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REVIEW

# Achieving operational tolerance in transplantation: how can lessons from the clinic inform research directions?

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## Summary

Since the first solid organ transplant between the Herrick twins in 1954, transplantation immunology has sought to move away from harmful immunosuppressive regimens towards tolerogenic strategies that promote long-term graft survival. This has required a concerted multinational effort with scientists and clinicians working towards a common goal. Reports of immunosuppression-free kidney and liver allograft recipients have provided the proof-of-principle, but intentional generation of tolerance in clinical transplantation is still only achieved infrequently. Recently, there have been an increasing number of encouraging developments in the field in both experimental and clinical studies. In this article, we review the latest advances in tolerance research and consider possible future barriers and solutions in achieving reliable graft acceptance in the long term.

## Introduction

The rejection of major histocompatibility complex (MHC)-mismatched organs after transplantation necessitates the use of immunosuppressive drugs to prevent allograft rejection. However, such regimens are associated with serious side effects such as the development of life-threatening infections, an increase in the risk of cancer, and nephrotoxicity. These drugs therefore significantly reduce patient quality of life and are associated with poor adherence [1]. This is of clinical significance as nonadherence to immunosuppression is one of the leading causes of graft loss in renal transplant patients on a par with chronic allograft dysfunction and death with a functioning graft [2]. Moreover, although current immunosuppressive therapies are effective in preventing acute rejection with 1-year renal graft survival rates of over 90%, they are less useful against chronic rejection and graft dysfunction [3]. In the UK, 5-year graft survival for renal transplant patients varies between 84% and 91%, with patient survival rates of 88–96%. For liver transplants, patient survival is approximately 87% at 3 years [4]. In the USA, the Scientific Registry of Transplant Recipients reports a 10-year probability of graft failure of 40% (living donor) and 60% (deceased donor) for kidney transplants and a 50% probability of graft failure for liver transplants [3]. Box 1 summarizes the current conventions for immunosuppression after renal and liver transplants [5–8].

There have been reports of a small number of transplant patients that have discontinued immunosuppression – either owing to nonadherence or to physician-led intentional weaning – and who, surprisingly, have not suffered from rejection. The state that develops in these patients is termed 'operational tolerance', in that there is long-term survival of the allograft in the absence of immunosup-

**Box 1: Current conventions in immunosuppression regimes after renal and liver transplantation in the UK and USA [5–8].**

**Renal**

**Kidney Disease Improving Global Outcomes Guidelines (summarised) [5]**

• Induction: Recommend a biologic agent as part of initial IS medication

First line induction therapy: recommend using an interleukin 2 receptor antagonist (IL2-RA).

Induction therapy for high immunologic risk: recommend using lymphocyte-depleting agent.

• Maintenance: Recommend using a combination including a CNI and an antiproliferative agent, with or without corticosteroids

Suggest using tacrolimus as the first-line CNI.

Suggest using mycophenolate as the first-line antiproliferative agent

Suggest that in patients who are at low immunological risk and who receive induction therapy, corticosteroids could be discontinued during the first week after transplantation.

Suggest using the lowest planned doses of maintenance IS medications by 2 to 4 months after transplantation, if there has been no acute rejection

Continuation of CNI is suggested over CNI withdrawal.

If using prednisone beyond 1 week after transplantation, continuation is suggested over withdrawal.

The SRTR database analysis for the USA confirms that induction therapy is normally a T cell depleting agent with tacrolimus and mycophenolate ± steroids for initial and subsequent maintenance.

**United Kingdom National Institute for Health and Clinical Excellence Guidelines (summarised) [6]**

• Induction: Basiliximab or daclizumab, used as part of calcineurin-inhibitor-based immunosuppression

• Initial/maintenance: Tacrolimus is considered as an alternative to ciclosporin when a calcineurin inhibitor is indicated

• If intolerant to calcineurin inhibitors and should avoid/minimise -> Mycophenolate mofetil

• If intolerant to calcineurin inhibitors and should withdraw -> Sirolimus

**Liver**

The picture is less clear-cut for liver transplantation. The most recent SRTR report [3] shows that commonly there is no induction therapy, or if used, it is an IL2-RA or a T cell depleting agent. There is also more variation in maintenance therapy with the most common options being tacrolimus and mycophenolate, only tacrolimus, or variations of cyclosporine, azathioprine and mTOR inhibitors. As with renal transplantation, steroids play a role. A recent commentary [8] highlighted the current variation in consensus with work suggesting tacromlimus and mycophenolate beter than tacrolimus alone [7].

pression. Although operational tolerance may share some features of true tolerance (which in turn may be defined as sustained donor-specific unresponsiveness), it appears that operational tolerance represents a spectrum of immunological states in which the allograft is allowed to survive at a level that provides adequate clinical function in the absence of immunosuppression. Indeed, it is possible that a clinically undetectable immune response to the organ may be occurring, making 'prope tolerance' a more accurate definition of the state. The ease of attainment of operational tolerance appears to be closely related to the type of organ transplanted, with liver allograft recipients dominating reports in the literature published to date.

In this review, we will consider in parallel, clinical reports of operational tolerance in patients together with the current basic science research into possible underlying mechanisms of tolerance, thereby providing an insight into how this state may be induced therapeutically in the future.

## What is tolerance?

As discussed, tolerance may be defined experimentally as donor-specific nonreactivity. This may be further confirmed by the adoptive transferability of tolerance and acceptance of a second donor allograft with rejection of a novel third-party allograft [9]. The earliest experimental proof that tolerance to an allograft is possible stems from seminal work by Medawar [10], Hasek and Hraba [11] and Owen [12]. In these experiments, the immune system

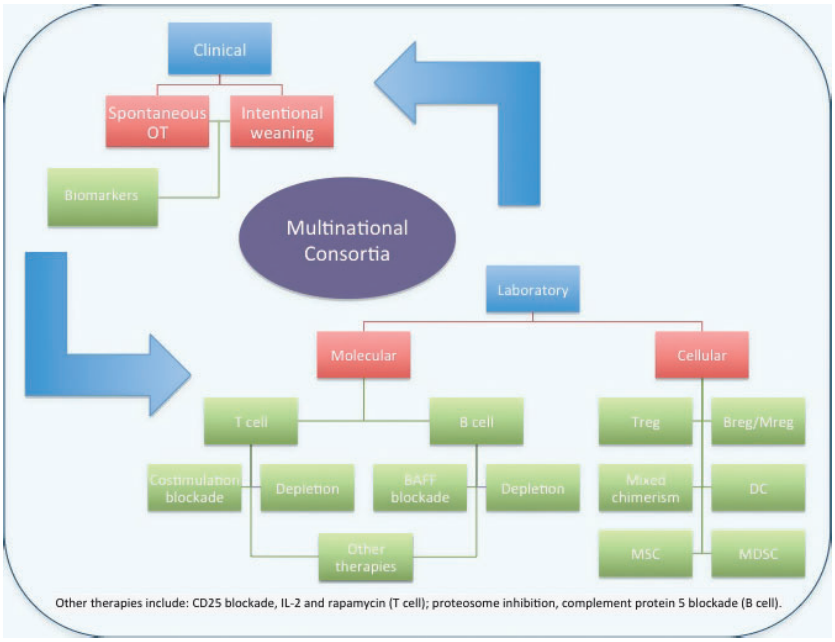


**Figure 1** The relationship between laboratory and clinical tolerance research. Importantly, the two streams work together, co-ordinated by multinational consortia. Clinical data from spontaneously tolerant patients informs laboratory research. Laboratory research provides new approaches to testing tolerance induction in the clinical setting. BAFF, B cell activating factor; Breg, Regulatory B cell; DC, dendritic cell; ITN, immune tolerance network; MDSC, myeloid-derived suppressor cell; MSC, mesenchymal stromal cell; OT, operational tolerance; RISET, Reprogramming of the Immune System for the Establishment of Tolerance; Treg, regulatory T cell.

'treats' foreign antigen as self by the establishment of mixed blood chimerism after neonatal recipient exposure to donor blood/leucocytes. Subsequent skin allografts from the blood/leucocyte donor are accepted without rejection. A similar state occurs in certain dizygotic twins, but the intentional generation of such tolerance from a neonatal age in the clinical setting is not possible in most circumstances (although neonatal heart transplants in infants who are yet to produce antibodies to major-blood group antigens have been successful [13]). Recreating a 'neonatal' environment in the immune system in adults requires conditioning regimens that have side effects and risks that are not appropriate for the majority of patients on transplant waiting lists. Thus, although the molecular mechanisms underlying rejection [14] and potential therapeutic avenues to induce tolerance from *in vitro* and *in vivo* work are becoming clearer [15], the translation of these successes to the clinical setting has proved challenging.

The quest to identify tolerogenic therapies has been a bipartite approach with reports of spontaneously tolerant patients providing a serendipitous proof-of-principle that tolerance may be possible in humans and thus driving clinical research. Simultaneously, experimental *in vitro* and *in vivo* animal model research aims to devise new tolerogenic therapies (Fig. 1). These efforts have been aided significantly by two research consortia (*see later*), the Indices of Tolerance/Reprogramming of the Immune System for the Establishment of Tolerance study and the Immune Tolerance Network [17,18].

## Clinical reports of operational tolerance

A practical definition of *operational* tolerance in the clinical setting is 'a well-functioning graft lacking histological signs of [acute or chronic] rejection in the absence of any immunosuppressive drugs (for at least 1 year), in an immunocompetent host capable of responding to other challenges including infections' [19,20]. Operational tolerance has been achieved infrequently and the incidence varies by allograft type, with the most common reports being in liver transplants followed by renal transplants. Orlando and colleagues reviewed both fields and summarized that out of 461 liver recipients in whom weaning of immunosuppression was attempted, this was successful in 163 and of these, 100 (22%) remained immunosuppression free 1 year after withdrawal of drugs [20]. In kidney transplantation, over 200 cases of operational tolerance persisting for over 1 year were reviewed [21]. The finding that operational tolerance is most commonly seen in liver transplant recipients in as much as 20% of patients [22] may be partly explained by a unique venous endothelial system that is repopulated by bone-marrow-derived cells [23]. Aside from liver and

kidney cases, there is one case of operational tolerance reported in a lung transplant [24] and one in a heart transplant [25], but never in other solid organ transplants such as pancreas or intestine.

Conceptually, clinical cases of operational tolerance can be divided into three types (based on Orlando *et al.*, 2010 [21]), the long-term goal being to use knowledge from the first two categories to increase the successful application of intentional tolerogenic protocols:

1. Spontaneous tolerance after patients were noncompliant with immunosuppression.
2. Planned weaning under supervision as a result of toxicity, intolerable side effects/complications from immunosuppression, or likelihood of tolerance in liver cases.
3. Cases in which operational tolerance is the therapeutic aim and tolerogenic protocols are applied on an individual basis.

## Reports of spontaneous operational tolerance

The liver provides the best available paradigm of operational tolerance. The liver itself is considered an 'immunoregulatory' solid organ with specialized venous endothelial turnover, a high number of extramedullary haemotopoietic stem cells and the ability to produce numerous immunoregulatory substances [26]. Hypotheses such as the role of microchimerism in hepatocytes after liver transplantation are being called into question as the percentage of chimeric hepatocytes as analysed using PCR do not reveal any correlation with allograft outcome [27]. The privileged state of the liver in transplantation is highlighted by the relatively lower need for HLA or blood-group matching, as well as the lower incidence of acute rejection. In addition, there is a low incidence of chronic rejection and interestingly, protection of other organs in combined transplants [28].

Operational tolerance in liver patients can be achieved in approximately 20% of individuals [20], although multiple factors determine the success of the weaning protocol including the underlying cause of liver failure and the time after transplantation. Although this is a significant number of patients, there is a reluctance to attempt weaning in a greater number of patients because of the risk of rejection. In addition, the majority of reports of operational tolerance in liver transplantation detail nonrandomized studies with extremely stringent exclusion criteria, meaning that physicians are unable to extend their findings easily to all patient groups. Methods that may help identify patients that are amenable to weaning may, therefore, potentially increase the proportion of tolerant liver transplant recipients. Indeed, in a recent study of paediatric patients by Feng and colleagues [29], the proportion of tolerant paediatric liver transplant recipients may be closer to 60% compared with the 20% of adult patients thought initially [30]. In a prospective multicentre trial, 20 selected paediatric parental living-donor liver transplant recipients with stable liver function had immunosuppression withdrawn and tolerance assessed by liver biopsy. Twelve of the 20 enrolled developed evidence of operational tolerance. While encouraging, this study highlights the problems of small patient numbers and particular exclusion criteria (in this case autoimmune or infective causes of liver failure) that prevent wider interpretation of results. The question of whether there are factors correlating with tolerance was explored in a retrospective historical cohort analysis of 134 paediatric semi-allogeneic living-related donor liver transplant patients. In this study, the absence of early rejection, HLA-A match and an increased presence of regulatory T cells (Treg) in the peripheral blood over 10 years post-transplant were predictive of operational tolerance [31]. Other recent developments in identifying mechanisms that preclude the development of operational tolerance include the finding that the presence of anti-donor HLA antibodies predicts the absence of future operational tolerance in paediatric living-donor liver recipients [32].

Despite the increasing frequency of operational tolerance in liver allografts, the underlying mechanisms have not been fully elucidated and, likewise, the findings from liver transplantation cannot be easily extrapolated to other organs. Indeed, operational tolerance in renal transplantation has been reported in only just over 100 cases [21]. The majority of these cases, however, were in noncompliant patients or in those who had a bone marrow transplant for myeloma or other haematological disorders and subsequently received a kidney allograft from the same donor. Despite the evidence that it is possible, intentional induction of tolerance has been frustratingly unsuccessful and, unlike liver transplants, rejection episodes frequently progress to graft loss.

## Clinical trials of tolerance induction

Induction strategies initially focussed on leucocyte depletion or costimulatory blockade to induce tolerance. The classical trial is that of Starzl and colleagues [33] in which a leucocyte depletion strategy followed by low-dose tacrolimus-based immunosuppression was employed in kidney, pancreas and intestine transplant recipients. However, despite a reduction in immunosuppression requirements, operational tolerance was not achieved, which may be explained by the lack of depletion of memory T cells during induction [34].

More successful have been cases with prior bone marrow transplantation resulting in generation of mixed chimerism and subsequent renal transplantation from the same donor [35–37]. Importantly, the renal transplant in these cases was performed many years after bone marrow transplantation for haematological malignancy, and thus not with the

intention of inducing tolerance. Cases of simultaneous bone marrow transplantation and renal transplantation for myeloma-induced renal failure have been reported from Massachusetts General Hospital [38,39] and Stanford [40]. The results of these trials are promising in that they show evidence of at least transient chimerism and weaning of immunosuppression with stable graft function during follow-up (four of five patients in the Massachusetts 2008 series and eight of 12 in the Stanford 2011 series). Perhaps, most exciting is the series of the Stanford Group of 16 renal transplant patients conditioned with total lymphoid irradiation and anti-thymocyte globulin followed by a transplant of $CD34^+$ haematopoietic progenitor cells and T cells from HLA-matched donors. Peripheral blood analysis revealed an increase in the proportion of Treg and chimerism in 15 of 16 patients, with eight persisting for 1–3 years and withdrawing from chemotherapy and four experiencing recurrence of disease or rejection.

A subsequent series by the Massachusetts Group showed that long-term donor-specific tolerance was possible in patients with end-stage renal failure without co-existing malignancies. Five patients had nonmyeloablative conditioning and subsequently received combined kidney and bone marrow transplantation from haploidentical donors. Four of the five patients had long-term organ survival without immunosuppression. Blood tests within the first year suggested a suppressive mechanism of tolerance, but after 3 years, anergy or deletional mechanisms seemed to be responsible [41].

Although the number of patients in such case series is small and the studies nonrandomized, they provide useful catalysts for the necessary larger scale multicentre studies once tolerogenic protocols are refined. A significant caveat is the difficulty in performing this in cadaveric transplantation. Furthermore, we must bear in mind the two major barriers to clinical translation of such therapies, namely homeostatic proliferation (the need to create haematological 'space' to allow engraftment of transfused cells) and heterologous immunity (previous antigen exposure causing alloreactive memory, potentially against the graft antigens) [15,42,43].

The translation of tolerogenic strategies from animal models to clinical settings has been hampered by the difficulty of achieving chimerism across HLA-mismatched recipients. The role of the thymus in the development of mixed chimerism in clinical studies remains unclear [44]. In addition, the propensity for induction of graft versus host disease (GVHD) secondary to donor lymphocytes perceiving recipient antigens as foreign poses a significant problem. However, the recent demonstration that bioengineered mobilized cellular product enriched for haematopoietic stem cells and tolerogenic graft facilitating cells combined with nonmyeloablative conditioning can be used to achieve donor-specific unresponsiveness and durable chimerism in five of eight HLA-mismatched recipients at 1-year follow-up is intriguing [45]. Although the importance of the facilitating cellular component of this protocol and the safety profile on a large scale remains to be elucidated [46], this result is extremely encouraging in that the protocol was able to achieve high levels of donor chimerism using an acceptable regimen, without GVHD and permitting graft tolerance. Interim-term results of this study are equally promising. Of 11 reported cases, durable chimerism was reported in six and transient chimerism in three. Those displaying only transient chimerism have been maintained on tacrolimus monotherapy, whereas those demonstrating durable chimerism have been weaned off all immunosuppression. Of note, one patient had graft loss secondary to sepsis and another did not display any evidence of chimerism. Importantly, the authors raise the concept of using T cell chimerism as a biomarker of tolerance rather than donor-specific hyporeactivity [47].

Recently, Brouard and colleagues published details from a long-term follow-up of renal transplant recipients [48]. In this study, 27 tolerant patients were compared with patients under immunosuppression or patients that had stopped immunosuppression and subsequently rejected their kidney transplant. They found that tolerant patients received induction immunosuppression less frequently (possibly attributable to closer HLA matching), were older at time of transplantation and weaning, and were less susceptible to infection. Clearly, the generation of tolerance is a multifactorial process and numerous patient and treatment factors will need to be taken into account. However, it is reassuring that tolerance does seem to persist as a robust phenomenon for many years, although graft dysfunction may eventually occur in some patients (eight of the 27 that were studied).

Transplant acceptance inducing cells (TAICs) are preparations containing an active component that is now known to be regulatory macrophages (Mreg) [49]. These have been studied in two trials, TAIC-I and TAIC-II [50,51]. TAIC-I was a phase I/II trial in which 12 patients received renal grafts from deceased donors together with a perioperative infusion of TAICs. Three patients safely completed their immunosuppression minimization [50]. Subsequently, a second trial was conducted in five living related donor recipients. Whereas three patients had immunosuppression weaned to tacrolimus monotherapy, only one had immunosuppression successfully withdrawn. Unfortunately, this patient subsequently rejected the graft at 34 weeks [51]. The possibility of TAIC infusion in a trial setting can be concluded from the above, but differences in TAIC preparation, infusion amount, induction strategies and timing mean further conclusions regarding the utility and safety are difficult without further studies. More recently, work by

the same group on refined Mreg showed excellent graft function in two living related renal transplant recipients who received preoperative infusion of donor-derived Mreg [52].

## Biomarkers

The Biomarkers Definitions Working Group defines a biomarker as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention' [53]. Epigenetic mechanisms may also play a role and contribute to biomarker profiles [54,55]. The importance of identifying biomarkers of operational tolerance is twofold. Firstly, this would allow the effective identification of candidate patients for minimization and potential discontinuation of immunosuppression. Secondly, both the markers identified and the patients identified can provide hypotheses for testing underlying mechanisms of tolerance and to derive novel tolerogenic therapies. There are thus an increasing number of studies addressing this issue, summarized in Table 1 (see review by Londono *et al.*, 2012 and Roedder *et al.*, 2012 [56,57])

A significant step forward was provided by Brouard *et al.* [58], who studied the peripheral blood of 75 renal transplant recipients including those with operational tolerance, acute/chronic rejection and stable allograft function, and compared these with healthy controls. Using quantitative polymerase chain reaction (Q-PCR) and microarrays, they identified 49 genes of which 33 correctly segregated tolerance and chronic rejection phenotypes with 99% and 86% specificity. However, this tolerant signature was also shared with 1 of 12 and 5 of 10 stable patients on triple immunosuppression and low-dose steroid monotherapy respectively. When the genes were further analysed in terms of their function, the expression pattern suggested a functional phenotype of reduced costimulatory signalling, immune quiescence, apoptosis, and memory T cell responses. Subsequently, the Indices of Tolerance/RISET and ITN consortia reported their landmark findings in tolerant renal transplant patients with the tolerant kidney recipients in RISET having a characteristic peripheral B lymphocyte signature, corroborated by the ITN findings of expansion of B cells and expression of B-cell-related genes [17,18]. Overall, the European team was able to identify operationally tolerant patients with 0.964 specificity and 0.933 sensitivity.

Recently, Li and colleagues used microarrays and Q-PCR on peripheral blood samples in liver transplant patients to identify a common set of genes in operationally tolerant patients [59]. Of note, 13 genes were highly expressed in NK cells and this profile was evident in both adult and paediatric transplant recipients, regardless of cause of liver failure. Furthermore, the gene profile of tolerant renal transplant patients showed increased expression of *FOXP3, GATA3* (GATA binding protein 3), *TGFB1* and *TGFBR1* suggesting involvement of Treg and T helper (Th) 2 cells in tolerance [60].

Intriguingly, comparison of the profiles for kidney and liver patients does not reveal any significant overlap in biomarkers [61]. More specifically, liver patients express a high number of natural killer (NK) cell genes, whereas kidney patients express B cell genes, suggesting organ specific mechanisms of tolerance, potentially with Treg as a common end effector as highlighted by the increase in *FOXP3* expression [62]. Further support comes from work in paediatric living-donor liver transplantation where analysis of peripheral blood revealed an increased frequency of $CD4^{+}CD25^{high}$ T cells [63,64]. Interestingly, there is also an increase in $V\delta1\gamma\delta T$ cells (a subset of $\gamma\delta T$ cells that produce IL-10 and are involved in generating fetomaternal tolerance in pregnancy). However, in these studies, the frequency of NK cells was decreased in tolerant patients highlighting the complexity of the underlying tolerant pathways. Another aspect to consider is the role of the innate immune system in tolerance and rejection [65]. Work has demonstrated that there is lower expression of TLR4 on monocytes in the peripheral blood of renal transplant patients that are operationally tolerant, with increased expression in chronic rejection [66].

Thus far, the majority of biomarker studies have relied on peripheral blood sampling for analysis and profiling [67]. However, an elegant study by Cobbold and colleagues [68] offers some warnings regarding biomarkers. Biomarkers of tolerance were explored in three different murine skin graft models by examining the graft, spleen and draining lymph node. Syngeneic grafts and allografts that became tolerant had a similar gene expression profile, which differed from the gene expression of a rejecting allograft. However, no differences were seen in the spleen or draining lymph node. The authors argue that the tolerance mechanisms in allograft acceptance could be akin to those that maintain self-tolerance in an inflammatory environment and by extension, were a biomarker profile of tolerance to exist, the location most likely to reveal this is in the allograft. This sentiment is supported by Brouard and colleagues [69], who argue that blood transcriptomic measurements may be insufficient to detect tolerant phenotypes, and furthermore, may be confounded by exposure to immunosuppression.

Studies such as that by Becker and colleagues [70] are useful in clarifying the biomarker profile of tolerance in the allograft. In this study, biopsies were taken from kidney allografts in four patient groups (operationally tolerant, borderline changes, interstitial rejection and stable function). When analysed, there was a distinct pattern of immunohistochemical expression of the signal transducer Phosphoino-

**Table 1.** A summary of selected clinical studies of tolerance biomarkers in renal and liver transplant recipients. The outlined biomarkers are chosen from those that have been highlighted as particularly significant from the analysis in the studies (adapted from Londono *et al.*, 2012 [56] and Roedder *et al.*, 2012 [57]).

| Clinical study | Allograft | Tissue sample | Methodology | Biomarkers identified |
|---|---|---|---|---|
| Brouard *et al.*, 2007 [58] | Renal | Blood | Microarray, qPCR | CD9, FOXP3, MAPK9, NKG7, TK1, TNFRSF7 |
| Braud *et al.*, 2008 [113] Sivozhelezov *et al.*, 2008 [114] | Renal | Blood | Microarray | RB1, POLR2B, CREBBP, GTF2F1, JAK3, SFRS1, SF3B1, HNRPH1, EGFR, MAPK14, PIK3CA, HNRPD, CPSF3, HNRPH2, CASP3, SFRS3, MADH4, FYN, HNRPR, SNRPA1, SF3A2, PIK3R1 |
| Newell *et al.*, 2010 [17] | Renal | Blood and urine | Microarrays | IGKV4-1, IGLL1, IGKV1D-13 |
| Sagoo *et al.*, 2010 [18] | Renal | Blood | Flow cytometry, microarrays, RT-PCR | $CD4^+CD25^{int}$ T cells, FOXP3/α-1,2-mannosidase ratio, CD79B, TCL1A, HS3ST1, SH2D1B, MS4A1, TLR5, FCRL1, PNOC, SLC8A1, FCRL2 |
| Brouard *et al.*, 2011 [115] | Renal | Blood | qPCR | RHOH, BUB1B, TMTC3, MS4A1, GAGE, C1S, RAB30, PLXNB1, AKR1C1, CCL20, NCAPH, AKR1C2, CDC2, SPON1, RGN, RBM9, DEPDC1, HBB, SYNGR3, CHEK1 |
| Lozano *et al.*, 2011 [61] | Renal and Liver | Blood | Microarray, qPCR | Various including NK-cell- and B-cell-related transcripts |
| Hoshino *et al.*, 2012 [116] | Renal | Blood | Luminex | DSA |
| Danger *et al.*, 2012 [117] | Renal | Blood | Microarray | SNPs in PARVG |
| Martinez-Llordella *et al.*, 2007 [118] | Liver | Blood | Microarray, qPCR | Vcδ1+ T cells, $CD4^+CD25^+$ T cells, CD94, IL1, IL23, ICAM1, TNF-α, NKG2D, CD160 |
| Pons *et al.*, 2008 [62] | Liver | Blood | qPCR | $CD4^+CD25^+$ T cells, FOXP3 mRNA |
| Martinez-Llordella *et al.*, 2008 [119] | Liver | Blood | Microarray, qPCR | KLRF1, SLAMF7, NKG7, ILR2B, KLRB1, FANCG, GNPTAB, CLIC3, PSMD14, ALG8, CX3CR1, RGS3 |
| Scandling *et al.*, 2008 [40] | Liver | Blood | – | $CD4^+CD25^{high}FOXP3^+$ cells, DC2:DC1 ratio, γδT cells (Vδ1/Vδ2 ratio), NK cell, γδT cell, CD8-cell receptors, cytokine gene polymorphisms (TNF-α, IL-10), soluble HLA-G |
| Castellaneta *et al.*, 2011 [120] | Liver | Blood | Monoclonal antibody staining and flow cytometry | HLA-G |
| Li *et al.*, 2012 [59] | Liver | Blood | Microarrays, qPCR | SENP6, FEM1C, ERBB2, AKR1C3, MAN1A1, UBAC2, GPR68, NFKB1, MAFG, BTG3, ASPH, PTBP2, PDE4DIP |
| Bohne *et al.*, 2012 [75] | Liver | Tissue | Microarrays, qPCR, flow cytometry | HAMP, TFRC, FTHL12, FTHL8; SH2D1B, CLIC3, PSMD14, NCAM1, IL2RB, PDGFRB, GZMB, NCR1, GNG2, KLRF1, KLRC4 |

AKR(x)C(n), Aldo-keto reductase family (x) member C (n); ALG8, asparagine-linked glycosylation 8; ASPH, aspartate beta-hydroxylase; BTG, B cell translocation gene; BUB1B, Budding uninhibited by benzimidazoles 1 homolog beta; C1S, complement component 1 s subunit; CASP, caspase apoptosis-related cysteine peptidase; CCL20, chemokine (C-C motif) ligand 20; CD, cluster of differentiation; CDC2, cell division control protein 2; CHEK1, checkpoint kinase 1; CLIC, chloride intracellular channel; CPSF, cleavage and polyadenylation specific factor; CREBBP, cAMP response element-binding protein binding protein; CX3CR, CX3C chemokine receptor; DC, dendritic cell; DEPDC1, DEP domain containing 1; DSA, donor-specific antibodies; EGFR, epidermal growth factor receptor; ERBB, human epidermal growth factor receptor; FANCG, Fanconi anaemia complementation group G; FEM1C, Fem-1 homolog c; FOXP3, Forkhead box protein 3; FTHL, Ferritin, heavy polypeptide-like, FYN, FYN oncogene related to SRC, FGR, YES; HNRPR, heterogeneous nuclear ribonucleoprotein R; GNG2, Guanine nucleotide-binding protein (G protein), gamma 2; GNPTAB, GlcNAc-1-phosphotransferase alpha and beta; GPR, G protein-coupled receptor; GTF2F1, general transcription factor IIF subunit 1; GZMB, granzyme B; HAMP, hepcidin antimicrobial peptide; HBB, haemoglobin beta; HLA, human leucocyte antigen; HNRP, heterogeneous nuclear ribonucleoprotein; ICAM1, intercellular adhesion molecule 1; IGKV, immunoglobulin kappa variable; IGLL, immunoglobulin lambda-like polypeptide; IL, interleukin; IL2RB, interleukin 2 receptor subunit beta; PDGFRB, platelet-derived growth factor receptor beta type; JAK, Janus kinase; SFRS, splicing factor, arginine/serine-rich; KLR(x) Killer cell lectin-like receptor subfamily x member; MADH, mothers against decapentaplegic homolog; MAFG, musculoaponeurotic fibrosarcoma oncogene homolog G; MAN1A, Mannosyl-oligosaccharide 1,2-alpha-mannosidase; MAPK, mitogen-activated protein kinase; MS4A1, membrane-spanning 4-domains, subfamily A, member 1; NCAM, neural cell adhesion molecule; NCAPH, non-SMC condensin I complex, subunit H; NCR, natural cytotoxicity triggering receptor; NFKB, nuclear factor kappa b; NK, natural killer cells; NKG(n)(x) Natural killer group (n), member (x); PARVG, gamma parvin; PDE4DIP, phosphodiesterase 4D interacting protein; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; PIK3R, phosphatidylinositol 3-kinase regulatory subunit; PLXNB1, Plexin B1; POLR2B, Polymerase (RNA) II (DNA directed) polypeptide B; PSMD14, proteasome (pro-

some, macropain) 26S subunit, non-ATPase, 14; PTBP, polypyrimidine tract binding protein; RAB30 Member RAS oncogene family; RB Retinoblastoma protein; RBM9 RNA-binding protein, fox-1 homolog 2; RGN, regucalcin; RGS, Regulator of G-protein signalling; RHOH, Ras homolog gene family, member H; SENP Sentrin-specific protease 6; SF(x)(n), Splicing factor (x) subunit (n); SH2D1B, SH2 domain-containing protein 1B; SLAMF, signalling lymphocytic activation molecule family; SNPs, single nucleotide polymorphisms; SNRPA U2, small nuclear ribonucleoprotein polypeptide A′; SPON1, spondin 1; SYNGR3, synaptogyrin 3; TFRC, transferrin receptor; TK, thymidine kinase; TMTC3, transmembrane and tetratricopeptide repeat containing 3; TNF, tumour necrosis factor; TNFRSF, tumour necrosis factor receptor superfamily member; UBAC, ubiquitin-associated domain-containing protein.

sitide 3 kinase (PI3K) and the NF-kB subunit c-Rel (proto-oncogene encoded by *REL*). Specifically, these were significantly decreased in tolerant patients compared with other groups. The importance of biopsy assessments has now been recognized clinically with the Banff working group publishing guidelines on biopsy monitoring of liver allografts in operationally tolerant patients [71].

There is thus a pressing need for validation of current putative biomarkers in prospective weaning trials, as well as identification of novel markers in multicentre longitudinal studies with adequate numbers of patients and a combined approach comprising transcriptomics, cellular, proteomic, clinical and phenotypic data [69].

## Mechanisms of operational tolerance

Despite the wealth of experimental and clinical data concerning operational tolerance, the mechanisms require further elucidation. Much circumstantial evidence has come from the biomarker studies identifying phenotypes correlating with operational tolerance. Although a role for Treg is heralded by a wealth of experimental and clinical data [72,73] other cells are also likely involved with B cells featuring increasingly prominently in newer studies. Analysis of peripheral blood mononuclear cell microRNA (miR) profiles of tolerant renal transplant patients revealed overexpression of miR-142-3p. When interrogated in Raji B cells, miR-142-3p modulated close to 1000 genes relating to B cell responses and a negative feedback loop involving TGF-β [74]. Mechanisms may also involve the iron homeostasis pathway. Bohne and colleagues [75] analysed sequential liver biopsies and blood tests in a prospective multicentre immunosuppressive drug withdrawal trial and found that tolerant patients had increased expression in the graft of genes involved in iron homeostasis, increased iron deposition in hepatocytes, and raised serum levels of hepcidin and ferritin. The authors suggest that mechanisms such as the iron-hepcidin, adenosinergic and suppressor of cytokine signalling 1 (SOCS1) immunomodulatory pathways play crucial roles in operational tolerance.

It is worth expanding on the mechanisms underlying the function of regulatory cells [72]. Pre-existing naturally occurring Treg can cross-react with donor alloantigen from the graft [76]. Subsequently, Breg and tolerogenic dendritic cells can stimulate development of induced Treg [77]. These work by numerous routes, including secretion of IL-10 [78] and TGF-β [79], as well as modifying the amino acid microenvironment and energy bioavailability in the graft [80]. Other cells such as T regulatory type 1 cells (Tr1), $CD8^+$ Treg and $CD4^-CD8^-$Treg also work by various mechanisms that often end by suppressing the activity of antigen-presenting cells [81–84]. Cells of the innate immune system also contribute. Myeloid-derived suppressor cells suppress the proliferation of T cells, B cells and NK cells through various mechanisms, including the expression of inducible nitric oxide synthase (iNOS) and arginase [85]. MSCs function through mechanisms that involve prostaglandin E2 (PGE2), transforming growth factor-β (TGFβ) and matrix metalloproteinases (MMPs) [86]. Mreg produce large amounts of interleukin-10 (IL-10) and can, therefore, create a microenvironment that is permissive for the generation of Treg [87].

## Tolerogenic therapies

The vast majority of drug-based tolerogenic therapies have failed to induce operational tolerance and emphasis is now on cellular therapies and the induction of mixed chimerism. A recent review by Page and colleagues [88] summarizes current therapeutic attempts (Table 2).

The classical strategy of tolerance – that of chimerism – is now experiencing a resurgence [39,89]. Initially demonstrated by Medawar, Billingham and Brent [10] in their now classical mouse experiments, mixed chimerism is the concept of both recipient and donor haematopoietic stem cells co-existing to provide a constant supply of antigen to generate tolerance through central deletional mechanisms. The appeal of chimerism has been tempered by the severe risks associated with the induction protocols and emphasis is now on attempts to derive noncytoreductive nonirradiative protocols that create haematological space for bone marrow/stem cell engraftment. Although there have been successes in animal models and early clinical trials (reviewed by Sykes 2009 [90]), the requirement for large numbers of donor cells has further precluded their widespread use. In addition, minimizing the potential for GVHD post-transfusion has also proved difficult [91]. Nevertheless, reports are increasing in the literature of successful induction of tolerance using a mixed chimerism strategy in small selected patient groups [39,89,92,93].

**Table 2.** The current range of tolerogenic strategies in experimental and clinical settings (taken from Page *et al.*, 2012 [88]).

| Category | Therapeutic | Mechanism |
|---|---|---|
| T cell depletion | Anti-thymocyte globulin (ATG) | Depleting polyclonal antibodies to thymocytes that express multiple target antigens; possible induction of regulatory T cells. *Clinical* |
| | Alemtuzumab | Depleting mAb to CD52, on T, B, NK cells, some monocytes. *Clinical trials* |
| Costimulation blockade | Abatacept | CTLA-4 Ig, blockade of CD28:CD80/86 costimulatory pathway. *Clinical trials* |
| | Belatacept | CTLA-4 Ig, blockade of CD28:CD80/86 costimulatory pathway. *Clinical trials* |
| | Efalizumab | Blockade of LFA-1:ICAM-1 costimulatory pathway. *Clinical trials – withdrawn* |
| Other T cell therapies | Basiliximab | Blockade of CD25 (interleukin 2 receptor $\alpha$ chain). *Clinical* |
| | Aldesleukin + rapamycin | Interleukin 2+ rapamycin, to increase regulatory T cell proliferation and survival, and stabilize the expression of Forkhead box P3 (FoxP3). *Clinical trials* |
| B cell therapeutics | Rituximab | Depleting mAB to CD20. *Clinical trials* |
| | Belimumab | Blockade of B cell activating factor (BAFF), causing depletion of follicular and alloreactive B cells, decrease in alloantibody response, and promotion of immature/transitional B cell phenotype and a regulatory cytokine environment. *Clinical trials* |
| | Atacicept | Blockade of BAFF and APRIL. *Clinical trials* |
| | BR3-Fc | Blockade of BAFF, causing decrease in peripheral, marginal zone, and follicular B cells. *Clinical trials – withdrawn* |
| | Bortezomib | Proteosome inhibitor, causing apoptosis of mature plasma cells. *Clinical trials* |
| | Eculizumab | Blockade of complement protein C5, to prevent complement-mediated injury caused by circulating alloantibody. *Clinical trials* |
| Cellular therapy | Mixed chimerism | Infusion of donor bone marrow into myoablated/immune-conditioned recipient, to produce co-existence of donor and recipient cells. *Case series* |
| | Regulatory T cells | Infusion of expanded regulatory T cells, to inhibit inflammatory cytokine production, down- regulate costimulatory and adhesion molecules, promote anergy and cell death, convert effector T cells to a regulatory phenotype, and produce suppressive cytokines IL-10, TGFβ, and IL35. *Murine models. HSCT trials* |
| | Regulatory T cells + IL-2 | As above, plus the addition of IL-2 to promote Treg survival, development, and expansion. *Murine models* |
| | Dendritic cells | Immunomodulatory effects include their ability to acquire and present antigen, expand and respond to antigen-specific Tregs, constitutively express low levels of MHC and costimulatory molecules, produce high IL-10 and TGFβ and low IL-12, resist activation by danger signals and CD40 ligation, resist killing by natural killer or T cells, and promote apoptosis of effector T cells. *Murine models* |
| | Macrophages | Immune suppression mediated through the enrichment of CD4+ CD25+ Foxp3 cells and cell contact- and caspase-dependent depletion of activated T cells. *Clinical trials* |
| | Mesenchymal stromal cells | Inhibition of T cell activation and proliferation, potentially as a result of production of IL-10, NO, and IDO, and suppression of IFNγ and IL-17. *Clinical trials* |

CTLA-4, Cytotoxic T lymphocyte antigen 4; IDO, indoleamine 2,3-dioxygenase; IFNγ, Interferon γ; IL-10, interleukin 10; LFA-1, lymphocyte function-associated antigen 1.

Since the discovery of Treg as modulators of the immune system, adoptive transfer strategies have proven successful in murine models and early studies in patients are also producing optimistic results [94,95]. The ability of Treg to promote donor-specific tolerance explains the wealth of research being undertaken. This could potentially allow suppression of the cells responsible for graft rejection alone, without compromising remaining immune function [72,96]. Current methods involve *ex vivo* expansion of Treg using magnetic beads coated with CD3 and CD28 and subsequent adoptive transfer into an allograft recipient. Our group has demonstrated the ability of such a strategy to promote graft acceptance in humanized mouse models [94,97]. The generation of antigen-specific Treg is now an active research area with strategies including splenocyte stimulation, dendritic cell co-culture, MHC-peptide multimers and lentiviral-based T cell receptor (TCR) gene transfer [98–101]. Although no clinical trials of Treg in solid organ transplantation have been undertaken yet, promising data are emerging from early trials of Treg therapy for the prevention of GVHD posthaematopoietic stem-cell transplantation [102–104]. Our group will be participating in trials as part of the ONE Study in a Europe wide consortium to investigate cellular therapies for tolerance (www.onestudy.org).

Combined with the findings of comparative studies of operational tolerance profiles in different allografts, it is increasingly likely that different organs become accepted via different routes and therefore a unifying therapy may never be realized, with patient-specific strategies designed

based on allograft type. Conversely, the generation of Treg may be the common end pathway as highlighted by work on maturation resistant rapamycin conditioned dendritic cells promoting experimental tolerance to cardiac allografts by preferentially modulating Treg [105] as well as the generation of peripheral Treg in mixed chimerism [39]. Likewise, *in vitro* culture analysis of MSCs has shown an ability to increase Treg [106] perhaps explaining their ability to prolong vascularized skin grafts in rats [107] and their ability to alleviate GVHD in 39 of 55 patients with steroid refractory forms in a Phase II trial [108]. However, the lack of success in subsequent phase III trials is disappointing [109].

It is evident that cellular tolerogenic therapies offer much promise in the experimental and early clinical trials reported, but reliable and robust clinical translation faces significant challenges, not least logistical ones of cost and cellular expansion [88,110–112].

## Conclusion

Despite clinical proof that operational tolerance is possible, historically, the majority of reports are of spontaneous tolerance resulting from nonadherence or as an unintended secondary benefit, for example after bone marrow transplantation for haematological malignancies. However, the outcomes from recent studies in patients without prior bone marrow transplantation are encouraging. Liver transplants provide the best paradigm we currently have available and a combined approach with clinical and experimental research is required to allow us to eventually clarify the mechanisms of operational tolerance and generate novel tolerogenic therapies. These efforts must be co-ordinated by multinational consortia to facilitate reliable well-powered trials and to ensure that primary safety concerns for new treatments strategies are addressed. This highlights a key caveat to be borne in mind, namely that of experimenting on 'tolerance up-front' when the short-term graft survival for many transplants is excellent. In addition, costing is currently nearing on prohibitively expensive for cellular therapies. Nevertheless, it is likely that the combined costs of lifelong immunosuppression and the treatment of conditions arising from immunosuppression or graft loss will be significantly more expensive than a single effective tolerogenic therapy.

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