Transplant International

REVIEW

Regulatory T-cell therapy in liver transplantation

Gavin P. Whitehouse[1], Andrew Hope[2] & Alberto Sanchez-Fueyo[1]

1 Division of Transplantation Immunology and Mucosal Biology, Institute of Liver Studies, Medical Research Council Centre for Transplantation, Faculty of Life Sciences and Medicine, King's College London, London, UK
2 CRF GMP Unit, NIHR Biomedical Research Centre at Guy's and St Thomas' NHS Foundation Trust and King's College London, London, UK

Correspondence
Professor Alberto Sanchez-Fueyo, James Black Centre, King's College London, Denmark Hill Campus, SE5 9NU London, UK.
Tel.: +44 (0)20 7848 5884;
fax: +44 (0)20 7848 5084;
e-mail: Sanchez_fueyo@kcl.ac.uk

SUMMARY

Modern immunosuppression drug regimens have produced excellent short-term survival after liver transplantation but it is generally accepted that the side effects of these medications remain a significant contributing factor for less satisfactory long term outcomes. The liver has unique tolerogenic properties as evidenced by the higher rates of operational tolerance seen in liver transplant recipients compared to other solid organ transplants, and therefore, liver transplantation offers an attractive setting in which to study tolerizing therapies. $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs) are crucial for maintenance of self-tolerance and prevention of autoimmune disease and are therefore an appealing potential candidate for use as a tolerizing cell therapy. In this review, we summarize the evidence from drug withdrawal trials of spontaneous operational tolerance in liver transplantation, the unique immunology of the hepatic microenvironment, the evidence for the use of $CD4^+CD25^+FOXP3^+$ regulatory T cells as a tolerance inducing therapy in liver transplantation and the challenges in producing clinical grade Treg cell products.

Transplant International 2017; 30: 776–784

Key words
immunosuppression, liver transplantation, tolerance, Tregs

Received: 7 February 2017; Revision requested: 27 March 2017; Accepted: 7 June 2017

Introduction

The liver has long been recognized to hold unique tolerogenic properties, and consequently, clinical liver transplantation has been an attractive setting in which to study transplantation immunology and more recently tolerance inducing strategies in humans.

A number of regulatory immune cell populations are under investigation as potential immunotherapies, but in this review, we will focus on $CD4^+CD25^+FOXP3^+$ regulatory T-cell (Treg) therapy and provide a brief introduction to the early evidence for tolerance in liver transplantation, the evidence for the role of Tregs in transplantation tolerance and the clinical trials, which have shown proof of principle for the efficacy of Treg therapy in this setting.

Immunosuppression and side effects

Short-term survival rates following the first liver transplant in 1963 progressively improved in the following decades through advances in surgical techniques, and a further leap was observed with the introduction of calcineurin inhibitor (CNI) immunosuppression in the early 1980s, leading to the 1-year survival rates of 80–85% that are routinely seen in transplant centres around the world today [1]. However, over the next 3 decades, it has become evident that the improvement in short-term survival has not translated into medium/long-term survival and information obtained from a UK data set of 2702 adult liver transplant recipients found these patients lost on average 7 life years compared to an equivalent non-transplant population [2]. In a study of

doi:10.1111/tri.12998

299 patients analysing deaths following liver transplantation in those recipients surviving more than 3 years, 58% of late deaths were due to non-hepatic causes with the majority attributed to malignancies and cardiovascular disease [3]. Over the longer term, chronic renal failure is also a significant cause of morbidity and mortality in this population, with rates of severe renal dysfunction occurring in up to 18.1% of those recipients surviving more than 13 years [4]. The fact that non-hepatic causes of mortality predominate in recipients more than 3 years post-transplant has led to the general acceptance that CNI's and other immunosuppressive drugs significantly contribute to this mortality and has steered focus to the investigation of novel immunosuppression minimization strategies, in particular those potentially leading to the complete withdrawal of immunosuppression [3,5,6].

Spontaneous operational tolerance and immunosuppression withdrawal trials

Operational tolerance is defined as stable graft function in a recipient off immunosuppressant drugs and in whom no clinically significant detrimental immune response or immune defects are detected [7–10]. In the absence of robust biomarkers of tolerance, the only reliable way of confirming the establishment of tolerance is by intentionally withdrawing immunosuppression. When exploring the use of tolerizing cell therapies in liver transplantation, it is important to consider the occurrence and prevalence of spontaneous operational tolerance, which has long been recognized in this setting and occurs with greater frequency than in the context of any other solid organ transplant. Therefore, it is important to establish the factors favouring spontaneous operational tolerance when utilizing liver transplant recipient populations, in which this phenomenon may occur frequently and potentially be a confounding factor for end points of therapeutic success.

Since early anecdotal reports of spontaneous tolerance in non-compliant patients and the subsequent retrospective analyses and single-centre trials [11–19], several prospective multicentre immunosuppression withdrawals trials have helped to establish the rates of tolerance, in both the paediatric and adult liver transplant populations. Furthermore, they have clarified the patient and clinical factors that determine the likelihood of spontaneous tolerance. The first prospective multicentre trials of immunosuppression withdrawal in the paediatric population by Feng *et al.* in 2012 and in adult liver transplant recipients by Benitez *et al.* in 2013 reported rates of operational tolerance of 60% and 41%, respectively, in patients more than 3 years (adult) or 4 years (paediatric) post-transplantation, without a history of autoimmune liver disease, and not more than minimal inflammatory infiltrates in their liver biopsies [20,21]. Both these studies therefore utilized a highly selected group of liver transplant recipients and in those medically supervised drug withdrawal trials with less selective inclusion criteria, lower rates of spontaneous operational tolerance of between 5.6% and 38% were observed [11,12,14–19,22,23]. These trials identified that the time elapsed from transplantation was the most significant factor associated with the frequency of spontaneous tolerance. Patients underwent weaning according to strict protocols, and in this setting when rejection did occur, it was usually easily treated, and the overall strategy was considered safe. While these prospective drug withdrawal trials were not designed or powered to show a reduction in the long term effects of IS, longer term follow-up data derived from previous single-centre studies, suggest that the minimization of IS exposure in tolerant patients could reduce deterioration in renal function and metabolic parameters [5,24,25].

Unique characteristics of the hepatic immunological microenvironment

Most notably, clinical human liver transplantation is distinguished from other solid organ transplants in that it does not require preconditioning induction immunosuppression regimens in the face of a positive crossmatch, does not require HLA matching and allows the safe application of lower blood levels of immunosuppression than both those employed in other solid organ transplant recipients and those recommended by pharmaceutical manufacturers [26,27].

The liver is continuously presented with a large volume of foreign antigens via the portal venous system and selectively reacts to blood-borne pathogens, while preventing excessive immunological activation against an array of nonpathogenic antigens derived from the translocation of food and bacterial degradation products [28,29]. A prime example is the response to chronic exposure to bacteria-derived lipopolysaccharide (LPS) recognized by Toll-like receptors, in which the normal proinflammatory response through activation of intracellular signalling cascades leads to anti-inflammatory responses and the production of inhibitory cytokines, for example IL-10 [30,31].

Following allogeneic organ transplantation, proinflammatory responses are triggered by the recognizing

of donor MHC molecules by recipient T cells and animal models demonstrate this arises whether the organ is eventually rejected or tolerance occurs. However, in the case of tolerance, the inflammation is short-lived through the inactivation/deletion of graft infiltrating lymphocytes [32–34]. In addition, the liver can act as a secondary lymphoid organ, activating and retaining $CD4^+$ and $CD8^+$ naïve T cells following antigen presentation by an array of conventional and nonconventional APCs including dendritic cells, macrophages, B lymphocytes, stellate cells (HSC), hepatocytes and liver sinusoidal endothelial cells [35–39]. In particular, when hepatocytes act as APCs, the result is transient clonal expansion of naive $CD8^+$ cells but due to a lack of costimulation this is followed by apoptosis [39–42]. In addition, *in vivo* murine models show an increase in the frequency of Tregs following the adoptive transfer of HSC suggesting that APCs drive differentiation to an immunosuppressive Treg phenotype [43].

The description of a thymus-derived suppressor $CD4^+$ T-cell population by Hall *et al.* in 1990 was further characterized in 1995 by Sakaguchi *et al.* [44,45] by the expression of the IL2 receptor α (CD25) that was critical for self-tolerance and avoidance of the development of autoimmune disease. Further characterization proved the FOXP3 transcription factor was required for the generation, maintenance and function of Tregs and has subsequently become the most widely used, albeit not entirely specific, marker for this lymphocyte subpopulation [46,47].

Tregs are capable of suppressing $CD4^+$ and $CD8^+$ T-cell immune responses through a number of mechanisms including inhibitory cytokine production, for example IL-10 and TGF β, cytolysis, induction of effector T-cell apoptosis and inhibition of APCs [48]. The significant role for Tregs in preventing rejection and inducing tolerance has been demonstrated in rodent models of liver transplantation [49–51].

Several studies in human liver transplantation have described an increased frequency of Tregs in the peripheral blood of operational tolerance recipients [52,53]. Dynamic analysis during weaning of immunosuppression showed a significant increase in the relative frequency and absolute Tregs numbers in patients who proved tolerant compared to those that rejected [16]. These observations need to be interpreted with caution given the confounding effects of CNIs, which decrease the number of circulating Tregs and are not always adequately accounted for. Furthermore, Tregs are known to migrate to sites of inflammation, which results in their accumulation in the liver during episodes of liver allograft rejection (where their frequency as compared to that of effector T cells tends to be higher than in noninflamed grafts), with a corresponding reduction in their peripheral blood numbers [16,54–56]. In this regard, data derived from sequential liver biopsies performed in patients undergoing IS withdrawal has been more informative, as it indicates that tolerance is associated with a transient intragraft inflammatory response that includes enrichment in Tregs [16,54,55], which closely resembles what has been described in experimental animal models.

Proinflammatory immune responses are important for protection from viral and bacterial pathogens and there is evidence to suggest that the immunosuppressive effects of Tregs could be potentially detrimental in acute and chronic infections. Mechanisms include limiting $CD8^+$ viral destruction, $CD8^+$ T-cell proliferation and recruitment of virus-specific $CD8^+$ T cells, therefore creating an immunological environment favouring persistent viral infection [57–61].

In the context of bacterial infections, Tregs have also been shown in humans to expand in line with effector T cells in mycobacterial tuberculosis and in mouse models, Treg depletion led to a lower bacterial burden, and co-transfer of Tregs led to an increase in bacterial burden in the lungs [62–65].

The unquestionable pivotal role of Tregs in the establishment of tolerance in animal models as described above, the circumstantial evidence indicating that Tregs are likely to be involved in the regulation of intrahepatic inflammatory responses in human liver transplant recipients, and the fact that it is possible to generate large numbers of human Tregs both *ex vivo* and *in vivo*, makes them ideal candidates for use as a potential tolerizing cell therapy. Tregs therefore offer the promise of targeted allograft tolerance in contrast to the indiscriminate inhibition of alloimmune responses seen with current immunosuppression medications.

Ex vivo expanded regulatory T cells

In the context of allogeneic solid organ transplantation, it is recognized that the excess of alloreactive effector T cells overwhelms the relatively smaller number of functional regulatory T cells in favouring rejection and graft destruction [66]. In animal models, it is possible to suppress alloreactive effector T-cell responses and to induce tolerance by providing an excess of Tregs. Thus, utilizing Tregs as a strategy to induce tolerance relies on shifting the balance in favour of Tregs, either through diminishing the number or function of effector T cells

or increasing the relative frequency or function of Tregs or both [67–69]. For this review, we will focus on the following therapeutic approaches to achieve a favourable effector T-cell/Treg ratio in humans:

1 Delivery of *ex vivo* expanded Treg cellular therapy
2 The pharmacological *in vivo* induction/expansion of Tregs.

In animal transplant models, a ratio of Tregs to effector T cells of 1:2 has been shown to be required for prevention of rejection [67,70–72]. It has been estimated that based on an 70-kg human, collection of the maximum number of 8×10^9 lymphocytes by a single leukapheresis procedure would only provide 0.2×10^9 Tregs. Even employing therapeutic lymphodepletion therapy such as the use of Thymoglobulin, which has less effect on depleting Tregs than effector T cells [73,74], it has been estimated that infusion of this number of Tregs would only provide a 3.7% increase to 16.7% of $CD4^+$ T cells [75]. Thus, *ex vivo* expansion of purified Tregs is required to achieve the predicted $49–79 \times 10^9$ cells to reach a therapeutic 33–55% of the $CD4^+$ population [75]. These estimations are however made on the use of polyclonal Tregs and up to 10 times less may be required if alloantigen-reactive Tregs, which are more potent than polyclonal Tregs are used [76–78].

The production of clinical grade Tregs is challenging and will be reviewed below. Tregs can be reliably identified by a panel of cell surface markers with cells expressing CD4, high levels of CD25 (the high affinity IL-2 receptor α chain of the IL-2 receptor) and low levels of the CD127 (IL-7 receptor). However, utilizing FOXP3, the most accurate phenotypic marker of Tregs is limited as it is an intracellular protein and therefore cannot be used for the purification of intact cells. Furthermore, transplant recipients and patients with chronic liver disease tend to be lymphopenic and may have reduced Treg numbers and/or function, which may compromise cell isolation and expansion. Despite this, protocols for the successful production of clinical grade polyclonal and antigen-specific Tregs have been reported [79,80].

Preclinical animal models demonstrated the infusion of *ex vivo* expanded recipient Tregs can produce indefinite acceptance of heart allografts in murine models and the delay in islet allograft rejection in a humanized murine model [77,81]. Early trials in human subjects have largely utilized the setting of bone marrow transplantation and type 1 diabetes, which have provided limited data in terms of efficacy but reassuringly a good safety profile [82–85].

The tolerance inducing efficacy of a regulatory T-cell therapy has recently been shown in living donor liver transplantation utilizing an *ex vivo* generated Treg-enriched cell product in 10 consecutive adult patients [86]. In this Japanese study, seven of the recipients were male with a median age of 56 years old and with a median meld score of 15. The indications for transplant were NASH, HCC (NASH, HBV), alcohol, HCV, PBC and PSC. All patients underwent splenectomy at the time of transplantation and received methylprednisolone, mycophenolate and CNI immunosuppression. Methylprednisolone and mycophenolate were weaned and stopped within the first postoperative month. A single dose of cyclophosphamide (40 mg/kg) was given on day 5, and a single infusion of the cell product was then given on day 13 post-transplantation. Donor and recipient lymphocytes were procured by leukapheresis prior to transplantation. Recipient lymphocytes and splenocytes were co-cultured with irradiated donor lymphocytes in the presence of costimulatory blockade (anti-CD80/CD86) to enrich for anergic and/or regulatory donor-specific T cells. The T-cell-enriched cell product was a mixture of $CD4^+$, $CD8^+$ lymphocytes accounting for 58.6% and 16.9% of infused cells, respectively, with a varying composition of monocytes, NK cells, B cells, myeloid DCs, plasmacytoid DCs and granulocytes. The number of $CD4^+CD25^+FOXP3^+$ T cells infused varied between patients ranging from 0.23×10^6/kg to 6.37×10^6/kg and with a mean of 3.39×10^6/kg (± 2.12 SD) [86].

Seven patients with nonimmunological liver diseases successfully achieved uneventful weaning and completed cessation of IS. At the time of the publication of the study, these patients had been off immunosuppression between 16 and 33 months. Significantly, the doses of cells infused were much lower than those estimated to be required on the basis of animal studies. This study therefore provides a proof of principle of the efficacy of Treg therapy in liver transplantation. Furthermore, it confirms previous observations made in the setting of drug withdrawal trials indicating that those patients with a history of immune-mediated liver disease aetiology are very unlikely to develop allograft tolerance.

Treg therapy trials in progress

Several clinical trials are currently in progress around the world utilizing a number of Treg therapies in liver transplantation (Table 1). An *ex vivo* expanded polyclonal regulatory T-cell therapy is being utilized in the ThRIL trial at King's College Hospital, UK [clinical trials.gov NCT02166177], the DeLTA and ARTEMIS trials at University of California, San Francisco, USA, are

using donor-alloantigen-reactive regulatory T cells (darTregs) [NCT02188719] NCT02474199, and a further collection of trial designs at Nanjing Medical University, China, is utilizing donor-antigen-specific Tregs in patients at early and late time points following liver transplantation [NCT01624077].

Clinical Grade (GMP) Treg production challenges and future perspectives

Many clinical trials in solid organ transplantation have utilized a living donation transplant setting as this provides access to prospective identification of the donor and access to donor tissue ahead of a planned procedure. As such the cellular product can be manufactured ahead of the transplant operation and infusion of the cell product in the early post-transplant period. However, the overwhelming majority of liver transplant programmes across the world utilize allografts from deceased donors, which only provides donor identification and therefore access to donor tissue a few hours prior to the procedure. In order for future *ex vivo* expanded cell therapies to have universal and wide spread application in liver transplantation, cell production processes will need to obviate the requirement for access to donor tissue or utilize immediate peri-transplant procurement of both donor and recipient cellular starting materials, which will inevitably lead to infusion of the cell product at relatively later time points.

The current (2017) manufacturing procedures for Treg Investigational Medicinal Products are resource intensive. Expansion times can take up to 6 weeks in clean-room facilities and often require batch segregation when the patient from which the starting materials were procured is serologically positive for viral markers. The challenge is made bigger because there are so few licensed sites with the capability to make Tregs under GMP conditions. This will be a major factor in the adoption of Tregs therapy as part of standard care for transplant recipients should it prove to be safe and beneficial. These issues could be addressed with enhanced expansion procedures. Current static culture systems could be replaced with bioreactors using, for example, rocking motion to reduce expansion times and increase scale, and thus shortening clean-room occupancy times.

Clean-room occupancy also comes with a cost; the most costly part of Treg production is the raw materials. Upstream processing, selection of target cells using magnetic or fluorescent antibodies, uses highly specialized reagents and sterile tubing sets. This, coupled with

Table 1. Summary of clinical trials utilizing *ex vivo* expanded Tregs in liver transplantation.

Clinical trials.gov identifier	Liver transplant setting	Country	Study design	Age of participants (years)	Target Treg dose	Multiple Treg injections	Immunosuppression withdrawal
NCT01624077	Chronic rejection in liver transplantation	China	Open label single group assignment	10–60	Donor-alloantigen-reactive Tregs 1×10^6 cells/kg	Yes	Yes
NCT02474199	Tolerance induction in living donor liver transplantation	USA	Open label single group assignment	18–70	Donor-alloantigen-reactive Tregs (darTregs) 300–500 $\times 10^6$ cells	No	Yes
NCT02166177	Tolerance induction in deceased donor liver transplantation	UK	Nonrandomized, Open label single group assignment	18–70	Autologous polyclonal Tregs Cohort 1: 0.5–1.0 $\times 10^6$ cells/kg Cohort 2: 3.0–4.5 $\times 10^6$ cells/kg Cohort 3: 5.0–6.5 $\times 10^6$ cells/kg	No	No
NCT02188719	Tolerance induction in deceased donor liver transplantation	USA	Nonrandomized, open label parallel assignment	21–70	Donor-Alloantigen-Reactive Regulatory Tregs Cohort 1: No Tregs Cohort 2: 25–60 $\times 10^6$ cells Cohort 3: 100–240 $\times 10^6$ cells Cohort 4: 400–960 $\times 10^6$ cells	No	Yes

cell culture reagents and cytokines required, typically amounts to a current cost of approximately £30,000 per batch. Wider adoption of Treg therapy could reduce these costs with the economies of scale in supply of consumables, but it remains the responsibility of the manufacturers of cell therapies to address cost-of-goods so that the financial burden on healthcare providers is minimized. This issue is particularly important for Treg production, because unlike other cell therapies there is no economy possible through the allogeneic-production route; each autologous batch is unique, and the demands are for scale-out, not the more easily cost-efficient scale-up.

There is a drive to develop Treg production procedures that will yield antigen-specific suppressive cells, in order to minimize off-target immune suppression. These procedures are not well defined currently, and there may be a need to introduce antigen presenting cells (APCs) into a Treg-culture system that will be both effective and cause no contamination of the product downstream. One approach could be to render the functional APCs unable to divide with irradiation. Alternatively, downstream purification procedures might be adopted, like antibody-mediated methods used in upstream processing; either method is likely to be costly.

The purity of Treg products has been difficult to control, due partly to contaminating cells selected in upstream processes. Magnetic selection has benefits of scale and throughput, but is crudely binary, and positive selection can occur just once. If the upstream process reduces CD8 cells and selects cells that are CD25-immunoreactive, the resulting culture can start with a low Treg purity. We have used selective culture conditions (e.g. media containing rapamycin) to suppress the growth of contaminating cells, but this has not always been sufficient. A better approach in the future will be to obtain highly pure cells at the outset, and this will require scalable fluorescently activated cell sorting (FACS) in aseptic closed systems, which is currently under development.

Purity is necessary to exclude contaminating (and potentially counterproductive) cells and to enable potency. Methods of determining potency of a Treg product require a better understanding of Treg function. There are numerous modes of action (inhibitory cytokines, cytolysis, metabolic disruption and APC targeting) [87], and not all can be tested. This presents the quality-control analyst with a challenge, as the choice of potency assay might not predict efficacy. And relying on cell-based assays, like suppression of T-effector growth, can yield unpredictable results; the future will require simpler methods for potency assays.

In short, manufacturers of Tregs cell therapy products will supply future markets by shortening expansion times, reducing costs-of-goods, delivering antigen-specificity, enhancing cell purity and controlling potency.

In vivo Treg expansion

Calcineurin inhibitors (CNIs) are the mainstay of immunosuppression in liver transplantation and have proven very effective in reducing the activity of Th1- and Th17-mediated tissue allograft damage. The CNIs impede the activity of effector T-cell function and IL-2 transcription by inhibiting the T-cell receptor translocation of nuclear factor of activated T cells (NFAT) into the nucleus [88–90]. Tregs depend on IL-2 signalling for their development, survival and function and respond to very low levels of IL-2 due to the high expression of the IL-2 Receptor α chain (CD25). It is therefore recognized that CNI therapy also has a undesirable effect on Tregs which are dependent on IL-2 and NFAT to efficiently express FOXP3 and curb the transcription of proinflammatory genes [91–93]. As a result, transplanted patients on CNI therapy exhibit a lower number of Tregs [88,94].

Several clinical trials have demonstrated clinical efficacy of low dose IL-2 in patients with steroid refractory chronic graft versus host disease and HCV related vasculitis by preferentially expanding Tregs [95,96]. The use of IL-2 resulted in an up to 8 times expansion of Tregs without a significant increase in conventional T cells. This provides the promise of expanding the *in vivo* Treg pool without the requirement for expensive and large-scale production facilities required to produce clinical grade Tregs.

The potential beneficial effects of regulatory T cells are to be investigated in the setting of liver transplantation in the LITE Trial (NCT02949492) at King's College London, which is due to start recruiting early 2017.

Conclusion

Clinical immunosuppression withdrawal trials demonstrate the spontaneous achievement of allograft tolerance in the setting of liver transplantation due to the unique hepatic immunological environment. However, spontaneous operational tolerance occurs late after transplantation, and in order for the potential benefits of immunosuppression withdrawal to be realized, tolerance induction strategies will be required to promote tolerance at earlier time points. Regulatory T cells play a crucial role in tolerance and the first trial utilizing an *ex vivo* expanded regulatory T-cell therapy in the setting of liver transplantation has shown therapeutic promise.

The manufacture of clinical grade regulatory T-cell products currently requires specific and expensive infrastructure, and trials in other transplant settings offer the advantage of pharmacological *in vivo* expansion of the Treg pool with the prospect of wider and economically more attractive application.

Funding

The authors' research is supported by the Medical Research Council Centre for Transplantation (reference J006742/1) and by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' National Health Service (NHS) Foundation Trust and King's College London. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

Conflict of interest

No conflict of interests to declare.

REFERENCES

1. Registry TET. European Liver Transplant Registry. Data Analysis Booklet, 2012.
2. Barber K, *et al.* Life expectancy of adult liver allograft recipients in the UK. *Gut* 2007; **56**: 279.
3. Pruthi J, *et al.* Analysis of causes of death in liver transplant recipients who survived more than 3 years. *Liver Transpl* 2001; **7**: 811.
4. Gonwa TA, *et al.* End-stage renal disease (ESRD) after orthotopic liver transplantation (OLTX) using calcineurin-based immunotherapy: risk of development and treatment. *Transplantation* 2001; **72**: 1934.
5. Pons JA, *et al.* Immunosuppression withdrawal improves long-term metabolic parameters, cardiovascular risk factors and renal function in liver transplant patients. *Clin Transplant* 2009; **23**: 329.
6. Boudjema K, *et al.* Reduced-dose tacrolimus with mycophenolate mofetil vs. standard-dose tacrolimus in liver transplantation: a randomized study. *Am J Transplant* 2011; **11**: 965.
7. Girlanda R, Kirk AD. Frontiers in nephrology: immune tolerance to allografts in humans. *J Am Soc Nephrol* 2007; **18**: 2242.
8. Lerut J, Sanchez-Fueyo A. An appraisal of tolerance in liver transplantation. *Am J Transplant* 2006; **6**: 1774.
9. Demetris AJ, *et al.* Monitoring of human liver and kidney allograft tolerance: a tissue/histopathology perspective. *Transpl Int* 2009; **22**: 120.
10. Brouard S, *et al.* The natural history of clinical operational tolerance after kidney transplantation through twenty-seven cases. *Am J Transplant* 2012; **12**: 3296.
11. Devlin J, *et al.* Defining the outcome of immunosuppression withdrawal after liver transplantation. *Hepatology* 1998; **27**: 926.
12. Mazariegos GV, *et al.* Weaning of immunosuppression in liver transplant recipients. *Transplantation* 1997; **63**: 243.
13. Ramos HC, *et al.* Weaning of immunosuppression in long-term liver transplant recipients. *Transplantation* 1995; **59**: 212.
14. Tisone G, *et al.* Complete weaning off immunosuppression in HCV liver transplant recipients is feasible and favourably impacts on the progression of disease recurrence. *J Hepatol* 2006; **44**: 702.
15. Pons JA, *et al.* Endothelial cell chimerism does not influence allograft tolerance in liver transplant patients after withdrawal of immunosuppression. *Transplantation* 2003; **75**: 1045.
16. Pons JA, *et al.* FoxP3 in peripheral blood is associated with operational tolerance in liver transplant patients during immunosuppression withdrawal. *Transplantation* 2008; **86**: 1370.
17. Assy N, *et al.* Randomized controlled trial of total immunosuppression withdrawal in liver transplant recipients: role of ursodeoxycholic acid. *Transplantation* 2007; **83**: 1571.
18. Tryphonopoulos P, *et al.* The role of donor bone marrow infusions in withdrawal of immunosuppression in adult liver allotransplantation. *Am J Transplant* 2005; **5**: 608.
19. Takatsuki M, *et al.* Weaning of immunosuppression in living donor liver transplant recipients. *Transplantation* 2001; **72**: 449.
20. Feng S, *et al.* Complete immunosuppression withdrawal and subsequent allograft function among pediatric recipients of parental living donor liver transplants. *JAMA* 2012; **307**: 283.
21. Benitez C, *et al.* Prospective multicenter clinical trial of immunosuppressive drug withdrawal in stable adult liver transplant recipients. *Hepatology* 2013; **58**: 1824.
22. Eason JD, *et al.* Tolerance: is it worth the risk? *Transplantation* 2005; **79**: 1157.
23. Girlanda R, *et al.* Long-term outcome of immunosuppression withdrawal after liver transplantation. *Transplant Proc* 2005; **37**: 1708.
24. Orlando G, *et al.* The Tor Vergata weaning off immunosuppression protocol in stable HCV liver transplant patients: the updated follow up at 78 months. *Transpl Immunol* 2008; **20**: 43.
25. Tryphonopoulos P, *et al.* Long-term follow-up of 23 operational tolerant liver transplant recipients. *Transplantation* 2010; **90**: 1556.
26. Rodriguez-Peralvarez M, *et al.* Early tacrolimus exposure after liver transplantation: relationship with moderate/severe acute rejection and long-term outcome. *J Hepatol* 2013; **58**: 262.
27. Calne RY. Immunological tolerance – the liver effect. *Immunol Rev* 2000; **174**: 280.
28. Jenne CN, Kubes P. Immune surveillance by the liver. *Nat Immunol* 2013; **14**: 996.
29. Thomson AW, Knolle PA. Antigen-presenting cell function in the tolerogenic liver environment. *Nat Rev Immunol* 2010; **10**: 753.
30. John B, Crispe IN. TLR-4 regulates CD8+ T cell trapping in the liver. *J Immunol* 2005; **175**: 1643.
31. Knolle P, *et al.* Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge. *J Hepatol* 1995; **22**: 226.
32. Kamada N. The immunology of experimental liver transplantation in the rat. *Immunology* 1985; **55**: 369.
33. Steger U, *et al.* Exhaustive differentiation of alloreactive CD8+ T cells: critical for determination of graft acceptance or rejection. *Transplantation* 2008; **85**: 1339.
34. Li Y, *et al.* The presence of Foxp3 expressing T cells within grafts of tolerant

human liver transplant recipients. *Transplantation* 2008; **86**: 1837.
35. Bertolino P, *et al.* Antigen-specific primary activation of CD8+ T cells within the liver. *J Immunol* 2001; **166**: 5430.
36. Wuensch SA, Pierce RH, Crispe IN. Local intrahepatic CD8+ T cell activation by a non-self-antigen results in full functional differentiation. *J Immunol* 2006; **177**: 1689.
37. Klein I, Crispe IN. Complete differentiation of CD8+ T cells activated locally within the transplanted liver. *J Exp Med* 2006; **203**: 437.
38. Tay SS, *et al.* Intrahepatic activation of naive CD4+ T cells by liver-resident phagocytic cells. *J Immunol* 2014; **193**: 2087.
39. Bowen DG, *et al.* The site of primary T cell activation is a determinant of the balance between intrahepatic tolerance and immunity. *J Clin Invest* 2004; **114**: 701.
40. Bertolino P, Trescol-Biemont MC, Rabourdin-Combe C. Hepatocytes induce functional activation of naive CD8+ T lymphocytes but fail to promote survival. *Eur J Immunol* 1998; **28**: 221.
41. Herkel J, *et al.* MHC class II-expressing hepatocytes function as antigen-presenting cells and activate specific CD4 T lymphocyutes. *Hepatology* 2003; **37**: 1079.
42. Holz LE, *et al.* Intrahepatic murine CD8 T-cell activation associates with a distinct phenotype leading to Bim-dependent death. *Gastroenterology* 2008; **135**: 989.
43. Jiang G, *et al.* Hepatic stellate cells preferentially expand allogeneic CD4+ CD25+ FoxP3+ regulatory T cells in an IL-2-dependent manner. *Transplantation* 2008; **86**: 1492.
44. Sakaguchi S, *et al.* 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.
45. Hall BM, *et al.* Specific unresponsiveness in rats with prolonged cardiac allograft survival after treatment with cyclosporine. III. Further characterization of the CD4+ suppressor cell and its mechanisms of action. *J Exp Med* 1990; **171**: 141.
46. Mantel PY, *et al.* Molecular mechanisms underlying FOXP3 induction in human T cells. *J Immunol* 2006; **176**: 3593.
47. Walker MR, *et al.* Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+ CD25- T cells. *J Clin Invest* 2003; **112**: 1437.
48. Thompson C, Powrie F. Regulatory T cells. *Curr Opin Pharmacol* 2004; **4**: 408.
49. Li J, *et al.* The dynamic changes of Th17/Treg cytokines in rat liver transplant rejection and tolerance. *Int Immunopharmacol* 2011; **11**: 962.
50. Fujiki M, *et al.* Induced tolerance to rat liver allografts involves the apoptosis of intragraft T cells and the generation of CD4(+)CD25(+)FoxP3(+) T regulatory cells. *Liver Transpl* 2010; **16**: 147.
51. Li W, *et al.* CTLA4 engagement is required for induction of murine liver transplant spontaneous tolerance. *Am J Transplant* 2005; **5**: 978.
52. Martinez-Llordella M, *et al.* Multiparameter immune profiling of operational tolerance in liver transplantation. *Am J Transplant* 2007; **7**: 309.
53. Li Y, *et al.* Analyses of peripheral blood mononuclear cells in operational tolerance after pediatric living donor liver transplantation. *Am J Transplant* 2004; **4**: 2118.
54. Demirkiran A, *et al.* Intrahepatic detection of FOXP3 gene expression after liver transplantation using minimally invasive aspiration biopsy. *Transplantation* 2007; **83**: 819.
55. Taubert R, *et al.* Hepatic infiltrates in operational tolerant patients after liver transplantation show enrichment of regulatory T cells before proinflammatory genes are downregulated. *Am J Transplant* 2016; **16**: 1285.
56. Taubert R, *et al.* Enrichment of regulatory T cells in acutely rejected human liver allografts. *Am J Transplant* 2012; **12**: 3425.
57. Dittmer U, *et al.* Functional impairment of CD8(+) T cells by regulatory T cells during persistent retroviral infection. *Immunity* 2004; **20**: 293.
58. Robertson SJ, *et al.* In vitro suppression of CD8+ T cell function by Friend virus-induced regulatory T cells. *J Immunol* 2006; **176**: 3342.
59. Zelinskyy G, *et al.* Kinetics of CD8+ effector T cell responses and induced CD4+ regulatory T cell responses during Friend retrovirus infection. *Eur J Immunol* 2006; **36**: 2658.
60. Suvas S, *et al.* CD4+ CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses. *J Exp Med* 2003; **198**: 889.
61. Fulton RB, Meyerholz DK, Varga SM. Foxp3+ CD4 regulatory T cells limit pulmonary immunopathology by modulating the CD8 T cell response during respiratory syncytial virus infection. *J Immunol* 2010; **185**: 2382.
62. Guyot-Revol V, *et al.* Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis. *Am J Respir Crit Care Med* 2006; **173**: 803.
63. Chen X, *et al.* CD4(+)CD25(+)FoxP3(+) regulatory T cells suppress Mycobacterium tuberculosis immunity in patients with active disease. *Clin Immunol* 2007; **123**: 50.
64. Kursar M, *et al.* Cutting Edge: regulatory T cells prevent efficient clearance of Mycobacterium tuberculosis. *J Immunol* 2007; **178**: 2661.
65. Scott-Browne JP, *et al.* Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. *J Exp Med* 2007; **204**: 2159.
66. Hamano K, *et al.* Evidence that the continued presence of the organ graft and not peripheral donor microchimerism is essential for maintenance of tolerance to alloantigen in vivo in anti-CD4 treated recipients. *Transplantation* 1996; **62**: 856.
67. Graca L, *et al.* Both CD4(+)CD25(+) and CD4(+)CD25(−) regulatory cells mediate dominant transplantation tolerance. *J Immunol* 2002; **168**: 5558.
68. Feng G, *et al.*, Functional regulatory T cells produced by inhibiting cyclic nucleotide phosphodiesterase type 3 prevent allograft rejection. *Sci Transl Med* 2011; **3**: 83ra40.
69. Feng G, Wood KJ, Bushell A. Interferon-gamma conditioning ex vivo generates CD25+ CD62L+ Foxp3+ regulatory T cells that prevent allograft rejection: potential avenues for cellular therapy. *Transplantation* 2008; **86**: 578.
70. Hara M, *et al.* IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J Immunol* 2001; **166**: 3789.
71. Golshayan D, *et al.* In vitro-expanded donor alloantigen-specific CD4+ CD25+ regulatory T cells promote experimental transplantation tolerance. *Blood* 2007; **109**: 827.
72. Nishimura E, *et al.* Induction of antigen-specific immunologic tolerance by in vivo and in vitro antigen-specific expansion of naturally arising Foxp3+ CD25+ CD4+ regulatory T cells. *Int Immunol* 2004; **16**: 1189.
73. Lopez M, *et al.* A novel mechanism of action for anti-thymocyte globulin: induction of CD4+ CD25+ Foxp3+ regulatory T cells. *J Am Soc Nephrol* 2006; **17**: 2844.
74. Morelon E, *et al.* Preferential increase in memory and regulatory subsets during T-lymphocyte immune reconstitution after Thymoglobulin induction therapy with maintenance sirolimus vs cyclosporine. *Transpl Immunol* 2010; **23**: 53.
75. Tang Q, Lee K. Regulatory T-cell therapy for transplantation: how many

cells do we need? *Curr Opin Organ Transplant* 2012; **17**: 349.
76. Sanchez-Fueyo A, *et al.* Specificity of CD4+ CD25+ regulatory T cell function in alloimmunity. *J Immunol* 2006; **176**: 329.
77. Tsang JY, *et al.* Conferring indirect allospecificity on CD4+ CD25+ Tregs by TCR gene transfer favors transplantation tolerance in mice. *J Clin Invest* 2008; **118**: 3619.
78. Sagoo P, *et al.* 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.
79. Safinia N, *et al.* Successful expansion of functional and stable regulatory T cells for immunotherapy in liver transplantation. *Oncotarget* 2016; **7**: 7563.
80. Putnam AL, *et al.* Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation. *Am J Transplant* 2013; **13**: 3010.
81. Xiao F, *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 IL2rgammanull mice. *PLoS ONE* 2014; **9**: e90387.
82. Trzonkowski P, *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.
83. Di Ianni M, *et al.* Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* 2011; **117**: 3921.
84. Brunstein CG, *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.
85. Marek-Trzonkowska N, *et al.* Administration of CD4+ CD25highCD127- regulatory T cells preserves beta-cell function in type 1 diabetes in children. *Diabetes Care* 2012; **35**: 1817.
86. Todo S, *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.
87. Vignali DAA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* 2008; **8**: 523.
88. Akimova T, *et al.* Differing effects of rapamycin or calcineurin inhibitor on T-regulatory cells in pediatric liver and kidney transplant recipients. *Am J Transplant* 2012; **12**: 3449.
89. Hermann-Kleiter N, Baier G. NFAT pulls the strings during CD4+ T helper cell effector functions. *Blood* 2010; **115**: 2989.
90. Steward-Tharp SM, *et al.* New insights into T cell biology and T cell-directed therapy for autoimmunity, inflammation, and immunosuppression. *Ann N Y Acad Sci* 2010; **1183**: 123.
91. Li X, *et al.* Function of a Foxp3 cis-element in protecting regulatory T cell identity. *Cell* 2014; **158**: 734.
92. Schreiber SL, Crabtree GR. The mechanism of action of cyclosporin A and FK506. *Immunol Today* 1992; **13**: 136.
93. Wu Y, *et al.* FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* 2006; **126**: 375.
94. San Segundo D, *et al.* Reduced numbers of blood natural regulatory T cells in stable liver transplant recipients with high levels of calcineurin inhibitors. *Transplant Proc* 2007; **39**: 2290.
95. Koreth J, *et al.* Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med* 2011; **365**: 2055.
96. Saadoun D, *et al.* Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. *N Engl J Med* 2011; **365**: 2067.