Chapter

Regulatory T Cells in the Mosaic of Liver Transplantation Tolerance

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Abstract

The success of transplantation depends on multiple factors, but the establishment of immune tolerant milieu is of critical importance. Hepatic environment consists of different cellular populations with prominent capacity to tolerate a huge range of antigens. Among them, regulatory T cells (Tregs) play an important role. They control the strength of immune reactions against non-self antigens and were shown to have an impact on the establishment of immune tolerance in the post-transplantation period. Furthermore, they impact a particular state after transplantation – operational tolerance. The abundant data show that Tregs might be manipulated, which suggests their further implementation as a treatment strategy. Tregs are also a very attractive target as a biomarker in the monitoring of post-transplantation period. Here, we review the particular role of Tregs among the broad spectrum of immune tolerance mechanisms of the liver in the light of the current directions of medical research.

Keywords: liver transplantation, regulatory T cells, immune tolerance, biomarker, operational tolerance

1. Introduction

Transplantation is the most beneficial approach to treat diseases, manifested by irreversible changes of the liver parenchyma. The success of transplantation depends on both the surgical operation and the development of an immune tolerant milieu in the post-transplantation period. In solid organ transplantation, the immunological mechanisms that are naturally dedicated to the defense from foreign antigens (microbial, viral etc.), are directed towards HLA (MHC) molecules and allo-antigens of the graft. This powerful immune reaction may destroy the graft and compromise the beneficial effect of the transplant. In the routine clinical practice, the control of effector immune function is achieved through immune suppressive therapy. However, in kidney and liver transplantation a spontaneous development of immune tolerance where a particular T cells subset – regulatory T cells (Tregs) is supposed to play important role [1].

Thus, recent achievements in transplantation research motivate the focus on the immunological mechanisms in two directions. From one side, this is the continuous investigation on new and more relevant biomarkers for the monitoring of the post-transplantation period and prediction of graft rejection. From the other side, is the need of new therapeutic opportunities that might be influenced by the scientific research on the fine immune mechanisms [2].

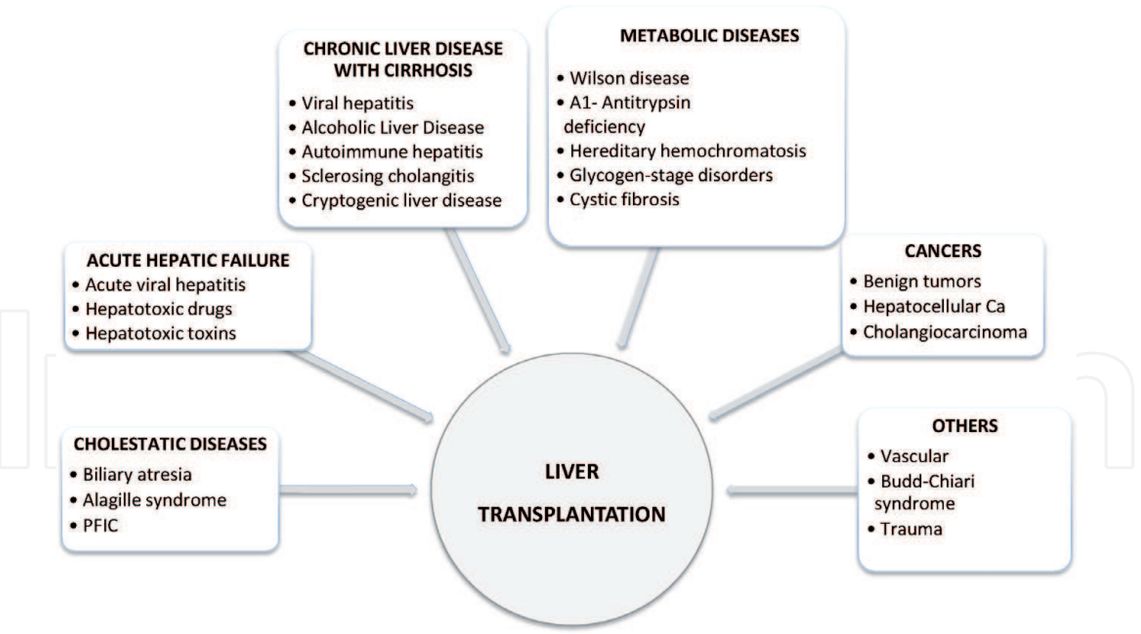


Figure 1.
Principal medical conditions that require liver transplantation.

Studies on Tregs are particularly intense in the field of transplantology precisely in connection with their suppressive function. Lots of data in the literature on their behavior in transplantation of solid organs as well as stem cells is present. While in kidney, heart and other transplants already have outlined trends in the dynamics and even the therapeutic application of Tregs [3], the situation with liver transplantation (LT) is more special.

Patients with life-limiting liver disease, which may present in the form of acute liver failure, end-stage chronic liver disease, hepatic malignancy, or inborn metabolic disorders need LT - liver function is heavily impaired as a result of irreversible morphological changes (**Figure 1**).

Whatever the cause, the outcome of liver transplantation depends on three main factors: the clinical approach, the immune characteristics of the liver, and the therapeutic provision of immunological tolerance.

2. Clinical aspects of liver transplantation

From a clinical point of view, the outcome of transplantation depends on the general condition of the recipient (MELD score in adults and PELD score in children) before surgery and the quality of the graft, surgical technique, postoperative care, immunosuppressive therapy. The operation is one of the largest in volume and complexity in surgery in general. Most often in Europe and the United States the so-called "standard" LT is performed, in which an entire organ is transplanted - whole liver graft. Deceased liver transplantation (DLT) is not common in Asia and part of a living donor organ is used [4, 5].

In deceased, an organ donation is possible when the graft is from a donor who has been registered as brain dead (brain dead donor) and donation after cardiac death.

Liver transplantation from a living donor (LDLT) offers some advantages over cadaveric donation: determining the time of the operation, the graft is from a healthy person and is in optimal condition, the cold ischemia time is shortened. It is suitable for children due to the possibility of precise selection of the graft in

accordance with the patient's weight. Choosing a donor candidate is sometimes difficult due to the presence of arterial variations combined with additional abnormalities in other vessels and the biliary tract [6]. LDLT is suitable in cases of rare diseases in patients under 1 year of age. Of the pediatric liver transplantations performed at Lozenets University Hospital, about 65.5% of the patients are in this age group, and between 10 and 18 years of age they are significantly fewer [7].

Another type is domino transplantation, but it is rarely performed. Indication for it is Familial amyloid polyneuropathy (FAP). The disease affects extrahepatic organs and liver function is preserved. This allows the liver of the FAP patient to be given to another patient, from whom (in turn) receives the damaged organ (domino effect) [8]. The main requirement for the FAP recipient is to be over 55–60 years old, in order to minimize the risk of developing the disease.

Partial transplantation is performed as a matter of urgency in two specific situations. The first is in acute liver failure, in order to support the damaged organ until its recovery. The graft is then removed and the immunosuppressive therapy is stopped. The second case is in patients with congenital functional or metabolic disorders that affect the liver. Implantation of the partial graft preserves its own organ, corrects metabolic abnormalities and does not require whole liver transplantation [9]. In both situations, the transplant can be orthotropic or heterotropic.

A variant of the partial transplantation is the split-transplant, in which the two lobes are distributed between two recipients. In recent years, due to the increased number of patients on the waiting list and the small number of potential donors, the technique of split-liver transplantation has been applied in which in vivo /in situ or ex-vivo/ex-situ the liver is divided into two parts - right for adult transplantation and left for pediatric transplantation. In some cases, it is possible to use the split-technique for transplantation of two adults. It is preferable to perform split-LT in-vivo, which reduces the risk of biliary complications, hemorrhage and significantly reduces the cold ischemia time of the graft [4, 5]. The main condition is the ratio between the weight of the graft and the patient, which must be at least 0.8% [4, 10]. The aim is to ensure the long-term vital functions of the recipient.

The complexity of the operation creates preconditions for the occurrence of complications during and after the operation. In the postoperative period, the leading are vascular and biliary complications, stenosis of the anastomosis, risk of infection and others.

In the long term, the outcome of transplantation depends largely on the establishment of optimal post-transplant immune tolerance. Here the immunological features of the liver, which distinguish it from other organs, play a significant role.

3. Tolerogenic milieu of the liver

The liver is a metabolic organ with a principal role of the detoxification and nutrient storage, but also protein synthesis and production of biochemicals required for the digestion and growth.

Without a doubt, the liver is also an important element of the immune system. A broad range of parts of innate and adaptive immunity is synthesized inside like acute-phase proteins, cytokines, complement components etc. Indeed, the cells that populate the liver encompass not only those with metabolic function. A great variety of immune cells are found in the parenchyma. Liver sinusoidal endothelial cells (LSECs), Kupffer cells, dendritic cells, hepatic stellate cells (HSCs), natural killer (NK) cells, NKT cells and T cells are present in the liver interstitium. In addition, hepatic cells express surface receptors immanent for the innate immunity [11]. Altogether, they participate in the establishment of a particular milieu that

from one hand tolerates a broad range of gut-derived antigens continuously passing across and on the other hand, retain the capacity to set up an immune response against pathogens like bacteria and viruses.

The questions of how this community maintains an immunotolerant environment to nutritional antigens, provide effector response to pathogens and guarantee liver transplantation are of particular interest. Moreover, the graft rejection is relatively rare in LT as compared to other SOT.

The combination of particular anatomy, variety of cells, specific expression of HLA molecules [12] and sustained antigenic stimulation makes the liver a unique immunologic structure. The blood delivered by vena portae is rich in alimentary and other antigens, which in fact are tolerated by the healthy liver. Indeed, the basal levels of pro- and anti-inflammatory cytokines are constant, but change under pathologic conditions in etiology-dependent manner [13–15].

The liver primarily is a metabolic organ and this function significantly impacts its immunologic reactivity. The metabolism of carbohydrates and lipids has a particular impact. Absorbed by hepatocytes, they are collected as glycogen and lipoproteins. Further on, cholesterol, triglycerides and other intermediate metabolites can trigger TLR signalization and inflammasome activation. The final result is the increased pro-inflammatory cytokines secretion followed by the initiation of different pathologic phenomena, liver fibrosis for example [16]. Another example demonstrates that metabolic variations in hepatocytes during hepatitis B and C infection can raise viral replication [17, 18].

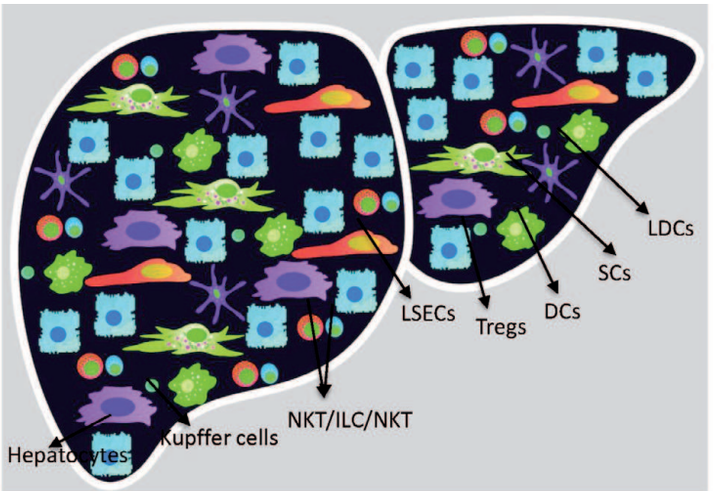
Together with hepatocytes, dendritic cells and macrophages also participate in the hepatic cytokine regulation. The oxidative phosphorylation may switch to anaerobic glycolysis (effect Warburg) leading to stimulation of pro-inflammatory mediators synthesis [19, 20]. Succinate dehydrogenase can additionally influence the cytokine balance. Its high levels may activate Hypoxia induced factor-1 (HIF-1) and production of IL-1β, thus providing evidence for the direct communication between the cellular metabolism and inflammatory response [21]. IL-1β per se is an important player in the control of homeostasis by regulating sleep, feeding, temperature in healthy conditions [22]. In pathologic situations, IL-1β is a critical mediator of the inflammatory response through processing of pro-IL-1β by Caspase-1 via inflammasome [23, 24]. Other mechanisms can be also involved in the cleavage of pro-IL-1β into biologically active IL-1b. Among them are serine proteases-neutrophil elastase, proteinase 3, cathepsin G in neutrophils [25–27] and the spontaneous release of IL-1β following pyroptosis and necroptosis [28]. Not surprisingly, IL-1β levels were found increased in other, relatively frequent medical conditions like NAFLD [29].

4. Tolerogenic properties of liver cells

Hepatic cells represent a heterogeneous population where different liver residents have their own tolerogenic approach (**Figure 2**).

4.1 Hepatocytes

Parenchymal hepatocytes are the major population in the liver. Although being involved in metabolism, toxin neutralization and glycogen synthesis, they function also as immune cells by expressing immune-associated molecules like pattern recognition receptors (PRRs), adhesion and major histocompatibility complex (MHC) molecules [30, 31]. The latter permit hepatocytes to act as antigen-presenting cells for CD8+ T cells and to trigger their activation and proliferation [32]. However, the particular hepatic environment does not ensure the required survival factors



Cell type	Tolerogenic capacity
Hepatocytes	Expression of PRRs, MHC molecules, antigen presentation, , induction of Tregs
Kupffer cells	Receptors for complement and antibodies, TLRs, cytokines
Liver Sinusoidal Endothelial Cells	Expression of TLRs, RIGs and B7-H1, antigen presentation,
Stellate Cells (SCs)	Antigen presentation, PD-L1, B7-H7, Fas/Fas-L expression, induction of Tregs
Liver Dendritic Cells (LDCs)	Generation of Tregs, immunosuppressive cytokines
NKT/ILC/NKT	Limited response to stimulation vie TCR, weak suppressive potential
Regulatory T cells (Tregs)	Production of suppressive cytokines, expression of inhibitory molecules, Potential to be introduced as immune therapy after transplantation

Figure 2.
The mosaic of tolerogenic cells in the liver. On the table below are shown main tolerogenic mechanisms, employed by every population.

for CD8+ cells and they rapidly undergo activation-induced apoptosis [33]. Interestingly, the inflammatory response can be accompanied by the expression of MHC class II molecules followed by antigen presentation to CD4+ T cells [34, 35]. Depending on the differentiation status of helper cells, they may undergo Th2 differentiation of uncommitted CD4 T cells, or abrogated ability of previously differentiated Th1 to secrete interferon-γ, and finally - switch of CD4 + T effector cells towards induced regulatory T cells (FoxP3 + CD25+) [33, 36] . Ergo, liver parenchymal cells have substantial tolerogenic potential directed to both CD4+ and CD8+ T cells. Whether all would be launch together or not need to be elucidated.

4.2 Liver sinusoidal endothelial cells

Liver sinusoidal endothelial cells (LSECs) are an important part of the reticuloendothelial system. They are highly specialized and form the lining of the hepatic sinusoids. Their characteristic morphology (abundant fenestrae) and the permanent exposition to the blood flow permit them to filter out blood antigens. From an immunological viewpoint LSECs are liver-resident antigen-presenting cells that might be considered as bridge between the innate and adaptive immunity. It is evidenced by the expression of TLRs and RIGs, which under stimulation, turn on the production of proinflammatory cytokines, upregulation of costimulatory molecules and release of cytokines, that affect T cells [13, 37]. In parallel, LSECs may present antigens to T cells although not being professional APCs as shown by the expression of MHC class I on the LSECs surface [15]. The question about MHC

class II expression is still not well clarified, although under specific conditions, LSECs may present antigens to CD4+ T cells inducing immune tolerance [38] . LSEC actively participate in the induction of tolerance. For example, in naïve CD8+ T cells, the cognate interaction triggers the expression of co-inhibitory B7-H1 but not the co-stimulatory CD80/86 molecules exclusively on LSEC but not DC, which together with increased costimulation via CD28 is critical for the induction of CD8+ T cell tolerance by LSEC [39].

4.3 Kupffer cells

Kupffer cells (KCs) are particular subset of macrophages, settled in the liver. They represent appr. 35% of the non-parenchymal liver cells and 90% of all tissue macrophages [40]. They are located in the sinusoids, thus being systematically exposed to gut-derived antigens, circulating immune cells and pathogens. The principle function of KCs is pathogen killing. However, KCs are armed with scavenger receptors, TLRs, complement receptors and antibody receptors, secrete cytokines and chemokines and express broad range of receptor molecules [41]. Thus, KCs not only participate in antimicrobial killing, but also are an active player in the immune network. Their primary function is the antigen presentation. They express MHC class I and class II molecules which together with other costimulatory molecules activate T cells. In healthy conditions, KCs promote immune tolerance in several ways – lower expression of MHC class II, B7–1, B7–2, CD40, PD-1/PD-1 L and possible involvement of IL-10, nitric oxide, TGF-b [38, 42]. Other mechanisms include the ability of KCs to absorb and clear alloreactive antibodies in liver transplantation [43]. Conversely, when stimulated (through TLRs for ex.), KCs become potent activators of T cells and NK cells [44]. In animal models, depletion of graft Kupffer cells was beneficial for the graft acceptance [45]. The double sword performance of KCs needs to be investigated in details, especially in humans. In any case, the current knowledge clearly indicates the KCs impact heavily on the reactivity of the liver immune system.

4.4 Stellate cells

Hepatic Stellate cells (HSCs) are another subset with immune function. This relatively small population (5–10% of liver parenchymal cells) resides in the space of Disse and is primarily involved in the storage of retinoid droplets and vitamin A and regulation of blood flow in the sinusoids [32, 46, 47]. In the intact liver, they are quiescent cells. Once activated, they differentiate to myofibroblast and participate in hepatic fibrosis pathogenesis [48]. The second direction of their function is the immunosuppression. Study in animals and humans show that they are effective antigen presenting cells with tolerogenic capacity because of the expression of PD-L1, B7-H7 and Fas/Fas-L pathways [49–51]. When activated, HSCs induce myeloid derived suppressor cells and Foxp3+ regulatory T cells by production of retinoic acid [52] In addition, they produce a broad range of cytokines, like TGF-b, IL-6 etc., and are able to respond to them, thus actively participate in the immune-mediated network in the liver [48].

4.5 Liver dendritic cells

Dendritic cells (DCs) are professional antigen-presenting cells. Following the antigen processing, they activate T cells and unlock the adaptive immune reaction. The liver-resident DCs are distinct population as compared to blood DCs. While DCs expressing CD1c and CD14 represent 95% of blood DCs, in liver they are 70%

and those expressing CD141 increase up to 30% [53]. It is still unclear in details how these cells contribute to the development of immune tolerance. Although some evidences that DCs may enhance graft rejection by increase of CD80 and CD86 expression [54, 55], the depletion of donor DCs is followed by graft rejection [56]. The investigations of Bamboat et al. demonstrate that liver DCs generate more suppressive $CD4^+CD25^+$FoxP3+ T regulatory cells and IL-4-producing Th2 cells via an IL-10-dependent mechanism [57]. Another particular feature of LDCs is their low endocytosis capacity and weak capacity to stimulate T cells. Conversely, they produce high levels of the immunosuppressive IL-10 [58, 59]. The comparison with their splenic match reveal important differences: lower secretion of type I interferons, "lipid-based dichotomy" – lipid contents dependent antigen presentation, plasmacytoid DC ($B220^+$) account for 19% of liver DC, but only 5% of spleen DC [60–62].

4.6 NK, ILC cells and NKT cells

Natural killer cells represent 30–50% of hepatic lymphocytes [63]. They differ from conventional NK cells and are closer to innate lymphoid cells (ILCs). In mouse they are closer to ILCs1, because of the expression of NK1.1 (CD161), CD69, CD49a NKp46, TRAIL. Both in mouse and human, these cells express CD49a and CD69 and secrete IFN-g and TNFa, but have weak suppressive capacity [64]. The third subset NKT cells are well presented in the liver. Different subsets are differentially presented in mice and humans, but have similar function – support immune homeostasis, control autoimmune reactions and immune responses to microbial and viral infections and cancer [65]. Of particular interest are invariant NKT and mucosal-associated invariant T cells (MAIT). They are $CD3^+CD4^-CD161^+V\alpha7.2^+$ cells and have robust IFN-g and granzymes B response to inflammatory signal, but limited responsiveness when stimulated directly via TCR [66, 67].

Therefore, the proper hepatic cells have dual function. On one hand they are involved in metabolic processes in the liver and on the other – they participate in immune-mediated reactions per se and by carrying out the function of a bridge between biochemical reactions and immune pathways.

Hepatic cells interact with local immune cells and thus actively participate in the establishment of a sustained immune tolerant milieu. Zheng and Tian (2019) analyzing current data, highlight the death of effector cells and the "education" of regulatory cells as key processes leading to the development of liver tolerance. Additionally, they describe a broad spectrum of baseline immune mechanisms responsible for the state of hyporesponsiveness – clonal deletion, clonal anergy, clonal deviation, T cell dysfunction/exhaustion, education etc. [68].

5. Regulatory T cells – conditio sine qua non for liver transplantation tolerance

5.1 General characteristics of regulatory T cells

Regulatory T cells are considered as the effector cellular arm of immune tolerance. Since the first publication of Kojima (1976), there is a constantly growing interest towards Tregs – cellular properties and medical applications [69]. Along with their unique suppressive phenotype (CD25 + FoxP3 + CD4+ T cells) [68, 69], Tregs express broad range of molecules that mirror their affiliation to the population of T cells and are widely applied in research and medical

practice. Similarly to conventional T cells, they may be differentiated as naïve and memory, based on the expression of CD45RA, recent thymic emigrants (CD31), activated (HLA-DR) etc. [70, 71]. Their trafficking is ensured by the expression of chemokine receptors [72, 73].

The hallmark of Tregs is the expression of the transcription factor FoxP3 [68]. It controls the transcription program of Tregs by regulating several genes – increases expression of *Il2ra* (CD25), *Ctla4* (CTLA-4), *Tnfrsf18* (GITR), but inhibits those of *Il2*. At the same time *Foxp3* is subject of a tight regulation, where STAT5 signaling pathway is probably of key importance [74–77]. One may say that this is as two step process, starting with the generation of $CD25^{hi,}$ but $FoxP3^{-}$ Tregs-precursors, followed by the induction of FoxP3 through cytokine/STAT5-dependant signals involving HDAC [74, 78, 79]. Blocking of JAK/STAT pathway downmodulates Foxp3 expression [80]. In addition, a group of studies indicate that the maintenance of Tregs suppressive function is dependent on the epigenetic regulation of foxp3 locus by the Polycomb repressive complex 2 (PRC2). PRC2 consists of four subunits, primarily of enhancer of zeste homolog 2 (EZH2), EED, SUZ12, and RbAp48, where EZH2 is of particular interest [81]. The function of EHZ2 differs among different T-cell populations, leading to variations in H3K27me3 levels and silenced genes. In FoxP3 negative cells, EZH2 deficiency is associated with autoimmune diseases, reduced number of Tregs and expansion of memory T cells [82, 83].

The origin of Tregs in periphery is still a hot topic. Clear evidences show that a subset of Tregs - thymic Tregs (tTregs), come directly from the thymus during the process of intrathymic maturation of T cells [84–86]. Their selection occurs predominantly in the medulla during the negative selection by the high-avidity interactions between mTECs and thymocytes [87, 88], although some studies indicate that the process starts earlier, in the cortex [89]. Under specific conditions, like increased concentration of TGF-β, hormonal changes or continuous antigenic stimulation, Tregs can arise from naïve CD4+ T cells in periphery – inducible Tregs (iTregs) [90–92].

5.2 Tregs are armed by different suppressive mechanisms

Independently of the origin, Tregs are powerful immune suppressors. Both subsets use several approaches to regulate the strength of the immune response. Roughly, they are based on the expression of particular molecules, secretion of cytokines and consumption of IL-2 and might be categorized as contact-dependent and contact-independent.

Early studies demonstrated that the contact-dependent way is effectuate by the constitutive expression of CD152 (CTLA-4) by Tregs [93, 94]. The engagement of CD80/CD86 pathway activates tryptophan catabolism and expression of indoleamin 2,3 dioxygenase (IDO) [95]. Another mechanism involves PD-1/PD-L1 [95]. It is effective both against autoreactive B cells [96] and T lymphocytes [97]. Dilek et al. using alloreactive human T cells and blocking antibodies, evidenced by live cell dynamic microscopy that CD28, CTLA-4, and PD-L1 differentially control velocity, motility and immune synapse formation in activated Teff versus Tregs [94]. Although natural, their expression on the Tregs surface is inducibly increased and ensures the negative regulation of different receptors mediated signaling cascades in the target cells [98, 99]. Thus, Tregs directly attenuate cellular proliferation and activation.

The second line is facilitated by the production of different soluble factors upon activation. Among them are the immunosuppressive cytokines IL-10, TGF-b [100, 101], IL-34 [102] and IL-35 [103, 104]; perforins and granzymes [105]. It should be also considered that Tregs are target of cytokines like the proinflammatory TNF-α. The exact effect needs to be precised because current data are

controversial. The study of Valencia et al. evidenced that treatment with anti-TNF antibody (infliximab) increases FOXP3 mRNA and protein expression by $CD4^{+}CD25^{hi}$ Tregs and restored their suppressive function [106]. Later on, Chen et al. shows that upon in vitro activation with plate-bound anti-CD3 Ab and soluble anti-CD28 Ab, Foxp3 expression by highly purified mouse Tregs is markedly downregulated. TNF partially abrogates this effect and stabilizes Foxp3 expression as this effect of TNF can be blocked by anti-TNFR2 Ab, but not by anti-TNFR1 Ab [107, 108]. In any case, the role of TNF-a needs to be further investigated because TNF-a plays important role in the inflammatory reactions, where Tregs are expected to be also involved.

Upon activation, effector T cells produce IL-2, which stimulates T cells proliferation and the expansion of the immune response. At the same time, Tregs are distinguished by the their high expression of CD25 [109]. These facts suggest that Tregs need IL-2 to survive [110, 111]. Placed in an activated milieu, Tregs may compete with effector cells and as a result, decrease the levels of IL-2 in the environment. In fact, this is the third approach that Tregs apply to achieve a state of suppression [112, 113].

Regulatory T cells apply all of the above-mentioned approaches for the establishment of immune-tolerant milieu. Still, there are no evidences about the mechanism preferred by nTregs or iTregs. Similarly, despite the abundance of data, no specific mechanisms can be attributed to a particular pathologic condition. Probably, the modus operandi of Tregs depends on the finetuning of T-cell receptor-antigen recognition and interaction, the target cell characteristics and the cytokine spectrum in the surrounding milieu.

5.3 The impact of regulatory T cells on the operational tolerance

The establishment of immune tolerance after solid organ transplantation (SOT) is the key therapeutic challenge in the post-operative period. In most cases, it is induced by the continuous application of immunosuppressive therapy. However, in some patients, a discontinuation of the regimen arrives, due to infections, cancer etc. Surprisingly, in some cases changes in immunological parameters, indicating the development of a state of immune tolerance were found despite the lack of immune suppression. This particular situation is defined as operational tolerance and is characterized by the absence of any clinical and histological signs of rejection in therapy free patients [114]. The operational tolerance (OT) is reported after transplantation of different solid organs, but is frequent in LT [115, 116]. It is supposed that tolerogenic properties of the liver and residential lymphocytes play a key role in this process [117]. The exact mechanisms are not fully clarified, but the intensive research highlights that it depends on multiple factors. Among them are regulatory T cells, particular gene expression profile and serum levels of HLA-G.

Regulatory T cells are the first parameter associated with OT. In patients with spontaneous tolerance they are increased independently of the age of recipient [118]. According to the study of Koshiba et al., not only the proportion CD4 + CD25high + T cells was increased in the tolerant patients' peripheral lymphocytes and suppressed MLR specifically to the donor antigen, but also FOXP3 expressing cells were present within the tolerant liver [119]. Pons and colleagues describe sustained increase in CD4 + CD25+ and CD4 + $CD25^{hi}$ cells in patients with operational tolerance in comparison with non-OT patients in a bimonthly evaluation intervals until M16 [1].

Interestingly, in LT Tregs show a particular dynamic. In one of the earliest studies, pre-transplantation levels of Tregs were higher in patients than in controls. Lowest levels were observed at month 3 after Tx, followed by a relative increase at 12 months and at later time points [120].

The main question is why these cells are elevated in recipients? In fact, up to now reports discussing this topic in the literature are insufficient. The detailed study of Demirkiran and colleagues examined the presence and allosuppressive activity of CD4 + CD25 + Foxp3+ Tregs in perfusates of human liver grafts and monitored the cells presence in the circulation of recipients after liver Tx. The authors show an increased proportion of CD4 + CD25 + CTLA4+ T cells compared with healthy control blood. The increased percentages of Foxp3+ cells, which were negative for CD127, confirmed the enrichment of Tregs in perfusates. They suppressed proliferation and IFN-γ production of donor and recipient T cells. In vivo within the first weeks after Tx, up to 5% of CD4 + CD25 + CTLA4+ T cells in recipient blood were derived from the donor liver, indicating that a substantial number of donor Tregs detach from the liver graft during perfusion and continue to migrate into the recipient after Tx. These donor Tregs suppress the direct pathway alloresponses and may in vivo contribute to chimerism-associated tolerance early after liver Tx [121]. In a small number of patients, we found a pick in the percentage of Tregs at day 7 (D7) followed by a decrease until D30 being always around and above healthy controls values independently of the diagnosis or age. The simultaneous routine measurement of liver function laboratory parameters revealed that the increase in Tregs precedes albumin synthesis restoration [122]. In another study Baumann et al. evidence that the benign clinical course of subclinical rejection (SCR) compared to acute clinical rejection (ACR) is associated with intrahepatic T cell infiltration patterns showing less cytotoxic T cells and more CD4 + FOXP3+ Tregs. They demonstrate that in patients with SCR the pattern of infiltrating T cells is characterized by a stronger accumulation of CD4+ cells, an increasing CD4+/CD8+ ratio, and an increasing CD4+ forkhead box P3 (FOXP3) + regulatory T cell (Treg)/CD8+ ratio, which was not seen in acute clinical rejection. These intrahepatic T cell patterns were not reflected in the peripheral blood [123]. Cumulatively, these data suggest the presence of particular sort of cellular chimerism, associated with liver transplantation.

The chimerism is a specific phenomenon characterized by the presence of cells from one individual in another. Microchimerism is reported in hematopoietic stem cells transplantation as a result of migration of passenger lymphocytes from the graft into recipient and in pregnancy (foetus). In SOT, microchimerism is described in mice first [124]. Among multiple reports showing than donor mononuclear cells migrate from the graft in the recipient, Jonsson et al. find that the peak levels of chimerism are within the first 48 hours after transplantation and the range reaches 20% of total peripheral blood mononuclear cells [125, 126]. In a concise and very interesting review, Abrol and colleagues assume that increased expression of chemokines in the liver attracts alloreactive T cells that are subsequently destroyed by coming in contact with various liver cells inherently programmed towards tolerance induction [127]. Donor specific hypo-responsiveness, down regulation of T helper type I cytokine (IFN-γ) and no change in T helper type 2 cytokine (IL10) in the *in vitro* mixed lymphocyte reaction in recipients who achieved operational tolerance were also reported [128]. Indeed, this topic needs more studies directed to the detailed evaluation of different cellular subsets. Probably, Tregs might be part of passenger leucocytes or they might be secondary induced by the modified hepatic environment.

6. Other tolerogenic strategies

Tregs are not the only approach, involved in tolerance establishment in liver transplantation. Other cells also participate in by expressing particular molecules or changing their genetic pattern.

In 16 operationally tolerant liver recipients, 16 recipients requiring on-going immunosuppressive therapy, and 10 healthy individuals by microarray profiling Martinez-Llordella et al. identified a gene expression signature that could discriminate tolerant recipients from immunosuppression-dependent patients with high accuracy. This signature included genes encoding for γδ T-cell and NK receptors, and for proteins involved in cell proliferation arrest. In addition, tolerant recipients exhibited significantly greater numbers of circulating potentially regulatory T-cell subsets (CD4 + CD25+ T-cells and Vδ1+ T cells) than either non-tolerant patients or healthy individuals [129].

The human leucocyte antigen-G (HLA-G) is a non-classical HLA class I molecule with prominent tolerogenic properties. It inhibits cytotoxicity and proliferation, but stimulates the development of regulatory T cells. HLA-G is present as a membrane-associated form and a soluble one. Interestingly, the uptake of HLA-G by some resting but mostly in activated CD4 and CD8 T cells leads to the instant generation of a new type of regulatory cells that initially act through cell-surface molecules that they temporarily display but do not express themselves [130–132]. This mechanisms is defined as trogocytosis and seems to play important role for the establishment of immune tolerance [133]. Several groups provides evidences about the role of HLA-G in kidney and heart transplantation [134–136]. In healthy conditions in adults, HLA-G is weakly expressed in liver, but it might be transmitted through transendothelial migration and/or trogocytosis from circulating cells under particular circumstances like cytokines, hypoxia etc. [137–139]. In all cases, elevated levels of sHLA-G is associated with reduced risk of rejection and better survival [140, 141].

7. Clinical relevance of regulatory T cells for liver transplantation

Slowly, but without doubt, regulatory T cells are getting involved in the diagnostic process of many pathological conditions, expressed by deviations in immune tolerance – autoimmune [142, 143], tumors [144, 145], recurrent pregnancy loss [146, 147], primary immune deficiencies [110, 148] etc.

Current data indicate that Tregs have the potential to be a potential biomarker for the monitoring of the posttransplantation period [2]. Specifically, in liver transplanted patients Tregs are object of intensive research mostly because they are inherently involved in the operational tolerance and are part of natural liver toleragenic mechanisms. Despite the abundant data showing the benefit of Tregs determination during posttransplantation period, there are still many unresolved questions. Some of them are related to the definition of Tregs phenotype that should be used. Although identified as FoxP3 + CD4+ T cells, the expression of FoxP3 was demonstrated less informative than the promotore demethylation because it distinguishes true Tregs from transiently FOXP3+ activated T cells [149]. For diagnostic purposes, Tregs are often defined as CD127-CD25 + CD4+, but recent advances in the field showed a population CD25- [150], which is not fully characterized regarding CD127. Another direction that needs to be elucidated are Tregs in biopsies and peripheral blood. In Barcelona consensus (2016) several studies are shown with ambiguous results [151], that does not provide clear evidences for the relevance of Tregs measurement in posttransplantation period. The third direction is the significantly decreased expression of CD25 in relation to the immunosuppressive therapy and the consecutive inability to find out $CD25^{hi}$Treg cells in the periphery. Although Tregs are highly informative regarding the operational tolerance [152], the question is still unresolved and more studies are required to determine the value of Tregs in the monitoring of post-transplantation period.

It seems, that Tregs are more promising as a therapeutic approach for the control of immune activation and development of a state of immune tolerance. Early experimental studies demonstrated association between them and the delay in islet allograft rejection and long term survival [153, 154]. During last five years, together with other approaches [155], Tregs attract the medical interest in the field of GVHD and solid organ transplantation [156, 157]. Todo et al. in 2016 report phase I results clinical trial with ex vivo expanded recipient polyclonal Tregs in living kidney transplants. Despite variability in recipient's renal disease, the expansion protocol produced Tregs which met all release criteria, expressing >98% CD4 + CD25+ with <1% CD8+ and CD19+ contamination and > 80% FOXP3 expression with stable demethylation in the FOXP3 promoter. Within recipients, expanded Tregs amplified circulating Treg levels in a sustained manner. Clinically, all doses of Treg therapy tested were safe with no adverse infusion related side effects, infections or rejection events up to two years post-transplant [158]. Another study undertook a direct comparison of the *in vitro* and *in vivo* functional activities of the different memory and naïve Treg subpopulations showing that the naive Treg is the Treg population that exhibits the ideal biological features of a Treg therapeutic while the highly suppressive memory Tregs should be purposefully excluded from a Treg therapeutic due to their low lineage delity, low proliferative capacity, and greater pro-inflammatory potential [159]. Recently published results from The ONE study demonstrates that regulatory cell therapy is achievable and safe in living-donor kidney transplant recipients, and is associated with fewer infectious complications, but similar rejection rates in the first year. Therefore, immune cell therapy is a potentially useful therapeutic approach in recipients of kidney transplant to minimize the burden of general immunosuppression [160].

Finally, the immune capacity of the liver depends on the local hepatic and immune cells that transitory populate it. The current research provides abundant data about the intercellular tolerogenic mechanisms. However, some points need to be better clarified from the scientific and medical point of view. Among them are the fine-tuning of common immunosuppressive therapeutics regarding regulatory T cells, biochemical mechanisms of interactions between hepatocytes and immune cells, whether immune parameters of activation/suppression might provide information in advance about the liver function, the impact of individual immunogenetic variations on the recovery and operational tolerance etc. We think that the immune system should be considered as important player in the liver tolerance network and involved in the post-transplantation period monitoring.

8. Conclusion

The liver is a unique structure, where immunological mechanisms meet metabolic processes. The tightly regulated collaboration between them creates particular tolerogenic milieu that impacts the homeostatic state. Regulatory T cells are shown to play important role in these events. Their dynamic and function are promising for the further development of new biomarkers and treatment strategies in liver transplantation.

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Conflict of interest

The authors declare no conflict of interest.

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