite improvements in short-term outcome, long-term survival remains suboptimal because of the morbidity and mortality associated with long-term use of immunosuppression. There is, therefore, a pressing need to devise protocols that induce tolerance in order to minimize or completely withdraw immunosuppression in transplant recipients. In this review we will discuss how regulatory T cells (Tregs) came to be recognized as an attractive way to promote transplantation tolerance. We will summarize the preclinical data, supporting the importance of these cells in the induction and maintenance of immune tolerance and that provide the rationale for the isolation and expansion of these cells for cellular therapy. We will also describe the data from the first clinical trials, using Tregs to inhibit graft-versus-host disease (GVHD) after haematopoietic stem cell transplantation and will address both the challenges and opportunities in human Treg cell therapy.

Keywords: cellular therapy, transplantation, Tregs

Introduction
Advances in surgical techniques and the introduction of T cell-directed immunosuppressive agents has made solid organ transplantation a well-established treatment for end-stage failure of several major organs. Despite improvements in short-term outcome, long-term patient and graft survival remain suboptimal due to the toxic side effects associated with long-term use of these drugs. A major goal of transplantation research is, therefore, to promote ‘tolerance’, a state in which the host’s immune system can be reprogrammed and then guided to accept a transplant without the need for long-term immunosuppression. In this pursuit, clinically applicable protocols aim to tip the balance in favour of regulation by either the in-vivo expansion of T cells with regulatory activity or the infusion of ex-vivo expanded cells.

The past two decades have seen the discovery of many different types of regulatory T cells, including: CD8– T cells [1], CD4–CD8– double-negative T cells [2], CD8–CD28– [3], natural killer (NK) T cells [4] and γδ T cells [5], but these are less well studied compared to CD4+ regulatory T cells (Tregs). In this review we will focus on the potential for clinical application of CD4+ Tregs, characterized by high and stable expression of surface interleukin (IL)-2 receptor α chain (IL-2Rα, CD25hi) and the transcription factor, forkhead box protein 3 (FoxP3) [6]. These CD4+CD25+FoxP3+ cells are thymus-derived, referred to as natural Tregs (nTregs), compared to their counterparts that are generated in the periphery and whose activation requires T cell receptor engagement and cytokines, the induced Tregs (iTregs) [7,8].

In comparison to iTregs, studies support the more potent and stable role of nTregs (referred to hereafter as Tregs) in maintaining self-tolerance and preventing autoimmunity [9]. The ability to expand such cells has, therefore, become an attractive prospect in modulating immune responses not only in the context of solid organ transplantation, but also in autoimmunity and prevention of graft-versus-host
Regulatory T cells (T\textsubscript{reg})

Markers used for isolation

Aside from the expression of CD25 [14] and FoxP3 (outlined above), human T\textsubscript{reg} also express CD27 [15], CD45RA [16], CD39 [17], CD122, cytotoxic T lymphocyte antigen-4 (CTLA-4 or CD152) and the glucocorticoid-induced tumour necrosis factor receptor (GITR) family-related gene [18,19]. However, most of these cell surface markers are not exclusive to T\textsubscript{reg} with some of these markers also expressed by non-regulatory CD4\textsuperscript{+} T cells, posing a challenge during the isolation process. As an example, data support the key role of FoxP3 in the development, maintenance and function of T\textsubscript{reg} with supporting evidence that point mutations in the FoxP3 gene leads to a functional T\textsubscript{reg} deficit that is evident in patients with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) [20].

Despite this, FoxP3 is not a sufficient marker for the isolation of T\textsubscript{reg} as many activated effector T cells also express FoxP3 without having a regulatory phenotype [21]. Moreover, being an intracellular protein, this marker cannot be used to isolate T\textsubscript{reg}. What complicates the story even further is that human T\textsubscript{reg} are heterogeneous. In contrast with mice, the combination of the marker CD45RA and the level of expression of FoxP3 delineates the human T\textsubscript{reg} compartment into naive or resting T\textsubscript{reg} (CD45RA\textsuperscript{hi}FoxP3\textsuperscript{low}), effector T\textsubscript{reg} (CD45RA\textsuperscript{lo}FoxP3\textsuperscript{hi}), both of which are suppressive in vitro, and the non-suppressive, cytokine secreting non-T\textsubscript{reg} (CD45RA\textsuperscript{low}FoxP3\textsuperscript{lo}) [22,23].

The search for further surface markers to aid in the unique identification of T\textsubscript{reg} has led to studies showing that T\textsubscript{reg} also express low levels of CD127, the ß-chain of the IL-7 receptor [24]. Accordingly, the constitutive high expression of CD25 but low expression of CD127 has been used to discriminate T\textsubscript{reg} from activated effector T cells [25]. However, the combination of CD25 and CD127 is still not sufficient to isolate functionally pure T\textsubscript{reg}, bearing in mind that not all the ex vivo-isolated FoxP3\textsuperscript{+} T\textsubscript{reg} are regulatory.

Such studies, therefore, highlight the fact that despite all the efforts to identify T\textsubscript{reg} markers, the quest continues and we have yet to find markers that define ‘pure’ T\textsubscript{reg} populations for the purposes of cellular therapy.

Mechanism of action

Several mechanisms of suppression by T\textsubscript{reg} have been proposed. T\textsubscript{reg} can suppress the functional ability of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells directly by preventing their differentiation, activation and proliferation via either cell–cell contact or a contact-independent route, which includes inhibitory cytokines such as IL-10, transforming growth factor (TGF)-ß and recently IL-35 [26–28]. They can also kill effector T cells directly in a perforin-dependent and granzyme-dependent manner or suppress their activation [29,30]. Furthermore, T\textsubscript{reg} have been shown to express galecitrin-1, with blockade of galecitrin-1 binding to activated T cells being shown to reduce the T\textsubscript{reg} inhibitory effect [31].

Moreover, T\textsubscript{reg} may mediate their suppressive function by acting directly on dendritic cells (DCs), attenuating their antigen-presenting and co-stimulatory functions. In support of this, Fassbender et al. [32] showed that the co-culture of murine DCs with T\textsubscript{reg} led to an increase in DC cyclic adenosine monophosphate (cAMP), which was responsible for the down-regulation of the co-stimulatory molecules, CD80/CD86. Other mechanisms include the role of cytotoxic T lymphocyte antigen 4 (CTLA-4), a negative co-stimulatory molecule on T\textsubscript{reg}, in either up-regulating indoleamine 2, 3-dioxigenase (IDO) expression on DCs which, in turn, down-regulated immune responses [33], or acting as an effector molecule to inhibit
CD28 co-stimulation by the cell-extrinsic depletion of co-stimulatory ligands [34].

As evident from the studies outlined, therefore, it becomes clear that the precise mechanism of suppression by T<sub>reg</sub> has yet to be fully elucidated.

**Preclinical data in support of adoptive T<sub>reg</sub> cell therapy**

The term ‘adoptive immunity’ was first coined in 1954 byBillingham et al. [35], who were able to show that passive transfer of primed immune cells can generate immunity in the recipient. Subsequently, numerous animal studies have demonstrated the effectiveness of this adoptive transfer of immunity towards cancer and infectious disease [36,37]. Moreover, the use of IL-2 permitted, for the first time, the ex-vivo culture and expansion of T cells in humans [38]. In addition, many transplant researchers found that CD4<sup>+</sup> T cells were responsible for donor-specific tolerance, and it was the study by Hall et al. [39] which concluded that transplant tolerance was mediated by CD4<sup>+</sup>CD25<sup>+</sup> cells. In this study they showed that in cyclosporin-treated rats with long-term cardiac allograft survival, the adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup> T cells resulted in tolerance.

The application of T<sub>reg</sub> in the context of organ transplantation is supported further by the seminal work by Sakaguchi et al. [6], who showed that T<sub>reg</sub> from naive mice prevented rejection of allogeneic skin grafts in T cell-deficient nude mice given CD25<sup>-</sup> T cells. Subsequently, a series of preclinical rodent models of skin and cardiac transplantation demonstrated that T<sub>reg</sub> present in the recipient at the time of transplantation are critical in the induction and maintenance of tolerance (reviewed in [40]). In support of such studies we have also generated T<sub>reg</sub> lines in vitro, and shown that these T<sub>reg</sub> are very effective at inducing survival of MHC-mismatched heart allografts [41]. Furthermore, in a murine skin transplant model following thymectomy and partial T cell depletion, we have demonstrated previously the ability of in-vitro-expanded T<sub>reg</sub> in inducing donor-specific transplantation tolerance in this system [42].

The importance of adoptive T<sub>reg</sub> therapy in transplantation is supported further in mouse models of bone marrow transplantation, where the transfer of freshly isolated T<sub>reg</sub> together with the bone-marrow allograft has been shown to ameliorate GVHD and facilitate engraftment [43]. GVHD was also the first model in which it was shown that the adoptive transfer of ex-vivo-expanded donor T<sub>reg</sub> was highly effective in preventing acute or chronic GVHD [44]. Moreover, the adoptive transfer of T<sub>reg</sub> has been shown to prevent rejection of pancreatic islet [45] and other organ allografts [46,47].

The use of currently available humanized mouse models of GVHD and allotransplantation [48,49] has reinforced further the importance of T<sub>reg</sub> in these settings. These models are based on the reconstitution of immunodeficient mice with human immune cells. More recently we have also shown the efficacy of human T<sub>reg</sub> in preventing alloimmune dermal tissue injury in a humanized mouse model of skin transplantation [50]. Furthermore, Nadig et al. [51] developed a human vessel graft model to study the in-vivo function of T<sub>reg</sub>. Their results showed convincingly that grafts from mice reconstituted with peripheral mononuclear cells (PBMCs) alone exhibited extensive vasculopathy, whereas the co-transfer of T<sub>reg</sub> prevented this process.

Such adoptive transfer experiments in rodents, therefore, support the notion that tolerance requires ‘tipping the balance’ between reactivity and regulation. Despite such data generated in preclinical animal models, showing successfully that T<sub>reg</sub> can induce and maintain transplantation tolerance, we currently face many challenges in the laboratory that have hindered the widespread application of T<sub>reg</sub> cell therapy in the transplant setting. In addition, a number of different strategies have been proposed for the isolation and expansion of T<sub>reg</sub> for cellular therapy. However, there is no consensus on the optimal process, and many such processes have their limitations (discussed below).

**Clinical application of human T<sub>reg</sub>: challenges with large-scale manufacture**

Isolation and expansion

One of the obstacles in the implementation of clinical protocols using T<sub>reg</sub> is their low frequency, 1–3% of total peripheral blood CD4<sup>+</sup> T cells, and data (from animal models) which suggest that, for these cells to suppress immune responses, high doses of T<sub>reg</sub> in relation to effectors is required [52,53]. This means that for cellular therapy, it will almost certainly be necessary to use a polyclonal stimulus to expand T<sub>reg</sub> in vitro. In this regard, the large-scale ex-vivo expansion of human T<sub>reg</sub> by stimulation with anti-CD3 and anti-CD28 monoclonal antibody-coated beads and high-dose IL-2 has been demonstrated successfully [54]. However, effectors have the potential to proliferate vigorously under such conditions, so that even a trace of effectors in the starting population can be expanded in high numbers. The injection of such cells would, therefore, be detrimental to the patient and may lead to rejection. Thus, it is essential to either initiate the expansion culture with highly purified T<sub>reg</sub> (a challenge in view of the absence of a T<sub>reg</sub>-specific cell surface marker) or create culture conditions that favour T<sub>reg</sub> cell growth.

Two different combinations of markers appear to be promising for T<sub>reg</sub> isolation. The first seeks to isolate CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> but with the addition of an antibody to select for CD45RA<sup>+</sup> cells and so eliminate antigen-experienced or memory T cells [16]. The second combination also uses the CD4<sup>+</sup>CD25<sup>+</sup> phenotype, but includes CD127 expression. The rationale for using CD127 as a
marker for T_{reg} isolation (as explained in earlier sections) is on the basis that in human T_{reg} there is a reciprocal expression of CD127 and FoxP3, and thus CD127 provides a sortable surrogate marker for FoxP3^{+} T_{reg} [24]. Moreover, the so-called ‘naive’ T_{reg} population based on the co-expression of CD4 and CD45RA yield T_{reg} with a greater suppressive capacity than total CD25^{hi} cells [55]. The reason for this became clear when Miyara et al. [22] noted the subpopulations of human FoxP3^{+} T cells and discovered that the CD25^{+}CD45RA FoxP3^{hi} cells contain many Th17 precursors. Furthermore, after 3 weeks of in-vitro expansion the CD45RA^{-}-expanded T_{reg} remained demethylated (compared to the CD127^{+} T_{reg} that became methylated) at the T_{reg}-specific demethylation region (TSDR), which is a conserved region upstream of exon 1 within the FoxP3 locus [completely demethylated in natural T_{reg} but methylated fully in induced T_{reg} and effector T cells (T_{eff})] [55,56]. Such studies, therefore, support the isolation of T_{reg} based on CD45RA^{+} expression, bearing in mind that they are the most stable population for expansion and have the greatest expansion potential [16]. Despite such studies, one drawback is that the number of naive T_{reg} declines in the peripheral blood with age [57], and hence isolation based on CD127 expression may still be a practical approach. Moreover, two elegant studies by Kathryn Wood’s group [51,58] support the in-vitro superiority of the CD4^{+}CD25^{hi}CD127^{low} T_{reg} at regulating alloreactivity compared to T_{reg} isolated based on the expression of CD4 and CD25 alone. Such studies have important implications for the design of future clinical studies.

The search for further surface markers to aid the isolation of purer or more potent T_{reg} populations led to studies investigating markers such as CD121a/CD121b, TGF-β/latency associated peptide (LAP) [59] and CD39 [60]. However, all these proteins are expressed only on activated T_{reg}, and would be of use only to re-isolate T_{reg} after expansion. This may not be feasible, in view of the costs of re-isolating billions of T_{reg} on a per-patient basis. Other studies complicate the story even further. Ito et al. [61] showed that FoxP3^{+} T_{reg} could be grouped into two subsets based on the expression of the inducible T cell co-stimulator (ICOS). They showed that while ICOS^{+} FoxP3^{+} T_{reg} mediate their suppressive function via TGF-β, ICOS^{−}FoxP3^{+} T_{reg} additionally secrete IL-10. Therefore, depending on the type of immune response to be suppressed, it may be useful to isolate subsets of T_{reg} which have specific mechanisms of action. Moreover, a recent study by Ukena et al. [62] compared different T_{reg} isolation strategies in order to define the most promising T_{reg} target cell population for cellular therapy. They compared CD4^{+}CD25^{hi} enrichment, CD4^{+}CD25^{lo} enrichment and depletion of CD127, enrichment of CD4^{+}CD25^{hi}CD45RA T cells, depletion of CD49d^{+} (a marker of proinflammatory cytokine-producing effector T cells) and CD127^{+} T cells and enrichment of CD4^{+}CD25^{*} ICOS^{+} and ICOS^{−} T_{reg}. They concluded that while CD4^{+}CD25^{hi}CD127^{−} and CD4^{+}CD25^{lo}ICOS^{−} T_{reg} are the most promising T_{reg} for fresh cell infusions in clinical trials with respect to cell yield, phenotype, function and stability, the CD4^{+}CD25^{lo} T_{reg} qualify as the best candidate for in-vitro expansion. Such studies, therefore, paint a complicated picture that when choosing the T_{reg} marker for cell isolation we should also bear in mind other factors other than simply purity, i.e. isolating potent cells with a mechanism of action to suppress the immune response of interest and cells with the desired expansion profiles.

Despite this, however, what limits choice when devising a clinically applicable protocol is that isolation techniques need to be good manufacturing practice (GMP)-compliant, and GMP purification reagents for all the various markers outlined above are not yet available. The clinical T_{reg} selection protocols used to date in the United Kingdom have used a combination of depletion and positive selection steps, with the isolation tools involving mainly the automated CliniMACS plus system (Miltenyi Biotec, Bisley, UK). This enables GMP-compliant cell selection by magnetic bead activated cell sorting [63]. More specifically, for the production of CD4^{+}CD25^{lo} T_{reg} the GMP-grade antibodies available enable the depletion of CD19/CD8-expressing immune cells followed by a positive selection of CD25-expressing T cells [64]. The major drawback with such techniques is that this process does not guarantee the selection of CD25^{lo} cells compared to the fluorescence activated cell sorter (FACS) sorter, which allows the important distinction to be made between the CD4^{+}CD25^{lo} and CD25^{hi} cells. In addition, the process does not allow the selection of T_{reg} based on multiple parameters and the ~60% purity of the isolated cells [65] is not comparable with the >95% purity achieved using the FACS sorter [56].

In addition to the automated CliniMACS plus system (Miltenyi Biotec), there are two other commercially available methods for GMP-grade T cell isolation and expansion. Life Technologies Ltd (Paisley, UK) produces the DynaMag™ CTS™ system, which is a magnetic device used in combination with the Dynabeads® CTS™ and Dynabeads® ClinExVivo™ to positively isolate bead bound cells or deplete unwanted cell types. Dynabeads® CD3/CD28 CTS™ are used to positively isolate T cells; these beads are also able to activate the bound T cells and when cultured in the presence of IL-2 result in a 100–1000-fold expansion of the isolated T cells. The T cells are purified by labelling cells with mouse immunoglobulin (Ig)G1 antibodies and using the Dynabeads® IgG1 Binder CTS™ for positive isolation, negative isolation or cell depletion.

Stage Cell Therapeutics (Göttingen, Germany) is a cell therapy company that manufactures Streptamer® reagents for isolation of defined lymphocytes. In view of isolating purer T_{reg} populations, their system involves three positive selection steps by magnetically tagged Fab-Streptamers.
Following each labelling and positive selection step, the tagged cells are liberated completely from the magnetically tagged Fab-Streptamers by incubation with a competing Streptactin ligand D-biotin that causes disruption of the Fab-multimer complex, dissociation of the Fab-Streptamer label from the target cell surface and complete removal upon washing. The first positive isolation step involves anti-CD4-Fab-Streptamer labelling, followed by anti-CD25-Fab-Streptamer labelling, and finally anti-CD45RA-Fab-Streptamer labelling is used to isolate a triple-positive Treg cell preparation that is CD4+CD25+CD45RA+.

Interestingly, however, the study by Marek et al. [66] showed that regardless of the initial phenotypic markers used for isolation (i.e. CD25+CD127low, CD45RA-, CD45 RA+) during the expansion process, Treg were transforming into effector/memory-like cells which produced inflammatory cytokines. They proposed that independent of the phenotypic markers used for Treg isolation, the only variable to help maintain the Treg phenotype and function was limiting the expansion time to 2 weeks. Based on such studies, therefore, it is of particular importance to ensure that the stability of the Treg is maintained during the expansion process.

Basu et al. [67] described a method for the expansion of Treg in the presence of the immunosuppressant, rapamycin (sirolimus). They showed that rapamycin inhibits preferentially the proliferation and function of CD25+ conventional effector T cells and thus permits the expansion of Treg even from a mixed starting population [67,68].

Furthermore, and in support of such a study, Tresoldi et al. [69] showed that only the expansion cultures in the absence of rapamycin are contaminated by the CD4+CCR6+CD161+ T helper type 17 (Th17) precursor cells. Despite this promise, adding rapamycin to Treg cultures has its own disadvantages in view of diminishing overall Treg expansion [70]. The addition of rapamycin may, therefore, necessitate extended expansion times in order to achieve the therapeutic numbers – a problem, bearing in mind studies showing loss of FoxP3 expression in human Treg upon repetitive stimulation (mentioned earlier [55]). It is also important to consider that target doses of expanded Treg may not always be reached, as reported in a clinical trial by Brunstein et al. [71], even when using protocols without the addition of rapamycin. Such trials used anti-CD3/CD28 beads for stimulation and expansion of the Treg lines, the only GMP reagents available (with a safety record in humans). However, stimulation with cell-based artificial APCs (aAPCs), expressing the co-stimulatory molecule CD86 and an Fc receptor (FcR) for loading of anti-CD3 monoclonal antibody (mAb), has also been used to expand Treg [72] with approximately fourfold superiority over the use of anti-CD3/CD28 beads.

These studies, therefore, highlight the many obstacles that we still need to overcome to refine further the current protocols for the isolation and expansion of Treg to ensure safe and efficacious application in the clinical setting.

Despite these hurdles in the laboratory, there is still much debate over the specifics of the clinical protocol (outlined below).

**Clinical protocol**

**Immunosuppression and timing of Treg**

Most transplant recipients are treated with a combination of immunosuppressive drugs and biological agents to control rejection and/or GVHD responses. The combination of drugs used varies depending on the type of organ being transplanted as well as the protocols used by individual transplant centres. For example, some countries use induction therapy with monoclonal or polyclonal antibody preparation such as alemtuzumab or anti-thymocyte globulin (ATG) at the time of transplantation. This treatment markedly depletes most of the leucocyte populations in the peripheral blood. Interestingly, leucocyte depletion has the potential to tip the balance in favour of immune regulation by creating a situation whereby regulatory immune cells outnumber the effector cells. However, whether or not induction therapy is used, when devising clinical protocols to incorporate Treg it is crucial to take into account the influence of the various immunosuppressants on the Treg in vivo.

Taking the example of ATG, the data indicate that in both adults and children ATG causes a rapid decrease in naive CD4 and CD8 T cells which persist usually for at least 2 months [73]. In addition, it has been shown that treatment with ATG is associated with the expansion of FoxP3+ T cells in vivo and suggests a shift in Treg to a Teff ratio. Despite this, CD4+ and CD8+ memory cells are resistant to depletion by ATG and these cell subsets expand over the initial 6 months post-transplantation [73]. The fact that memory cells survive depletion may explain why patients do not suffer opportunistic infections post-ATG therapy. However, these cells can contribute to early graft injury and loss and, importantly, these cells are more resistant to suppression by Treg than naive T cells [74]. However, to limit memory T cell expansion (post-induction therapy), transplant recipients are maintained on other immunosuppressive drugs, most commonly a calcineurin inhibitor (CNI) such as tacrolimus or cyclosporin A, and an anti-proliferative agent such as mycophenolate mofetil. It has been proposed that both types of drug inhibit the generation and function of Treg. Despite this, in animal models in the context of autoimmunity it has been shown that for Treg to exert their suppressive function tissue inflammation needs to be controlled [75]. It seems that for Treg to expand in vivo and exert their suppressive function they require a tolerogenic milieu. In support of this, a recent study analysing the dynamics of the alloimmune response in vivo demonstrated a rapid invasion of effector cells in the grafts followed by the delayed arrival of Treg that were ineffective at controlling...
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tissue damage [76]. In contrast, when the recipient mice were treated with anti-CD40L mAb and rapamycin, effector T cell infiltration was delayed and more than 30% of the graft infiltrating T cells were T<sub>reg</sub>. Of note, there is good evidence in the literature indicating that rapamycin is superior to tacrolimus for the thymic export and survival of T<sub>reg</sub> [77,78]. In contrast to CNIs, rapamycin appears to be tolerance-permissive by selectively inducing apoptosis or necrosis of alloreactive effector cells while promoting T<sub>reg</sub> induction [79], expansion [78] and function [80]. This may suggest that rapamycin is the ideal candidate for short-term therapy post-depletion in humans. However, rapamycin monotherapy post-depletion is associated with a high risk of acute rejection [81], and it is not yet clear whether the concomitant therapy with T<sub>reg</sub> would be sufficient to prevent this or whether further immunosuppression will be required in the short term.

The use of combinations of immunosuppressive agents in the clinical setting highlight the challenge associated with designing protocols that include the infusion of T<sub>reg</sub>. Thus, the competing actions of each immunosuppressive drug may have to be considered together with the key question of the timing of cell injection.

Most of the preclinical T<sub>reg</sub> therapy studies published thus far administered T<sub>reg</sub> prior to or at the time of transplantation, in contrast to studies of autoimmune disease where it has been shown that T<sub>reg</sub> therapy after disease initiation can effectively reverse the disease course [82]. The intensity of the anti-allograft response and the fragility of the transplanted organ may explain the lack of efficacy when T<sub>reg</sub> infusion is delayed. However, if T cell-depleting reagents such as ATG are used as induction therapy, it may be possible to delay T<sub>reg</sub> infusion until lymphocyte numbers start to recover 2 months or more after transplantation. This might tip the balance between T<sub>reg</sub> and T<sub>eff</sub> cells and help to promote a tolerant state.

Location of T<sub>reg</sub> function and site of T<sub>reg</sub> injection

An additional consideration regarding T<sub>reg</sub> therapy is the site of action of T<sub>reg</sub> and, consequently, the desired homing properties of injected cells. In the transplant setting, T<sub>reg</sub> lymph node homing and their ability to traffic to grafts are both required for their protection against graft rejection [83]. Interestingly, in a mouse islet transplant model, therapeutic T<sub>reg</sub> function initially at the graft site (preventing the exit of donor-derived DCs) and then traffic to the draining lymph node and continue to exert their suppressive function there [84]. In so doing, they prevent the exit and migration of donor-derived DCs to the lymph nodes, thereby reducing alloimmune priming. The translation of such a study to the clinic may mean that to ensure that T<sub>reg</sub> exert their suppressive function we need to either inject the cells at the graft site or ensure that the cells reach the graft/lymph node due either to their alloantigen specificity or homing receptor expression. Bearing in mind the serious complications associated with injection of the cells at the graft site, i.e. the risk of bleeding if cells are injected via the portal vein (in the case of liver transplantation), the favoured option is infusion via a peripheral vein.

Studies have shown antigen-specific T<sub>reg</sub> to be more potent than polyclonally activated T<sub>reg</sub> cells [85–87]. Moreover, T<sub>reg</sub> with direct specificity are very potent in preventing acute rejection early after transplantation, while T<sub>reg</sub> with indirect specificity seem to be crucial to prevent chronic rejection [42,46]. In addition, using antigen-specific T<sub>reg</sub> would have additional advantages; first, their action would be limited to the site of alloantigen source and immune activation [88,89]; and secondly, this may avoid the undesirable pan-suppression, mediated by polyclonal cells, resulting in an increased risk of infections and cancers. However, although the expansion of direct pathway allospecific human T<sub>reg</sub> has been achieved [90,91], expansion of indirect pathway T<sub>reg</sub> has proved more difficult, posing further challenges [92,93]. It is important to note, however, that despite the evidence supporting the importance of antigen-specific T<sub>reg</sub> in preventing solid organ rejection, in GVHD the transfer of T<sub>reg</sub> enriched for alloantigen specificity showed only moderately improved efficacy when compared to the polyclonal T<sub>reg</sub> cell population [94]. Although such studies emphasize the lack of antigen-specific requirement for the transferred T<sub>reg</sub>, interestingly, a recent study discussed the importance of homoing receptor expression in this transplant setting.

Ukena et al. [95] showed that tolerant patients without GVHD after haematopoietic stem cell (HSC) transplantation expressed significantly higher levels of the chemokine receptors transplantation. This may suggest that homing of T<sub>reg</sub> to secondary lymphoid tissue and sites of inflammation may play an important role in the control of GVHD, despite some studies suggesting that GVHD is a systemic disease and the concentration of T<sub>reg</sub> at a localized site is not required.

These types of study, therefore, support the notion that therapeutic strategies using T<sub>reg</sub> have to take into account the fact that these cells not only need potent suppressive function, but also need appropriate tissue trafficking to enable contact with their target cells. Therefore, if the T<sub>reg</sub> are to be injected via a peripheral vein then it is important that they express the molecules such as CD62L and CCR7 that are crucial for their migration to the lymph nodes and other chemokine receptors, e.g. CXCR3 for liver homing [96]. Moreover, T<sub>reg</sub> vary in their expression of trafficking and homing receptors according to their individual histories and state of activation. They have been shown to variously express CCR2, CCR4, CCR7, CCR8, CCR9, CXCR1 and CXCR4 (reviewed in [97]). In addition, it is now known that within the pool of FoxP3-expressing cells functionally diverse T<sub>reg</sub> subsets can be identified on the basis of chemokine receptor expression [98]. In view of the impor-
Dose of Treg and number of injections

Aside from the timing of injection and the site of injection, what is of paramount importance is to decide the dose of Treg that is needed (recently reviewed in [100]).

The trials to date (outlined below) of Treg therapy in the context of bone marrow transplantation will inform us of the doses that are safe and tolerated in patients. However, the first trials of Treg therapy in solid organ transplantation will need to start with a dose escalation study to assess the safety and tolerability of Treg at various doses (minimum determined by the trials to date and the maximum number determined by the maximum number of Treg reached during the in-vitro expansion process). In addition, whether polyclonal Tregs or antigen-specific Tregs, are used will influence the dose. Of note, studies using antigen-specific Tregs showed that lower numbers were able to achieve the same functional efficacy as larger numbers of polyclonal Tregs [86,87]. Finally, whether a single injection or multiple injections are required is a matter of debate and may be determined in a Phase II efficacy study, where patient outcomes should also be measured and an in-depth patient monitoring planned.

The use of molecular diagnostic tools can help to assess the increased expression of biomarkers of operational tolerance in patients receiving cellular therapy and whether these can be used as surrogate end-points of efficacy [101–103]. The same approach can be used to define whether or not the patients have decreased expression of biomarkers of acute rejection [104,105]. Furthermore, phenotypic analysis of patient PBMCs, using flow cytometric analysis, can determine whether or not the number of Treg has increased or the composition of the T cell compartment has changed as a result of the intervention [106]. Using the same analysis, the cytokine profile of the cells that have been phenotyped can be analysed to establish their plasticity. Finally, lymphocyte compartments can be monitored after specific interventions, as has been shown useful when associating expansion of lymphocyte subsets, in this case naïve B cells, in peripheral blood of patients in whom better outcomes were noted [107].

First results of clinical trials

In spite of the potential concerns and controversies outlined with regard to Treg isolation and expansion protocols and the optimal clinical protocol, clinical trials are under way to test the therapeutic potential of Treg.

Beneficial effects of Treg infusions on allograft survival were first reported in bone marrow transplantation models in which donor Treg reduced the incidence of GVHD. The first human trial using Treg cell therapy by Trzonkowski et al. [108] involved two patients. The first patient had chronic GVHD 2 years post-bone marrow transplantation. After receiving 0·1×10^6/kg FACS purified ex-vivo-expanded Tregs from the donor, the symptoms subsided and the patient was withdrawn successfully from immunosuppression without evidence of recurrence. The second patient had acute GVHD at 1 month post-transplantation, which was treated with several infusions of expanded donor Treg. Despite initial and transitory improvement, the disease progressed and resulted ultimately in the patient’s death. This was the first report to show that adoptive transfer of Treg is well tolerated and thus was a major breakthrough.

Results of a larger Phase I/II study were reported in which a total of 23 patients receiving umbilical cord blood (UCB) stem cell transplants were enrolled into a Treg escalation trial [71]. CD4^+CD25^hi Tregs were isolated from a third-party UCB graft and expanded by anti-CD3/CD28-coated beads and recombinant IL-2 over a period of 18 days.

Patients received expanded Treg at doses ranging from 1×10^5 to 30×10^5/kg. Of note, the targeted Treg dose was achieved only in 74% of cases. Compared with the 108 historical controls, there was a reduced incidence of grades II–IV acute GVHD (from 61 to 43%; P=0·05), although the overall incidence of GVHD was not significantly different.

In a third trial (Phase I/II), conducted by Di Ianni et al. [109], 28 patients were enrolled who underwent haematopoietic stem cell transplantation for haematological malignancies. Patients received donor Treg without ex-vivo expansion and donor conventional T cells (Tcons) without any other adjuvant immunosuppression. Different dose regimens were used, ranging from 5×10^6/kg Tcons with 2×10^6/kg Treg to 2×10^6/kg Tcons with 4×10^6/kg Treg. As two patients receiving the latter regimen developed acute GVHD, compared with none of the other patients, the authors concluded that a dose of 1×10^6/kg Tcons with 2×10^6/kg Treg is safe. Moreover, patients receiving Treg demonstrated accelerated immune reconstitution, reduced cytomegalovirus (CMV) reactivation and a lower incidence of tumour relapse and GVHD when compared to historical
controls. However, it is also important to note the disappointing patient survival, with only 13 of the 26 patients surviving, but this may have been because of pre-existing fungal infections and the harsh conditioning regimens that were used.

With the results from stem cell-treated patients showing that Treg therapy is well tolerated, it is now time to initiate trials in solid organ transplantation. In this regard, the ONE Study, a multicentre Phase I/II study funded by the European Union FP7 programme, will investigate the safety of infusing ex-vivo-expanded Treg cells (among other regulatory cells) into kidney transplant recipients. Moreover, clinical trials to test the safety and tolerability of polyclonally expanded or donor alloantigen-specific Treg cell therapy in cal trials to test the safety and tolerability of polyclonally expanded or donor alloantigen-specific Treg cell therapy in combination with depletion of alloreactive T cells and short-term immunosuppression in liver transplant patients are currently being planned.

**Conclusion**

The first results of clinical trials applying Treg, in stem cell transplantation are very encouraging, and provide a basis for future trials in solid organ transplantation. Such trials should involve a small number of patients, aiming at evaluating the safety of increasing doses of Tregs. In addition, the clinical protocol for such trials should be based on a Treg-supportive immunosuppressive regimen, not only to protect against rejection, but also to create the tolerogenic milieu to maximize the potential efficacy of the exogenously administered Tregs. Recent progress in understanding Treg biology and the development of experimental mouse models has highlighted how heterogeneous and plastic murine as well as human Tregs can be. This supports the importance of a careful design of purification and expansion protocols for generating Tregs for clinical application with release criteria set with the most current understanding of Treg biology. Moreover, it is of paramount importance to ensure a comprehensive patient immune monitoring plan and the use of biomarkers that can predict the successful induction of immune tolerance, which would allow for the safe minimization or even withdrawal of immunosuppression.

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**Disclosure**

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