# From immunosuppression to tolerance

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## Summary

The past three decades have seen liver transplantation becoming a major therapeutic approach in the management of end-stage liver diseases. This is due to the dramatic improvement in survival after liver transplantation as a consequence of the improvement of surgical and anaesthetic techniques, of post-transplant medico-surgical management and of prevention of disease recurrence and other post-transplant complications. Improved use of post-transplant immunosuppression to prevent acute and chronic rejection is a major factor in these improved results. The liver has been shown to be more tolerogenic than other organs, and matching of donor and recipients is mainly limited to ABO blood group compatibility. However, long-term immunosuppression is required to avoid severe acute and chronic rejection and graft loss. With the current immunosuppression protocols, the risk of acute rejection requiring additional therapy is 10–40% and the risk of chronic rejection is below 5%. However, the development of histological lesions in the graft in long-term survivors suggest atypical forms of graft rejection may develop as a consequence of under-immunosuppression. The backbone of immunosuppression remains calcineurin inhibitors (CNI) mostly in association with steroids in the short-term and mycophenolate mofetil or mTOR inhibitors (everolimus). The occurrence of post-transplant complications related to the immunosuppressive therapy has led to the development of new protocols aimed at protecting renal function and preventing the development of *de novo* cancer and of dysmetabolic syndrome. However, there is no new class of immunosuppressive drugs in the pipeline able to replace current protocols in the near future. The aim of a full immune tolerance of the graft is rarely achieved since only 20% of selected patients can be weaned successfully off immunosuppression. In the future, immunosuppression will probably be more case oriented aiming to protect the graft from rejection and at reducing the risk of disease recurrence and complications related to immunosuppressive therapy. Such approaches will include strategies aiming to promote stable long-term immunological tolerance of the liver graft.

## Introduction

In the early era of liver transplantation, it was felt that rejection was less problematic than in other organ transplantation. Experimental and clinical studies suggested that the liver was less sensitive to rejection than the skin, heart, lungs or kidney, a consequence of the liver's tolerogenic potential. This was based on a large body of observational and experimental evidence. For instance in pigs and some rat strains, liver transplants are accepted without the need for immunosuppressive drugs and furthermore the liver may induce tolerance for other transplanted organs and decrease the risk of rejection of associated heart or kidney transplants [1]. Clinically hyperacute rejection of liver transplants is an extremely rare event despite the lack of major histocompatibility complex (MHC) matching and successful grafts are possible across ABO blood groups and in the presence of positive cross matches between donor and recipient which is not true of other organ transplants [2].

During the early years of liver transplantation, serious surgical and infectious complications dominated the post-operative period although it rapidly became apparent that although rejection is usually less severe in liver compared with other organ transplants it is still a major issue requiring immunosuppressive therapy that is life-long in most patients [3–6]. Thus, before the advent of cyclosporine, the rate of acute rejection at post-transplantation day 5–7 was around 80% and although, as stated above, liver transplants can survive in the face of ABO incompatibility or positive cross matches, these situations were associated with a risk of severe life threatening acute rejection mixing cellular and humoral rejection [2]. Thus in order to achieve high long-term graft survival rates, rejection should be efficiently prevented and treated. In this review we will discuss the evolution of

immunosuppressive protocols, their current objective, the mechanisms of acute and chronic rejection, the basis of tolerance and the possibility to achieve tolerance in clinical practice.

## Immunosuppression in liver transplantation: evolution of protocols

### Immunosuppressive drugs used in liver transplantation

#### Corticosteroids

Steroids have always been used in liver transplantation. They are administered to prevent acute rejection and commonly started per-operatively, and given as high bolus doses to treat acute rejection. Their widespread use in transplantation reflects their many potent actions but this also leads to severe toxicity and numerous side effects such as diabetes, infection, hypertension, Cushing's syndrome, poor wound healing, osteoporosis and accelerated cardiovascular disease. This has promoted clinicians to try to minimize their use and with the knowledge of liver transplantation evolving, the way steroids are being used has changed. Firstly, the cumulative dose of steroids given during the first weeks has been drastically reduced. Many patients still receive a loading dose initially of 500 mg, but then the dose of steroids is rapidly tapered. In addition, in many centres steroids are discontinued after a few weeks or months except in some particular cases, such as patients transplanted for autoimmune diseases, although even here there is evidence that in many cases steroid withdrawal is safe [7]. Therefore in many centres, patients are free of steroids after the first 6 months and some centres have advocated steroid free regimes. In most centres corticosteroids continue to be the first line treatment for acute rejection although the total amount of steroids given as treatment of rejection is much less than in the early years of liver transplantation. This reflects the increasing realization that a lymphocytic infiltrate on liver biopsy in the first 10 days after transplantation does not always result in clinical rejection and that patients should only be treated if they have biochemical evidence of graft dysfunction. Furthermore, it is now appreciated that whereas the effect of steroids is impressive for early acute rejection it is much less so in cases of persistent late acute rejection and chronic rejection [8]. Thus clinicians have realized the importance of assessing the inflammatory activity of rejection more carefully by not continuing to treat with high-dose steroids once the condition has evolved into the chronic phase, which is much less responsive to steroids. Interestingly this change in practice has been associated with a dramatic fall in the incidence of chronic rejection so that it is now a relatively rare event in liver transplantation, leading to the need for re-transplantation in less than 5% of patients [9,10]. Steroids have a Dr Jekyll and Mister Hyde character: as an example in hepatitis C patients, steroids increase the serum viral load and boluses of steroids increase fibrosis progression but in contrast, the rapid discontinuation of steroids after transplantation has been associated with an immune rebound deleterious for the graft. Moreover, steroid free protocols do not lead to reduced graft fibrosis at 2 years probably due to the fact that the lack of steroids was compensated for by more aggressive immunosuppression using other agents [11].

#### Purine inhibitors

*Azathioprine.* In the very early years of liver transplantation azathioprine and corticosteroids were the mainstay of immunosuppression before the discovery of cyclosporine [4–6,12]. Long-term survivors from those programmes show the efficacy of azathioprine. This is particularly demonstrated in renal transplantation where some units switched to calcineurin inhibitors (CNI) relatively late because of the risks of renal toxicity, and despite this, have large cohorts of long-term survivors on azathioprine-based non-CNI protocols. Since the 1980s and early 1990s azathioprine has been used in combination with steroids and CNIs to prevent the development of rejection. Azathioprine has never been used to treat acute rejection. Over the past 20 years the use of azathioprine has progressively reduced and been replaced by the newer purine inhibitor mycophenolate [13,14].

#### Mycophenolic acid (MPA) prodrugs

*Mycophenolate mofetil (MMF).* MMF is a more potent immunosuppressive agent than azathioprine, although one can argue that azathioprine's relatively weaker effect makes it a safer drug to use in combination with CNIs. MMF is undoubtedly associated with a reduced risk of marrow suppression and squamous cell carcinoma of the skin but the fact that azathioprine is safe during pregnancy whereas MMF is teratogenic in animals is in azathioprine's favour in women of child bearing age. The group at the Royal Free Hospital have proposed the use of azathioprine in patients with hepatitis C virus (HCV) infection claiming it is associated with a slower progression of graft fibrosis [15]. However, the consensus of opinion from other studies and particularly from renal transplantation is that azathioprine is less effective than MMF and it is much less widely used than previously. MMF is a prodrug of mycophenolic acid (MPA) and an inhibitor of inosine-5′-monophosphate dehydrogenase (IMPDH) an enzyme used in the *de novo* synthesis of guanosine nucleotides necessary for lymphocyte activation. Another prodrug of MPA *enteric-coated mycophenolate sodium*, is also used as an immunosuppressant. Their main side effects are leukopenia and the occurrence of abdominal pain and particularly diarrhoea, which affects about 20% of patients but often resolves on reducing the dose. These mycophenolate acid prodrugs are now widely used in transplantation mainly in association with CNI. Like azathioprine they are not potent enough or rapidly acting to allow them to be used to treat acute rejection and the evidence suggests that in most cases they are not potent enough to be used as a sole immunosuppressant. However, in combination with CNIs they allow immunosuppression to be maintained while minimizing the dose of CNI and thus reduce the risk of CNI-induced renal toxicity [13,14].

#### Calcineurine inhibitors

*Cyclosporine.* Most people considered that the advent of cyclosporine was a therapeutic breakthrough, a true revolution that led directly to the improved survival after liver transplantation from its introduction in 1984 [4,5,16]. In fact this is only partially true. Cyclosporine by its potent immunosuppressive effect decreased both the incidence and severity of acute rejection and was thus a major cause of improvement in the results of liver transplantation. However the advent of cyclosporine coincided with unprecedented improvements in the surgical, anesthesiological, and medical management of liver transplantation and these may have contributed as much if not more than the introduction of cyclosporine to the extraordinary development of liver transplantation [3–5,17]. Cyclosporine became the basis of modern immunosuppression in combination with steroids or in

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earlier years in combination with corticosteroids and azathioprine. Cyclosporine is able to prevent the occurrence of acute rejection but is not effective in its treatment. The cyclosporine-based immunosuppression protocols used in the early 1990s gave a rate of acute rejection of 30–40% and a rate of chronic rejection of 10–15% allowing a reduction of the use of high doses of steroids and therefore a reduction in infectious complications and steroid related complications. A drawback of cyclosporine A was its low bioavailability, due to its lipophilic structure, and a high incidence of side effects particularly the serious complications of arterial hypertension and nephrotoxicity but also hirsutism and gum hypertrophy which, although not life threatening, are unacceptable to some patients. In the early 1990s cyclosporine was replaced by cyclosporine microemulsion, which was more stable, with a significantly better bioavailability [18]. C2 serum level (cyclosporine blood level 2 h after ingestion of cyclosporine microemulsion) was shown to be a better monitoring of cyclosporine activity than through blood level of cyclosporine [18].

*Tacrolimus.* Tacrolimus is a macrolide with strong immunosuppressive activity based on a property it shares with cyclosporine calcineurin inhibition, despite their very different chemical structure. It was added to the armentarium of immunosuppression in the early 1990s, ten years after cyclosporine and was initially developed for liver transplantation [19,20]. The immunosuppressive potency of oral tacrolimus is greater than cyclosporine and its bioavailability is excellent, it has become the main CNI used in most immunosuppressive protocols often in combination with corticosteroids [21]. Following the introduction of these protocols of immunosuppression, the rate of acute rejection fell progressively to 20% and the rate of chronic rejection to 5% and most cases of acute rejection are now controlled either by an increase of tacrolimus or by boluses of steroids with a reduced total immunosuppressive load and consequent reduction in infectious complications. Although tacrolimus is effective in the treatment and prevention of rejection it has side effects, particularly those it shares with cyclosporine as a consequence of calcineurin inhibition, arterial hypertension, renal toxicity and diabetes [10].

## Monoclonal antibodies
*Anti-IL-2 receptor antibody.* Monoclonal antibodies that block the IL-2 receptor and thereby prevent IL-2 dependent expansion of effector T cells have been shown to prevent the development of graft rejection in organ transplantation including liver transplantation. Two products have been licensed: *basiliximab*, a chimeric antibody, and *daclizumab*, a humanized antibody that has since been removed from the market. Both antibodies target the alpha chain of the IL-2 receptor and prevents the expansion of activated lymphocytes in the initial cascade of acute rejection. For this reason, they should be given during the first hours post-transplantation and are usually administered until day 5. They are not effective against established acute rejection and because the IL-2 receptor is expressed at high levels on regulatory T cells they could in theory disrupt established tolerance. They are non-nephrotoxic leading to their use particularly after transplantation in patients with renal failure and in protocols with delayed introduction of CNI to decrease the rate of renal failure [22].

*OKT3.* This antibody targets the T cell receptor and is extremely potent. It works in the context of transplantation by inducing the apoptosis of activated T cells but unfortunately is non-specific also destroying memory T cells involved in protection against viral infections and immune surveillance leading to a high incidence of opportunistic infections and an increased risk of lymphomas, which has led to its withdrawal from clinical use [23,24].

*Anti-thymocyte globulin or anti-lymphocytic serum.* This polyclonal antibody from rabbit or horse origin is effective in prevention and treatment of rejection. Due to severe complications such as lymphoma or opportunistic infections, the duration of administration and therefore the overall amount administered has been reduced. It is now administered over a maximum of 3–7 days. It is non-nephrotoxic and therefore can be used in patients with renal failure. It also has a place in patients with positive cross matches, ABO incompatible liver transplantation and patients with high risk of rejection [10].

## mTOR inhibitors
Two mTOR inhibitors have been used in liver transplantation: *rapamycin* (*sirolimus*) is licensed in kidney transplantation but not in liver transplantation due to concerns about vascular thrombosis in the post-operative period in early trials [25]. Although these vascular complications have not been confirmed in later trials rapamycin is not used routinely in liver transplantation. *Everolimus*, another mTOR inhibitor, has recently been licensed for the prevention of rejection in liver transplantation, in specific indications it is started 1 month after the initial operation due to its effects on wound healing [26,27]. mTOR inhibitors have major immunosuppressive activity through their intracellular binding to FKBP12 and inhibition of mTORC1 which blocks cell cycle progression and IL-2 signalling in T cells. Thus they allow T cell activation but prevent cells from proliferating in response to IL-2. mTOR inhibitors are strong immunosuppressant's with low nephrotoxic effects. Their efficacy is enhanced with CNI, but they may increase the nephrotoxicity of CNI and the combination should be used with caution using a lower dose of CNI and careful monitoring. Although usually well-tolerated, mTOR inhibitors have many side effects such as hypercholesterolemia, delayed wound healing, thrombocytopenia, mouth ulcers and leg oedema. In addition, some cases of severe interstitial pneumonia have been reported. mTOR inhibitors are interesting because in addition to their immunosuppressive properties they may promote immune tolerance (see later) and also have anti-proliferative and anticancer properties. It has been shown in kidney transplantation that the introduction of rapamycin reduced the risk of skin cancer recurrence in renal transplant patients. By extension, many authors are supporting the use of mTOR inhibitors in patients transplanted for hepatocellular carcinoma with the aim of reducing recurrence. They are now used in patients with CNI related nephrotoxicity and switching from CNI based immunosuppression to everolimus has been associated with reversibility of renal failure in some patients [25,28,29].

## New immunosuppressive drugs
It is surprising and at the same time disappointing that despite the rapid evolution of immunosuppression during the 1980s and 1990s the last generation of immunosuppressive drugs entered in clinical practice are the mTOR inhibitors and in 2015 CNI are still the backbone of immunosuppression. The co-stimulator blocker belatacept has failed to demonstrate a benefit in liver transplantation, in contrast to kidney transplantation, and

other immunosuppressive drugs including leflunomide and the sphingosine 1 phosphate inhibitor FTY720, have not been successfully taken up in liver transplantation.

Efalizumab, a non-depleting humanized leukocyte function associated antigen 1 (LFA1; CD11a) specific antibody that inhibits LFA-1 functions such as T cell antigen presenting cells (APC), is still in evaluation for its stabilization and antigen presentation [10].

*Protocols of immunosuppression*

The evolution of immunosuppression in liver transplantation is shown in Fig. 1. The backbone of immunosuppression remains the CNI associated with steroids. Over the years several concepts have emerged: the overall amount of steroids given has been dramatically reduced. In most immunosuppression protocols, after an initial high-dose of steroids administered peri-operatively, the daily dose of steroids has been reduced to 20 mg prednisolone during the first weeks post-transplantation and then rapidly tapered until full discontinuation within the first post-transplant months. Some centres have advocated immunosuppression without steroids from the start to reduce steroids related side effects. However this policy is not used in most centres because it puts patients at an increased risk of acute rejection requiring high-dose corticosteroid boluses, which negate any benefit of a steroid free immunosuppression protocol. Furthermore, effective immunosuppression in the absence of corticosteroids may require a higher dose of CNI leading to increased CNI toxicity. Steroid free immunosuppression was tested in patients at risk of hepatitis C recurrence based on the theoretical hypothesis that steroid use increases viral replication and fibrogenesis. However, in a randomized protocol including a steroid free arm, the degree of fibrosis was not reduced in the steroid free group [11].
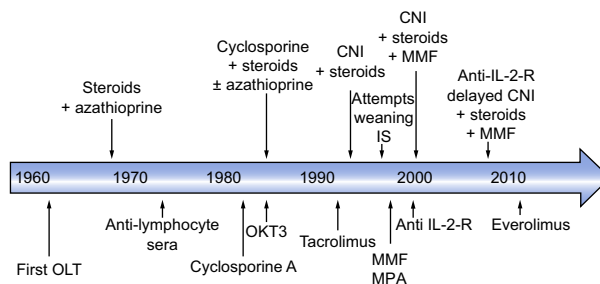


**Fig. 1. Timeline of immunosuppression and immunosuppressive protocols.** CNI: Cyclosporine A, Tacrolimus, Cyclosporine microemulsion, Tacrolimus released; MMF: mycophenolate mofetil; MPA: mycophenolic acid; Anti-IL-2-R: IL-2 receptors antibodies.

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**Key Points 1**

- The current rates of acute and chronic rejection are 10-40% and 5% respectively

- Rejection severity can be graded histologically using the Banff classification

- The backbone of immunosuppression remains CNI

- Medium and long-term complications of immunosuppression are a major concern and include renal, metabolic, cardiovascular disease and *de novo* cancer

- Renal function sparing regimens are in current use including immunosuppression combining low dose CNI with anti-IL-2-R Ab, MPA prodrugs or everolimus

- There are currently no new major immunosuppressive drugs in clinical trials

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The concept of "renal protection" is an important factor in designing immunosuppression protocols. A significant proportion of liver transplant patients develop renal failure during follow-up and a major cause is CNI toxicity [30]. The CNI effect is fixed

during the first post-transplant weeks and is mostly irreversible leading to the need to reduce CNIs to a minimum. Therefore several schemes have been proposed during the post-transplant period: a triple drug combination with CNI, steroids and MPA prodrugs to avoid a too high dose of CNI; a delayed introduction of CNI around day 5 with an induction with IL-2 receptor antibody [22,31]. In the long-term it has been proposed to further reduce CNI administration by introducing everolimus either combined with low dose CNI or alone or with MPA prodrugs [27,28].

The treatment of acute rejection remains mostly unchanged based on an increase of immunosuppression, a switch from cyclosporine to tacrolimus and boluses of corticosteroids according to the severity of histological rejection and to the response to anti-rejection therapy. Perhaps the main advance in the treatment of acute rejection is the recognition that a portal infiltrate in itself is not an indication to treat and that many early infiltrates resolve spontaneously without the need for increased immunosuppression. Treatment should be confined to patients with evidence of graft injury in whom other causes have been excluded and rejection confirmed by liver biopsy. It should be noted that apart from the advent of everolimus there is no major change in the immunosuppression protocols over the last decade [32].

*Specific aspects of immunosuppression*
*Hepatitis C patients.* The immunosuppressive regimen should be potent enough to prevent acute rejection but not so excessive that it exacerbates HCV disease progression through increasing viral replication and the associated accelerated fibrosis. Current data have failed to show differences in the incidence or severity of HCV recurrence using tacrolimus or cyclosporine. However, several studies reported that cyclosporine inhibits HCV replication in a cell-based replicon model and has a favourable impact on response to interferon therapy as compared with tacrolimus. Cyclosporine may act as an inhibitor of cyclophilin B during antiviral therapy. The effect of corticosteroids on the recurrence of hepatitis C is unclear, and seems to differ according to how they are used. Several studies have shown that the use of steroid boluses to treat rejection episodes increases HCV viremia by 1–2 log and worsens the severity of disease recurrence resulting in increased patient and graft loss. By contrast, the rapid and early withdrawal of steroids after transplantation may be deleterious and lead to rapid development of fibrosis [33–35]. However, studies of steroid free immunosuppression regimens have found no difference in the outcome of HCV recurrence [11]. Data on the

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effect of other immunosuppressive agents, such as MMF, azathioprine, antibodies to the IL-2 receptor and mammalian target of rapamycin inhibitors, on HCV recurrence remain controversial. Thus the optimal immunosuppression protocol remains to be determined.

*Long-term complications of immunosuppression*

With the improvement of survival after transplantation it has become more important to improve the long-term quality of life of transplant patients and to reduce the side effects of immunosuppression.

Patients are exposed to several risks:

A) A dysmetabolic syndrome characterized by the occurrence of diabetes, arterial hypertension, and obesity [36,37].
B) Cardiovascular complications, which are now the main cause of long-term mortality.
C) An increased risk of *de novo* cancer. Transplant patients have a 100 fold increased risk of skin cancers such as basocellular or squamous cell carcinoma, they have also a 3–5 fold increased risk of non-skin cancers in comparison to the general population particularly blood cancers and gastrointestinal tract cancer [38,39].
D) Renal complications: chronic renal failure is one of the main causes of long-term morbidity after liver transplantation. The rate of severe renal failure is estimated at 20–25% at 5 years. As said above, the main cause of chronic renal failure is CNI toxicity and therefore, strategies to protect the kidney of liver transplant patients are essential [30,40,41].

## Immunological basis of rejection in liver transplantation

*Allograft rejection*

*Acute rejection*
Allograft rejection is graft damage arising as a consequence of an immunological reaction to foreign antigens on the graft. Alloantigens are potent activators of immunity although in the context of liver transplantation and immunosuppression a minority of patients develop clinical symptoms and it is important to distinguish between the immune response to the graft and clinical rejection because only the latter requires additional immunosuppression [42]. The incidence of clinically significant rejection is 10–40% in most series usually within the first month after transplantation [43] when liver injury results in elevated levels of bilirubin, transaminases and alkaline phosphatase [44]. The diagnosis is confirmed by the characteristic histological findings of portal inflammation, bile duct injury and venous inflammation often associated with centrilobular necroinflammation involving hepatic venules and surrounding hepatocytes [45]. Because milder forms of rejection can be managed without additional immunosuppressive therapy it is important to define the severity using for instance the Banff classification [46,47]. Late acute rejection occurring more than three months after transplantation is often a consequence of inadequate immunosuppression. It is associated with a more hepatitic appearance and more markedly elevated serum transaminases in contrast with the cholestatic picture which is characteristic of portal-based acute

rejection. It is also less responsive to immunosuppression and more likely to become chronic [8,48] Fig. 2.

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**Key Points 2**

- MHC antigens remain the most important alloantigens in graft rejection

- The liver is a tolerogenic organ and its microanatomy, cellular composition and cytokine microenvironment contribute to the easier acceptance of liver versus other solid organ transplants

- Rejection is a T cell driven immune response that predominantly targets bile ducts

- Preservation and reperfusion injury can contribute to the breaking of tolerance and triggering of immune mediated injury

- Chemokines have a role in compartmentalising infiltrating leukocytes and the balance of local effector and regulatory cells recruited determines graft outcome

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*Chronic rejection*
With advances in immunosuppressive therapy there has been a progressive fall in the prevalence of chronic rejection, which now accounts for <2% of cases of graft failure. In its most severe form it is characterized by severe bile duct damage leading to bile duct loss and an obliterative arteriopathy with rapid progression to graft failure although more indolent forms also occur particularly in cases that present more than twelve months after transplantation [49]. Chronic rejection can progress to bridging fibrosis and cirrhosis [45,50].

*Antibody-mediated rejection*
Antibody-mediated rejection is seen in ABO incompatible transplants [51] and in the presence of preformed lymphocytotoxic antibodies [52]. Its most severe form, hyperacute rejection, is extremely rare and characterized by widespread haemorrhage, microvascular thrombosis and hepatocyte necrosis as a consequence of preformed antibodies binding to endothelial antigens [52–54]. It can be prevented in recipients of ABO-I grafts by the use of anti-B cell directed immunosuppression. Antibody-mediated mechanisms are suggested by the presence of C4d deposits in graft endothelium and may be involved in both acute and chronic rejection and associated with more severe graft dysfunction as well as a failure to respond to immunosuppression [55]. In addition to the very rare cases of "pure" hyperacute or acute antibody-mediated rejection cases (described above), results from recent retrospective single-centre reports suggesting that the presence of preformed or *de novo* anti-human leukocyte antigen (HLA) antibodies could also be involved in a number of additional negative immunological outcomes. Thus, anti-HLA donor-specific antibodies (DSA), mostly anti-HLA class II, have been associated with; i) increased risk of rejection and liver and kidney graft loss in simultaneous liver-kidney transplant recipients [56]; ii) with a higher prevalence of advanced fibrosis or cirrhosis in paediatric liver transplant recipients [57]; iii) with an increased risk of
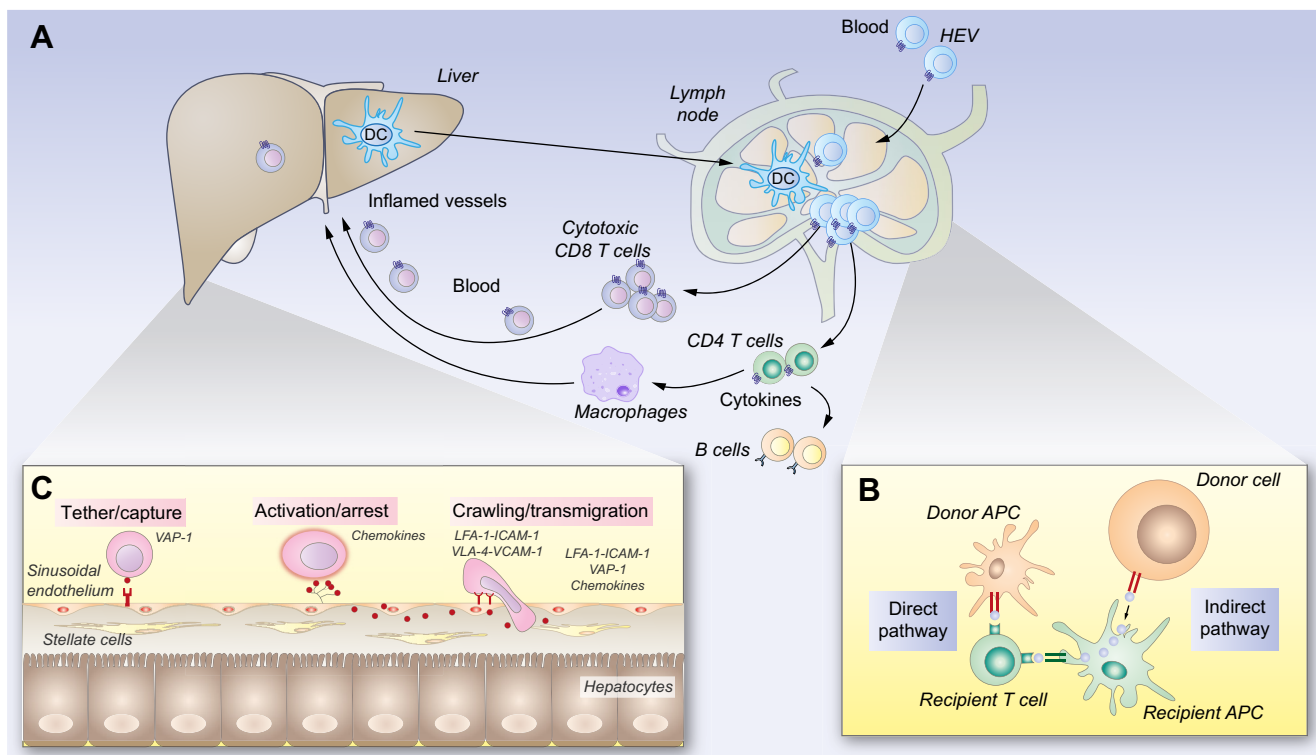
**Fig. 2. Mechanisms of graft rejection.** (A) Damage to the graft during preservation and reperfusion activates the innate immune system including local NK and NK T cells that secrete proinflammatory cytokines. Dendritic cells (DC) migrate from the graft to recipient lymphoid tissue where they activate alloreactive T cells that enter lymphoid tissues from blood via high endothelial venules (HEV). Responding T cells proliferate, differentiate and secrete cytokines that promote activation of other effectors including monocytes/macrophages and eosinophils. Effector cells are recruited to the graft from the blood via activated hepatic endothelium and destroy donor cells, particularly bile duct cells and hepatocytes, by direct lysis (cytotoxic T lymphocytes and NK cells) cytokine-mediated effects or involvement of other cells including macrophages. Activated T cells provide help for alloantibody production by B cells leading to antibody-mediated graft damage and complement deposition on graft endothelium. (B) There are two types of allorecognition. Direct allorecognition occurs when T cells recognize intact foreign major histocompatible complex (MHC) molecules on donor antigen presenting cells (APC). This pathway dominates in acute graft rejection. T cells also recognize donor peptide fragments processed by and presented on self-MHC molecules (indirect allorecognition). This is important in chronic graft rejection but may have conflicting effects: providing T cell help for B cells leading to anti-graft antibodies but also activating regulatory T cells that limit graft damage and promote tolerance. Liver resident cells including sinusoidal endothelial cells and hepatocytes also present antigens but when they interact with naïve T cells the results is tolerance and in the case of endothelium a stop signal which prevents recruitment. (C) Effector cells are recruited to the graft by sequential interactions with activated hepatic sinusoidal endothelium. A brief tethering capture phase is followed by chemokine triggering of integrin (LFA-1 and VLA-4) mediated adhesion to ICAM-1 and VCAM-1 on activated endothelium which leads to stable adhesion and arrest followed by migration on and through the endothelial barrier into the graft, a process that involves chemokines, integrins and the atypical adhesion receptor vascular adhesion protein-1 (VAP-1) which has also been implicated in sialic acid dependent tethering.

post-transplant HCV-induced advanced liver fibrosis in adult recipients [58]; and iv) are more frequent in patients with chronic rejection than in those with normal graft function [59]. The extent to which DSA influences liver allograft pathology is still difficult to estimate. Large prospective clinical trials will be required to clarify their exact pathogenic role. In the meantime, implementation of routine anti-HLA antibody monitoring or at least biobanking of serum samples to do so retrospectively if required, should be encouraged as a means to confirm or dispute existing associations.

*Underlying mechanisms of rejection*

*Allorecognition and immune activation.* Rejection results from activation of immune pathways by alloantigens the most important of which are the MHC antigens (Ags) [60]. Immune recognition of mismatched donor HLA results in both cellular and humoral immune activation and allograft rejection. Although antibody-mediated hyperacute rejection is very rare in liver

transplantation, DSA to donor MHC class I can drive complement dependent and independent pathways that may contribute to tissue damage in acute and chronic rejection [61–63]. Donor HLA-specific antibodies are associated with portal capillary and stromal C4d staining, indirect evidence of antibody-mediated graft injury, and with an increased incidence of chronic rejection of liver allografts [58]. However, the question of whether antibodies are a cause or a consequence of rejection is unproven and the relationship between DSA and graft damage is more complex in the liver than in the case of other transplanted organs. This is due to the fact that: i) markers of antibody-mediated damage (i.e. circulating DSA and graft deposition of complement protein C4d) are often present in the setting of typical acute cellular rejection [63]; ii) the liver can absorb large amounts of anti-HLA antibodies (particularly anti-class I) without undergoing detectable immunological damage; iii) the liver has a much lower, although variable, expression of HLA class II antigens than other allografts such as kidneys or hearts, which is not often taken into account when considering the pathogenic role of

anti-HLA class II DSA; iv) intrahepatic C4d staining has a low specificity as a marker of antibody-mediated rejection; and v) in contrast to other allografts, identification of intrahepatic NK cells or NK-derived transcripts does not seem to be associated with antibody-mediated rejection [64–66]. Clinical studies have shown that pre-existing donor HLA class I specific antibodies or their early appearance post-transplant are associated with subsequent rejection suggesting a causative role. Furthermore, rejection is associated with anti-class I specific antibodies which is consistent with a functional role because class I antigens are strongly expressed on bile ducts, the principle targets in liver rejection [63]. However, not all patients with pre-existing DSA or who develop *de novo* DSA develop rejection making it difficult to justify routine monitoring in the post-transplant patient. Nonetheless, the strong evidence implicating DSA in the pathogenesis of rejection suggests that testing for HLA-specific antibodies at the time of acute rejection might help determine the likelihood of chronic rejection and thereby target patients for more aggressive immunosuppression. The use of anti-T cell induction therapy with daclizumab or OKT3 reduces the risk of rejection in patients who are DSA positive suggesting that early T cell activation is important in the generation of antibody-mediated rejection [63]. Therapeutic targeting of B cells is a logical approach and pre-transplant depletion of B cells protects from rejection in experimental models [67]. However, the most widely available B cell therapy, rituximab, targets CD20 which is absent from plasma cells and memory B cells that will already be primed in patients with pre-existing DSA. The treatment of established antibody-associated chronic rejection is more difficult and may require different immunosuppression protocols.

### Basis of acute cellular rejection

*Acute rejection* is a T cell driven immune response to donor antigens [42], which may be recognized via three pathways termed direct, indirect and semi-direct. In the direct pathway, intact allogeneic MHC molecules on donor APCs are recognized by recipient T cells without the need for processing. This is the dominant pathway in acute rejection whereas indirect antigen presentation, in which allogeneic antigens are taken up and processed by APCs and presented on recipient MHC, dominates in chronic rejection and later immune responses to the graft. The semi-direct pathway is less well understood and involves recipient APCs that express intact donor MHC molecules as a consequence of fusion with donor exosomes [68]. The initial T cell response is characterized by infiltration of the graft by CD4+ and CD8+ T cells but myeloid cells and innate lymphoid cells are involved and may determine the outcome of allorecognition [69]. The nature of the antigen presenting cell and the site of antigen presentation also have a major effect on outcome. Immune responses in the liver differ from those in other organs as a consequence of its unique microanatomy and cellular composition [70]. The liver contains several cell types capable of processing and presenting antigen and activating lymphocytes although these interactions frequently result in tolerance rather than effector responses. Tolerogenic donor dendritic cells (DCs) within the graft donor migrate into donor lymphoid tissues after transplantation where they may persist and maintain tolerance. However, graft injury or damage or the presence of infection can activate both donor and recipient DCs to drive effector

responses explaining how preservation injury, for example, can be associated with more severe rejection [71].

*Cross presentation of antigens* by hepatic sinusoidal cells usually leads to a failure of CD8 T cells to develop full effector function and this may contribute to tolerance as well as interactions between recipient T cells and donor hepatocytes [72]. Consequently under many circumstances the outcome of T cell activation in the liver is tolerance [73]. However, strong effector responses can be generated under the correct conditions as demonstrated by the strong immune response to hepatitis A virus for example. The outcome depends in part on the site of immune activation with activation by DCs in draining lymph nodes usually leading to a vigorous immune response whereas local activation by sinusoidal endothelial cells or hepatocytes leads to tolerance [74,75].

*The inflammatory microenvironment in the liver allograft* affects the outcome of allorecognition. The activation of CD4 T cells by alloantigens is strongly influenced by the cytokine environment in which activation takes place and this contributes to the balance between rejection and tolerance of the allograft. CD4 T cells can differentiate into effector or regulatory phenotypes depending on the cytokines present during activation. In the presence of IL-12, CD4+ cells become T helper 1 (Th1) cells which secrete interferon gamma and contribute to tissue destruction. Whereas the presence of retinoic acid and TGFβ leads to the induction of the transcription factor Foxp3 and formation of regulatory T cells (Tregs) and the combination of IL-6 with TGFβ leads to the development of IL-17 producing T cells (Th17) rather than Tregs. The presence of IL-4 and relative lack of IL-12 drives differentiation into T helper 2 (Th2) cells which produce IL-4, IL-5, and IL-13 [76]. Th1 cell differentiation in response to high intragraft levels of IFNγ is an important driver of rejection [77] although interferon is also required for the development of tolerance in liver transplantation [78]. Recent studies implicate Th17 cells in liver allograft rejection in both experimental models and clinical transplantation and the balance between Th17 cells and Tregs may be particularly important in determining outcome [79,80].

*Recruitment of effector cells to the rejecting allograft.* Hepatic inflammation as a result of allorecognition leads to activation of resident immune cells and the recruitment of leucocytes from the circulation mediated by combinations of adhesion molecules and chemotactic cytokines that drive transendothelial migration into liver tissue [81]. In the case of the liver, direct visualization of leucocyte recruitment using intravital microscopy has shown that leucocytes are capable of adhesion and migration across different regions of the hepatic microvasculature but the majority of leukocyte migration occurs across the hepatic sinusoids [82]. The hepatic sinusoidal endothelium has a unique structure and phenotype and this together with the relatively low levels of shear stress in the sinusoids has led to the involvement of distinct combinations of receptors including vascular adhesion protein-1 (VAP-1), which has been demonstrated to mediate lymphocyte recruitment to the liver during graft rejection [83].

Activation of graft endothelium by injury or local inflammation leads to increased adhesion molecule expression and chemokine secretion leading to increased infiltration of lymphocytes into the graft. Chemokines are critical for leucocyte recruitment and the gamma interferon inducible chemokines CXCL9 and CXCL10 are induced during liver allograft rejection and recruit effector cells expressing their cognate receptor CXCR3 [84].

Furthermore this axis is activated early during preservation reperfusion injury and may be critical for initiating the effector response during allograft rejection [85].

Chemokines also have an important role for the compartmentalization of infiltrating leukocytes during liver allograft rejection. Cholangiocytes, the primary targets of acute cellular rejection, secrete several chemokines including not only CXCL9 and CXCL10 but also CCL20 which selectively recruits IL-17 secreting Th17 and Tc17 cells to the bile ducts which may be an important step in targeting immune damage at intrahepatic bile ducts during rejection [86]. Thus the microenvironment of the liver graft is critical in determining the outcome of allorecognition and this will be affected by many factors including donor age and underlying conditions such as steatosis, the severity of preservation reperfusion injury and the presence of infection all of which affect the inflammatory state of the graft.

*Mechanisms of hepatocyte and bile duct destruction in rejection*

In graft rejection cytotoxic T cells and other effector leukocytes bind to bile ducts and hepatocytes, which they then kill, using several molecular mechanisms. Cytolytic T cells can kill targets through the granzyme/perforin pathway, which is activated following engagement of the CD8 T cell receptor/MHC complex. However, hepatocytes are relatively resistant to this type of killing and the dominant mechanism of both hepatocyte and cholangiocyte killing involves members of the TNF superfamily [87]. During liver inflammation, including allograft rejection, the TNF superfamily receptors CD40, TNFR1, TNFR2, and TRAIL receptors are increased on hepatocytes and cholangiocytes allowing CD8+ T cells, macrophages, and NK cells, which express ligands for these receptors to participate in liver cell killing [88,89]. In addition cholangiocytes are susceptible to replicative senescence. This results from ischemia and oxidative stress during graft preservation and reperfusion injury, which sensitizes them to subsequent immune-mediated injury during rejection [90,91]. An important factor in irreversible bile duct loss is the microvasculature that supplies the intrahepatic biliary tree. The microvasculature itself is a target of the alloresponse in rejection and can thus be destroyed as part of the rejection process. Even severe damage to bile ducts is reversible as long as the microvasculature is preserved but once this is lost irreversible bile duct loss ensues [92]. Together, these pathways drive cholangiocyte destruction, ductular reaction and periportal fibrosis culminating in the vanishing bile duct syndrome that is characteristic of chronic rejection. The clinical consequence is progressive jaundice, liver failure and in some circumstances the development of graft cirrhosis.

## Clinical aspects

*Acute rejection*

In clinical practice, acute rejection occurs around day 5 post-transplantation. However, evidence of a lymphocytic infiltrate of portal tracts can be present histologically as early as day one but such infiltrates may not represent rejection and may in fact be part of graft recognition leading to eventual immune tolerance. Currently it is considered that histological features of rejection without any biological consequences should not be treated and for this reason, routine protocol biopsies are no longer carried out in most centres. In contrast, any clinical suspicion of acute rejection needs to be confirmed histologically to assess histological severity and to exclude other causes (drug toxicity, ischemic hepatitis, infection, recurrent disease) before treatment is started. Acute rejection usually has few clinical symptoms and the diagnosis is based on increases of liver enzymes ALT, AST, GGT and alkaline phosphatase. The increase in INR is usually minimal or mild. Ultrasound doppler of the liver shows an absence of dilation of intrahepatic biliary tree, and patent hepatic artery and portal vein. In severe forms of rejection, the portal flow can be slow due to an increase in vascular resistance through the inflamed liver, and portal oedema may be detected on CT scanning.

Histologically acute rejection is now classified according to the Banff criteria (Table 1), which classify the grade of inflammatory infiltrate in the portal space, around biliary ducts, and in vessel walls. The decision to treat acute rejection is based on the severity of biochemical abnormalities and the Banff criteria. Fine needle biopsies are no longer used for the diagnosis of acute rejection. Minimal and mild acute rejection can be treated with an increase of immunosuppression dosing. Severe acute rejection usually requires boluses of high-dose steroids.

*Hyperacute rejection or humoral rejection*

In ABO incompatible transplants or those done in the presence of a positive cross match, a particular form of rejection with features of hyperacute or humoral rejection can be seen. This event is not immediate and is usually delayed for a few days occurring at any time during the first ten days. Clinically, it can be extremely severe with dramatic increase of AST and ALT and increase in INR associated with acute liver failure and progression to multiple organ failure. The liver is engorged with areas of haemorrhagic necrosis. Histologically there is a marked portal infiltrate with haemorrhagic lobular necrosis and endothelial damage [93]. In

**Table 1**. Histological features of acute rejection according to Banff criteria and calculation of rejection activity index.

| Category | Criteria | Score |
|---|---|---|
| Portal inflammation | Lymphocytic infiltration in few triads | 1 |
| | Mixed infiltrate in most triads | 2 |
| | Marked expansion of all triads by a mixed infiltrate | 3 |
| Bile duct inflammation and damage | Minority of ducts infiltrated by inflammatory cells | 1 |
| | Most of the ducts infiltrated by inflammatory cells with degeneration | 2 |
| | As above to 2, most ducts showed degenerative changes | 3 |
| Venous endothelial inflammation | Subendothelial lymphocytic infiltrate in a minority of portal/hepatic venules | 1 |
| | Subendothelial lymphocytic infiltrate in most of portal/hepatic venules | 2 |
| | As above for 2 with moderate or severe inflammation with perivenular necrosis | 3 |

Total score: some of the components.
Score 1–3 minimal rejection; 4–6 mild rejection; 7–9 severe rejection.

# Review

the 1990s, ABO incompatible liver transplantation was performed for some emergency cases but was associated with a high incidence of not only humoral rejection but also a particular form of chronic graft dysfunction characterized by progressive destructive cholangitis leading to graft failure [51,54]. More recently, ABO incompatible transplantations have been attempted again particularly in living donor transplantation initially in transplantation for children, then for adults. With living donation transplantation it is possible to prepare the recipient using plasmapheresis of immunoabsorption to deplete antibodies and prevent B cell activation and thus reduce the risk of hyperacute and humoral rejection [94].

## Chronic rejection

End-stage chronic rejection is characterized by immune destruction of bile ducts leading to a cholestatic state. The diagnosis is suspected clinically by the exclusion of other causes of cholestasis: biliary obstruction, recurrent cholestatic hepatitis C. Usually chronic rejection is preceded by episodes of acute uncontrolled rejection. However this is not always the case. Histologically chronic rejection is characterized by destruction of interlobular bile ducts (vanishing bile duct syndrome) associated with cholestasis. It is frequently associated with centrilobular inflammation and necrosis and with foam cell lesion within intrahepatic arterial branches. The different types of chronic rejection and histological definitions have been classified according to Banff criteria (Table 2). The prevalence of chronic rejection is around 5–15% according to definitions. Fortunately in liver transplantation, chronic rejection leading to re-transplantation is relatively rare (5%). However chronic rejection is probably underestimated due to the absence of liver biopsies performed routinely on the long-term in most centres and because other long-term graft pathology may represent atypical forms of chronic rejection e.g. graft hepatitis and even graft fibrosis and cirrhosis of unknown cause [95,96]. Centres in which routine liver biopsies are performed have shown that histological signs of chronic rejection can be present and frequently worsen with time [95,96].

## Atypical forms of rejection

### De novo allo- or auto-immune hepatitis on the graft
Clinically it is characterized by an increase in AST and ALT with histological features of autoimmune hepatitis associate in some cases with the emergence of autoantibodies and of elevated immunoglobulin G levels. This hepatitis is frequently sensitive to reintroduction or increase of steroidal use [97–99].

### Idiopathic post-transplant hepatitis (IPTH)
This form of hepatitis of unexplained cause has been described by centres performing routine liver biopsies. In most cases patients also have low level transaminases suggesting that long-term patients should be biopsied if they have persistently abnormal liver function tests. In some cases IPTH can progress to fibrosis and even cirrhosis. The effect of steroids on IPTH is controversial. It is unclear if IPTH is an atypical form of chronic rejection [95,100].

### Long-term graft biopsies
Centres who performed routine long-term liver biopsies have shown the presence of histological lesions in some patients who have persistently normal liver enzymes. In some patients these lesions may evolve over time with the development of histological features such as ductopenia, IPTH, alloimmune hepatitis, venoocclusive disease [101], suggesting the occurrence of subclinical atypical rejection raising the issue of the need for long-term immunosuppression (see later) [95,96,100].

## Tolerance in liver transplantation

### Mechanisms and basic aspects

The liver exhibits a unique immunologic microenvironment and responds differently to rejection and immune-mediated injuries compared to other organs. The two most well-known manifestations of this process are: 1) that antigen presentation within the liver, which is mediated by a variety of cell types such as dendritic cells, Kupffer cells, sinusoidal endothelial cells, and hepatocytes, leads to tolerization of T cells rather than T cell–mediated immunity; and 2) that pattern recognition receptors such as Toll-like receptors (TLRs) expressed by liver cells often respond differently to pathogen associated molecular patterns than what is observed in other tissues (e.g. lipopolysaccharides exerts immunosuppressive effects in the liver, while it behaves as a potent immunostimulatory molecule elsewhere). This environment has evolved to support the key immune surveillance functions exerted by the liver, which is responsible for clearing

**Table 2**. Histological features of early and late chronic liver allograft rejection, adapted from Demetris *et al.* Hepatology 2000; 31: 792–799.

| Structure | Early CR | Late CR |
|---|---|---|
| Small bile ducts | Degenerative changes<br>Involving a majority of ducts<br>Bile duct loss <50% | Degenerative changes<br>Loss in ≥50% of portal tracts |
| Terminal hepatic venules and zone 3 hepatocytes | Inflammation<br>Zone 3 necrosis and inflammation | Focal obliteration<br>Bridging fibrosis |
| Portal tract hepatic arterioles | Loss <25% of portal tracts | Loss >25% of portal tracts |
| Other | Spotty hepatocyte necrosis | Sinusoidal foam cell accumulation<br>Marked cholestasis |
| Large perihilar hepatic artery | Intimal inflammation<br>Focal cell deposit without lumen cell compromise | Luminal narrowed by subintimal foam cells |
| Large perihilar bile ducts | Inflammation damage<br>Focal cell deposition | Mural fibrosis |

CR, chronic rejection.

blood-borne pathogens without eliciting undesirable destructive immune responses against food-derived antigens and intestinal bacterial degradation products delivered by the portal venous flow. In spite of the default bias towards immunoregulation and tolerance, the liver can promote powerful effector immune responses when exposed to live pathogens, large quantities of danger-associated molecular patters, or in the presence of dysfunctional immunoinhibitory pathways (i.e. autoimmunity). The mechanisms ultimately responsible for the switch from tolerance to effector immunity are not entirely understood and are likely the result of a complex interplay between immune and non-immune liver cells [102,103,82] Fig. 3.

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**Key Points 3**

- Immunosuppression weaning can be achieved in 20% of selected transplant patients

- HCV eradication is recommended before attempting immunosuppression weaning in HCV recipients

- The role of anti-HLA DSA in liver graft rejection is still partly unknown in contrast to other organs and requires further studies

- Infusion of *ex-vivo* expanded donor-specific suppressor T cells to induce tolerance is in investigation

- MSC infusions to induce tolerance is in investigation

- Machine organ perfusion may reduce immunogenicity and play a role in immunosuppression management

- Conventional immunosuppressive drugs will probably remain the backbone of future immunosuppression, but an "à la carte" immunosuppression strategy is mandatory

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The liver's unique environment is distinctly apparent in the setting of liver transplantation, in which it is responsible for the spontaneous acceptance of liver allografts observed in many animal models [104,105]. This was first demonstrated in pigs, and subsequently in rats (in which spontaneous tolerance or rejection occur depending on the donor-recipient strain combination) and in mice. Animals spontaneously accepting MHC-mismatched liver allografts develop donor-specific tolerance, which allows them to accept skin grafts from the same donor without the need for therapeutic immunosuppression. Rodent models of spontaneous liver allograft tolerance have been particularly useful in providing mechanistic information. A key aspect of the development of spontaneous liver allograft tolerance is the observation that, shortly after transplantation, recipient lymphocytes infiltrate the transplanted liver and accumulate in the portal and central areas. Liver-infiltrating lymphocytes become activated and induce a mild degree of parenchymal damage with increased serum aminotransferases, but instead of destroying the graft they are gradually cleared from the liver by a phenomenon that likely involves lymphocyte apoptosis and/or T cell degradation within hepatocyte lysosomes (suicidal emperipolesis) [75,106–109]. At least in the mouse model this process is dependent on CD4+CD25+Foxp3+ regulatory T cells, as depletion of this lymphocyte subset using an anti-CD25 antibody before transplantation prevents the tolerance development and results

in alloimmune graft destruction [110]. However, spontaneous liver transplant tolerance in other models cannot be transferred by Tregs in the first few weeks following transplantation implying that Tregs alone cannot always induce tolerance [111].

*Tolerance and weaning of immunosuppression*

The outcome following clinical liver transplantation clearly differs from what is observed in animal models, in that graft rejection rapidly occurs unless patients receive strong pharmacological immunosuppression. As compared with other clinical transplantation settings, however, it is clear that human liver allografts also display unique immunological features. Thus, liver transplantation can be performed across a positive cross match, requires less vigorous immunosuppressive regimens, does not significantly benefit from HLA matching, and is associated with less frequent chronic rejection. While some of these characteristics can be attributed to the unmatched regenerative capacity of liver grafts, liver transplantation is the only setting in which a significant proportion of patients can eventually discontinue maintenance immunosuppression without undergoing rejection, a phenomenon known as spontaneous operational tolerance. The observation that liver transplant recipients can occasionally maintain stable graft function for a long period of time in the absence of immunosuppressive medication was originally reported in the early 1990s in Pittsburgh [112–114]. Following these original reports, several single centres described their experiences with the discontinuation of immunosuppression due to patient non-compliance, in the setting of cancer or severe infections, or performed intentionally under medical supervision [115–122]. Taken together, these observations suggested that approximately 20% of selected liver recipients could safely discontinue their maintenance immunosuppression [123]. More recent data derived from multi-centre immunosuppression withdrawal trials, have contributed to clarify the prevalence and natural history of spontaneous operational tolerance. A US multi-centre paediatric trial enrolled 20 recipients of parental living donor liver grafts at least 4 years after transplantation [124]. Drug withdrawal was successful in 12 patients, who have now been off immunosuppression for more than 6 years. Successful drug withdrawal was defined as 1 year off immunosuppression with normal liver function tests. Protocol liver biopsies, performed at enrollment, and 2 and 4 years after complete drug discontinuation, did not show clinically significant histological changes. The only clinical parameter correlating with successful drug withdrawal was time since transplantation. A European multi-centre adult trial enrolled 102 recipients of cadaveric liver grafts, out of whom 41 reached the primary endpoint, defined as stable biochemical and histological graft status for 1 year after complete drug discontinuation [125]. Liver biopsies were obtained at baseline and 1 and 3 years post-withdrawal. Operationally tolerant patients have now been off drugs for more than 5 years, and reinstitution of immunosuppression has not been required in any case. At enrollment, and as compared with patients who rejected, operationally tolerant recipients had an increased time after transplant, were older and predominantly male. The effect of time was striking: only 13% of patients transplanted for less than 6 years successfully withdrew their immunosuppression, while this occurred in 79% of recipients enrolled in the study more than 11 years after transplantation. In liver recipients who were 6–11 years post-transplant, the
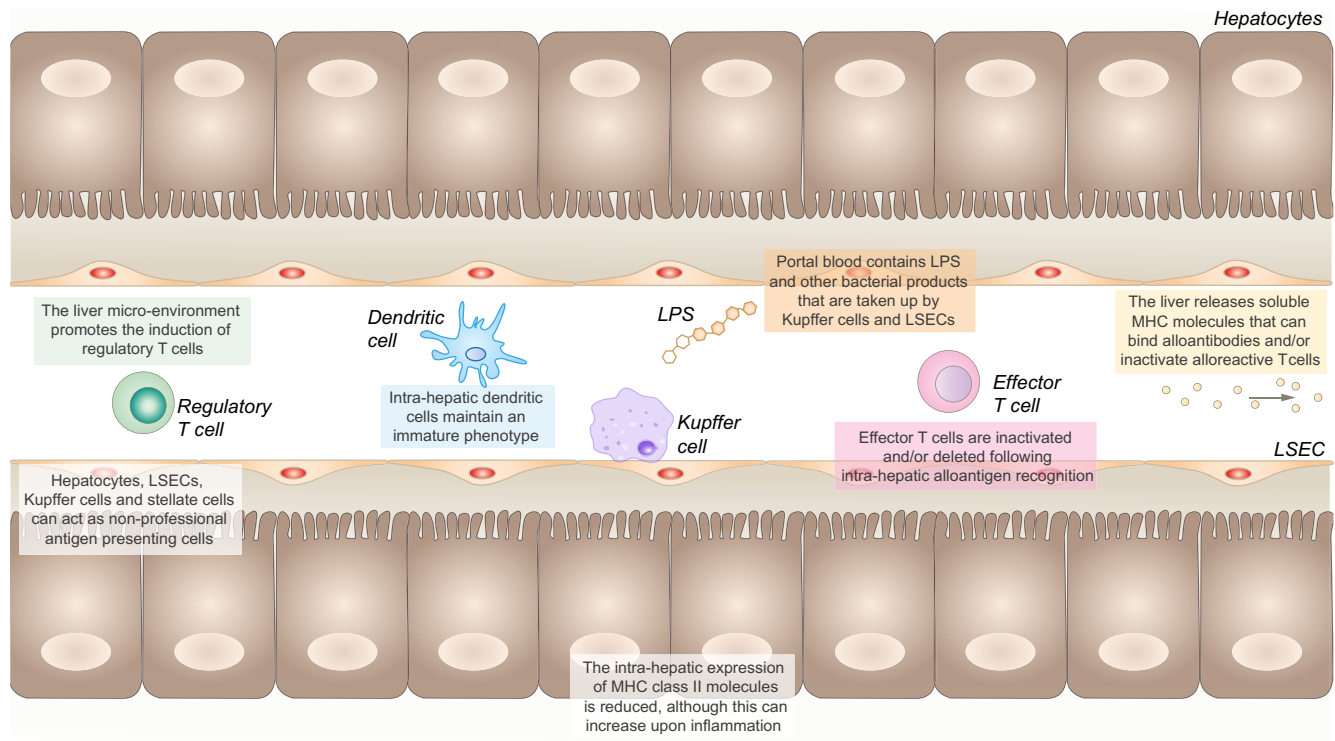
**Fig. 3. Mechanisms of liver allograft tolerance.** The liver contains multiple subsets of dendritic cells, which are professional antigen presenting cells, but that tend to maintain an immature and non-immunogenic phenotype. Kupffer cells, liver sinusoidal endothelial cells (LSEC), stellate cells and hepatocytes can also act as non-professional antigen presenting cells, but tend to deliver suboptimal T cell priming and induce dysfunctional T cells. Continuous exposure to lipopolysaccharides (LPS) by cells expressing Toll-like receptor-4 (TLR4), such as Kupffer cells and LSEC, results in downregulation of co-stimulatory molecules and production of immunosuppressive cytokines. Spontaneous liver allograft tolerance requires the presence of regulatory T cells, whose generation is promoted by the liver microenvironment. The liver releases substantial amounts of MHC molecules (particularly class I), which can exert immunomodulatory properties.

success rate was 38%. In addition to these two trials, preliminary data from an on-going US randomized adult trial reported in 2011, revealed that out of 18 patients in whom immunosuppression withdrawal was attempted during the second year post-transplant, only two succeeded. This further supports the notion that time after transplantation is a key parameter associated with spontaneous operational tolerance. How representative of the overall transplant recipient population are the patients enrolled in these immunosuppression withdrawal trials remains to be fully clarified. Patients with a history of autoimmune liver disease are typically excluded, as they are known to have a higher risk of rejection and/or disease recurrence. In addition, HCV-negative recipients exhibiting clinically significant graft inflammation and/or fibrosis are also considered non-eligible. Among recipients with normal liver function tests, approximately 20–30% of adult recipients would not meet histological eligibility criteria, but this could be 50% or more in paediatric recipients. HCV-infected recipients can also be weaned off immunosuppression [126]. Indeed, in a recent clinical trial in which 25 liver recipients with chronic HCV hepatitis discontinued their immunosuppression, success was associated with immunological parameters previously known to be associated with HCV immune evasion, suggesting that in some HCV-infected recipients the virus facilitates the discontinuation of immunosuppression by modifying the liver's immunological microenvironment [127]. Although feasible, drug withdrawal in HCV-infected recipients is considerably more complex than in HCV-negative patients, as

early detection of rejection in the presence of abnormal liver function tests and/or chronic hepatitis may be difficult. Consequently, eradication of HCV with direct antiviral agents is likely to become the preferred option before considering immunosuppression withdrawal.

A number of clinical trials are currently in progress both in Europe and the US. These will serve to confirm the high prevalence of tolerance among long-term surviving recipients, help clarify the mechanisms relevant to human liver allograft tolerance, and determine whether it is possible to prospectively identify tolerant recipients on immunosuppression by employing diagnostic biomarkers.

## The future of immunosuppression

Immunosuppressive drugs are a double-edged sword, prolonging the functional life of liver allografts but at the same time shortening the life of transplant recipients, as compared to the non-transplanted general population. There is still a need therefore for drugs with less toxicity that will be easier to adhere to. Newer immunosuppressive drugs, particularly biological agents such as monoclonal antibodies and fusion proteins, continue to be tested within industry-sponsored clinical trials. Most of these trials, however, are focused on autoimmune diseases and not in organ transplantation. An additional limitation in the field of liver transplantation is the lack of well-accepted surrogate markers of

hard clinical endpoints such as graft or patient survival, which hampers the performance of reasonably sized clinical trials. In this scenario, the development of novel immunosuppressive strategies is mostly undertaken within academic investigator-driven studies. Immunomodulatory cell therapy with *ex vivo* expanded regulatory T cells is currently being tested to intentionally induce liver transplantation tolerance early after transplantation. Of these trials, the most advanced shows the infusion of recipient *ex vivo* expanded donor-specific suppressor T cells allowing for the successful discontinuation of immunosuppression in 6 out of 10 living donor liver transplant recipients, although aggressive recipient conditioning with cyclophosphamide and splenectomy was required. [128]. The effects of mesenchymal stromal cell (MSC) infusions are also being explored within a variety of clinical trials, which aim at improving preservation injury, preventing ischemic cholangiopathy, or facilitating immunosuppression minimization. While these are very promising pilot studies, key issues related to the clinical use of immunomodulatory cell therapy (e.g. dosing, timing, most appropriate adjunctive immunosuppression) will need to be

clarified before large scale clinical applications can be considered. The use of machine perfusion to preserve liver allografts is likely to influence how immunosuppressive drugs are employed as well, given that in experimental animal models machine perfused liver allografts exhibit substantially reduced immunogenicity [129]. Advances in organ bioengineering and xenotransplantation could also radically modify the management of immunosuppression following transplantation. The use of acellular liver scaffolds repopulated with patient derived cells is far from being a fantasy, and is likely to be tested within pilot clinical trials over the next decade [130]. Another approach is to develop xenochimeric livers by injecting pluripotent human stem cells into animals with specific organogenesis defects, or into xenogeneic embryonic primordium [131]. Looking into the future, conventional immunosuppressive drugs will likely remain the mainstay of the management of liver transplant recipients. The current "one size fits all" strategy however will certainly be superseded. Selected recipients will require no immunosuppression at all as a result of intentionally-induced or spontaneously developed tolerance. In the remaining patients immunosuppression will be managed

**Table 3**. Overview on current and future strategies to optimize immunosuppression in clinical liver transplantation.

| Objective | Potential strategies | Current challenges | Ref. |
|---|---|---|---|
| Development of targeted immunosuppressive regimens | Small molecules, antibodies or fusion proteins directed against molecules involved in T cell activation, or targeting specific immune cell subsets (e.g., anti-CD40, anti-CD28, anti-CD20, anti-IL-12, anti-CD2, bortezomid) | • The relatively small patient population, increased costs of clinical trials, and lack of clinically relevant short-term endpoints prevent systematic investigation of new reagents in liver transplantation | [132] |
| Development of biomarkers to miminize immunosuppression | Anti-HLA antibodies | • Large prospective studies required to confirm pathogenicity and the effects of different immunosuppressive regimens | [56] [57] [58] [59] |
| | Non-invasive transcriptional and/or serological markers of rejection (e.g., CXCL10, CXCL9, miRNA) | • Biomarkers not yet validated in liver transplantation | [133] |
| | Donor specific cellular assays (e.g., ELISpot, flow cytometry functional assays) | • Biomarkers not yet validated in liver transplantation | [133] |
| Identification of spontaneous operational tolerance | Use of biomarkers to select patients for drug withdrawal | • Biomarker validation in independent larger cohorts of patients required <br> • Only selected recipients long time after transplant likely to benefit | [127] [134] |
| Induction of tolerance | Adoptive transfer of *ex vivo* expanded regulatory T cells | • Very high cost <br> • Requires very complex infrastructure particularly if donor-specific cells are being generated | [128] [135] [136] |
| | Adoptive transfer of mesenchymal stromal cells | • Heterogenous results in experimental animal models <br> • Not investigated for this indication in clinical organ transplantation | [137] [138] |
| | Induction of donor-type hematopoietic chimerism | • Requires aggressive recipient conditioning <br> • Currently unfeasible outside of living donor transplantation | [139] |
| | Expansion of endogenous regulatory T cells (e.g., low-dose IL-2 therapy) | • Not tested in organ transplantation | [140] [141] |
| Modulation of the liver allograft immunogenicity | Use of *ex vivo* machine perfusion of liver allografts to modulate inflammatory responses | • Effects beyond the early post-transplant period still not investigated | [129] |
| Generation of bioengineered liver grafts | Use of acellular liver scaffolds repopulated with patient derived cells. | • Experimental strategies not yet investigated in relevant pre-clinical models | [130] [131] |
| | Development of xenochimeric liver grafts | | [142] |

according to the quality of the transplanted organ, recipient co-morbidity profile, underlying graft inflammatory status, and degree of cellular and/or humoral sensitization (Table 3).

## Conclusion

Immunosuppression took a great part in the dramatic improvement in the results of liver transplantation. The future should concentrate on the reduction of side effects due to immunosuppressive drugs in the aim to improve long-term quality of life with preservation of the long-term viability of the liver graft. Tolerance is an achievable goal in a minority of patients. Processes to increase tolerance of the liver allograft have to be developed.

## Conflict of interest

DS: Consultancy for Astellas, BMS, Gilead, Janssen-Cilag, LFB, MSD, Novartis, Roche, Biotest. DHA and AS-F: nothing to declare.

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