

Immunological aspects of liver cell transplantation

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Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Supported by Grants of the German Research Foundation (DFG, SFB 738; projects B3, C11) and BMBF 01EO1302.

Conflict-of-interest statement: No potential conflicts of interest.

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Received: July 31, 2015
 Peer-review started: August 1, 2015

First decision: October 13, 2015

Revised: October 21, 2015

Accepted: December 7, 2015

Article in press: December 8, 2015

Published online: March 24, 2016

Abstract

Within the field of regenerative medicine, the liver is of major interest for adoption of regenerative strategies due to its well-known and unique regenerative capacity. Whereas therapeutic strategies such as liver resection and orthotopic liver transplantation (OLT) can be considered standards of care for the treatment of a variety of liver diseases, the concept of liver cell transplantation (LCTx) still awaits clinical breakthrough. Success of LCTx is hampered by insufficient engraftment/long-term acceptance of cellular allografts mainly due to rejection of transplanted cells. This is in contrast to the results achieved for OLT where long-term graft survival is observed on a regular basis and, hence, the liver has been deemed an immune-privileged organ. Immune responses induced by isolated hepatocytes apparently differ considerably from those observed following transplantation of solid organs and, thus, LCTx requires refined immunological strategies to improve its clinical outcome. In addition, clinical usage of LCTx but also related basic research efforts are hindered by the limited availability of high quality liver cells, strongly emphasizing the need for alternative cell sources. This review focuses on the various immunological aspects of LCTx summarizing data available not only for hepatocyte transplantation but also for transplantation of non-parenchymal liver cells and liver stem cells.

Key words: Liver cell transplantation; Cell-based therapy; Hepatocyte transplantation; Transplant immunology; Regenerative medicine

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Core tip: Failure of durable engraftment of transplanted hepatocytes despite application of immunosuppression is mainly attributed to the remaining recipient's immune responses against these allogenic grafts. Immune responses significantly differ from those observed for transplantation of whole livers and other solid organs. Innate immunity in combination with adaptive immune responses by T- and B-cells have to be taken into account for liver cell transplantation-specific immunosuppressive strategies. Possible clinical solutions to these obstacles will involve new combinations of novel and established immunosuppressive and anti-inflammatory drugs, co-transplantation of other liver cell types or regulatory immune cells. In the future, also (syngenic) liver stem cells will be an option.

Oldhafer F, Bock M, Falk CS, Vondran FWR. Immunological aspects of liver cell transplantation. *World J Transplant* 2016; 6(1): 42-53 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v6/i1/42.htm> DOI: <http://dx.doi.org/10.5500/wjt.v6.i1.42>

INTRODUCTION

Liver cell transplantation (LCTx) constitutes a promising approach for the treatment of various acute and chronic liver diseases^[1,2] as well as surgically induced small-for-size syndrome^[3]. In addition, LCTx also offers the option for cell therapeutic intervention using genetically modified liver cells with repair functions introduced^[4].

Mature hepatocytes were regarded the most obvious cell type to be applied in LCTx since the hepatocyte itself has been identified as a central functional unit of the liver. Albeit established in many small animal models, state-of-the-art protocols for LCTx in humans still have not resulted in the expected clinical successes^[5,6]. Failure of durable engraftment of transplanted hepatocytes mainly can be attributed to the recipient's immune responses against these allogenic cells^[7] and the severe competition with fully integrated organ-resident cells in a non-preconditioned environment^[8]. Furthermore, despite of using immunosuppression, long-term graft acceptance after LCTx has not yet been achieved in humans^[9]. This is in contrast to established small animal models (mice and rats) for LCTx that often rely on the use of genetically modified animals^[10,11] and/or hepatotoxic damaging^[12] of the recipient liver for pre-conditioning but cannot be transferred to the clinics. The broad clinical use of LCTx is further hampered by limited proliferative capacities of currently applied primary human hepatocytes (PHH), and cells suitable for transplantation purposes under GMP compliant production procedures remain scarce^[13].

Consequently, considerable research efforts are ongoing to optimize clinical protocols for LCTx as well as to identify reliable sources of liver cells suitable for LCTx. Use of alternative cell types such as stem cells or

stem cell derived hepatocytes might not only solve the problem of shortage in donor organs for hepatocyte isolation but - also by including options for autologous cell transfer - could overcome the existing hurdle of graft rejection by the recipient's immune system.

Hepatocyte rejection has been an underestimated problem, since from experiences with whole liver transplantations, the liver is considered an immune-privileged organ: Animal studies demonstrated long-term survival of liver allografts without the need for immunosuppression in strain combinations that would rapidly reject kidney or cardiac allografts^[14,15]. In addition, patients usually require smaller doses of immunosuppressive drugs after orthotopic liver transplantation (OLT) compared to other solid organs^[16]. Thus, the initial assumption was that transplantation of allogenic hepatocytes would also profit from this immune-privilege defined as low alloreactivity against liver grafts. However, allogenic hepatocyte transplants were not "invisible" or resistant to the recipient's immune system since *in vivo* a rapid rejection of purified transplanted allogenic hepatocytes is observed^[17]. This discrepancy between a potentially tolerogenic organ, *i.e.*, the liver, and isolated hepatocytes implies that either other hepatic cells like stellate cells or liver sinusoid endothelial cells (LSEC) contribute to this liver-specific tolerance^[18] or that singularized hepatocytes lose their tolerogenic potential in an allogenic environment accompanied by an inflammatory process.

Therefore, detailed knowledge of the immune responses induced by transplanted liver cells is instrumental for an improvement of cell engraftment and long-term acceptance of liver cell grafts. Nevertheless, to date there is still only limited literature available on these issues. This review aims at summarizing the *in vitro* and *in vivo* data addressing the immunological aspects of LCTx.

CLINICAL APPLICATION AND OUTCOME

The experience with clinical application of hepatocyte transplantation in humans is still limited to about 140 cases^[19]. Hepatocyte transplantation has been performed as an alternative to OLT to treat inborn errors of liver metabolism, chronic or acute liver failure or to maintain liver function as a bridge to OLT^[20]. In the former case, most pediatric patients suffered from urea cycle defects like Ornithine transcarbamylase deficiency or Citrullinemia. Clinical observation of these transplanted individuals demonstrated the safety of this procedure and patients showed clinical improvement and/or partial correction of the underlying metabolic disease. However, in the majority of the cases sustainable and significant benefits were not observed, and so far there is no report about a patient with a metabolic disease which has been completely cured^[21]. In patients with acute liver failure clinical improvement such as a reduction of ammonia and bilirubin levels

were observed, but the clinical outcome in the course of cell transplantation still was not significantly affected. In few individuals hepatocyte transplantation has been applied to treat patients with chronic liver disease: Here the outcomes likewise were very heterogeneous and overall comparable to the results reported for pediatric patients^[20]. Major hurdles hampering the success of hepatocyte transplantation seem to be rejection of transplanted cells by the recipient's immune system as well as insufficient engraftment of the donor cells within the recipient's liver.

TRANSPLANTATION OF PRIMARY HEPATOCYTES

Rejection of hepatocytes by the innate immune system

The innate immune system plays a critical role in the early immune response after hepatocyte transplantation. Both syngenic and allogenic transplanted liver cells have been shown to be targeted by the innate immune system in *in vitro* experiments^[22,23]. For further characterization of these immune responses experiments have been performed in mouse models showing that cells of the innate immune system such as granulocytes and macrophages cells infiltrate areas surrounding the transplanted hepatocytes in an early phase after transplantation (1-3 d)^[24]. Overall, it has been reported that up to 70% of transplanted hepatocytes may be eliminated by this initial innate immune response^[24]. Most interestingly, there were no differences in quantity or quality of infiltrating immune cells when comparing transplantation of allogenic vs syngenic hepatocytes, suggesting that stimulation by alloantigen does not seem to be a prerequisite for induction of an innate immune reaction. At present, three major mechanisms have been proposed which might explain this distinct phenomenon:

The first molecular mechanism postulated by Olszewski *et al.*^[25] suggests that uncovered intercellular surface adhesion molecules (cadherins) are recognized as "non-self" by granulocytes and monocytes/macrophages and subsequently provoke lysis of the transplanted cells. These adhesion molecules are hidden in the hepatic trabeculae and, thus, normally are inaccessible for immune cells in healthy liver tissue. However, they become exposed during the process of liver cell isolation applying collagenase for enzymatic digestion of the liver tissue and can subsequently be recognized by immune cells which, in turn, initiate the cytotoxic process leading to elimination of transplanted cells. Blocking of these molecules with monoclonal antibodies (mAb) resolved the effect in this experimental setting.

Bennet *et al.*^[26] described an additional mechanism termed "instant blood-mediated inflammatory reaction" (IBMIR), a reaction which has also been shown following islet cell transplantation^[26]. In their study with

fresh hepatocytes, they showed that PHH exposed to human blood induced a rapid loss of platelets from the blood, an extensive generation of thrombin-antithrombin complexes and a concomitant increase in the complement component C3a, followed by a drop in the polymorphonuclear leukocyte (PMN) count^[27]. Examination of the clots by confocal microscopy revealed infiltrating PMNs and platelets surrounding the PHH. This inflammatory reaction might explain why Kupffer cells are rapidly surrounding the transplanted cells after LCTx^[28]. Overall, this reaction with its main features resembled the IBMIR originally defined in clinical islet cell transplantation^[26].

The third mechanism was described by Gupta *et al.*^[24] assuming that portal occlusion by cell emboli of transplanted hepatocytes may induce perfusion-reperfusion injury, oxidative stress and impairment of cell viability. This, in turn, results in recruitment of inflammatory cells and eventually depletion of transplanted hepatocytes^[24]. This mechanism mainly leads to an activation of non-parenchymal cells such as Kupffer and stellate cells. In consequence, the survival of transplanted hepatocytes could be considerably increased *in vivo* by pretreatment of graft recipients with gadolinium chloride, known to significantly impair the function of Kupffer cells^[28].

Natural killer (NK) cells represent another key player of innate immunity. In the context of organ transplantation, NK cells were suggested for a long time to belong primarily to the first line of innate defence against pathogens and this pro-inflammatory effector concept was also applied for allograft rejection^[29]. NK cells have the potential of allo-specific recognition of transplanted cells by the so-called "missing self concept"^[30] which is based on the presence of inhibitory receptors specific for self-MHC that protect autologous tissue. In case of missing self-MHC molecules either in allogeneic situations or down-regulation of MHC by pathogens, the lack of protective inhibitory signals results in NK cell activation, *i.e.*, cytotoxicity and cytokine secretion. Despite this capacity of allorecognition, NK cells have not yet been investigated in hepatocyte transplantation and, therefore, their potential involvement in rejection of transplanted PHH remains to be defined.

More information is available for whole organ liver transplantation focusing rather on consequences of liver transplantation on NK cell repertoire and function than on a potential tolerogenic effect of PHH or other hepatic cells on NK cell alloreactivity. For example, alterations of the peripheral NK cell repertoire were observed in pediatric liver transplant recipients^[31]. A special role of the liver in NK cell generation was demonstrated by the observation of an infiltration of peripheral c-kit-positive NK cell precursors into the liver and the local development of an hepatic NK cell repertoire^[32]. Furthermore, donor NK cells derived from the grafted liver, *i.e.*, passenger leukocytes, were

detected in the periphery of pediatric liver recipients during the first month after transplantation^[33]. In a rat model of allogenic liver transplantation, no direct evidence for an involvement of donor-derived NK cells in liver transplant tolerance could be demonstrated^[34]. In addition, expression profiling of peripheral blood derived from tolerant liver transplant recipients revealed NK cell-related signatures in addition to other iron metabolism signatures^[35-37], suggesting that NK cells may rather be involved in an establishment of tolerance than in rejection of allogenic tissue. This differential view on the role of NK cells in organ and, especially in hepatocyte transplantation, demonstrates the need for further investigations of these innate immune cells in transplantation.

Rejection of hepatocytes by the adaptive immune system

In addition to the innate immune response, transplanted hepatocytes also face rejection mediated by the adaptive immune system, *i.e.*, T- and B-cells. Bumgardner *et al.*^[38] developed an animal model of hepatocyte transplantation to analyze rejection of transplanted cells *in vivo*. Hepatocytes of a transgenic mouse line expressing the human α -1-antitrypsin (*hA1AT*) gene were transplanted into the recipient by intrasplenic injection and the survival of the transgenic hepatocytes was determined by detection of secreted hA1AT protein in the recipient's serum. This group performed a series of experiments to characterize the rejection of allogenic hepatocytes: First, hepatocytes were transplanted into completely T-cell, selectively CD4⁺ or CD8⁺ T-cell, or B-cell deficient mice. Only recipients deficient of T-cells showed long-term survival of transplanted hepatocytes (> 16 wk). Transplantation of allogenic hepatocytes into recipients deficient of B-cells, CD4⁺ or CD8⁺ T-cells alone resulted in a loss of hA1AT by day 10 after transplantation^[38], demonstrating that immunologic rejection of allogenic hepatocytes is mediated primarily by T-cells.

T-cell mediated rejection and more specifically CD4⁺ T-cell mediated rejection is well known from transplantation of allogenic hearts and pancreatic islet allografts. Heart and islet allograft survival was significantly prolonged by treatment with anti-CD4-mAbs^[39,40], whereas the outcome of hepatocyte transplantation was not improved in this setting. When hepatocytes and heart allografts were transplanted simultaneously with a short-term medication of anti-CD4-mAbs, hepatocytes were destroyed by day 10 post-transplantation while most hearts survived more than 60 d^[41], further underlining the different intensity of graft rejection between solid organs and allogeneic hepatocytes.

To further dissect this T-cell response, allogenic hepatocytes were transplanted into mice pretreated with mAb against CD4, CD8 or the combination of both. The median survival time of hepatocytes in graft

recipients only pretreated with a single mAb against CD4 or CD8 showed a mean survival of only 10 and 14 d (10 d in the untreated control group), respectively. In recipients treated with the combination of anti-CD4-mAb and anti-CD8-mAb, hepatocyte survival was prolonged to approximately 35 d. This study confirmed that hepatocytes can be highly immunogenic and stimulate a strong cell-mediated immune response by both CD4⁺ and CD8⁺ T-cells^[42].

Also, when allogenic hepatocytes were transplanted into CD4 knock-out (KO) or CD8 KO mice without any further treatment, the mean survival time of transplanted cells were 10 and 14 d, respectively. However, when CD4 KO mice were treated with anti-CD8-mAb and CD8 KO mice with anti-CD4-mAb, respectively, hepatocellular allografts survived for 35 d in both groups. The reported studies collectively demonstrate that both CD4⁺ and CD8⁺ T-cells can independently promote hepatocyte rejection^[43].

As mentioned above, the importance of CD4⁺ T-cell mediated rejection is well known from other solid organ transplantation models^[39]. However, rejection of hepatocytes may also be initiated solely by CD8⁺ T-cells due to MHC class I -specific alloreactivity. When both CD4- and CD8-dependent pathways are available, the latter pathway seems to predominate, suggesting that direct MHC class I - and indirect MHC class II -specific T-cell activities may cooperate in hepatocyte rejection.

In concordance with these observations, Allen *et al.*^[44] reported about a patient with Crigler-Najjar syndrome type 1 undergoing hepatocyte transplantation. Despite initial successful engraftment of transplanted allogenic liver cells, there was a continuous loss of graft function due to strong CD8⁺ T-cell alloreactivity, predominately directed against a particular HLA class I alloantigen. Hence, in the absence of any evidence for humoral rejection, the authors concluded that cell-mediated rejection was the most likely cause of graft loss in this patient.

Bumgardner *et al.*^[17] summarized their experimental data to three possible mechanisms of hepatocyte allograft rejection. The first is a CD4⁺ T-cell dependent CD8⁺ T-cell mediated hepatocyte rejection. In this case, CD4⁺ T-cells become activated by host APCs and produce pro-inflammatory cytokines which permit activation and maturation of CD8⁺ precursor cytolytic effector T-cells (pCTL). These recognize MHC class I molecules on donor hepatocytes, become activated and target hepatocytes for apoptotic cell death *via* Fas/FasL, granzyme/perforin, TNF or other cytotoxic effector molecules.

The second mechanism is also CD8⁺ T-cell-mediated but CD4⁺ T-cell independent. CD8⁺ cytolytic T-cells directly recognize allogenic MHC molecules on donor hepatocytes. In a CD40-dependent process as substitute for CD4⁺ T-cell help, allospecific cytolytic T-cells are activated and target donor cells for apoptotic cell death also *via* the same mediators

mentioned above such as Fas/FasL, granzyme/perforin or TNF.

The third mechanism is CD8⁺ T-cell-independent CD4⁺ T-cell-mediated hepatocyte rejection. Donor hepatocyte MHC class I alloantigens are shed and subsequently scavenged by both host APC and host B-cells, which cross-present allogenic peptides *via* host MHC class II to host CD4⁺ T-cells in a B7 (CD80)- and CD40-dependent manner. CD4⁺ T-cells become activated and produce pro-inflammatory cytokines stimulating the activation and maturation of B-cells to produce alloantibodies that finally mediate the various mechanisms involved in antibody-mediated rejection.

Apart from T-cell mediated rejection, some data also suggest an involvement of humoral components, *i.e.*, antibodies, in rejection of allogenic hepatocytes. Horne *et al.*^[45] studied the acute damage of allogenic liver parenchymal cells by the CD4-dependent pathway and showed that this pathway is mediated by alloantibodies. This alloantibody-mediated acute rejection is targeting transplanted allogenic hepatocytes *via* macrophage-mediated cytotoxic immune damage^[46]. However, donor-reactive alloantibodies were only produced in significant quantities in hepatocyte recipients with lack of CD8⁺ T-cells or impaired cytotoxic effector mechanisms^[45].

Zimmerer *et al.*^[47] showed that CD4⁺ T-cells significantly upregulate IL-4 and downregulate IFN- γ in the absence of CD8⁺ T-cells. When CD4⁺ T-cells are transferred into CD8-depleted IL-4 KO mice that cannot produce any post-transplant alloantibodies on their own, high antibody levels are observed following hepatocyte transplantation, suggesting that IL-4-producing CD4⁺ T-cells are critical for post-transplant alloantibody production. In addition, CD8⁺ T-cells have the ability to reverse this IL-4-dominated cytokine profile by upregulating IFN- γ and, therefore, they can negatively regulate alloantibody production^[47]. Moreover, CD8⁺ T-cells also appear to directly downregulate alloantibody production by eliminating alloprimed B-cells through perforin- and FasL-mediated cytotoxicity^[48]. These data suggest that there might be a distinct subset of CD8⁺ cytotoxic T-cells that recognize primed B-cells and inhibit humoral rejection, which is an interesting paradox due to the previously reported CD8⁺ T-cell mediated rejection *via* the same cytotoxic molecules.

Horne *et al.*^[49] conclude that when hepatocytes activate both CD4- and CD8- dependent immune responses, the CD8-dependent response predominates CD4-dependent and B-cell-dependent immune pathways.

Role of co-stimulatory signals for rejection of allogenic hepatocytes

Effective T-cell activation on one hand requires antigen-specific signals to the T-cell receptor by the MHC/peptide complex on APCs and, on the other hand, depends on non-antigen-specific co-stimulatory signals to T-cells. The CD28/B7 (CD80) and CD40L/

CD40 co-stimulation pathways play critical roles in the activation of T-cells after allogenic transplantation of solid organs, kidney in particular, and their inhibition can lead to prolonged allograft survival^[50,51]. In kidney transplantation, costimulation blockade by a mutated fusion protein of CTLA-4-Ig (Belatacept/Nulojix[®]) was clinically approved with remarkable improved long-term outcome regarding kidney function^[52,53]. To determine the role of these co-stimulation pathways for the rejection of allogenic hepatocytes, mice were treated with either anti-CD40L-mAb or CTLA4-Ig to block CD40L/CD40 or CD28/B7 signaling, respectively. Administration of anti-CD40L-mAb caused significant prolongation of hepatocyte allograft survival whereas the application of CTLA4-Ig showed no significant effects. Thus, the CD40L/CD40 system plays a critical part in T-cell mediated rejection of allogenic hepatocytes, whereas the CD28/B7 co-stimulatory pathway may just play a subsidiary role^[54].

Gao *et al.*^[55] further studied the role of these co-stimulatory pathways in CD4 KO and CD8 KO mice and showed unexpectedly that treatment with CTLA4-Ig, ineffective in wildtype C57BL/6 mice, significantly prolonged the survival of allogenic hepatocytes in CD8 KO mice. These data implicate that both CD8⁺ and CD4⁺ T-cells may utilize the CD40L/CD40 co-stimulation pathway during hepatocyte rejection, but only the CD4⁺ T-cells also can promote rejection of hepatocytes *via* the CD28/B7 pathway^[55].

However, even the combination of CD28/B7 and CD40L/CD40 co-stimulatory pathway inhibition leads to only slightly prolonged survival of allogenic hepatocytes, while being capable of inducing immunologic tolerance to heart and pancreatic islet cell allografts. CD4⁺ and in particular CD8⁺ T-cells can still reject hepatocytes in absence of CD40L/CD40 signaling^[55], indicating that further co-stimulatory pathways may be involved in T-cell mediated rejection of hepatocytes.

One example for alternative co-stimulation pathways could be the blockade of LFA-1/ICAM-1 interaction that has been reported to prolong survival of several allografts and allogenic hepatocytes expressing ICAM-1. This adhesion molecule promoted the development of allospecific cytolytic effector T-cells (CTL) *in vitro* and *in vivo*, which could be inhibited by the application of anti-ICAM-1-mAb^[56,57].

Wang *et al.*^[58] demonstrated the importance of the LFA-1-mediated co-stimulatory pathway showing that 70% of the hepatocytes survived more than 60 d when transplanted into a CD4 KO mice with simultaneous suppression of LFA-1 signaling, pointing towards the importance of LFA-1 co-stimulation on CD8-dependent rejection. Moreover, targeting both the LFA-1/ICAM-1 pathway and CD40L/CD40 co-stimulation resulted in synergistic effects, thus, survival of hepatocytes could be achieved for more than 60 d in 100% of mice in both CD4- and CD8-dependent T-cell rejection^[58].

TRANSPLANTATION OF NON-PARENCHYMAL LIVER CELLS

The role of hepatic non-parenchymal cells for the induction of rejection or tolerance

As described above, hepatocytes can be acutely rejected *via* the innate and adaptive immune system, but at least in animal models, solid liver allografts are spontaneously accepted in many species without immunosuppression^[16]. This might suggest that liver non-parenchymal cells such as stellate cells, Kupffer cells and liver endothelial cells also could play an important role protecting allogenic hepatocytes from rejection.

Hepatic stellate cells

Hepatic stellate cells (HSC) are known to possess the ability to differentiate into myofibroblasts for the production of extracellular matrix leading to hepatic fibrosis^[59]. However, HSC have also demonstrated a strong T-cell inhibitory activity in *in vitro* and *in vivo* studies:

Charles *et al*^[60] demonstrated *in vitro* that IFN- γ stimulated HSCs express B7-H1 (PD-L1), in a dose-dependent manner as well as produce the suppressive cytokines IL-10 and TGF- β . The formation of PD-1/PD-L1 complexes transmits an inhibitory signal which reduces the proliferation of CD8⁺ T-cells. Hence, HSCs can markedly inhibit T-cell responses elicited by either allogenic APCs or CD3/CD28-beads, which was associated with an increase in activated CD4⁺ and CD8⁺ T-cell apoptosis. In addition, the B7-H1-blocking antibody significantly reversed the inhibitory effect suggesting that inhibition *via* the PD-1/PD-L1 pathway plays an important role for the immunosuppressive effect of stellate cells^[60]. However, PD-L1 might not be the only relevant protein in this context, since neutralization of the latter by anti-B7-H1-mAb only partially reverses HSC-induced inhibition of T-cell proliferation^[60].

Yang *et al*^[61] analyzed several death molecules in HSC by qPCR finding that only the TNF-related apoptosis-inducing ligand (TRAIL) was upregulated following IFN- γ stimulation. Moreover, they showed that HSCs from TRAIL KO mice largely lost their capacity to protect co-transplanted islet cell allografts. Thus, TRAIL might be involved in the immune-regulatory function of HSCs, which is likely mediated by TRAIL receptor-triggered death of activated T-cells^[61].

In addition, in a mouse model of islet cell transplantation, co-transplanted HSCs were seen to protect islet allografts from rejection^[62]. The underlying mechanism for this immunomodulatory effect seems to include not only elimination of activated specific CD8⁺ T-cells as shown by the *in vitro* studies stated above, but also expansion of regulatory T-cells (T_{reg}). The expansion of T_{reg} due to HSC co-transplantation cannot finally be explained by this study, but the

authors postulate that HSC influence APCs that process alloantigens from islet cells and induce T_{reg} in the draining lymphnodes^[63].

Recently, Dusabineza *et al*^[64] showed that HSC can improve engraftment of PHH in a mouse model of transplantation of hepatocytes co-cultured with HSC into immunodeficient SCID mice. Due to the lack of T- and B-cells, adaptive immune responses have no influence in this setting. Nevertheless, co-transplantation of hepatocytes with HSC did not generate fibrosis but significantly improved hepatocyte engraftment, probably by supporting hepatocytes to cross the sinusoidal-endothelial barrier. The authors state that HSCs may protect hepatocytes from dying while entrapped in the sinusoidal network or promote adhesion to the endothelial wall. A further explanation could be that HSCs produce vasoactive peptides that may increase endothelial permeability and improve crossing and homing of hepatocytes^[64].

Kupffer cells

Kupffer cells are the largest population of tissue-resident macrophages and play an important role as tolerogenic APCs shown to induce tolerance after liver transplantation^[65,66]. However, from our knowledge, no data exists on the administration of allogenic Kupffer cells and the resulting immunological effects. Nevertheless, when Kupffer cells function as APCs, they have been described to either promote tolerogenic effects *via* IL-10 and TGF- β production and proliferation of T_{reg} or to enhance pro-inflammatory effects through the activation of NK T-cells *via* CD1-dependent antigen presentation^[67-70].

Furthermore, Kupffer cells are of special interest in the setting of ischemia/reperfusion injury after liver transplantation. In several studies, depletion of Kupffer cells was shown to worsen the transplantation outcome compared to control groups. This effect seems to correlate with the secretion of the potent anti-inflammatory cytokine IL-10 by Kupffer cells, which is necessary to balance the cytokine milieu towards Th₂-mediated protection^[71,72].

A possible role of Kupffer cells in LCTx thus needs to be evaluated in future studies.

LSEC

In a hemophilia KO mouse model (hemophilia A), Follenzi *et al*^[73] demonstrated that LSEC have the capability to repopulate the livers of mice with healthy endothelial cells and to rehabilitate plasma factor VIII activity with correction of the bleeding phenotype. This study shows that transplantation of LSEC can be safely performed in a mouse model and that transplanted cells may integrate and function in the recipient's liver.

Multiple studies have shown an immunoregulatory effect of LSEC when functioning as APCs, for example during liver transplantation^[74]. *In vitro* studies have shown that allogenic LSEC possess an

immunoregulatory effect due to induction of allospecific T-cell hyporesponsiveness^[74,75]. Banshodani *et al.*^[76] also recently published *in vivo* experiments showing that LSEC also have immunoregulatory effects *via* the PD-1/PD-L1 pathway in a mouse model of LSEC transplantation.

In conclusion, many studies describe immunoregulatory effects of non-parenchymal liver cells, most often in the context of whole liver transplantation and chronic liver inflammation. In general, tissue based immunomodulation is a widespread property of many tissues. However, there are only few studies that analyzed the effect of allogenic transplanted non-parenchymal liver cells on the immune system with further studies urgently required.

TRANSPLANTATION OF STEM CELLS AND HEPATOCYTE-LIKE CELLS

Liver stem cells (LSC) can be seen as the optimal future source for LCTxs. On one hand, they would be capable to proliferate *in vitro*, thus, provide an unlimited cell source. On the other hand, if derived from patient's own liver biopsies and propagated *in vitro*, autologous liver stem cell transplantation could become a therapeutic option for a number of indications where the patients are not in acute need for cell and gene therapy - without any immunological complications as opposed to allogenic cell transplantation. Thus, intense research for (human) LSC are ongoing worldwide for more than 30 years without clinically useful definitions of a liver-specific stem cell phenotype. Also, numerous attempts are being made to derive transplantable, functional hepatocyte-like cells from other unlimited sources like embryonic stem (ES) or induced pluripotent stem (iPS) cells, so far with only moderate success^[77].

Recently, considerable progress was made regarding the transplantation of murine^[78] and the generation of potential human LSC^[79], own unpublished data). So far, only murine^[78] and rat^[80] LSC were successfully transplanted, albeit in autologous settings. Thus, no data exist so far regarding immunogenicity of allogenic LSC. However, some findings from allogenic stem cell transplantations in combination with other organ systems such as bone^[81], retinal epithelium^[82] and endothelium^[83] indicate at least immune-privileged properties of stem cells compared to mature tissue cells upon transplantation. At first thought, the reduced immunogenicity of transplanted stem cells appears to delay but not to prevent the onset of immune-recognition. The importance of the immature state is underlined by the observation that cell maturation during engraftment towards terminally differentiated cells is associated with a loss of their immune-privileged state. However, there is some evidence that tolerance, developed towards transplanted allogenic stem cells, extends later to their differentiated progeny^[84]. Furthermore, for epithelial tissue types

like the liver, transplanted cells might be immune-privileged initially during tissue repair (associated with full immune exposure), whereas later immunogenic properties on the surfaces of matured engrafted cells maybe partially invisible to the immune system within the fully reformed tissue.

Taken together, little is known about the potential effects of LSC transplantations with respect to immunological aspects and liver regeneration. Nevertheless, one can safely assume that allogenic LSC transplantation will certainly be associated with reduced immunological consequences as compared to transplantation of mature hepatocytes.

IMMUNOSUPPRESSION/IMMUNOMODULATION

Conventional immunosuppressive drugs

Most centers performing hepatocyte transplantation simply adapted protocols used for OLT, consisting of steroids and calcineurin-inhibitors (CNI) (Tacrolimus/Cyclosporin). Continuous and effective immunosuppression with CNI seems to be of particular importance since patients with low levels of Cyclosporin displayed acute rejection of transplanted hepatocytes^[85]. Several studies have demonstrated that CNI improve hepatic regeneration^[86,87] and the administration of Cyclosporin or Tacrolimus increased the mitotic index in the regenerating liver of adult rats^[88]. These effects seem to be even more important after hepatocyte transplantation as compared to OLT, since engraftment and proliferation of liver cells are fundamental for the success of LCTx. Immunosuppressive regimens without steroids or with low doses of CNI have been recommended, especially in patients affected by urea cycle disorders, because of their catabolic effects^[85]. The complete removal of CNI has been achieved by the addition of drugs such as mycophenolate mofetil (MMF) or mTOR-inhibitors such as Rapamycin. However, some data suggest that Rapamycin is associated with an increased risk of graft loss, death and sepsis after OLT when compared to the use of conventional-dose Tacrolimus alone^[89]. Furthermore, mTOR-inhibitors might inhibit liver regeneration^[90] and, therefore, could potentially delay hepatocyte engraftment and proliferation.

Wu *et al.*^[91] compared Tacrolimus, Rapamycin and MMF in a rat hepatocyte transplantation model and showed that mTOR-inhibition could be beneficial during the phase of engraftment of transplanted cells. However, it may be advisable to avoid Rapamycin or other mTOR-inhibitors during the anticipated period of transplanted cell proliferation. CNI and MMF could serve as alternatives during this phase of transplantation. Later, when proliferation of transplanted cells has been completed, Rapamycin could possibly be used again if required^[91].

As mentioned before, the co-stimulation blockade

has been clinically approved for kidney transplantation but not for other solid organ transplantations. Belatacept is a high affinity fusion protein that binds to B7.1 (CD80) and B7.2 (CD86) on human APCs. Regarding a possible tolerogenic effect of co-stimulation blockade using Belatacept for the use in OLT, no association with operational tolerance was observed^[92]. Since in animal experiments a beneficial effect of CTLA-4-Ig on CD4⁺ T-cell mediated rejection of hepatocytes *via* the CD28/CD80 (B7) pathway was found^[55], Belatacept, nevertheless, might be of interest for the use in LCTx and should be investigated in the future.

Novel anti-inflammatory drugs

After delivery of transplanted hepatic cells to liver sinusoids, several steps follow before cells are fully integrated in to the tissue architecture. During these steps, including entry into sinusoids and passage into the liver parenchyma, 70%-80% of initially transplanted cells are destroyed mainly due to sinusoidal effects, oxidative stress and cytokine-mediated toxicity^[13]. Novel strategies, hence, have been developed to optimize engraftment and minimize early hepatocyte cell loss early after transplantation. The majority of these strategies aims at pre-treating recipients prior to cell transplantation to either minimize the vascular and inflammatory changes induced by transplanted cells or to reduce the endothelial barrier between liver sinusoids and parenchyma or to activate HSC to release beneficial substances: The COX-2-specific inhibitors Naproxen and Celecoxib were shown to increase the number of engrafted hepatocytes by activation of HSC. These drugs induce HSC to express cytoprotective genes, vascular endothelial and hepatocyte growth factor, matrix-type metalloproteinases and tissue inhibitor of metalloproteinase-1, which regulate hepatic remodeling^[93].

Furthermore, transplanted hepatocytes promote IBMIR and, therefore, the treatment with anti-inflammatory drugs like the TNF antagonist Etanercept seems to downregulate this IBMIR. In a rat model of hepatocyte transplantation, Etanercept showed beneficial effects by blocking the synthesis of inflammatory cytokines, chemokines as well as their appropriate receptors leading to enhanced cell survival and engraftment of transplanted cells into the recipient's liver^[94]. Similar to Etanercept, the dual endothelin-1 receptor blocker Bosentan improves cell engraftment, independently of hepatic ischemia or inflammation, but without improving liver repopulation. However, incubation of hepatocytes with Bosentan protected cells from cytokine toxicity *in vitro* and produced superior cell engraftment and proliferation *in vivo*^[95].

Immunomodulation with Treg

To prevent rejection in hepatocyte transplantation currently continuous treatment with immunosuppressive medication is needed, which may be harmful due to

nephrotoxicity, increased risk of infections and cancer just to name the most important ones. Furthermore, despite the use of potent immunosuppressive agents, acute rejection remains the major cause of early allograft loss not only in solid organ transplantation but also in hepatocyte transplantation. An immunomodulatory regimen which improves patient and allograft survival and reduces the need for immunosuppressive drugs would be optimal and cell therapeutic approaches may be able to fulfill these requests. There are a number of lymphoid cell types with regulatory capacity that can promote tolerance induction in animal models of transplantation^[96]. Treg are the most widely studied and applied lymphoid cells for an immunomodulatory regimen. CD4⁺CD25⁺FoxP3⁺ Treg could be proven to control autoimmunity, inhibit graft versus host disease (GVHD) and prevent or delay allograft rejection in animal models^[97,98]. However, there are no studies concerning the effect of Treg in the context of hepatocyte transplantation. The only data available come from solid liver transplant studies in animals and human patients.

In a liver transplant rat model, Pu *et al.*^[99] could show that the adoptive transfusion of *ex vivo* donor alloantigen-stimulated CD4⁺CD25⁺ Treg combined with short-term Tacrolimus treatment prolonged the survival of liver allografts.

In humans, the frequency of circulating Treg is significantly decreased during acute rejection of liver allografts^[100]. Pediatric patients who achieved operational tolerance after liver transplantation showed increased levels of circulating Treg compared to patients who received immunosuppression^[101]. Therefore, an increased level of circulating Treg may be beneficial in particular for liver allograft survival. Yamashita *et al.*^[102] just recently conducted a clinical trial applying the infusion of donor antigen-driven Treg in 10 patients undergoing living donor liver transplantation. In 6 patients, immunosuppression was successfully withdrawn without causing allograft rejection and graft function was well maintained which may represent a landmark study for clinical application of cell therapy with Treg^[102].

In conclusion, the data from liver transplanted patients emphasizes that Treg could also have immunomodulatory potentials in hepatocyte transplantation.

CONCLUSION

Despite current hurdles concerning the engraftment and long-term acceptance of cellular allografts, LCTx still represents a very promising tool for the treatment of various liver diseases in the near future. Deeper knowledge of the immunological responses induced by transplanted cells though is a prerequisite for the success of this therapeutic approach. The available data clearly demonstrate that rejection of liver cell allografts is by far more complex than initially assumed and, most importantly, differs considerably from those

immune reactions observed following solid organ transplantation. Further immunological investigations *in vivo* and *in vitro* are desperately required - especially human data are still scarce.

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