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Running Title: Regulatory T-cell therapy in transplantation

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Abstract

Solid organ transplantation remains the treatment of choice for end-stage organ failure. Whilst the short term outcomes post-transplant have improved in the last decades, chronic rejection and immunosuppressant side effects remain an ongoing concern. Hematopoietic stem cell transplantation is a well-established procedure for the treatment of patients with haematological disorders. However, donor T-cells are continually primed and activated to react against the host causing graft-versus-host-disease (GvHD) that leads to tissue damages and death. Regulatory T-cells (Tregs) play an essential role in maintaining tolerance to self-antigens, preventing excessive immune responses and abrogating autoimmunity. Due to their suppressive properties, Tregs have been extensively studied for their use as a cellular therapy aiming to treat GvHD and limit immune responses responsible for graft rejection. Several clinical trials have been conducted or are currently

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ongoing to investigate safety and feasibility of Treg-based therapy. This review summarises the general understanding of Treg biology and presents the methods used to isolate and expand Tregs. Furthermore, we describe data from the first clinical trials using Tregs, explaining the limitations and future application of these cells.

Introduction

Solid organ transplantation is the treatment of choice for many end-stage organ failure [1], resulting in marked improvements in both morbidity and mortality. As a result of improved surgical technique, closer co-ordination between transplant centres and better immunosuppression, short-term results are excellent. Despite patient survival rates greater than 90% one year after surgery long-term acceptance still remain a challenge due to chronic rejection and the toxicity of the immunosuppressive drugs causing infections, organ failure and cancer [2–4]. "Operational tolerance" (OT) [5] remains the ultimate goal whereby patients achieve stable graft function without immunosuppression in an immunocompetent host. Whilst achieving a state of OT is rare; in the case of liver transplantation approximately 20% of recipients have been successfully weaned off immunosuppression [6] and this percentage increased with time from the transplant [7]. In reality, studies evaluating OT have been conducted on a selected group of patients while the large majority are maintained on immunosuppression lifelong. Due to ongoing concerns regarding immunosuppressant toxicity and chronic rejection, there is greater impetus to identify alternative immunosuppressant strategies.

Hematopoietic stem cell transplantation (HSCT) is an established procedure concerning the infusion of autologous, syngeneic or allogeneic stem cells for several high risk hematologic malignancies. The success of allogeneic HSCT depends on a multitude of parameters [8] type and stage of the underlying disease, age of the patients, human leukocyte antigen (HLA) disparity between donor/host and intensity of the pre-transplantation conditioning regimen [9,10]. The main side effect of HSCT is graft-versus-host disease (GvHD) where donor T-cells recognise the host minor and major histocompatibility antigens and proliferate, damaging target tissues [11] However, donor T-cells are key for graft-versus-leukemia (GvL) effect as well, and their depletion although abrogates GvHD abolishes the GvL effect. Patients undergoing GvHD receive an immunosuppressive regimen [12] responsible of many side effects but necessary to limit T-cell activation. GvHD can occur in acute and chronic forms according to time from transplantation and the type of response [13]. Although the post-transplant outcomes depend on the initial disease status only 50% to 80% of patients with acute GvHD [14] and 40% to 50% of patients with chronic GvHD respond to steroidal therapy [15]. As a result, there is a need for alternative and more effective strategies to modulate the ongoing immune response.

One identified approach involves the use of regulatory T-cells (Tregs) as a cellular therapy for the treatment of GvHD [16] and for limiting immune responses to allograft after solid organ transplantation [17].

In 1995, Sakaguchi and colleagues [18] identified for the first time a small population of CD4⁺ cells that expressed high levels of IL-2 α -chain receptor (CD25), whose depletion resulted in autoimmune diseases whilst their transfer to neonatally day 3 thymectomised mice prevented the disease. These cells called Tregs have a pivotal role in maintaining peripheral immunological tolerance, by preventing autoimmunity and chronic inflammation. In 2003, the transcriptional regulator forkhead box P3 (FoxP3) was discovered as a master control gene for mouse Tregs [19,20]. In recent years, preclinical studies have demonstrated how adoptive transfer of Tregs inhibited GvHD [21–23] and prevented/delayed allograft rejection [24,25]. In solid organ transplantation, we and others have

demonstrated that graft-specific Tregs displayed greater potency against graft rejection than polyclonal Tregs [26–28]. Together, these data supported the use of Tregs in the clinic and in 2009 the first trial using Tregs was published, opening a new field of investigation [29]. Herein, we provide an overview of human Treg heterogeneity/function and focus on the strategies used to isolate, expand and infuse Tregs under good manufacturing practice (GMP) conditions. Finally, we describe data from published papers and ongoing clinical trials using Tregs as cellular therapy, highlighting the limitations and future applicability of these cells within the transplant field.

Tregs: general overview

I) Heterogeneity of Tregs

The multiple subpopulations of Tregs are distinguished by the expression of different cell-surface markers, mechanisms of activation and how they function (reviewed by us in [30]). One of these subpopulations is the CD4⁺CD8⁻ Tregs which can suppress target cells by using a range of different mechanisms including the release of immunosuppressive cytokines and the induction of target cell death. However, despite the increasing progress to understand these cell types and their potential in solid organ transplantation [31], they are not currently available for clinical use. Thus, we will focus mostly on the best characterised Tregs which are the thymus-derived CD4⁺ Tregs (tTregs) which constitutively express CD25 and FoxP3 and represents 5-10% of all peripheral CD4⁺ T-cells [32]. While in the mouse the expression of neuropilin-1 has helped in distinguishing between tTregs and peripheral-derived Tregs (pTregs) [33,34] in human this is not possible [35]. Currently, the only way to distinguish tTregs is the evaluation of the Treg-specific demethylated region (TSDR), an evolutionarily conserved non-coding element within the FoxP3 gene locus, which is fully demethylated in tTregs [36]. However, the evaluation of TSDR methylation status can only be a tool in diagnosis or clinical trial monitoring but not used for Tregs isolation. The best marker to distinguish and isolate Tregs in combination with CD4 and CD25 is the α -chain of IL-7R (CD127) [37]; its expression inversely correlates with FoxP3 and suppressive Treg function [37]. In 2009, Miyara *et al.* demonstrated that human Tregs in peripheral blood are heterogeneous and consists of three main subpopulations based on their expression levels of CD45RA and FoxP3/CD25 [38]. Tregs can be divided into (1) naïve/resting and very stable cells expressing CD45RA⁺FoxP3^{low}; (2) effector Tregs expressing CD45RA⁻FoxP3^{high}; and (3) cytokine producing Tregs, expressing CD45RA⁻FoxP3^{low}. Naïve Tregs are considered the "real Tregs"; they are very suppressive and fully demethylated in the FoxP3 locus.

Among pTregs, arising from conventional CD4⁺CD25⁻ T-cells (Tconv) in the periphery under specific conditions, are the Th3 and the Tr1. The presence of TGF- β and IL-4 promotes the induction of Th3 cells which in turn predominately secretes immunosuppressive TGF- β [39], whereas, the presence of IL-10 and IFN- γ induces Tr1 cells which predominantly secretes IL-10 into the microenvironment [40,41]. Another type of pTregs are the induced CD4⁺CD25⁺FoxP3⁺ pTregs which are generated from peripheral CD4⁺FoxP3⁻ T-cells upon activation and in the presence of TGF β and IL-2 [42]; these Tregs display similar cell surface markers as tTregs and function by contact-dependent mechanisms and the release of immunosuppressive cytokines. TSDR methylation status is key to distinguishing between the thymus-derived CD4⁺CD25⁺FoxP3⁺ and the peripheral-derived CD4⁺CD25⁺FoxP3⁺ Tregs.

II) Treg Suppression Mechanisms

Tregs employ a plethora of contact and non-contact dependent mechanisms to exert their suppressive function on different cells like CD4⁺ and CD8⁺ T-cells, macrophages, dendritic cells (DCs), natural killer (NK) and B-cells. Thornton and Shevach demonstrated that Tregs require TCR stimulation in order to suppress in an antigen non-specific manner [43]. From a functional

perspective, the various potential suppressive mechanisms could be divided in four "modes of action": (A) metabolic interference, (B) inhibitory cytokine release, (C) cytolysis and (D) targeting antigen presenting cells (APCs) (extensively reviewed in [44]).

Briefly, (A) T-cells depend on IL-2 for survival and proliferation, Tregs constitutively express high levels of CD25 which depletes IL-2 from the microenvironment and limiting its availability for T-cell functions [18]. Additionally, CD39 and CD73 are ecto-enzymes found on the surfaces of Tregs. Firstly, CD39 converts pro-inflammatory extracellular adenosine triphosphate into adenosine monophosphate (AMP), secondly, CD73 converts AMP into anti-inflammatory adenosine [45]. (B) Tregs can release immunosuppressive cytokines such as IL-10, IL-35 and TGF- β to prevent T-cell proliferation and maturation of APC [46–48]. (C) Tregs secrete granzymes and perforins which cause apoptosis of target cells [44]. (D) Tregs are the only T-cell subpopulation that constitutively expresses CTLA-4; it binds CD80/CD86, the co-stimulatory molecules expressed by APCs, to block their binding to CD28, thus limiting T-cell activation. Furthermore, CTLA-4 can also down-regulate DCs' activity via trans-endocytosis or extraction of CD80 and CD86 resulting in diminished co-stimulation [49]. Very recently a novel mechanism of Treg suppression was discovered by us and others. It refers to the release of nano-sized vesicles called exosomes that are immunomodulatory. We demonstrated that Treg-derived exosomes inhibited T-cell proliferation *in vitro* via CD73 molecules found on the surfaces of these exosomes [50]. Additionally, Okoye *et al.* have shown that Treg-derived exosomes prevented autoimmune diseases *in vivo* which was attributed to the presence of inhibitory microRNA within these exosomes [51]. Another study demonstrated that the adoptive transfer of Treg-derived exosomes into a rat model of kidney transplantation prolonged the survival of the allograft [52]. Taken together, these studies demonstrated that Tregs can suppress the immune response via different mechanisms.

Treg manufacturing for clinical use

I) Source of Tregs and their isolation

Most pre-clinical studies source their Tregs cellular product from either peripheral blood (PB) or umbilical cord-blood (UCB). A pioneering study in 2006 by Hoffmann *et al.* described for the first time a GMP procedure for the isolation of CD4⁺CD25⁺ T-cells from standard leukapheresis product [53]. Isolation was carried out by CliniMACS (CliniMACS TM Instruments, Miltenyi Biotec) a clinical-scale magnetic enrichment of cells in a closed and sterile system. This was done in a two-stage method; firstly, depletion of CD19⁺ cells followed by an enrichment of cells expressing CD25 molecules. This has now become a well-established procedure for GMP isolation. Di Ianni *et al.* [54] applied this isolation procedure to 72 leukapheresis products. They isolated a mean of 263×10^6 Tregs and of these cells $79.8\% \pm 22.2$ were FoxP3⁺. Recently, our group published the first reports of the manufacture of clinical-grade Tregs from prospective liver and renal transplant recipients [55,56]. As an example, from 150mL of PB derived from patients with liver cirrhosis we were able to isolate $7.14 \times 10^6 \pm 0.938$ cells with high purity.

UCB has been used as an alternative source for the generation of Tregs for clinical use. Brunstein *et al.* [57] isolated a mean of 6.6×10^6 cells from one UCB with a mean purity of 66%.

Although the CliniMACS has been extensively used to isolate Tregs under GMP conditions [58,59] the purity of the cells obtained is not optimal as they are contaminated with CD25^{low} Tconv. This limitation has hampered the generation of antigen-specific Tregs production for which high purity of Tregs is needed. An alternative method for Tregs isolation is the flow cytometry-based purification. This offers the advantage of a highly pure cell product isolated using a combination of multiple surface markers (e.g. CD25 and CD127). Unfortunately, it presents considerable regulatory challenges in the EU (Directive 2003/94/EC and its Annex 2) and to date, only 1 group in Europe and 2 in the USA have obtained regulatory approval to use flow-sorted Tregs and published their clinical

strategy (University of Minnesota, USA [57]; University of California, USA [60] and University of Gdansk, Poland [29]).

II) Treg expansion

Considering the low number of Tregs present in both PB and UCB the infusion of a large number of freshly isolated Tregs is difficult to achieve [61]. In the setting of HSCT, Tregs are isolated from the donor and a larger number of cells can be obtained. However to increase the number of cells for infusion, both in GvHD and solid organ transplantation Tregs have been expanded *ex-vivo* using anti-CD3/CD28-coated beads in the presence of high dose of IL-2 (polyclonal expansion). One caveat of Treg isolation using immunomagnetic technique is that the resultant cells are contaminated with effector T-cells. To avoid the infusion of activated effector T-cells, we and others have developed Treg expansion protocols using drugs like rapamycin or *all-trans* retinoic acid (ATRA) [62,63]. The positive effect of rapamycin on Tregs viability and expansion has been observed firstly *in vivo*. Kidney transplanted patients receiving a rapamycin-based immunosuppression regimen presented an increased proportion of Tregs as compared to patients on calcineurin inhibitors [64]. *In vitro*, rapamycin significantly reduces the undesired expansion of effector T-cells allowing proliferation of Tregs that are independent from mTOR pathway for their cell cycle progression [65]. In addition, rapamycin confers to the expanded Tregs higher stability and suppressive capacity [66] as showed by us *in vitro* and in GvHD mouse models [63].

The alternative drug ATRA affects T-cell fate by contributing to Treg differentiation in combination with TGF- β [67,68]. Although its role in Treg induction is well established, the effects on tTregs are still controversial and for this reason no GMP expansion protocol have been developed yet.

After cell-sorting isolation, antigen-specific Tregs can be generated and expanded *ex-vivo* under GMP conditions. We in collaboration with Tang's group have recently published a preclinical protocol for the generation and expansion of antigen-specific Tregs [69]. Tregs were cultured with previously activated (by CD40 ligand) allogeneic B-cells in the presence of IL-2. These cells were more potent in suppressing alloimmune responses *in vitro* and *in vivo*, using a humanised mouse skin transplant model, when compared to polyclonally expanded Tregs.

Clinical trials using Tregs

At the end of October 2016 only few results from clinical trials have been published showing safety and feasibility of Treg infusion. However, there are several ongoing phase I/II clinical trials with Tregs in solid organ transplantation and HSCT (Table 1).

The first paper reporting the infusion of *in vitro* expanded Tregs was published in 2009 [29]. The authors described a procedure and first-in-man clinical effects of adoptive transfer of *ex vivo* expanded CD4⁺CD25⁺CD127⁻ cells for the treatment of 2 patients affected by acute and chronic GvHD, respectively. Due to the restricted patient number and the procedure to isolate and expand Tregs no conclusion about safety was drawn.

In 2011, Brunstein *et al.* published results from the first phase I clinical trial using expanded Tregs from third party UCB [57]. The study aimed to evaluate the safety and feasibility of UCB Tregs in 23 patients with acute GvHD. Patients received a dose-escalation of Tregs from 0.1 to 30 \times 10⁵ UCB Tregs/kg. No toxicities were observed after infusion, and Tregs were detected for 14 days. Although this was only a phase I clinical trial the authors affirmed that, compared with identically treated 108

historical controls, there was a reduced incidence of grade II-IV aGvHD with no deleterious effect on risks of infection, relapse, or early mortality.

In 2014, Martelli's group [70] published another study in which freshly isolated donor-derived Tregs were injected before HSCT to avoid the extensive *ex vivo* T-cell depletion of the graft. Between September 2008 and December 2012 they infused 43 patients with high-risk acute leukaemia. This study demonstrated for the first time that adoptive immunotherapy with Tregs protected from GvHD mediated by the infusion of high number of donor Tconv in patients undergoing full-HLA haploidentical transplantation. The surprising finding was the absence of GvHD in patients who received up to 10^6 Tcons/kg after an infusion of 2×10^6 Tregs. Furthermore, the immunological reconstitution was stronger and faster than the historical controls and after a median follow up of 45 months the leukaemia relapse in patients receiving Tregs was markedly reduced. In our opinion, this could be considered a proof that Tregs do not target GvL, however this data needs to be confirmed by other studies.

More recently, a clinical trial evaluating the adoptive transfer of allogeneic Tregs into patients with chronic GvHD has been published [71]. All the 5 patients selected for this trial were unresponsive to the standard therapy. To our knowledge, this is the first trial adopting a combined therapy using Tregs and low dose of IL-2. All the patients tolerated the Treg products combined with an increase of circulating Tregs and disease improvement or stability. Of note, the 3 patients receiving IL-2 showed an increased T-cell activation, however the clinical improvement suggests that the beneficial effects of low-dose IL-2 on Treg functions was able to control the possible expansion of effector subsets.

In another published clinical trial ("ALT-TEN"), Tr1 were used [72]. These cells have been infused into 12 patients with high-risk/advanced stage hematologic malignancies after chemotherapy conditioning and T-cell depleted haploidentical HSCT. Tr1 were infused when no spontaneous immune reconstitution was detectable. As highlighted by the authors this study had multiple limitations namely that 8 patients died so data was obtained from 4 patients only. A further problem was the percentages of Tr1 in the infused cell product. In fact, the infusion of 3×10^5 CD3⁺ cells/kg provoked GvHD grade III-IV in one patient, suggesting that the ratio between effector cells and Tr1 cells was too high. In our opinion, further trials are necessary to establish the safety of this cell product; and this will be done as part of "THE ONE STUDY" consortium (see below) who will test Tr1 cells as treatment after kidney transplant [73].

The only data regarding the use of Tregs in solid organ transplantation have been recently published from Okumura's group [74]. Between November 2010 and July 2012 they treated patients with end-stage liver failure who underwent transplantation from a living donor with a novel Treg-based cell therapy. Of note, all the patients were splenectomised. Recipient lymphocytes were enriched in regulatory cells after co-culture with irradiated donor cells in the presence of anti-CD80/CD86 antibodies for two weeks. The infused cell product contained a number of CD4⁺CD25⁺FoxP3⁺ cells ranging from 0.43×10^6 /kg to 6.37×10^6 /kg. The immunosuppression weaning started after 6 months post-transplantation followed by a complete weaning at 18 months. Noteworthy, results came from 10 consecutive patients although a total of 40 patients were initially planned. Unfortunately, this trial was suspended because of acute cellular rejection during weaning in 2 patients with primary biliary cirrhosis and 1 with primary sclerosing cholangitis. Seven patients were successfully weaned off immunosuppression while the 3 recipients with rejection were stabilised using low dose of tacrolimus and mycophenolate mofetil. In conclusion, this Treg-enriched product seems to be safe and the results promising. However, the effect of splenectomy in combination with Treg cell therapy has to be clarified and concerns remain about the presence of antigen-specific effector cells in the resultant cell product.

Between the ongoing clinical trials (Table 1), "THE ONE STUDY" is an EU Consortium aiming to test different regulatory cell products in kidney transplantation [73]. Our group together with the group in Oxford led by Andrew Bushell and Paul Harden has just completed the infusion of expanded autologous Tregs in 12 patients. Four doses of Tregs (1, 3, 6, 10×10^6 /kg) have been infused 5 days post-transplant in the presence of immunosuppressive drugs. Our group started at the same time of "THE ONE STUDY" another clinical trial called ThRIL (Table 1); investigating the safety of Tregs immunotherapy after liver transplantation. The clinical protocol involves ATG at time of transplantation, followed by tacrolimus with a switch to sirolimus at 2 months post-transplantation. Three Treg doses (same preparation of "THE ONE STUDY") are being tested: 1, 4.5 and 6×10^6 cells/kg at 3 months post-transplantation. Three patients have already been treated with the lowest dose of Tregs. Lastly, a clinical trial from the University of Liegi (Table 1) is aiming to assess the safety of the combination of donor Treg infusion and rapamycin administration (a non-standard immunosuppressor for this disease) in patients with steroid-refractory chronic GvHD. They will be firstly treated with rapamycin and after 3-4 weeks one infusion of Tregs will be administered.

Future Directions

As recently affirmed by KJ. Wood the infusion of Tregs in transplantation is at the "end of the beginning" [17]. This is because in the last 2 decades, Tregs have transformed from being an ideal candidate for OT induction and GvHD treatment/prevention to a population that can be isolated, expanded and infused *in vivo*. All published data so far indicate that Tregs are well tolerated even when high doses have been infused. However, this has also opened further lines of inquiry concerning: sources, isolation strategy, doses, timing of infusion, optimal immunosuppressive regimen and cell fate post-infusion.

The groups of MK. Levings and LJ. West have successfully isolated Tregs from discarded paediatric thymuses [75]. These Tregs have several advantages over their peripheral blood counterparts. The Treg yield in a single thymus exceeds the estimated Treg number in the entire circulating blood volume of an average-sized adult; moreover Tregs could be clearly distinguished from Tconv and after expansion they were more suppressive and stable than blood Tregs. However, this current source of Tregs is only from paediatric heart transplant patients.

Another step ahead for cell therapy using Tregs is the development of GMP-cell sorters. Using this strategy the following subsets of Tregs can be obtained: $CD4^+CD25^+CD127^-CD45RA^+$ for the isolation of naïve cells [38]; $CD4^+CD25^+CD127^-CD39^{high}$ [76] for Tregs presenting stronger stability and function under inflammatory conditions; $CD4^+CD25^+CD127^-CD226^-TIGIT^+$ [77] for the exclusion of unstable Tregs after *in vitro* expansion. However, a combination between CliniMACs and cell sorting are needed to obtain higher yields of cells.

Another issue is timing of Treg infusion that has to be programmed considering the immunosuppressive regimen adopted (extensively reviewed for solid organ transplantation by us in [78]), the type of patients and donors (death or living donor). The ongoing clinical trials are using new strategies combining Tregs infusion with the use of rapamycin as an immunosuppressive drug or, more recently, low dose of IL-2. These combined strategies could further prolong Treg survival and increase Treg stability *in vivo*, improving the outcome of cell therapy. Another advantage by prolonging Treg survival is to facilitate the induction of "infectious tolerance" [79,80], namely the capacity of Tregs to transmit tolerance from one population to another.

In view of improving the outcome of Treg therapy in the future it is important to understand the fate of the injected Tregs. Treg tracking in a non-invasive and safe way and suitable for GMP products remain undeveloped. A promising GMP-labelling protocol has been developed and tested in type-1

diabetic patients receiving polyclonally expanded Tregs [60]. During the expansion procedure, D-[6,6'-²H₂]glucose has been added in culture and incorporated in the DNA of replicating Tregs. After labelling, cells maintained their phenotype and function and could be detected in circulation 1 year post-infusion. This protocol allows the study of circulating Tregs *in vivo* and their stability, however to study Treg localisation in tissue and their homing capacity, new techniques are under development.

Conclusions

Although much work is still to be done, there is now concrete evidence to support Treg-based cell therapy in the clinical arena. Results coming from the ongoing clinical trials will give us additional information about the impact of these cells in the clinic. For this reason, we will only be able to conclude on their efficacy in a few years when longer-term data will become available.

References

1. Watson CJ, Dark JH. Organ transplantation: historical perspective and current practice. *Br J Anaesth* 2012; 108 Suppl 1: i29–42.
2. Bamoulid J, Staeck O, Halleck F, *et al.* The need for minimization strategies: current problems of immunosuppression. *Transplant International* 2015; 28: 891–900.
3. Söderlund C, Rådegran G. Immunosuppressive therapies after heart transplantation — The balance between under- and over-immunosuppression. *Transplantation Reviews* 2015; 29: 181–9.
4. Moini M. Review on immunosuppression in liver transplantation. *World Journal of Hepatology* 2015; 7: 1355.
5. Orlando G, Soker S, Wood K. Operational tolerance after liver transplantation. *Journal of Hepatology* 2009; 50: 1247–57.
6. Sánchez-Fueyo A. Hot-topic debate on tolerance: immunosuppression withdrawal. *Liver Transpl* 2011; 17 Suppl 3: S69–73.
7. Benítez C, Londoño M-CC, Miquel R, *et al.* Prospective multicenter clinical trial of immunosuppressive drug withdrawal in stable adult liver transplant recipients. *Hepatology* 2013; 58: 1824–35.
8. Singh AK, McGuirk JP. Allogeneic Stem Cell Transplantation: A Historical and Scientific Overview. *Cancer Res* 2016.
9. Fabricius WA, Ramanathan M. Review on Haploidentical Hematopoietic Cell Transplantation in Patients with Hematologic Malignancies. *Adv Hematol* 2016; 2016: 5726132.
10. Gyurkocza B, Rezvani A, Storb RF. Allogeneic hematopoietic cell transplantation: the state of the art. *Expert Rev Hematol* 2010; 3: 285–99.
11. Shlomchik WD. Graft-versus-host disease. *Nat Rev Immunol* 2007; 7: 340–52.
12. Garnett, Apperley, Pavlu. Treatment and management of graft-versus-host disease: improving response and survival. *Therapeutic Advances in Hematology* 2013; 4: 366–78.
13. Blazar B, Murphy W, Abedi M. Advances in graft-versus-host disease biology and therapy. *Nature Reviews Immunology* 2012; 12: 443–58.
14. Martin P, Rizzo D, Wingard J, *et al.* First- and Second-Line Systemic Treatment of Acute Graft-versus-Host Disease: Recommendations of the American Society of Blood and Marrow Transplantation. *Biology of Blood and Marrow Transplantation* 2012; 18: 1150–63.
15. Flowers, Martin. How we treat chronic graft-versus-host disease. *Blood* 2015; 125: 606–15.

16. Edinger M. Regulatory T cells for the prevention of graft- versus -host disease: Professionals defeat amateurs. *European Journal of Immunology* 2009; 39: 2966–8.
17. Juvet, Whatcott, Bushell, Wood. Harnessing Regulatory T Cells for Clinical Use in Transplantation: The End of the Beginning. *American Journal of Transplantation* 2014; 14: 750–63.
18. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995; 155: 1151–64.
19. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; 299: 1057–61.
20. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003; 4: 330–6.
21. Edinger M, Hoffmann P, Ermann J, *et al.* CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med* 2003; 9: 1144–50.
22. Jones SC, Murphy GF, Korngold R. Post-hematopoietic cell transplantation control of graft-versus-host disease by donor CD425 T cells to allow an effective graft-versus-leukemia response. *Biol Blood Marrow Transplant* 2003; 9: 243–56.
23. Trenado A, Sudres M, Tang Q, *et al.* Ex vivo-expanded CD4+CD25+ immunoregulatory T cells prevent graft-versus-host-disease by inhibiting activation/differentiation of pathogenic T cells. *J Immunol* 2006; 176: 1266–73.
24. Nadig SN, Wieckiewicz J, Wu DC, *et al.* In vivo prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells. *Nat Med* 2010; 16: 809–13.
25. Xiao F, Ma L, Zhao M, *et al.* Ex vivo expanded human regulatory T cells delay islet allograft rejection via inhibiting islet-derived monocyte chemoattractant protein-1 production in CD34+ stem cells-reconstituted NOD-scid IL2rynull mice. *PLoS ONE* 2014; 9: e90387.
26. 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.
27. Sagoo P, Lombardi G, Lechler RI. Relevance of regulatory T cell promotion of donor-specific tolerance in solid organ transplantation. *Front Immunol* 2012; 3: 184.
28. Jiang S, Tsang J, Game DS, Stevenson S, Lombardi G, Lechler RI. Generation and expansion of human CD4+ CD25+ regulatory T cells with indirect allospecificity: Potential reagents to promote donor-specific transplantation tolerance. *Transplantation* 2006; 82: 1738–43.
29. Trzonkowski P, Bieniaszewska M, Juścińska J, *et al.* First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127- T regulatory cells. *Clin Immunol* 2009; 133: 22–6.
30. Edozie FC, Nova-Lamperti EA, Povoleri GA, *et al.* Regulatory T-cell therapy in the induction of transplant tolerance: the issue of subpopulations. *Transplantation* 2014; 98: 370–9.
31. Guillonneau C, Picarda E, Anegon I. CD8+ regulatory T cells in solid organ transplantation. *Current Opinion in Organ Transplantation* 2010; 15: 751–6.
32. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+)/CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 2001; 193: 1285–94.
33. Weiss JM, Bilate AM, Gobert M, *et al.* Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. *J Exp Med* 2012; 209: 1723–42, S1.

34. Yadav M, Louvet C, Davini D, *et al.* Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. *J Exp Med* 2012; 209: 1713–22, S1–19.
35. Milpied P, Renand A, Bruneau J, *et al.* Neuropilin1 is not a marker of human Foxp3 Treg. *European Journal of Immunology* 2009; 39: 1466–71.
36. Toker A, Engelbert D, Garg G, *et al.* Active demethylation of the Foxp3 locus leads to the generation of stable regulatory T cells within the thymus. *J Immunol* 2013; 190: 3180–8.
37. Liu W, Putnam AL, Xu-Yu Z, *et al.* CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 2006; 203: 1701–11.
38. Miyara M, Yoshioka Y, Kitoh A, *et al.* Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* 2009; 30: 899–911.
39. Weiner HL. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol Rev* 2001; 182: 207–14.
40. Roncarolo MG, Gregori S, Bacchetta R, Battaglia M. Tr1 cells and the counter-regulation of immunity: natural mechanisms and therapeutic applications. *Curr Top Microbiol Immunol* 2014; 380: 39–68.
41. 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.
42. Kanamori M, Nakatsukasa H, Okada M, Lu Q, Yoshimura A. Induced Regulatory T Cells: Their Development, Stability, and Applications. *Trends Immunol* 2016.
43. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 1998; 188: 287–96.
44. Vignali D, Collison L, Workman C. How regulatory T cells work. *Nature Reviews Immunology* 2008; 8: 523–32.
45. Antonioli L, Pacher P, Vizi S, Haskó G. CD39 and CD73 in immunity and inflammation. *Trends in Molecular Medicine* 2013; 19: 355–67.
46. Chaudhry A, Samstein RM, Treuting P, *et al.* Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity* 2011; 34: 566–78.
47. Sawant D, Hamilton K, Vignali D. Interleukin-35: Expanding Its Job Profile. *Journal of Interferon & Cytokine Research* 2015; 35: 499–512.
48. Wan Y, Flavell R. “Yin–Yang” functions of transforming growth factor- β and T regulatory cells in immune regulation. *Immunological Reviews* 2007; 220: 199–213.
49. Qureshi OS, Zheng Y, Nakamura K, *et al.* Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* 2011; 332: 600–3.
50. Smyth LA, Ratnasothy K, Tsang JY, *et al.* CD73 expression on extracellular vesicles derived from CD4+ CD25+ Foxp3+ T cells contributes to their regulatory function. *Eur J Immunol* 2013; 43: 2430–40.
51. Okoye IS, Coomes SM, Pelly VS, *et al.* MicroRNA-containing T-regulatory-cell-derived exosomes suppress pathogenic T helper 1 cells. *Immunity* 2014; 41: 89–103.
52. Yu X, Huang C, Song B, *et al.* CD4+CD25+ regulatory T cells-derived exosomes prolonged kidney allograft survival in a rat model. *Cellular Immunology* 2013; 285: 62–8.
53. Hoffmann P, Boeld T, Eder R, *et al.* Isolation of CD4+CD25+ Regulatory T Cells for Clinical Trials. *Biology of Blood and Marrow Transplantation* 2006; 12: 267–74.
54. Di Ianni M, Del Papa B, Zei T, *et al.* T regulatory cell separation for clinical application. *Transfus Apher Sci* 2012; 47: 213–6.

55. Safinia N, Vaikunthanathan T, Fraser H, *et al.* Successful expansion of functional and stable regulatory T cells for immunotherapy in liver transplantation. *Oncotarget* 2016; 7: 7563–77.
56. Afzali B, Edozie FC, Fazekasova H, *et al.* Comparison of regulatory T cells in hemodialysis patients and healthy controls: implications for cell therapy in transplantation. *Clin J Am Soc Nephrol* 2013; 8: 1396–405.
57. Brunstein CG, Miller JS, Cao Q, *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.
58. 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.
59. Patel P, Mahmud D, Park Y, Yoshinaga K, Mahmud N, Rondelli D. Clinical grade isolation of regulatory T cells from G-CSF mobilized peripheral blood improves with initial depletion of monocytes. *Am J Blood Res* 2015; 5: 79–85.
60. Bluestone JA, Buckner JH, Fitch M, *et al.* Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Sci Transl Med* 2015; 7: 315ra189.
61. Tang Q, Lee K. Regulatory T-cell therapy for transplantation. *Current Opinion in Organ Transplantation* 2012; 17: 349–54.
62. Golovina TN, Mikheeva T, Brusko TM, Blazar BR, Bluestone JA, Riley JL. Retinoic acid and rapamycin differentially affect and synergistically promote the ex vivo expansion of natural human T regulatory cells. *PLoS ONE* 2011; 6: e15868.
63. Scottà C, Esposito M, Fazekasova H, *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.
64. Segundo D, Ruiz J, 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: 550–7.
65. Thomson AW, Turnquist HRR, Raimondi G. Immunoregulatory functions of mTOR inhibition. *Nat Rev Immunol* 2009; 9: 324–37.
66. Battaglia M, Stabilini A, Migliavacca B, Horejs-Hoeck J, Kaupper T, Roncarolo M-GG. 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.
67. Elias KM, Laurence A, Davidson TS, *et al.* Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. *Blood* 2008; 111: 1013–20.
68. Mucida D, Pino-Lagos K, Kim G, *et al.* Retinoic acid can directly promote TGF-beta-mediated Foxp3(+) Treg cell conversion of naive T cells. *Immunity* 2009; 30: 471–2; author reply 472–3.
69. Putnam AL, Safinia N, Medvec A, *et al.* Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation. *Am J Transplant* 2013; 13: 3010–20.
70. Martelli MF, Di Ianni M, Ruggeri L, *et al.* HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse. *Blood* 2014; 124: 638–44.
71. Theil A, Tuve S, Oelschlägel U, *et al.* Adoptive transfer of allogeneic regulatory T cells into patients with chronic graft-versus-host disease. *Cytotherapy* 2015; 17: 473–86.
72. Bacchetta R, Lucarelli B, Sartirana C, *et al.* Immunological Outcome in Haploidentical-HSC Transplanted Patients Treated with IL-10-Anergized Donor T Cells. *Front Immunol* 2014; 5: 16.

73. Geissler E. The ONE Study compares cell therapy products in organ transplantation: introduction to a review series on suppressive monocyte-derived cells. *Transplantation Research* 2012; 1: 10.
74. Todo S, Yamashita K, Goto R, *et al.* A pilot study of operational tolerance with a regulatory T-cell-based cell therapy in living donor liver transplantation. *Hepatology* 2016; 64: 632–43.
75. Dijke, Hoeppli, Ellis, *et al.* Discarded Human Thymus Is a Novel Source of Stable and Long-Lived Therapeutic Regulatory T Cells. *Am J Transplant* 2016; 16: 58–71.
76. Gu J, Ni X, Pan X, *et al.* Human CD39hi regulatory T cells present stronger stability and function under inflammatory conditions. *Cellular & Molecular Immunology* 2016.
77. Fuhrman, Yeh W-I, Seay, *et al.* Divergent Phenotypes of Human Regulatory T Cells Expressing the Receptors TIGIT and CD226. *The Journal of Immunology* 2015; 195: 145–55.
78. Safinia N, Scotta C, Vaikunthanathan T, Lechler RI, Lombardi G. Regulatory T Cells: Serious Contenders in the Promise for Immunological Tolerance in Transplantation. *Front Immunol* 2015; 6: 438.
79. Waldmann H. Tolerance can be infectious. *Nature Immunology* 2008; 9: 1001–3.
80. Jonuleit H, Schmitt E, Kakirman H, Stassen M, Knop J, Enk A. Infectious Tolerance. *The Journal of Experimental Medicine* 2002; 196: 255–60.

Ongoing clinical trials with Tregs in transplantation

Study ID	Phase	Product	Indication	Status
HSCT				
NCT01903473	II	Fresh tTregs	Steroid-Refractory cGvHD	Recruiting
NCT01911039	I	Fresh tTregs	Steroid Dependent/Refractory cGvHD	Recruiting
NCT00602693	I	Umbilical Cord Blood Tregs	GvHD Prevention	Ongoing but not recruiting
NCT02749084	I	Fresh tTregs	Severe Refractory cGvHD	Recruiting
NCT02526329	I	Fresh tTregs	aGvHD	Ongoing but not recruiting
NCT01937468	I	Fresh tTregs	Steroid-Refractory cGvHD	Recruiting
NCT01660607	I/II	Fresh tTregs	GvHD Prevention	Recruiting
NCT02385019	I/II	Fresh tTregs	Steroid-Refractory cGvHD	Recruiting
NCT01634217	I	Induced Tregs	Non-Myeloablative HLA Identical Sibling Donor	Recruiting
NCT01795573	I	Donor-alloantigen-reactive Tregs	aGvHD Prevention	Recruiting
SOT				
NCT02145325		Polyclonally Expanded tTregs	Living Donor Kidney transplant	Ongoing but not recruiting
NCT02129881	I/II	Polyclonally Expanded tTregs	Living donor kidney transplant	Recruiting
NCT02371434	I/II	Polyclonally Expanded tTregs	Living donor kidney transplant	Recruiting
NCT02244801	I/II	Donor-alloantigen-reactive tTregs	Living donor kidney transplant	Recruiting
NCT02091232	I/II	Belatacept-conditioned tTregs	Living donor kidney transplant	Recruiting
NCT02166177	I	Polyclonally Expanded tTregs	Liver transplant	Recruiting
NCT02188719	I	Donor-alloantigen-Reactive Tregs	Liver transplant	Recruiting
NCT02088931	I	Polyclonally Expanded tTregs	Living donor kidney transplant	Recruiting
NCT02474199	I	Donor-alloantigen-Reactive Tregs	CNI reduction in liver transplant	Not yet recruiting
NCT02711826	I	Donor-alloantigen-Reactive Tregs	Subclinical Inflammation in Kidney Transplantation	Recruiting
NCT01624077	I	Induced Tregs	Liver transplant	Ongoing but not recruiting

Haematopoietic stem cell transplantation (HSCT), Solid organ transplantation (SOT), acute Graft versus Host Disease (aGvHD), chronic Graft versus Host Disease (cGvHD), Calcineurin inhibitor (CNI).