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REVIEW

Tolerance in liver transplantation: Biomarkers and clinical relevance

Alberto Baroja-Mazo, Beatriz Revilla-Nuin, Pascual Parrilla, Laura Martínez-Alarcón, Pablo Ramírez, José Antonio Pons

Alberto Baroja-Mazo, Beatriz Revilla-Nuin, Pacual Parrilla, Laura Martínez-Alarcon, Pablo Ramírez, José Antonio Pons, Biomedical Research Institute of Murcia (IMIB-Arrixaca-UMU), 30120 Murcia, Spain

Pacual Parrilla, Pablo Ramírez, José Antonio Pons, Division of Gastroenterology and Hepatology and Liver Transplant Unit, University Hospital Virgen de la Arrixaca, 30120 Murcia, Spain

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Correspondence to: José Antonio Pons, MD, PhD, Division of Gastroenterology and Hepatology and Liver Transplant Unit, University Hospital Virgen de la Arrixaca, Ctra. Madrid-Cartagena, s/n, 30120 Murcia, Spain. joseapons.imib.arrixaca@gmail.com
Telephone: +34-968-369500
Fax: +34-968-369776

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Abstract

Transplantation is the optimal treatment for end-stage organ failure, and modern immunosuppression has allowed important progress in short-term outcomes. However, immunosuppression poorly influences chronic rejection and elicits chronic toxicity in current clinical practice. Thus, a major goal in transplantation is to understand and induce tolerance. It is well established that human regulatory T cells expressing the transcription factor FoxP3 play important roles in the maintenance of immunological self-tolerance and immune homeostasis. The major regulatory T cell subsets and mechanisms of expansion that are critical for induction and long-term maintenance of graft tolerance and survival are being actively investigated. Likewise, other immune cells, such as dendritic cells, monocyte/macrophages or natural killer cells, have been described as part of the process known as "operational tolerance". However, translation of these results towards clinical practice needs solid tools to identify accurately and reliably patients who are going to be tolerant. In this way, a plethora of genetic and cellular biomarkers is raising and being validated worldwide in large multi-center clinical trials. Few of the studies performed so far have provided a detailed analysis of the impact of immunosuppression withdrawal on pre-existing complications derived from the long-term administration of immunosuppressive drugs and the side effects associated with them. The future of liver transplantation is aimed to develop new therapies which increase the actual low tolerant *vs* non-tolerant recipients ratio.

Key words: Liver transplantation; Operational tolerance;

Regulatory T cells; Dendritic cells; Biomarkers

Core tip: Nowadays, the major goal in transplantation is to understand and induce tolerance. Although a plethora of genetic and cellular biomarkers is raising and being validated worldwide in large multi-center clinical trials, little is known about the impact of immunosuppression withdrawal on pre-existing complications derived from the long-term administration of immunosuppressive drugs and the side effects associated with them. The future of liver transplantation is aimed to develop new therapies which increase the actual low tolerant *vs* non-tolerant recipients ratio.

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INTRODUCTION

In 1953, Peter Medawar and his colleagues described in their key paper[1] that "acquired tolerance is due to a specific failure of the host's immunological response". Following on from this pioneering work of Medawar and his colleagues more than 50 years ago, extensive data obtained from rodents and large animal experimental transplantation models have led to a better understanding of the mechanisms leading to graft rejection and transplantation tolerance. In clinical transplantations since 1995, there has been increasing evidence to demonstrate that liver transplant recipients who cease to take immunosuppressive drugs maintain allograft function, suggesting that tolerance is already present[2,3]. Graft acceptance in the presence of significantly reduced immunosuppression (IS) requirements is referred to as "prope tolerance" or "minimal IS tolerance"[4]. In the clinical setting, "operational tolerance" (OT) is defined as the absence of acute and chronic rejection, and graft survival with normal function and histology in an IS-free, fully immunocompetent host, usually as an end result of a successful attempt at IS withdrawal[5]. Although complete immunosuppressive drug withdrawal has been rarely performed in an intentional manner, accumulated experiences from selected institutions indicate that this strategy is feasible in 20% of liver transplant recipients[6]. The achievement of immune tolerance to an allergenic donor has been a field of intense research over the last decades, fuelled by a critical need to avoid IS-related side effects (particularly nephrotoxicity, cancer, and cardiovascular events). Unfortunately, true immunologic tolerance has been difficult to achieve, in part, because allergenic engraftment is not a naturally occurring phenomenon and graft rejection is the most powerful and diverse immunologic response known. In recent years, the main endpoint of immunosuppressive therapy has shifted from the prevention of acute rejection toward the preservation of long-term graft function[7,8]. For instance, Foxp3-expressing regulatory T cells (Treg) critically prevent the occurrence of autoimmunity and suppress various immune responses. Some of the studies indicated that higher presence of Tregs correlated with better transplant outcomes, but some showed Tregs do not affect graft function and survival. The conclusion of each study might be limited to their study design or small sample size. Here, we review the development and function of Tregs, and how these cells are used to facilitate the induction of transplantation tolerance. Moreover, while dendritic cells (DC) are highly efficient antigen presenting cells (APC) for exerting allergenic immune responses, DC are also involved in establishing immune tolerance by deleting T cell clones or inducing Tregs[9], and we describe attempts of using tolerogenic DC as a therapeutic strategy to promote transplant tolerance. Likewise, we detail the implication of other cells in both the innate and adaptive immune system to diminish allergenic response.

In the other hand, development of new immunosuppressive drugs treating to minimize the adverse events while maintaining immunosuppressive efficacy are raising. The inhibitors of mechanistic target of rapamycin (mTOR), such as rapamycin and its derivate everolimus, are powerful non nephrotoxic agents with a different mechanism of action than calcineurin inhibitors (CNI), which blocking growth-factor-mediated cell proliferation in the cellular response to alloantigen[10,11], and could maintain an adequate level of IS while concomitantly promoting an immunologic profile which could favor tolerance to the graft.

Last but not least, a review of different attempts to establish a biomarker signature which define liver transplant recipients who are candidates to be subjected to a weaning protocol will be addressed in the last part of this overview.

REGULATORY T CELLS IN TRANSPLANTATION AND TOLERANCE

Regulatory cells are defined by their functional ability to suppress immune responses. In 1970, Gershon and Kondomade the seminal finding that T cells not only augmented but also dampened immune responses and that this down-regulation was mediated by T cells that were different from Th cells[12]. The term regulatory or suppressor cells was reintroduced in 1995 based on studies with mice thymectomized in the neonatal period that developed a fatal autoimmune disease[13].

They identified the CD25 molecule [the interleukin (IL)-2 receptor α-chain] as a Treg surface molecule candidate because CD25+ T cells, which constituted 5%-10% of peripheral CD4+ T cells (and less than 1% of peripheral CD8+ T cells) in normal naive mice, were confined in the CD25high and CD45RBlow fraction of CD4+ T cells. The identification of the forkhead box P3 (FoxP3) as a key transcriptional factor in Tregs has enabled us to determine a number of immunological characteristics of natural Tregs, including their function, stability and differentiation[14-16]. FoxP3 was initially identified as the responsible gene for an X-linked recessive inflammatory disease in scurfy mutant mice and then for the fatal autoimmune/inflammatory disease, immune dysregulation, polyendocrinopathy, enteropathy or X-linked syndrome (IPEX) in humans[17]. The indispensable role of FoxP3 for the control of these autoimmune and inflammatory disorders underlines the crucial importance of naturally arising FoxP3+CD4+ Tregs for self-tolerance and immune homeostasis[18].

Although other subsets of cells with regulatory functions have been described, CD4+CD25+FOXP3+ regulatory T-cells are the classic example. These cells are defined by FOXP3 expression, but they are not a homogeneous subset of cells. Regulatory T-cells can be subdivided into naturally occurring regulatory T-cells, which develop in the thymus, and adaptive or induced regulatory T-cells, which are converted from CD4+CD25- T-cells into cells with a characteristic molecular profile in peripheral blood[19]. Stable expression of FoxP3 in naturally occurring Tregs requires DNA methylation-based regulation[20]. Demethylation at a highly conserved region within the human *FoxP3* gene (Tregs-specific demethylated region, TSDR) was found to be restricted to Tregs when tested against all major peripheral blood cell types and a selection of non blood cells[21]. In addition to the high specificity for Tregs, it was also observed that FoxP3 TSDR demethylation occurred only in natural Tregs, but not in recently activated effector T cells transiently expressing FoxP3[22]. Some authors found that FoxP3 was fully demethylated in nTregs, partially demethylated in TGF-β-polarized iTregs and methylated in naïve and Th cells[21,23]. TSDR demethylation does not act as an on/off switch, but corresponds with stability of FoxP3 expression determined during the development in the thymus for naturally occurring Tregs. This data indicated that epigenetic modifications in the FoxP3 TSDR serve as a valuable marker for the identification of nTregs with a stable Tregs phenotype[24].

Although the exact mechanism of action of regulatory T-cells is still under debate, studies involving animal models have provided some insight. Such studies have shown, for example, that regulatory T-cells use several mechanisms to inhibit effector T-cells: modulation of APC function; metabolic disruption such as IL-2 deprivation or adenosine secretion; direct cytotoxicity toward effector T-cells; and secretion of inhibitory cytokines such as IL-10, IL-35 or TGF-β[25]. In the absence of regulatory T-cells, effector T-cells recognize alloantigens presented in the context of MHC molecules by APCs directly (donor APCs) or indirectly (recipient APCs). After T cells are activated by the binding of alloantigen-MHC to the TCR, and of CD80/86 to CD28, IL-2 is secreted by the effector T-cells, and through autocrine mechanisms leads to further T-cell activation, and proliferation and differentiation, ultimately causing allograft rejection. Regulatory T-cells inhibit APC function by down-regulating the expression of co-stimulatory molecules on APCs and by inducing APCs to produce immunoregulatory enzymes, such as indoleamine dioxygenase, that alter the metabolic microenvironment and depleting essential amino acids. In addition, the interaction between CD80/86 expressed by APCs and CTLA-4 expressed by regulatory T-cells is essential in mediating allograft tolerance. Once regulatory T-cells are activated, they secrete TGF-β, IL-10 and adenosine, which inhibit effector T-cells and render them unresponsive (anergy) or tolerant towards the graft. Collectively, these mechanisms protect the graft[25], such as Tregs are considered to be critical for the induction of transplant tolerance. Transplantation of MHC histoincompatible tissues elicits a strong, cytopathic, T cell-dependent immune response to donor tissues. In this T cell-dependent pathway to rejection, donor alloantigens are processed by donor (direct pathway of allorecognition) or recipient (indirect pathway of allorecognition) specialized APCs. The characteristics of the inflammatory environment in which donor-reactive CD4+ T cells recognize donor antigens determine the lineage commitment of these cells. Thus, depending on the cytokines present when antigen activation occurs, naïve CD4+ Th cells can acquire a variety of cytopathic and/or immunoregulatory phenotypes[26]. In the absence of proinflammatory cytokines, transforming growth factor TGF-β induces expression of FoxP3 and differentiation of CD4+ T cells into Tregs. In contrast, expression of TGF-β with IL-6 or IL-21 prevents development of the transplant-protective Tregs; instead, the antigen-reactive CD4+ T cells become IL-17-producing T cells (Th17), which are highly cytopathic[27-29]. Recent discoveries also revealed that, instead of being terminally differentiated, Th17 and Tregs have remarkable plasticity and are closely interlinked[30]. Thus, Tregs can differentiate into IL-17-producing cells in the presence of IL-2 and IL-1-β whereas in the presence of IL-27, Th17-producing cells also produce IL-10, an immunosuppressive cytokine that prevents them from functioning as destructive effector cells[31,32]. The current paradigm is that the outcome of transplant recipients, rejection or graft acceptance, is determined by the relative balance between cytopathic Th1 and Th17 CD4+ T cells *vs* rejection-blocking, cytoprotective Tregs; this balance depends on the level of inflammation in the microenvironment in which T-cell activation takes place[33].

Tregs have been used as a diagnostic tool in organ transplantation, and Tregs counts have been measured in blood, biopsy and urine samples after transplantation in many studies[34,35]. Although not unanimous, some studies have suggested that Tregs is associated with better outcome and can also serve as an immune marker to predict the individual risk of rejection and identify tolerant patients[36-41].

In liver transplantation (LT), several trials have been conducted to assess the feasibility of purposely discontinuing all immunosuppressive drugs under medical supervision[33]. Three studies reported the relationship between Tregs and transplant tolerance in LT[38,40,42,43] and demonstrated that Tregs content and function werenot lower in tolerance groups than chronic rejection group, stable group and control group, which suggested that Tregs may be associated with transplant tolerance. This Tregs increment was reported in retrospective studies where long-term operationally tolerant patients were compared with immunosuppressed patients. In the context of human LT, the dynamics of Tregs have not been extensively studied and may afford a means of identifying transplant recipients with a predilection to developing tolerance. Therefore, the immune process that occurs during the weaning off the IS was not analyzed. Our group carried out a prospective study to investigate the dynamic profile of the Tregs population in liver transplant patients during IS withdrawal and whether this profile could aid identification of patients who develop operational tolerance[44]. In this study the first evidence was provided to demonstrate that the increase of CD4+CD25high T cells and FoxP3 transcripts was associated with operational tolerance in liver transplanted patients during IS withdrawal.

Nowadays, Tregs are used as a cellular therapy for controlling rejection. *In vitro* and *in vivo* experimental models have demonstrated that production of Tregs in the periphery by FoxP3 transfection in naïve T cells can lead to tolerance induction and graft acceptance[45,46]. Nadig *et al*[47] have demonstrated that *ex vivo* expanded CD25hiCD4+ and CD127loCD25+CD4+ Tregs are very effective at inhibiting vasculopathy, with CD127loCD25+CD4+ cells being five times more efficient than T cells selected on the basis of high levels of CD25 expression prior to *ex vivo* expansion. These experimental data gave support to the potential use of Tregs in clinical transplantation. Many strategies exist for the *ex vivo* generation and /or expansion of Tregs[35,48-50]. Currently, three main approaches are being explored for Tregs expansion in the perspective of therapeutic protocols: *ex vivo* nTregs expansion, *ex vivo* conversion of naïve T cells to iTregs and *in vivo* expansion of nTregs and/or induction of iTregs[51-53]. Besides co stimulatory blockade, T-cell depletion induction therapies (*e.g.*, anti-CD3, anti-CD52 monoclonal antibodies or polyclonal anti-thymocyte globulins) are used in clinical solid organ transplant (SOT) to prevent acute rejection. These therapies induce profound and durable (weeks to months) reduction of circulating lymphocytes capable of mounting an alloresponse. Recent data suggests that T-cell depletion protocols allow preferential expansion of Tregs once lymphocytes gradually repopulate the host, thus skewing the Tregs/effector T cells ratio towards tolerance[54]. The "first-in-man" studies with expanded nTregs were carried out in patients who developed GVHD following bone marrow transplantation[55-58]. However, the use of antigen-specific Tregs at the time of transplantation may be limited if the donor is cadaveric, *i.e.*, not known in advance, as time is required to generate and expand *ex vivo* donor-specifc Tregs. In the contrary, if a living donor is available (HSCT, kidney, LT), recipient (or donor in the case of HSCT) T cells could be isolated in advance and manipulated *ex vivo* in the presence of donor-derived APC or peptides. Efficient isolation, expansion and cryopreservation strategies that comply with good manufacturing practice (GMP) guidelines are prerequisites for the clinical application of human CD4+CD25+CD127low FoxP3+ Tregs. Although the existence of Tregs is indisputable, using them for therapeutic purposes has not been straightforward; in fact the local microenvironment in which Tregs reside can have a considerable influence on their functional status[59]. In addition, one of the obstacles in the implementation of clinical protocols using Tregs is their low frequency in the peripheral blood leading to the need for *ex vivo* multiplication of the cells prior to their use *in vivo*[60]. While the transfer of Tregs prolonged allograft survival, it was not sufficient to induce robust tolerance on its own. This highlights the need for adjuvant immunomodulatory therapies to suppress strong immune activation and overcome the rapidly expanding pool of alloreactive T cells early after transplantation. Thus, the *in vivo* homeostasis, lifespan and stability of nTregs and iTregs need to be clarified before clinical trials on Tregs transfer can be considered.

DCs IN TOLERANCE

DCs act as surveillance for the immune system, sampling self and exogenous antigens in the peripheral tissues and presenting them to T cells in lymphoid organs. So, APCs serve as a bridge between antigens and lymphocytes. Likewise, DCs providing additional costimulatory signals and cytokines to stimulate the immune response. Functions of DCs stem from their high expression of surface major histocompatibility complexes (MHC) class Ⅰ and Ⅱ, costimulatory molecules and adhesion molecules[61]. Apart from their immunogenic roles, the influence of DCs on the immune system can also be tolerogenic or inhibitory in nature. DCs have been shown to be critical in maintaining central and peripheral tolerance through immune deviation, induction of T cell anergy, promotion of T cell apoptosis and induction of Tregs.

The importance of DCs in transplant rejection was highlighted by the finding that graft rejection was related to the migration of immunogenic passenger DCs into recipient lymphoid tissues, instigating rejection[62]. Donor derived DCs from allografts have the ability to directly migrate to recipient secondary lymphoid tissues to initiate immune responses[63], referred as direct pathway of allorecognition. Recipient DCs can be equally implicated in transplant rejection, through indirect pathway of allorecognition, as evidenced by the fact that skin allografts from MHC class Ⅱ$^{-/-}$ donors onto MHC class Ⅰ$^{-/-}$ recipients were still rejected[64]. However, more recently the view on recipient DCs as being solely potent stimulators of T cells has changed, based on evidence demonstrating the role of DCs in central and peripheral tolerance[65-67]. The overall tolerogenic or pathogenic capacity of DCs is dictated by: (1) the DC subset involved; (2) the maturation status; and (3) the microenvironment[61,68,69]. DC subsets differ on surface marker expression, tissue distribution and function. Human DCs subsets display a vast array of subsets: myeloid, plasmacytoid or follicular DCs and Langerhan's cells[70,71]. It is known that production by inmature DCs of indoleamine 2,3-dioxygenase (IDO), which catabolizes the essential amino acid L-tryptophan, evokes an amino acid deprivation, inhibiting antigen specific T cell proliferation while promoting Tregs development and tolerance[72,73]. The local microenvironment can have a significant impact on the development of DCs. Certain locations promote greater numbers of tolerizing DCs than others. Liver-derived DC progenitors were more suppressive than bone marrow derived DCs[74,75]. *In vivo* development of DCs in driven by hematopoietic growth factors c-Kit ligand and Fms-like tyrosine kinase 3 (FLT-3)[76-78], and production of granulocyte and monocyte colony stimulatory factors (GM-CSF) by activated T cells serves as maturation signal for DCs. Fully mature DCs produce proinflammatory cytokines and upregulate costimulatory molecules[79]. In contrast, immature DCs produce tolerogenic cytokine IL-10 and lack costimulatory signals for T cell activation.

Although the major mechanism of immune tolerance occurs in the thymus, DCs induce peripheral tolerance through: (1) deletion of alloantigen specific T cells; (2) induction of T cell anergy; (3) immune deviation; and (4) generation of regulatory T cells[9]. Transplantation of allogenic organs generates high frequencies of alloreative T cells, and deletion of donor-reactive T cells is critical in the induction of transplant tolerance. Elimination of donor-reactive T cells by DCs could be carried out through either inhibitory signaling or production of apoptotic factors[75,80,81]. The mechanisms through which immature DCs induce T cell anergy are not understood but thought to involve IL-10, directly through IL-10 receptor signaling or dependent on inducible T-cell costimulator (ICOS) signaling[82,83]. Another reason for considering tolerogenic DCs for tolerance therapies in transplantation is their ability to skew the cytokine profile in the direction of a Th2 phenotype[84]. DCs induce contrasting states of immunity or tolerance based on their maturation and subset. Both *in vivo* and *in vitro* evidence support the central role of IL-12 in the polarization of Th1 lymphocytes. Levels of IL-12 fluctuate during the different stages of DC development, therefore, DCs can differ in their immunogenicity depending on their maturation state. Tolerogenic DCs can in part mediate Tregs suppressive functions by promoting their development, principally by immature DCs[85]. Tolerogenic DCs may not only be involved in the induction of Tregs but may also play a role in the activation and maintenance of their suppressor functions. IDO has been shown to skew naive CD4+ T cell development towards the Treg lineage[86], and it was dependent on cell-cell contact mediated mechanisms[87]. Interestingly, Tregs may also promote the development of tolerogenic DCs from DC progenitors[88].

In this way, tolerogenic DCs are tempting from a clinical perspective because of their low capacity for T cell stimulatory functions and high capacity for inducing tolerogenicity. Tolerogenic DCs differ phenotypically and functionally from their mature DC counterparts. Downregulation of MHC class Ⅱ[89] and costimulatroy molecules as CD40, CD80, CD86 or CD83[90,91] or upregulation of inhibitory factors as B7-H1 or ICOS ligand[92] and death inducing ligands as FASL or TRAIL[93,94] on the surface of tolerogenic DCs compromises their ability to present antigen and activate T cells. In addition to contact dependent mechanisms of inhibition, tolerogenic DCs secrete effector molecules and regulatory cytokines (nitric oxide, heme-oxygenase-1, IL-10), thereby extending their suppressive effects[95-97].

In vitro propagation of DCs is necessary to generate the number of DCs required for therapeutic applications, due to DCs constituting a small fraction of leukocytes. In this way, several techniques have been carried out to manipulating DCs for therapeutic purposes. Maturation with GM-CSF[92] and tolerogenic cytokines, blocking the costimulatory pathway[74,98], using immunosupressive drugs as rapamycin[99] or by genetic engineering[100,101].

OTHER CELLS: B CELLS, MACROPHAGES AND NATURAL KILLER CELLS

B cells not only serve as an effector component of an immune response by generating antibodies, but they also present antigens to T cells and release immune cytokines. They may help to generate and expand Tregs as well as diminish antigen-specific T cell responses[102,103]. B cells also produce cytokines under inflammatory conditions. In particular, B cells produce large amounts of the immunosuppressive cytokine IL-10, which inhibits and reverses the progression of inflammation. Both CD5+ B1 cells and conventional B cells have been reported to produce IL-10[104]. These findings suggest that B cells may be critical regulators

in the process of tolerance induction. Clinical trials in renal transplantation revealed a significant increase of total B cell numbers and naive B cells in tolerant recipients[103,105]. Moreover, tolerant patients also had enhanced expression of B cell differentiation and activation genes such as TCL1A or VH4-34. It remains unknown whether the elevation of B cell numbers was a consequence of transplantation tolerance or whether the B cells were involved in promoting tolerance.

Other innate cell types exhibit similar features in tolerance induction. In certain settings, monocyte/macrophage can exert potent anti-inflammatory and immunosuppressive effects that help maintain peripheral tolerance[106]. The alternative activated M2 macrophages are capable of secreting anti-inflammatory cytokines, such as IL-10 and TGF-β that are involved in tapering immune responses and resolution of graft inflammation. In fact, some studies demonstrate that adoptive transfer of M2 macrophages can ameliorate the induction of experimental autoimmune encephalitis and prevent autoimmune colitis by inducing and expanding Tregs[107]. Additionally, adoptive transfer of donor-derived M2 macrophages in a cohort of human kidney transplant recipients allowed for significant reduction in the use of immunosuppressive drugs. Similarly, natural killer (NK) cells also employ different mechanisms to promote transplant tolerance. NK cells, guided by "missing self recognition", can eliminate graft-derived allergenic DCs, thus reducing T cell priming by the direct pathway of antigen presentation[108]. Killing of donor cells by NKs favours the indirect antigen presentation, which is implicated in tolerance induction. Also, some NK cells exhibit regulatory function through IL-10 dependent mechanisms and contribute to tolerance induction by tipping the balance towards regulation[109].

The striking dichotomy of innate immune cells in transplant settings (rejection *vs* tolerance) is most likely context dependent, representing opposite outcomes of the immune response to allotranasplants. Along this line, NK cells can be tolerogenic, and further NK maturation by IL-15 mediates rejection[110]. Likewise, M1 macrophages are pro-inflammatory and M2 macrophages are immunosuppressive. This context-dependent function of innate pathways and context-dependent regulation of innate immune cells constitute a major challenge in manipulating immune responses to allotransplants.

IMMUNOSUPPRESSIVE DRUGS IN TRANSPLANTATION TOLERANCE: RAPAMYCIN

CNI, such as tacrolimus and cyclosporine A, have become the principal immunosuppressive drug in solid organ transplantation[111]. Their use resulted in lower rejection rates and improved short-term allograft survival rates, although long-term improvements have been more difficult to achieve. The main reason is that prolonged CNI exposure is associated with nephrotoxicity[112], neurotoxicity[113], risk for cancer[114], metabolic complications[115], and hypertension[116]. Reducing CNI exposure is the main goal to lower these adverse events, maintaining immunosuppressive efficacy. The inhibitor of mTOR, such as rapamycin and its derivate everolimus, are powerful non nephrotoxic agents with a different mechanism of action than CNI. Meanwhile CNI block the production of proinflammatory cytokines leading to inhibition of T-cell activation, rapamycin reduce T-cell activation later in the cell cycle by blocking growth-factor-mediated cell proliferation in the cellular response to alloantigen[10,11], The distinct mechanism of action and favorable nephrotoxicity profile has led to rapamycin-containing regimens being developed with the aim of minimizing, eliminating, or avoiding exposure to CNI. mTOR is a protein kinase involved in the signal 3 pathway of lymphocyte activation[117]. More specifically, mTOR belongs to the PI3K pathway, which is involved in several fundamental cellular functions such as cell growth, proliferation, and survival. The mTOR protein interacts with several proteins to form two distinct complexes: mTOR complex 1 (mTORC1) and 2 (mTORC2)[118]. Rapamycin interact with and inhibits mTOR, but only when it is part of mTORC1 and not mTORC2[118].

Rapamycin mediates immunosuppressive effects through multiple immune cell types and processes. Inhibition of mTOR by rapamycin suppresses the immune response by preventing cell cycle progression from G1 to S phase, thereby blocking proliferation[119]. Likewise, rapamycin can promote T-cell anergy independently of the inhibition of proliferation even in the presence of TCR activation and co-stimulation by CD28 and IL-2[120,121]. Other important functions of rapamycin in the immune system are related to dendritic cells. Rapamycin inhibits the ability of dendritic cells to endocytose antigens, to express MHC class Ⅱ molecules and to express co-stimulatory molecules[122,123], thereby preventing these cells from fully maturing into APCs that can strongly stimulate T-cells. Furthermore, immature dendritic cells promote the expansion of regulatory T-cells thus promoting tolerance to the graft[124]. This is explained by the observation that the JAK/STAT signaling pathway is induced preferentially in regulatory T-cells, whereas the PI3K/AKT/mTOR signaling pathway is reduced relative to conventional T-cells[125]. In addition, rapamycin induces the expression of high levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL in regulatory T-cells; however, it downregulates the expression of such proteins in conventional T-cells[126].

Many studies have confirmed the beneficial effects of rapamycin or everolimus on regulatory T-cell biology[127-129]. Patients treated with rapamycin before an allergenic corneal transplant showed an increased percentage of regulatory T-cells after transplantation[130], these changes were associated with inhibition

Table 1 Biomarkers of tolerance in liver transplantation

Biomarker	Description	Study before or during IS withdrawal	Ref.
Dendritic cells			
pCD/mCD ratio	Tolerant patients have elevated pDC/mDC ratio. No differences between tolerant patients *vs* healthy controls	No	[134,150]
mDCs/pCD ratio	Elevated mDC/pCD associated with late rejection	No	[151]
PD-L1/CD86 ratio	Elevated PD-L1/CD86 expression on DCs in tolerant patients	No	[152]
DC HLA-G expression	Elevated on mDC	No	[153]
T cells			
Regulatory T cells	Increase of peripheral CD4+CD25high cells and RNA FoxP3 over time during weaning Increase in T regs in liver biopsy of tolerant patients	Yes	[40,43,44,133]
Natural killer	Increase in Tolerant patients	Yes	[154]
Soluble factors			
Serum HLA-G	Normal or elevated serum HLA-G levels associated to normal liver	No	[155-157]
Anti-donor antibodies	Absent in tolerant patients	No	[150]
Cell proliferation			
Phytohemagglutinin SI	SI < 20 and > 10 yr since LT 100% tolerance	Yes	[158]
Genetic profile			
Cytokine gene polymorphism	Low TNF-alpha and high/intermediate IL-10 production in OT	No	[159]
Gene transcripts	Enriched from NK, CD4+CD25+ FoxP3+, γδTCR+ and δ1TCR+	No	[42,43,160]
Genes related to iron homeostasis in liver graft	Enriched in tolerant patients (CDHR2, MIF, PEBP1, SOCS1, TRF)	Yes	[140]

DC: Dendritic cell; mDC: Monocytoid dendritic cell; LT: Liver transplantation; pDC: plasmacytoid dendritic cell; SI: Stimulation index.

of graft rejection. In another[128], patients treated with everolimus maintained constant levels of CD4+ T-cells during the treatment, but patients treated with CNI showed a decrease of these cells. Moreover, patients treated with everolimus had higher percentage of total CD4+ and naïve CD4 T-cells than those treated with CNI. With patients receiving IS, maintaining a pool of naïve T-cells is of great importance to protect against new infective agents. In addition, compared with cyclosporine A-treated patients, everolimus-treated patients had more regulatory T-cells and regulatory T-cells expressing CXCR3, a chemokine receptor that is responsible for the migration of T-cells to inflamed tissue such as the transplanted liver. Thus, everolimus seems to be more effective in preventing rejection by allowing regulatory T-cells to exert an effect *in situ*. Cyclosporine A-treated patients did not maintain the levels of regulatory T-cells that were present before LT.

The results of other studies of mice treated with rapamycin have suggested that antigen-specific T-cells responding to a pathogen express CD62L, which is associated with the development of a memory phenotype, whereas antigen-specific T-cells responding to a graft do not express this marker[131]. Thus, minimizing the generation of memory cells by treatment with an mTORi could decrease graft rejection responses, and indirectly promote an environment where tolerance could be established.

IDENTIFYING TOLERANT PATIENTS: A BIOMARKER SIGNATURE

A significant number of patients may become operationally tolerant after LT[6]; however, identifying tolerant patients before drug withdrawal is the purpose. Thus, researchers have focused on identifying biomarkers of tolerance that would aid the clinician in detecting tolerant individuals and help to elucidate molecular mechanisms of tolerance and provide therapeutic targets[132] (Table 1).

At the beginning, the studies performed to identify biomarkers of tolerance in LT employed immunophenotyping by flow cytometry and gene expression profiling of blood samples[40,42,43,133,134]. These studies were made in a retrospective and cross-sectional fashion, where operationally tolerant recipients defined as patients with stable graft function after IS withdrawal were compared to recipients under maintenance IS who suffered a rejection episode during drug weaning process (non-tolerant patients). Mazariegos *et al*[134] demonstrated a significant increase in the ratio of peripheral blood monocytoid dendritic cells (mDC) to plasmacytoid dendritic cell (pDC) precursors in tolerant patients compared to healthy controls and those on maintenance IS. In other reports, tolerant patients exhibited increased numbers of Tregs, and an increase in the vδ1/vδ2 T-cell subset ratio[133,135]. One of these groups showed, in their cohort of pediatric liver transplant recipients, that the γδT cell signature previously noted in peripheral blood mononuclear cells (PBMC) also characterized intragraft analysis and also showed significant accumulation of Treg in liver allograft biopsy samples of tolerant *vs* non-tolerant recipients[133]. These findings were corroborated recently in an adult cohort of tolerant subjects who underwent prospective withdrawal of IS[136].

While both peripheral blood immune cell phenotyping and cross-platform gene expression profiling showed tolerance to be associated with increases in B-cell-related transcripts, and in some reports, a skewing towards transitional and naïve B-cell repertoires[137,138], biomarkers associated with liver allograft tolerance are predominantly related to natural killer cells and γδT cells in blood, and genes related to iron homeostasis in the graft. Robust highly specific gene signatures have been developed as biomarkers associated with liver allograft tolerance[139]. The group of Martinez-Llordella *et al*[43] was the first to use microarray technology for the gene expression profiling of PBMC from operationally tolerant liver transplant recipients. They compared in a retrospective cross-sectional study gene expression profiles in the peripheral blood of tolerant and non-tolerant liver transplant recipients with healthy controls. They found that clinically tolerant patients could be identified not only by a signature of genes encoding several cell surface receptors expressed by NK, CD8+ and γδT cells as well as proteins involved in halting cell proliferation, but also by the expansion of CD4+CD25+Foxp3+ natural regulatory T cells (nTregs) and γδ TCR+ (especially vδ1 TCR+) T cells in the peripheral blood. This genomic and immunological footprint of operational tolerance was subsequently validated in an independent cohort of 23 additional recipients[42]. Our group reported one of the first prospective IS withdrawal studies analyzing the expression of FOXP3 in peripheral blood Tregs during withdrawal of IS in liver transplant recipients receiving cyclosporine A[44]. An increase in the frequency of CD4+CD25high cells was observed when IS was withdrawn in tolerant liver transplant recipients. Any significant difference in this population of cells was not observed in the non-tolerant group. In addition, tolerant patients exhibited an increase in *FOXP3* mRNA expression of peripheral blood mononuclear cells before complete IS withdrawal that continued even when IS therapy was stopped.

More recently, Bohne *et al*[140] reported the results of the first prospective IS withdrawal trial in liver transplant recipients including blood and liver tissue transcriptional biomarker studies. In that study, 98 liver recipients completed the trial: 57 experienced rejection and 41 were successfully weaned. Sequential blood and/or liver tissue samples from 75 recipients were analyzed with whole-genome microarrays and quantitative polymerase chain reaction. While PBMC gene analysis again corroborated the enrichment of natural killer and γδT cell transcripts, additionally, before initiation of drug withdrawal, operationally tolerant and non-tolerant groups differed in the intragraft expression of genes related to iron metabolism; tolerant patients also had higher serum levels of hepcidin and ferritin as well as increased iron deposition within hepatocytes. More important is the fact that certain hepatic tissue gene expression patterns had a high predictive value of the outcome of IS withdrawal in an independent set of patients. These results suggest a critical role for iron metabolism in the regulation of human intragraft alloimmune responses and provide a set of biomarkers to enroll the liver transplant patients into drug weaning trials with higher probability of success[140].

MicroRNAs (miRNAs) constitute a key regulatory component of immune system development and function. In a recent study, Vitalone *et al*[141] found in a rat experimental model of LT an increased expression of miR-142-5p and miR-181a in liver tissue and proposed that these miRNAs represented 2 potential biomarkers associated with tolerance. This study demonstrated the need for ongoing evaluation to delineate the role of individual miRNAs within the context of larger patient cohorts.

Recently, several promising biomarkers have been identified for determining patient alloreactivity and tolerance. A consensus document that aims to help tailor IS has been developed by the Biomarker Working Group of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology[142].

CLINICAL RELEVANCE OF TOLERANCE IN LIVER TRANSPLANTATION: DOES IMMUNOSUPPRESSION WITHDRAWAL REDUCE THE COMPLICATIONS RESULTING FROM ITS USE?

Regardless of the progress made in recent years in OT, it would be necessary to define in a controlled and prospective way different aspects that arise as questions from the patient's bedside: (1) Is it possible to withdraw IS in patients with LT? (2) Is it dangerous for patients to be subjected to an IS withdrawal protocol? (3) Is IS withdrawal beneficial for patients? and (4) Is there any parameter that during IS withdrawal process allows recognizing the group of patients who can be subjected to IS withdrawal? The first cases of OT after LT were documented by Starzl *et al*[143] in the early 1990s. Based on the finding that 11 LT patients had stopped taking IS medication due to lack of treatment adherence or post transplant lymphoproliferative disease, the authors designed a prospective study on intentional withdrawal of IS in LT patients and toxicity associated with IS[144]. In 18 (19%) of the 95 patients included in the study, IS could be completely withdrawn without causing alterations in liver function up to 2.2 years after inclusion. Since then, various studies have been published in which complete IS withdrawal in LT patients was attempted according to a pre-established protocol. Undoubtedly, intentional IS withdrawal protocols in LT without the use of presumably tolerogenic treatments are the largest in number and were the basis for establishing the proof of the concept of OT. Overall, OT was obtained in 23% of the patients without tolerogenic protocols, all of whom were selected for different reasons;

however, they were generally chosen because of adverse effects of immunosuppressive medication. The strategies investigated up to now, aimed at obtaining an IS-free state, are numerous and heterogeneous in terms of concept, rationale, patient age, underlying LT indications, objectives, type of LT (cadaver or living donor),duration of IS withdrawal period, duration of follow up, presence or absence of donor cell chimerism, tools used to measure tolerance mechanisms, *etc*. Nevertheless, the literature published to date can be summarized by maintaining that a permanent state of OT can be obtained in some patients undergoing LT for non-immunological underlying diseases and that those patients who do not achieve OT and experience rejection are not exposed to a greater risk of graft loss or death.

The first two prospective multicenter monitored clinical trials of IS withdrawal in pediatric and adult patients with LT have been recently published. The study of Benítez *et al*[145] is the first prospective multinational study of IS withdrawal in adults. This study included 102 patients out of 500 who were initially analyzed after IS withdrawal for a period between 6 and 9 mo. The primary goal of this study was to define the frequency of operational tolerance and the secondary objectives were based on mortality, graft loss, severity of rejection episodes, time between the start of IS withdrawal and rejection, histological liver changes after IS withdrawal, normalization of graft dysfunction after rejection onset and possible change in the side effects of IS followed by a 36 mo monitoring after inclusion in the study. Its main results and conclusions were:

A 40.2% (41/102) of patients with treatment intention or 41.8% (41/98) of patients by protocol compliance achieved OT, which was stable at least for 49 ± 7.7 mo of follow up.

Not all of the patients analyzed (500) were included in the study. Therefore, the applicability of this IS withdrawal strategy was only 20.4% (102/500).

The non-tolerant patients (57; 58%) were always this way during IS withdrawal and not after finishing withdrawal. Concerning these patients, rejection was mild or moderate in most of them and only severe in 5%. In addition, there were not any cases of chronic rejection and liver dysfunction was resolved in most cases with basal IS restoration or with association of low or moderate doses of steroids.

There were not any changes in comorbidities or in tolerant and non-tolerant patients after a follow-up period of 36 mo.

In tolerant patients, there was an increase of lobular inflammation beginning one year after IS withdrawal, not observed three years after such withdrawal.

One of the most important findings of this study is that the best tolerance predictor after LT is time. It is still more striking to notice that 79.2% of those who had had their graft for 10.6 years or more achieved IS withdrawal, indicating a propensity to develop tolerance over time. Nevertheless, the probability of tolerance was zero in patients with less than 6 years from LT and who were under the age of 49.

The results of this study demonstrate the real possibility of withdrawing immunosuppressive drugs in a higher proportion of patients with LT than the previously known of 20%, especially the more time has passed after the transplantation. The study has some limitations that the authors themselves acknowledge, as the possible bias because of the strict selection criteria that result in low applicability of this strategy. Furthermore, the lack of clinical benefit in terms of improvement of the side effects of IS requires a closer monitoring to study if it occurs. The findings of this study are consistent with the other large prospective study carried out in 20 pediatric transplant patients with parental living donor[146]. In this study, 60% of patients reached OT and these patients had been transplanted longer (median, 100.6 mo) than those non-tolerant patients (median, 73 mo). In addition, the study confirms a higher rate of OT in pediatric patients than in adults, as previously demonstrated.

The first two questions posed are clearly answered, both by Benítez *et al*[145], and by Feng *et al*[147], and it can be asserted that OT is possible and frequent the more time passes from LT and it is not particularly dangerous when done in a controlled way. Nevertheless, a longer follow up is necessary, since sometimes rejection can occur several years after IS withdrawal. However, it is more difficult to answer the question about whether IS withdrawal is beneficial for the patient, since neither study showed benefits.

A major focus of IS reduction or withdrawal has been the long-term effects. Few of the studies performed so far have provided a detailed analysis of the impact of IS withdrawal on pre-existing complications derived from the long-term administration of immunosuppressive drugs and the side effects associated with them (Table 2). In only one study the aim was to evaluate the feasibility of gradual withdrawal of IS in liver transplant recipients and to examine the impact of IS withdrawal on renal function and cardiovascular risk factors[148]. In this study, IS withdrawal was safely achieved in selected liver transplant patients and improved not only kidney function but also other CyA-associated side effects such as hypercholesterolemia, hyperuricemia, hypertension and diabetes control. However, longer follow-up periods are needed to confirm the benefits of IS withdrawal in liver transplant patients and to observe whether there are problems with chronic rejection after complete withdrawal of immunosuppressive drugs. Only one study has examined the effect of IS withdrawal in hepatitis C virus-positive recipients[146]. This study showed improvement in fibrosis after withdrawal, similar to that observed with successful post-LT interferon therapy. However, this preliminary study has not been replicated, and a follow-up study almost 3 years later did not show any histological differences.

Table 2 Impact of immunosuppression withdrawal on preexisting complications in liver transplantation

Author (year)	Ref.	No. of patients	Rational for IS withdrawal	Description of impact on preexisting complications	Impact on preexisting complications in tolerant patients
Mazariegos (1997)	[144]	95	Chronic IS-related toxicity	Yes	No changes in renal function or hypertension. Higher survey scores of patients well being
Devlin (1998)	[2]	18	Chronic IS-related toxicity	No	-
Takatsuki (2001)	[161]	63	30 PTLD	No	-
Eason (2005)	[162]	18	Patients who expressed a desire to discontinue IS	No	-
Tryphonopoulos (2005)	[163]	104	Role of DBMI in IS withdrawal in LT	No	-
Orlando (2008)	[146]	34	Impact of IS withdrawal on HCV disease in LT	Yes	Less cardiovascular or infectious diseases
Pons (2009)	[148]	22	Chronic IS-related toxicity	Yes	Renal function, hypertension, hypercholesterolemia, hyperuricemia, hypertension and diabetes control improved
Feng (2012)	[147]	20	Chronic IS-related toxicity	Yes	No changes in comorbidities
de la Garza (2013)	[158]	22	Chronic IS-related toxicity	No	-
Benitez (2013)	[145]	102	Chronic IS-related toxicity	Yes	No changes in comorbidities

IS: Immunosuppression; PTLD: Post-transplant lymphoproliferative disorder; HCV: Hepatitis C virus; LT: Liver transplantation; DBMI: Donor bone marrow infusion.

In this study, tolerant individuals were euglycemic and more intolerant individuals developed new onset diabetes that required specific treatment. Finally, significantly more intolerant patients were suffering from either cardiovascular or infectious diseases. Yoshitomi *et al*[149] found that grafts from operationally tolerant living donor LT recipients exhibited more fibrosis, ductular reactions, and decreased luminal diameter of bile ducts as compared to patients under IS treatment, and that these abnormalities improved after reintroduction of low-dose IS. However, these data should be cautiously interpreted due to the substantial difference in post transplantation time between the two groups.

A limitation of all withdrawal studies is the absence of prospectively followed, IS-maintained patients as control cohorts. Understanding the true clinical benefits of withdrawal rather than comparing long-term outcomes and IS-related effects in tolerant *vs* intolerant recipients is likely to be more useful when comparing such outcomes of intolerant *vs* IS-maintained or IS-minimized patients as control cohorts. The potential impact of IS minimization or withdrawal protocols on long-term subclinical histological graft damage (*e.g.*, idiopathic chronic hepatitis and/or progressive fibrosis) also remains to be properly investigated. This is relevant considering that most protocol biopsy studies have revealed substantial histological abnormalities in long-term surviving liver recipients with unremarkable liver function tests.

CONCLUSIONS AND FUTURE CHALLENGES

The future of LT should be focused on the reduction of side effects due to immunosuppressive drugs in order to improve quality of life with preservation of the viability of the liver graft. Tolerance is a reality in a reduced number of patients, so new treatments aimed to increase tolerance of the liver allograft have to be developed. Cell therapy with *ex vivo* expanded Tregs is currently being tested to induce LT tolerance. The effects of mesenchymal stromal cell infusions are also being explored, trying to improve preservation injury, preventing ischemic cholangiopathy, or facilitating IS minimization. While these are very promising studies, key issues related to dosing, timing or most appropriate adjunctive IS will need to be clarified before large scale clinical applications can be considered. Looking into the future, conventional immunosuppressive drugs will likely remain as principal therapy after LT. Some selected patients will not need IS due to induced or spontaneously developed tolerance. In the remaining recipients, IS will be administrated according to the quality of the graft, inflammatory status, or degree of cellular or humoral sensitization.

Defining new biomarkers to assess the individual immune status of a transplant patient to fine tune the immunosuppressive therapy is the key to improve graft and patient survival. Many biomarkers have not yet been validated in comprehensive prospective

clinical trials and the proposed clinical decision limits are frequently based on retrospective and single center experiences. Molecular profiling is evolving at unprecedented rates, as are the bioinformatic techniques required to enable the handling of the vast data pools generated. Definitive substantiation of the clinical utility of any of the discussed biomarkers rests on their successful application in prospective, randomized trials of biomarker-led IS weaning. Lastly, it is becoming evident that a single biomarker cannot be able to reflect all the alterations of the immune system associated with LT. Therefore, a panel of different biomarkers will be needed to properly evaluate the immunological suppression and to modify immunosuppressive treatment according to patient needs.

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