

# Transplantation tolerance

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Received: 24 July 2013 / Revised: 1 October 2013 / Accepted: 4 October 2013 / Published online: 10 November 2013  
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**Abstract** Although transplantation has been a standard medical practice for decades, marked morbidity from the use of immunosuppressive drugs and poor long-term graft survival remain important limitations in the field. Since the first solid organ transplant between the Herrick twins in 1954, transplantation immunology has sought to move away from harmful, broad-spectrum immunosuppressive regimens that carry with them the long-term risk of potentially life-threatening opportunistic infections, cardiovascular disease, and malignancy, as well as graft toxicity and loss, towards tolerogenic strategies that promote long-term graft survival. Reports of “transplant tolerance” in kidney and liver allograft recipients whose immunosuppressive drugs were discontinued for medical or non-compliant reasons, together with results from experimental models of transplantation, provide the proof-of-principle that achieving tolerance in organ transplantation is fundamentally possible. However, translating the reconstitution of immune tolerance into the clinical setting is a daunting challenge fraught with the complexities of multiple interacting mechanisms overlaid on a background of variation in disease. In this article, we explore the basic science underlying mechanisms

of tolerance and review the latest clinical advances in the quest for transplantation tolerance.

**Keywords** Allograft · Tolerance · Transplant · Kidney · Immunosuppression

## Introduction

Transplantation tolerance has a number of definitions. The original definition of Medawar in the 1950s referred to non-responsiveness to antigens [1]. In animal studies, tolerance may be defined as good longstanding graft function in the presence of a competent immune system, with no signs of graft immune injury when the animal is killed. The latter is obviously not useful in human transplantation and therefore “operational tolerance” is the term most widely used. Organ transplant recipients who have been successfully weaned from immunosuppression and have maintained stable graft function for 1 year or more are referred to as functionally or operationally tolerant [2, 3]. Although up to 20% of liver transplant recipients may be successfully withdrawn from immunosuppression [3–5], operational tolerance to renal allografts appears to be much less frequent [6, 7] and a predictive biomarker for success versus failure in weaning immunosuppression has yet to be identified and validated. As a result, subjects who fail the withdrawal of immunosuppression experience episodes of rejection leading to the re-initiation of immunosuppression after anti-rejection treatment. It is not known whether they experience compromised long-term graft survival as a result [8].

Current barriers to successful long-term allograft survival

Since the first human kidney transplant carried out by Dr. Joseph Murray in 1954 between identical twins, the regular development of new chemical immunosuppressants, as

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well as improved surgical and ancillary care, have led to dramatic increases in kidney allograft survival rates and enabled the transplantation of livers, hearts, pancreases, and lungs, as well as composite tissues [9]. Nonetheless, substantial problems remain in the fields of both adult and pediatric transplantation, and improvements in short-term (1-year) allograft survival have not been paralleled by gains in long-term graft survival (*US Organ Procurement and Transplantation Network. Annual Data Report 2011. Accessed July 17, 2013*). The reasons for graft loss are complex but can be broadly classified into three categories: inflammation-induced reactions against graft tissues, specifically ischemia–reperfusion (I-R) injury; immune-initiated reactions against graft tissues, and direct organ toxicity by the immunosuppressive drug. Strategies aimed at inducing operational tolerance in allograft recipients will address these last two modes of graft loss but, while it may be beyond the realms of this review, it is important to remember that if transplantation tolerance is ever to be achieved, the early inflammatory response to allograft tissue must also first be regulated.

Briefly, three alloimmune responses to transplanted tissues have been described: *hyperacute*, *acute*, and *chronic rejection*. When an alloantigen is recognized, the innate and adaptive immune systems respond synergistically to reject the allograft through non-exclusive pathways, including contact-dependent T-cell cytotoxicity, granulocyte activation by either T helper 1 (Th1)- or Th2-derived cytokines, natural killer (NK) cell activation, alloantibody production and complement activation [10]. With the introduction of prospective full immunological screening, *hyperacute* graft loss, which occurs when preformed donor-specific antibodies are present in the recipient's serum, is thankfully now rare. Equally, the use of potent immunosuppressive agents immediately following and in the maintenance phase after renal transplantation has seen a dramatic fall in the incidence of acute rejection, generally defined as rejection within the first year following transplantation.

As readers will know, the term *chronic rejection*, encompassing a multifactorial pathogenesis with alloimmune-dependent and -independent causative factors, is now considered meaningless in its lack of specificity and is no longer used in the Banff classification to renal allograft pathology [11]. Instead, the classification system allows for chronic antibody-mediated rejection and a score to reflect interstitial fibrosis and tubular atrophy [11]. The significance of the longevity of alloimmune mechanisms in the pathogenesis of chronic allograft damage is only now coming to the fore and substantial problems continue to hinder the realization of effective treatment strategies. In short, improvements in the short-term success of renal and extra-renal transplantation have had a minimal impact on long-term success and the rate of late graft loss is essentially unchanged [12, 13]. While there have been no studies directly comparing the outcome of tolerant patients to those who continue to receive immunosuppression, the intuitive

advantages associated with the avoidance of chronic immunosuppression continue to drive the enthusiasm for implementing approaches to induce tolerance to transplanted organ allografts [14].

#### Physiological self-tolerance

The ability of the immune system to avoid damaging self-tissues is referred to as self-tolerance, and failure of self-tolerance underlies the broad class of autoimmune diseases. Self-tolerance may be induced in immature self-reactive lymphocytes in generative lymphoid organs (central tolerance), or in mature lymphocytes in peripheral sites (peripheral tolerance). Central tolerance is itself made up of negative selection: the deletion of autoreactive thymocytes, and dominant tolerance: the generation of natural (n) T regulatory (Treg) cells with an avidity for self-peptide intermediate between that necessary for positive selection of conventional effector T (Tconv) cells and that needed for the deletion of autoreactive T cells [15]. However, central tolerance is incomplete and control of self-reactive cells that migrate to the periphery becomes the role of peripheral tolerance mechanisms. Peripheral tolerance can act at several levels. The simplest scenario involves ignorance of self-antigens, either because the latter are sequestered in sites not easily accessible to the blood/lymph-borne immune system [16, 17] or because the amount of antigen does not reach the threshold required to trigger an effector response [18]. Alternatively, T-cell encounters with self-antigen might lead to functional inactivation: anergy. Even when T cells become fully activated, effective tolerance can still be maintained if the nature of the response is such that pathogenic effects are avoided. Just as certain chemokines and cytokines promote pathogenicity, regulating where self-reactive T cells go and what they make represents another mechanism to prevent autoimmune destruction in the periphery. We now know that these mechanisms can be exploited for the prevention of transplant rejection [19], as well as the treatment of autoimmune and allergic disease, and antigens can be administered in ways that induce tolerance rather than immunity.

The balance of Treg cells and Tconv cells is key to immune homeostasis. Tregs are defined by their expression of the transcription factor FOXP3, in addition to surface phenotypic markers that are not restricted to Tregs, such as CD4, CD25, GITR, and cytotoxic T lymphocyte antigen 4 (CTLA4). Their importance is underscored by the lymphoproliferative and multi-organ autoimmunity phenotypes of Scurfy mutant mice and the human conditions *Immunodysregulation Polyendocrinopathy and Enteropathy, X-linked (IPEX)* syndrome and *X-Linked Autoimmunity-Allergic Dysregulation syndrome (XLAAD)*, which result from mutations in the *foxp3* gene and consequent lack of Tregs [20].

The majority of Treg cells develop naturally in the thymus (nTregs) and migrate to the periphery. In addition, TGF- $\beta$  and

IL-2 are able to promote, in response to T cell receptor (TCR)/CD28 co-stimulation, the differentiation of peripheral naive CD4<sup>+</sup> T cells into CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> cells, otherwise known as inducible (i) Treg cells, that possess T cell suppressive properties akin to those of nTregs [21–23].

#### Strategies for inducing transplantation tolerance

At the risk of oversimplification, there are two obligatory components to achieving transplantation tolerance: depletion of alloreactive Tconv cells and upregulation of alloreactive Treg cells. In recipients of solid organ transplants, the high frequency of alloantigen-reactive Tconv cells in the immune repertoire of the recipient compared with the relatively small number of Treg cells present at the time of transplantation means that the balance of cells is shifted towards allograft destruction [24]. This crucial balance between graft destruction and regulation can be shifted using strategies to inhibit the activity of Tconv cells and/or increase the relative frequency or functional activity of alloantigen-reactive Treg cells [25–29]. With this in mind, mixed chimeric and cellular tolerogenic therapies are being trialed where drug-based therapies have failed. A recent review by Page et al [30] summarizes current therapeutic attempts (Table 1). It is notable that most of the tolerogenic strategies that have been attempted experimentally and clinically include depleting agents, even when they are not named as the underlying strategy [31].

Recent work by Wu et al. identifies the innate immune system as a potential target through which to manipulate the tolerance-rejection immune balance [32]. After organ transplantation, Toll-like receptors (TLRs) drive innate immune responses as part of I-R injury and this leads to the subsequent initiation of adaptive alloimmune responses. Wu et al. found that mice deficient in the TLR adaptor protein MyD88 developed donor antigen-specific tolerance, which protected them from both acute and chronic allograft rejection and increased their survival after transplantation compared with wild-type controls. Administration of an anti-CD25 antibody to MyD88-deficient recipients depleted Treg cells and broke tolerance. In addition, defective development of Th17 immune responses to alloantigen both in vitro and in vivo occurred, resulting in an increased ratio of Tregs to Th17 effectors. The group concluded that MyD88 deficiency was associated with an altered balance of Tregs over Tconv cells, promoting tolerance instead of rejection.

#### Lymphodepletional strategies

Lymphodepletion, in the form of “induction therapy”, is an effective strategy for addressing the precursor frequency of alloreactive Tconv cells at the time of organ transplantation and preventing acute allograft rejection [33]. However, ongoing maintenance therapy during post-depletional cell

repopulation is still necessary to prevent T memory cells from driving rejection and alloantibody formation [31]. Agents have included monoclonal antibodies, radiation and cytotoxic drugs, and several preclinical studies have found that combining lymphodepletion with other modalities may be tolerogenic [31]. Work is now ongoing to better understand the process of homeostatic repopulation after lymphodepletion.

#### Cellular therapy

In 1990 Hall et al. demonstrated that the transfer of cells from rats accepting heart allografts following a short course of cyclosporine to naive rats conferred donor-specific tolerance [34]. Over the subsequent decades tremendous strides have been made in our understanding of regulatory cells and their roles in autoimmunity, infectious disease, cancer and transplantation. In addition to CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> nTregs and iTregs, we know that other cell populations also exhibit suppressive or regulatory properties. T regulatory type 1 (Tr1) cells produce large amounts of suppressive IL-10 [35]; T helper 3 (Th3) cells produce suppressive TGFβ [36]; and T regulatory type 35 (Tr35) cells produce IL-35, which is related to the IL-12 superfamily and acts to suppress immune response by a variety of mechanisms [37]. In addition CD8<sup>+</sup>CD28<sup>-</sup> cells [38], CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> cells [39] and NKT cells [40] have all been reported to exert regulatory effects on alloimmune responses. Until recently, the role of B cells in immunity was thought to be limited to effector responses through antibody production, antigen presentation or cytokine production, but more recently they have also been shown to serve a regulatory role [41]. However, unlike Tregs, there are no validated molecular or phenotypic markers to define Bregs and so they are currently defined on the functional basis of their IL-10 production [14].

As the fate of transplanted organs is in part determined by the balance between effector and regulatory activities, one approach to promoting tolerance is to enhance regulatory functions by transferring regulatory cells into transplant recipients post-operatively [14]. However, Tregs make up only 1–5% of the peripheral T cell pool so must be expanded in vitro following isolation, which is itself challenging because the FOXP3 marker is an intracellular protein requiring permeabilization of the cell prior to detection. Nevertheless, significant progress has been made in this field and experimental studies using MHC-mismatched combinations in murine skin and heart allotransplantation have shown that alloantigen-specific Tregs are more effective at inducing indefinite allograft survival than polyclonal Tregs [42–44]. In 2011, Sagoo et al. translated these observations into a human in vivo model [45]. Human Tregs were enriched from peripheral blood donations from healthy volunteers and stimulated with irradiated mature myeloid dendritic cells (DCs). The Tregs were then sorted and expanded if they expressed two activation markers,

**Table 1** The current range of tolerogenic strategies in experimental and clinical settings (reproduced from Page et al. 2012 [30], used with permission)

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
Costimulation blockade	Alemtuzumab	Depleting mAb to CD52, on T, B, NK cells, some monocytes
	Abatacept	CTLA-4 Ig, blockade of CD28:CD80/60 costimulatory pathway
	Belatacept	CTLA-4 Ig, blockade of CD28:CD80/60 costimulatory pathway
Other T cell therapies	Efalizumab	Blockade of LFA-1:ICAM-1 costimulatory pathway
	Basiliximab	Blockade of CD25 (interleukin 2 receptor $\alpha$ chain)
Aldesleukin + rapamycin	Aldesleukin + rapamycin	Interleukin 2 + rapamycin, to increase regulatory T cell proliferation and survival, and stabilize the expression of Forkhead box P3(FoxP3)
B cell therapeutics	Rituximab	Depleting mAb to CD20
	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 a regulatory cytokine environment.
	Atacicept	Blockade of BAFF and APRIL
	BR3-Fc	Blockade of BAFF, causing decrease in peripheral, marginal zone, and follicular B cells
	Bortezomib	Proteasome inhibitor, causing apoptosis of mature plasma cells
	Eculizumab	Blockade of complement protein C5, to prevent complement-mediated injury due to circulating alloantibody
Cellular therapy	Mixed chimerism	Infusion of donor bone marrow into myoablated/immune-conditioned recipient, to produce co-existence of donor and recipient cells
	Regulatory T cells	Infusion of expanded regulatory T cells, to inhibit inflammatory cytokine production, down-regulate costimulatory and adhesion molecules, promote energy and cell death, convert effector  T cells to a regulatory phenotype, and produce suppressive cytokines IL-10, TGF $\beta$ , and IL35
	Regulatory T cells + IL-2	As above, plus the addition of IL-2 to promote Treg survival, development, and expansion
	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 TGF $\beta$ and lowIL-12, resist activation by danger signals and CD40 ligation, resist killing by natural killer of T cells, and promote apoptosis of effector T cells
	Macrophages	Immune suppression mediated through the enrichment of CD4 <sup>+</sup> CD25 <sup>+</sup> Foxp3 cells and cell contact-and caspase-dependent depletion of activated T cells
	Mesenchymal stromal cells	Inhibition of T cell activation and proliferation, potentially due to production of IL-10, NO, and IDO, and suppression of IFN $\gamma$ and IL-17

CTLA-4, Cytotoxic T Lymphocyte antigen 4; IDO, indoleamine 2,3-dioxygenase; IFN $\gamma$ , interferon  $\gamma$ ; IL-10, interleukin 10; LFA-1, lymphocyte function-associated antigen 1

CD69 and CD71, after allospecific activation with the DCs in vitro. Transfer of these customized Tregs into a humanized mouse skin transplant model prevented transplant rejection and skin damage with higher efficacy than that associated with the transfer of nonspecific, polyclonal Tregs. No clinical trials of Treg therapy in solid organ transplantation have been undertaken as yet, but promising data are emerging from early trials using Tregs for the prevention of graft-versus-host disease (GVHD) post hematopoietic stem-cell (HSC) transplantation [46–48]. The ONE Study - a multicenter Phase I/II study funded by the European Union Seventh Framework Programme – has set about investigating the safety of infusing

ex vivo-expanded Treg cells into kidney transplant recipients ([www.onestudy.org](http://www.onestudy.org)) and the Russian State Medical University are currently recruiting for a phase 1 pilot study using autologous CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Tregs and Campath-1H to induce renal transplant tolerance in children ([www.clinicaltrials.gov/NCT01446484](http://www.clinicaltrials.gov/NCT01446484)).

A second approach to promoting tolerance is to select immunosuppressive agents that maintain the function of regulatory cells while suppressing effector cells [14]. This rationale explains the frequent avoidance of calcineurin inhibitors (CNI) that may interfere with the development of Tregs and tolerance [49], and the inclusion of sirolimus (Rapamycin)

that supports the development and maintenance of Tregs [50]. Biologic agents may also have dramatically differing effects on Tregs and Table 1 describes some of the currently available monoclonal antibody therapies thought to favorably alter the ratio of Treg to Tconv in the experimental or clinical setting [30].

### Chimerism

Mixed chimerism refers to a hybrid immune system whereby donor pluripotent HSCs engraft and coexist with recipient stem cells, giving rise to hematopoietic lineages in the recipient [51]. The establishment of hematopoietic chimerism leads to permanent and stable donor-specific tolerance in organ transplant recipients, and immunosuppression is usually not required to prevent graft loss once engraftment has occurred [52]. Tolerance induction by chimerism is the only approach that has been successful in all species in which it has been tested to date, including humans [8, 53].

It has been known since the early reports of Owen in 1945 [54] and Billingham et al. [1] in 1953 that chimerism induces tolerance to organ and tissue transplants. Owen observed that genetically disparate “freemartin” cattle twins sharing a common placenta were red blood cell chimeras, suggesting that each was reciprocally tolerant to the other sibling as evidenced by persistent chimerism after birth. Billingham, Brent, and Medawar extended these findings to demonstrate that infusion of hematopoietic-derived cells into newborn mice resulted in chimerism and was associated with acceptance of donor skin grafts [1]. In the ensuing years, significant efforts have focused on overcoming obstacles preventing translation of this approach to the clinical setting: GVHD, the requirement for HLA-matched bone marrow donors, and toxicity of myeloablative conditioning.

The achievement of transient chimerism, and weaning of immunosuppression with stable graft function during follow-up, in cases of simultaneous bone marrow and renal transplantation for myeloma-induced renal failure in patient cohorts from Massachusetts General Hospital [55, 56] and Stanford [57], led the way for clinical trials in patients with end-stage renal failure without malignancy [58]. However, the risk of toxicity from ablative conditioning that was acceptable for HSC transplantation in hematologic malignancy became unacceptable when applied to non-malignant situations such as solid organ transplantation, and reinforced an interest in the development of non-myeloablative or reduced-intensity conditioning approaches [59–61]. Observations from years earlier demonstrating equal tolerance in animals with 1% donor chimerism and those with 100% donor chimerism [52] led researchers to believe that complete replacement of the recipient hematopoietic system was not a prerequisite to tolerance induction, and indeed it is now possible to establish chimerism

and tolerance with non-myeloablative conditioning, substantially reducing the risk:benefit ratio pertaining to tolerance-induction efforts in solid organ transplantation.

In 2011, Scandling et al. reported on 16 patients who underwent kidney and HSC transplants in Stanford, USA, between 2005 and 2011 [62], but perhaps more promising is Leventhal et al.’s interim report published earlier this year which follows 15 patients who underwent living donor renal transplantation with HSC transplantation in Chicago between 2009 and 2012 [8]. All Leventhal’s subjects were HLA-disparate from their living kidney or HSC donor, ranging from five of six matched related to zero of six matched unrelated. Patients underwent non-myeloablative reduced intensity conditioning with fludarabine and cyclophosphamide on days –3 and +3; and 200 cGy of total body irradiation (TBI) on day –1 relative to the renal transplantation on day 0. Hemodialysis was performed after fludarabine and cyclophosphamide administration to avoid toxicities of these agents. Tacrolimus and mycophenolate mofetil (MMF) were started on day –3 and continued as maintenance immunosuppression. Kidney transplantation was performed without antibody induction therapy or oral corticosteroid cover. A bioengineered FDA-regulated HSC product enriched for facilitating cells was infused intravenously on the day following kidney transplantation. All but one patient demonstrated peripheral blood macrochimerism after transplantation. Chimerism was subsequently lost in three of those patients at 2, 3, and 6 months post-transplantation. All patients demonstrated donor-specific hyporesponsiveness and were weaned from full-dose immunosuppression. Complete immunosuppression withdrawal at 1 year post-transplant was achieved in those patients with durable chimerism. The group reported no GVHD and no engraftment syndrome.

Despite these successes, the question of whether chimerism-induced tolerance can be adopted more generally remains to be seen. A significant degree of expertise is required to carry it out and concerns remain regarding the potential morbidity of the conditioning regimen and the true incidence of rejection and longer-term graft loss that will emerge once a large number of patients are transplanted. However, the greatest barrier to making this approach more widely available is that it is currently only practical with live donors, and roughly 60% of kidney transplants performed in the US and UK (2010 data) use deceased donors. HSC transplant product must be infused within 48 h of being harvested or appropriate cryopreservation techniques need to be established [63]. Studies are underway to adapt this approach for deceased donors and subjects who have already had a living donor renal transplant, and have a donor willing and able to donate HSCs for transplantation ([www.clinicaltrials.gov/IND14900](http://www.clinicaltrials.gov/IND14900)). The HSC transplant sources from deceased donors would be the vertebral columns, as already validated in several clinical protocols [63].

## Mesenchymal stem cells in solid organ transplantation

Like HSCs, mesenchymal stem cells (MSC) are bone marrow populating cells that possess an extensive proliferative potential and the ability to differentiate into a diverse range of cell types. They occupy the stroma where they play a key role in the maintenance of bone marrow homeostasis and regulate the maturation of both hematopoietic and non-hematopoietic cells. In 2008, the MiSOT consortium was founded to enable the effective collaboration between research groups working towards the application of adherent stem cell products in solid organ transplantation [64]. Earlier this year, Reinders et al. reported on a phase I study using autologous bone marrow-derived MSCs for the treatment of allograft rejection post renal transplant and observed a donor-specific down-regulation of peripheral blood mononuclear cell proliferation [65]. In keeping with this, Perico et al. describe an increase in the ratio of Tregs to T memory cells observed during their pilot study using autologous MSCs in renal transplantation in 2011 [66]. MSC administration in clinical transplantation remains at the exploratory stage, with many authorities still skeptical and wary of safety concerns regarding the potential for MSC mal-differentiation and increased susceptibility to opportunistic infection. Nevertheless, the goal of reducing severe side effects of pharmaceutical immunosuppression justifies attempts to implement novel cellular therapies. Indeed, the combination of Tregs and MSC infusions is under consideration at several centers.

## Biomarkers of operational tolerance

Achieving transplantation tolerance requires a bi-directional approach. In addition to studying cellular therapies and chimerism induction in the quest for tolerance and improved long-term transplant outcome, robust parameters to define operationally tolerant transplant recipients amenable to drug minimization or withdrawal must be established. Furthermore, elucidation of the molecular pathways associated with the operational tolerance phenotype could provide novel targets for therapy.

In general, operationally tolerant kidney transplant recipients are extremely rare [67] and at present, they cannot be identified prospectively. This poses a major hurdle in reaching sufficient statistical power to perform meaningful analysis in the search for tolerance biomarkers. Nevertheless, the *Immune Tolerance Network* (ITN), sponsored by the National Institutes of Health, and *Indices of Tolerance* consortia have proved valuable in identifying a relatively large cohort of tolerant kidney transplant recipients to study. The best control group to define the immunological profile of tolerant patients would be those patients in whom drug weaning or withdrawal have been attempted without success [68], but immunosuppressive drug withdrawal in kidney transplant recipients is

associated with a significant risk of graft loss [67] and so clearly, for ethical reasons intentional weaning without intervention cannot be performed.

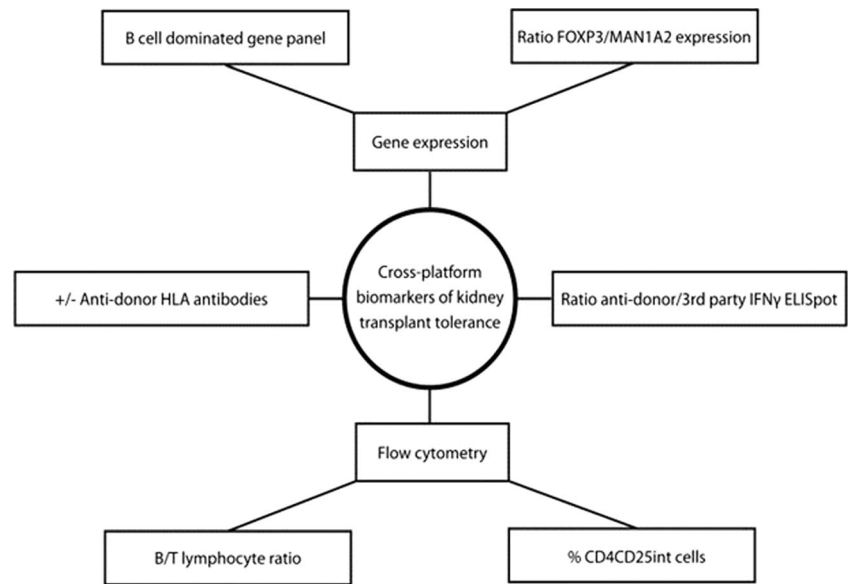
Thirty-six tolerant kidney transplant recipients were identified by the two consortia and fingerprints of kidney transplant tolerance have been developed [69, 70]. Stable patients receiving standard immunosuppression and healthy individuals, as well as patients with chronic rejection and stable function while receiving low-dose steroids only, were used as controls [68]. A large number of immunological parameters were determined, which resulted in a cross-platform signature of kidney transplant tolerance (Fig. 1) [68].

The liver is believed to have immunomodulatory properties, and there is growing evidence that operational tolerance can be achieved in a proportion of liver transplant patients significantly higher than that seen in other types of solid organ transplantation [2]. However, recent work by Waki et al. demonstrated a clear association between the presence of HLA class I and II antibodies post pediatric liver transplantation and future absence of operational tolerance [71]. These findings correlate with those published by Sarwal's group earlier this year in pediatric renal transplant patients [72]. They report a strong correlation between the development of de novo anti-HLA antibodies, most often after the first year post-transplantation, with significantly higher risks of graft injury and function loss. Further study is required to identify threshold levels and characteristics of HLA antibody specificities in relation to the development or absence of operational tolerance.

It is appealing to postulate that an increase in Tregs is mechanistically associated with tolerance and that monitoring the frequency of Tregs may identify tolerant recipients. However, Tregs infiltrate allografts in an attempt to suppress inflammation and thus may be present in all transplant recipients, including the small subset that are tolerant to the graft, but decreased in patients with chronic rejection. In keeping with this hypothesis, Louis et al. found no difference in the numbers of Tregs in tolerant individuals versus transplant recipients receiving immunosuppression who had stable graft function [73].

However, the same group did note an increase in total B cell numbers in the tolerant cohort, suggesting the possibility of B cell involvement [73], and more recently, a number of groups have reported the presence of a strong B cell signature in operationally tolerant transplant patients [69, 70, 74, 75]. One of these groups described a cohort of 25 operationally tolerant kidney transplant recipients and compared the phenotype and patterns of gene expression in peripheral blood and urine with those of kidney transplant recipients with stable function who were receiving immunosuppression, and samples from healthy volunteers [70]. Unexpectedly, there was a striking increase in the expression of B cell related genes as well as an increase in the actual number and frequency of B

**Fig. 1** Cross-platform biomarker signature of kidney transplant recipients determined by the Indices of Tolerance consortium (reproduced from Heidt and Wood 2012 [68], used with permission). The signature is dominated by B cell features, such as elevated expression of mainly B cell-specific genes, the absence of donor-specific HLA antibodies in the serum and elevated levels of (naive and transitional) peripheral B cells. Other markers that make up the tolerance signature are the ratio of FOXP3 to  $\alpha$ -1,2-mannosidase gene expression, a low donor-specific direct T cell response and decreased levels of activated T cells.



cells in the peripheral blood of the tolerant cohort. Analysis of microarray data demonstrated that 30 genes were differentially expressed between the tolerant group and the group receiving immunosuppression. However, all the studies of B-cell biomarkers of tolerance to date are based on the examination of long-term tolerant allograft recipients and not the prospective study of patients as they develop tolerance. Therefore, it is not possible to establish whether B cells are associated with the tolerant phenotype or are simply an epiphenomenon associated with other factors such as absence of immunosuppression. In contrast, natural killer cell transcripts seem to be the most robust markers of operational tolerance in liver transplantation, suggesting that different mechanisms operate in the two situations [76].

The link between chimerism and tolerance is well established and has led investigators to question whether the presence of chimerism could act as an independent biomarker of tolerance. The answer is “no”. A number of studies have recently reported a dissociation between tolerance and chimerism [77–80] and this dissociation is now thought to be caused by a lack of donor T-cell engraftment [80]. Production of donor T cells in murine chimeras correlates directly with tolerance to donor skin grafts, but chimeras without production of donor T cells reject donor skin grafts despite persistence of hematopoietic chimerism [51]. The role of donor T cells in tolerance induction and maintenance was in fact highlighted in Leventhal *et al*'s recent clinical study to induce tolerance to renal allografts through chimerism [81]. The mechanism behind the absence of donor T cell production in engrafted chimeras remains obscure but is likely to be affected by the conditioning approach used. Reliance on donor T cell chimerism as a biomarker for durable acceptance of hematopoietic grafts is not new to the HSC transplant community [82–84], and in the development of novel non-myeloablative

conditioning strategies to induce chimerism and tolerance, the solid organ transplant community will benefit from the lessons learned by our HSC transplant colleagues who focus on T cell chimerism as a primary end point [51].

Transplantation tolerance: an ongoing therapeutic goal

Strategies for inducing immune tolerance are fundamentally similar across a spectrum of immune-mediated disorders, including allergic disease, autoimmunity, and rejection of allografts. To that end, the quest to achieve transplantation tolerance has been aided significantly by two international research consortia, the *Immune Tolerance Network* and the *Reprogramming the Immune System for Establishment of Tolerance (RISET)/Indices of Tolerance* study. The results of pediatric renal transplantation have improved markedly in the last decade [85] but the same clinical problems remain in pediatric as adult renal transplantation: organ damage caused by chronic immune injury, long-term toxicity of immunosuppressive therapy, and difficulty in developing tolerance-inducing protocols. For that reason, The *Cooperative European Paediatric Renal TransplAnt INitiative* registry (CERTAIN; [www.certain-registry.eu](http://www.certain-registry.eu)) has been recently set up as a research network and platform on which to address unmet clinical needs [86].

Concluding remarks

Achieving reconstitution of immune tolerance in clinical medicine is a daunting challenge, but would have an enormous impact on both allograft and patient survival. A growing number of potential therapies provide a rich opportunity for matching selected interventions to appropriate patients, but

new insights into clinical stratification through use of biomarkers are required. International consortia are currently bringing strategic focus to this task, built upon the philosophy that diverse immune-mediated diseases, studied as a whole, will light the path towards immune tolerance.

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