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REVIEW

The CD8 T-cell response during tolerance induction in liver transplantation

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Both experimental and clinical studies have shown that the liver possesses unique tolerogenic properties. Liver allografts can be spontaneously accepted across complete major histocompatibility mismatch in some animal models. In addition, some liver transplant patients can be successfully withdrawn from immunosuppressive medications, developing 'operational tolerance'. Multiple mechanisms have been shown to be involved in inducing and maintaining alloimmune tolerance associated with liver transplantation. Here, we focus on CD8 T-cell tolerance in this setting. We first discuss how alloreactive cytotoxic T-cell responses are generated against allografts, before reviewing how the liver parenchyma, donor passenger leucocytes and the host immune system function together to attenuate alloreactive CD8 T-cell responses to promote the long-term survival of liver transplants.

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INTRODUCTION

Solid organ transplantation has become a common and key practice in modern medicine. Transplantation is, however, a very complex procedure, and generally the last available solution for patients with a damaged or defective organ. Subsequent lifelong immunosuppressive therapy is essential to prevent rejection of the allograft by the host immune system. However, prolonged treatment with immunosuppressive medications has significant side effects, including drug-related toxicity to other organs, increased rates of malignancies and increased risk of infection by a variety of pathogens.[1] Because of these undesirable side effects, achieving donor-specific immune tolerance in transplant recipients without the requirement for long-term administration of immunosuppressive drugs is the ultimate goal of modern transplantation.

Long-term tolerance in transplant recipients is difficult to achieve experimentally, but occurs spontaneously across major histocompatibility (MHC) barriers in many experimental models of liver transplantation, and has been documented clinically in a minority of liver transplant recipients. The intriguing observation that in the absence of immunosuppression liver transplants survived better than kidney or skin allografts was first made by Calne *et al.*[2] in outbred pigs. Acceptance of allogeneic liver transplants by recipients who were completely MHC mismatched with the donor was later confirmed in inbred rat and mouse strains.[3,4] More importantly, spontaneous acceptance of mismatched liver allografts in these models was associated with a state of immune tolerance that prevented or even stopped ongoing rejection of other solid organ grafts from the same donor.[4,5] As an immunosuppression mechanism, it has been noted that liver grafts are more potent than cyclosporin A, one of the most powerful nonspecific immunosuppressant agents.[6]

Similar observations have been made in the clinic for human liver transplantation. MHC matching does not influence human liver allograft survival.[7,8] In addition, unlike patients receiving other organs, who require lifelong immunosuppressive treatment, some liver transplant recipients can be weaned off immunosuppression without affecting the survival of the organ, nor altering its functions.[9] Immunosuppression can be withdrawn in up to 25% of carefully selected liver transplant recipients, with the potential for successful withdrawal guided by the preselection of patients via a range of clinical characteristics.[10] In addition, consistent with data from animal models, several clinical studies have reported reduced rejection rates of kidney[11–13] or heart allografts[14] in recipients cotransplanted with the liver from the same donor, implying a beneficial tolerogenic role of the hepatic allograft.

Elucidating the mechanisms by which the liver induces donor-specific tolerance is critical, as this knowledge could facilitate future strategies to induce acceptance of solid organ transplants in the clinic. Although they remain elusive, these mechanisms have been the subject of intense research in experimental rodent models. This review will focus on the fate of CD8 T cells during the early-phase post-liver transplantation. After canvassing the pathways by which cytotoxic CD8 T cells (CTLs) normally mediate allograft rejection, we will discuss why the same cells do not reject liver allografts and review the main mechanisms proposed to induce tolerance in the CD8 T-cell compartment in this setting.

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THE LIFE OF A CD8 T-CELL RESPONDING TO ITS COGNATE ANTIGEN

The main role of CD8 T cells is to control infections and to prevent tumour development. Although they have a beneficial role in immunity, CD8 T cells have a detrimental role in transplantation, as they are the main mediators of allograft rejection. CD8 T cells express unique T-cell receptors (TCRs) that recognise cognate peptide/MHC class I (MHC-I) complexes presented at the surface of antigen-presenting cells (APCs). TCR triggering initiates T-cell activation, proliferation and differentiation into a functional cytotoxic T cell able to secrete various proinflammatory cytokines, including interferon-γ (IFN-γ) and tumour necrosis factor-α, and lyse cells expressing the peptide/MHC-I complexes recognised by their TCRs.

CD8 T cells are commonly defined by their activation state: naïve (unexposed to their cognate antigen), effector (activated) and memory (returned to a resting state after activation). These three types of T cells express different homing receptors that dictate specific trafficking.[15] Naïve T cells recirculate through secondary lymphoid organs (SLOs) via blood and lymph. Within SLOs, they scan the surface of professional APCs, such as dendritic cells (DCs), for short periods of time.[16] When the TCR identifies a specific cognate peptide presented on MHC-I, T cells and DCs will form a stable interaction that initiates T-cell activation.[17] In addition to the TCR signal, optimal T-cell activation requires additional triggering of other signalling pathways. These include interactions with several T-cell costimulatory molecules (e.g., CD28 with CD80/CD86),[18] adhesion molecules (e.g., intercellular adhesion molecule-1 with lymphocyte function-associated antigen-1)[18] and proinflammatory cytokine receptors.[19] Following activation, effector CD8 T cells exit SLOs and enter the circulation before migrating into tissues to clear antigen-expressing cells. Following antigen clearance, most effector CTLs die, but a small population of memory T cells is maintained.

It is critical to dissect the respective contributions of naïve, effector and memory T cells in a transplant recipient, as they are all capable of mediating allograft rejection.

ACTIVATION OF NAÏVE ALLOREACTIVE T CELLS DURING TRANSPLANTATION

Following allograft transplantation, both donor parenchymal cells and donor passenger leucocytes (PLs) contained in the graft form a large source of MHC-mismatched cells and alloantigens potentially recognised as foreign by naïve recipient CD8 T cells. As naïve alloreactive T cells are activated within SLOs after pathogen infection, it is not surprising that this compartment is also the preferential site where naïve T cells are activated during allograft transplantation. In mice lacking SLOs, cardiac allografts survived indefinitely, even following transfer of naïve wild-type T cells that normally mediate acute graft rejection in the presence of SLOs.[20] However, transfer of wild-type lymphocytes pre-activated by donor splenocytes into SLO-deficient mice led to cardiac allograft rejection.[20] The key role of SLOs in acute allograft rejection mediated by naïve T cells has been confirmed for skin, heart and intestinal allograft models in other studies,[21,22]

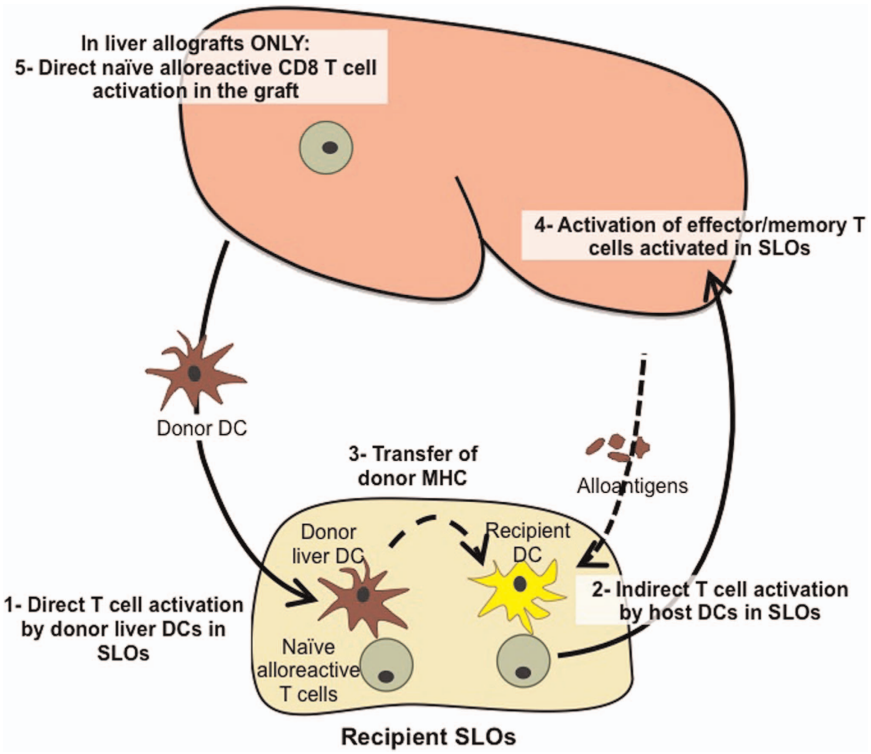

Figure 1 Pathways of T-cell activation after allograft transplantation. After allograft transplantation, alloreactive CD8 T cells can be activated by several presentation pathways. 1. Donor DCs and other PLs migrate very quickly out of the graft into recipient SLOs and can directly activate CD8 T cells (direct pathway of antigen presentation). Many of these T cells are aberrantly activated and undergo early apoptosis, but some might escape deletion and recirculate to the graft. 2. Recipient DCs in SLOs can also capture alloantigens from the graft and promote functional activation of CD8 T cells restricted by recipient rather than donor MHC (indirect presentation pathway). 3. Donor MHC can be transferred to recipient APCs and activate alloreactive CD8 T cells. 4. Pre-existing memory T cells and effector T cells activated in SLOs by both the direct and indirect presentation pathways recirculate to the allograft where they can be silenced or contribute to rejection. 5. Naïve alloreactive CD8 T cells can be recruited and directly activated in the allograft. This pathway would only occur for liver allografts as the liver is the only organ that allows activation of naïve CD8 T cells.

although a study in a mouse model of lung transplantation suggests that activation of alloreactive T cells by lung DCs may occur within the allograft.[23] Overall, the majority of these studies are consistent with a model in which naïve T cells require activation within SLOs to cause rejection of most solid organ allografts.

Recipient CD8 T-cell activation in SLOs is more complex in transplantation than during a response to a pathogen. Activation can result from either presentation via PLs that have recirculated from the graft (direct presentation pathway) or in response to presentation of alloantigens captured and presented by recipient DCs (indirect presentation) (Figure 1). In rodent models, donor passenger DCs have been shown to migrate into the SLOs of heart transplants recipients[24–26] where they interacted with T cells,[25] suggesting that they have a key role in activating recipient alloreactive T cells. The importance of direct presentation by donor DCs in rejection has been demonstrated by studies in which the same allogeneic kidneys were transplanted successively into secondary recipients syngeneic with the first.[27,28] After the first transplantation, most donor PLs within the transplanted kidneys were replaced by recipient leucocytes, promoting acceptance of retransplanted renal allografts by secondary recipients.[27] In contrast, injection of donor DCs into second recipients resulted in rapid rejection of the retransplanted grafts,[28] suggesting that donor DCs had a critical role in initiating T-cell activation that mediates organ rejection. Consistent with a key role of donor DCs in allograft rejection, heart allografts isolated from mice lacking DCs transplanted into wild-type allogeneic mice induced a less marked alloreactive T-cell response than in recipients of organs containing donor DCs, and promoted extended allograft acceptance.[26] This direct allorecognition pathway is thought to have an important role in killing donor parenchyma cells during acute graft rejection.[29]

In addition to direct activation by donor DCs, some specific DC subsets are also capable of CD8 T-cell activation via the presentation of exogenous antigens, a process known as cross-presentation.[30] Cross-presentation of alloantigens promoting activation of naïve alloreactive CD8 T cells has been shown to occur during transplantation.[31] T cells activated by the indirect pathway are usually less abundant compared with those directly stimulated by the direct pathway,[31,32] and have been shown to release proinflammatory cytokines that contribute to chronic graft rejection.[33]

More recently, a third mechanism of alloreactive T-cell activation has been identified, involving transfer of intact peptide/MHC-I complexes from donor cells onto host DCs (Figure 1). Although DCs have been shown to acquire intact peptide/MHC-I complexes expressed by other cells and promote naïve CD8 T-cell activation,[34,35] it was unclear whether this process occurred during transplantation. When a large number of donor cells carrying cognate peptide/MHC-I were transferred into MHC-I-mismatched recipient mice, host DCs could induce proliferation and effector differentiation of antigen-specific CD8 T cells, suggesting they acquired peptide/MHC-I complexes from donor cells.[34,35] To demonstrate that transfer of donor MHC molecules to DCs facilitated allorejection in mouse models, a recent study used a heart transplant model in which the cardiac allograft was acutely rejected by adoptively transferred transgenic CD8 T cells specific for donor MHC-I.[36] Depletion of recipient DCs resulted in a reduced allo-MHC-I-specific T-cell response and significantly delayed allograft rejection. Conversely, splenic DCs isolated from transplant recipients and transferred into secondary untreated recipients induced a CD8 T-cell response against donor MHC-I molecules. This study suggests that recipient DCs acquired intact MHC-I from donor cells after heart transplantation, and that this process contributed to allograft rejection.[36]

ACTIVATION OF ALLOREACTIVE EFFECTOR AND MEMORY T CELLS FOLLOWING TRANSPLANTATION

In addition to naïve T cells, recipient effector and memory T cells are important contributors to graft rejection. Alloreactive effector and memory T cells pre-exist in the recipient, generally following prior responses to pathogens. Alloreactivity of these cells in the absence of previous alloantigen exposure results from cross-reactive responses to peptide/donor MHC-I complexes. These unrelated donor-derived complexes may be structurally similar to cognate peptide/recipient MHC-I complexes normally recognised by these TCRs.[37] A single TCR can also adapt to different peptide/MHC-I complexes by slightly altering its conformation during interaction.[38] This crossreactivity is beneficial to the host, as it helps to control infections caused by unrelated pathogens, a phenomenon known as heterologous immunity.[39] However, in transplantation TCR crossreactivity is a major trigger of graft rejection.

Memory T cells cross-reactive to alloantigen have been identified in animal models.[40–42] Importantly, CD8 T cells specific for Epstein–Barr virus,[43] herpes simplex virus 2,[44] cytomegalovirus[45] and varicella-zoster virus[46] that crossreact against allo-MHC-I complexes (i.e., human leucocyte antigens) have also been identified in humans and potentially mediate transplant rejection.

Alloreactive effector and memory T cells in recipients are not always generated by crossreactivity. In some settings, including prior blood transfusion, pregnancy or organ transplantation, they are generated via previous exposure to the same alloantigen. In addition, some T cells may also carry two distinct TCRs and recognise more than one antigen. Such dual TCR T cells have been reported in both mice[47] and humans.[48] If memory T cells recognising a pathogen-derived determinant via one of their two TCRs express a second alloresponsive TCR,[49] they could contribute to allograft rejection. Increased numbers of dual-specific T cells have been recently linked to the development of acute graft-versus-host disease in patients who have undergone allogeneic haematopoietic stem cell transplantation.[50]

Owing to their lower activation threshold, the requirements for activation of alloreactive effector and memory T cells are not as stringent as for naïve T cells. Their activation is not restricted to interaction with DCs, but can also result following encounter with donor cells in the allograft (Figure 1). Memory cells are more readily able to proliferate and become polyfunctional after antigen encounter than naïve T cells.[51] They can also rapidly kill target cells without prolonged activation.[52] As a result, memory T cells are a significant mediator of allograft rejection.

A classical early study has shown that rats transplanted with a primary skin allograft displayed enhanced rejection of secondary skin grafts,[53] and transfer of memory CD8 T cells isolated from presensitized mice has been shown to lead to early destruction of donor skin grafts.[54] Donor-specific memory CD8 T cells have been demonstrated to infiltrate allografts faster than naïve cells,[55] and to mediate cardiac allograft rejection even in mice without SLOs, indicating that they do not require reactivation within SLOs to become functional.[56] More importantly, many studies have reported that alloreactive memory T cells are resistant to various immunoregulatory strategies, including costimulation blockade and regulatory T-cell (T_{reg}) induction, two treatments known to promote allograft acceptance by inducing tolerance in naïve alloreactive T cells.[54,57–59] In a preclinical nonhuman primate kidney transplant model, selective removal of memory T cells resulted in a better response to costimulation blockade and prevented rejection of renal allografts.[60] Pre-existing memory T cells have also been shown to correlate with the risk of kidney transplant rejection in humans.[61,62]

Role of other phenomena influencing the survival of organ allografts

Several factors can contribute to the survival of a transplanted allograft. As a detailed discussion of these determinants is beyond the scope of this review and has been covered elsewhere,[63] they will be briefly explained below:

Ischaemia–reperfusion injury increasing the risk of acute and chronic rejection. Cold and warm ischaemia, organ retrieval, handling and the surgery itself causes damage to the graft. This tissue damage affects both syngeneic and allogeneic grafts and is known as ischaemia–reperfusion injury (IRI). IRI is caused by the release of proinflammatory cytokines and chemokines by damaged cells. These molecules and cell debris activate various toll-like receptor signalling pathways and promote activation of various innate immune cells (DCs and macrophages, monocytes and neutrophils).[64] Activated innate immune cells release cytokines and chemokines that augment alloreactive adaptive immune responses and exacerbate graft rejection.[65] Macrophages and neutrophils further damage the graft by releasing large amounts of reactive oxygen species. IRI has a direct effect on allograft survival as it decreases the regenerative capacity of the organ and influences the adaptive immune response.[63]

Preformed antibodies causing hyperacute rejection. Hyperacute allorejection occurs within hours after organ reperfusion and is caused by pre-existing antibodies directed against incompatible blood group antigens and/or allo-MHC.[66] Antibodies directly kill cells in the graft by binding complement proteins or by activating phagocytes and natural killer cells. However, hyperacute rejection mediated by preformed antibodies is rarely observed in liver transplantation.

Role of newly formed antibodies in acute rejection. Rejection by newly generated antibodies against the graft is increasingly being recognised as having a role in liver transplant rejection, particularly in patients who are refractory to immunosuppressive therapies targeting T cells rather than B cells.[67]

CD8 T-CELL TOLERANCE IN THE LIVER: LESSONS FROM NON-TRANSPLANT STUDIES

Although induction of an alloreactive T-cell response results in rejection of most allografts, this response is dampened during liver transplantation, thus promoting liver allograft acceptance. The unique ability of the liver to induce donor-specific tolerance is not yet completely understood, but seems to require both liver parenchyma and liver PLs. To understand the mechanisms that promote acceptance of liver allograft acceptance, it is essential to first understand some of the unique properties of this organ in a non-transplant setting.

Studies investigating how CD8 T-cell respond to transgenic allo-MHC-I molecules or transgenically expressed antigens presented by various liver-resident cell types, including hepatocytes, liver sinusoidal epithelial cells (LSECs), Kupffer cells (KCs) and hepatic stellate cells (HSCs), have yielded invaluable information about how the liver could induce T-cell tolerance. These studies have revealed that unlike other non-lymphoid organs, the liver can support primary T-cell activation.[68,69] This unique property is facilitated by the

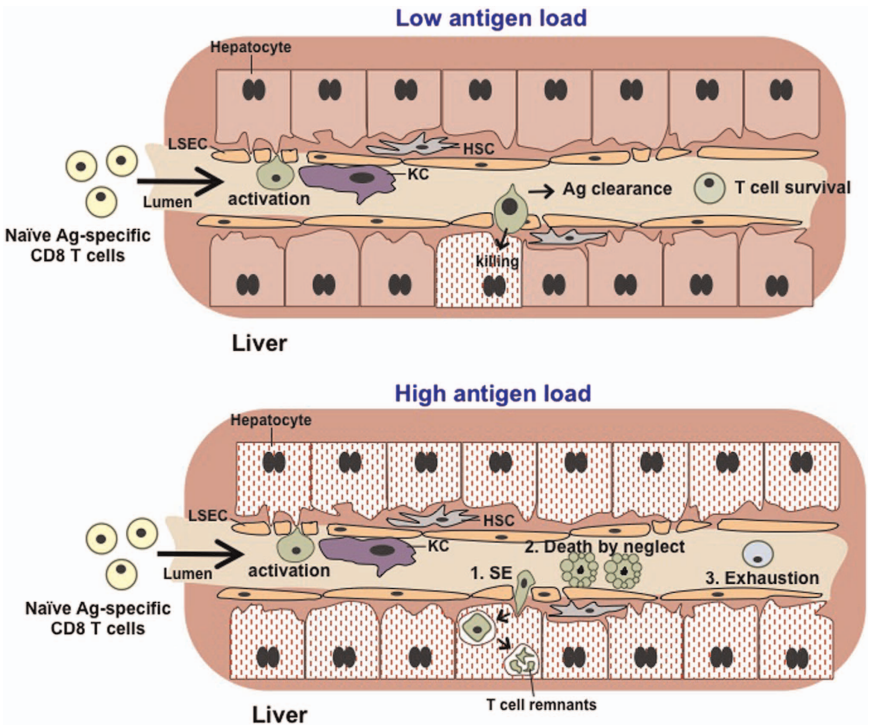

Figure 2 Mechanisms of CD8 T-cell tolerance in a non-transplant setting. When naïve alloreactive CD8 T cells recirculate to the liver, they interact with various liver-resident cells (hepatocytes, LSECs, HSCs and KCs) within the hepatic sinusoids. If CD8 T cells recognise their cognate peptide/MHC-I complexes, they are activated independently of lymphoid tissues. Recent experiments using hepatocytes suggest that the outcome of T-cell activation depends on the intrahepatic antigen load and/or number of antigen-expressing cells: when the antigen load is low and/or expression transient (top diagram), high-affinity CD8 T cells become CTLs, kill all antigen-expressing liver cells and survive in the long term. When the antigen load is high (bottom diagram), most CD8 T cells are tolerized. This tolerance is contributed by several mechanisms: 1. Invasion of T cells into hepatocytes and rapid non-apoptotic degradation in lysosomes (a process termed suicidal emperipolesis or SE) within the first few hours of activation. 2. Early apoptosis (death by neglect) of T cells surviving SE due to primary activation in the absence of costimulation. 3. Exhausted function of T cells surviving these early deletional processes.

permeable microarchitecture of the liver and the slow blood flow in the sinusoids, favouring stable interactions between circulating T cells and resident hepatic cells. Our recent findings[70] have shown that the intrahepatic antigen load and/or persistence have a key role in the fate of CD8 T cells activated in the liver. While persisting high levels of intrahepatic antigen expression (a situation akin to that associated with organ transplantation) are generally associated with tolerance (Figure 2, bottom panel), low levels of intrahepatic antigen expression (antigen expressed by low numbers of hepatocytes,[70,71] or transient intrahepatic antigen presentation following administration of exogenous peptide[72]) promote functional responses, antigen clearance and T-cell survival (Figure 2, top panel).

Several mechanisms have been shown to silence naïve CD8 T cells activated by hepatocytes or other liver APCs in the presence of a high antigen load. These mechanisms are listed below and illustrated in the bottom panel of Figure 2.

Death by neglect due to primary activation without costimulatory molecules

As liver-resident APCs do not normally express costimulatory molecules, naïve alloreactive T cells activated within the liver enter an incomplete differentiation programme that results in tolerance. Tolerance is also potentially contributed to by the presence of immunoregulatory cytokines known to be expressed in this organ.[73]

Using transgenic mouse models in which allo-MHC-I was restricted to hepatocytes or to hepatocytes and bone marrow-derived cells, we performed a series of studies to clarify how the type of T-cell activation dictated the fate of intrahepatic alloreactive T cells.[69,74,75] The alloreactive T-cell population expanded, but rapidly contracted when antigen presentation was strictly restricted to hepatocytes,[69] while continuing to increase when antigen presentation was mediated by both hepatocytes and bone marrow-derived cells. As intrahepatic T-cell recruitment and proliferation were similar in both settings,[69] this result suggests that CD8 T cells activated by hepatocytes were not as robustly activated as those undergoing activation in the lymph nodes, exhibiting a survival defect potentially because of the lack of costimulatory signals during primary activation by hepatocytes.[74] Consistent with the absence of sufficient survival signals, hepatocyte-activated T cells expressed higher level of the proapoptotic molecule B-cell lymphoma 2-interacting mediator (Bim) and activated caspase-3,[74] and underwent premature cell death or death by neglect (Figure 2). Bim appears to be a key mediator of T-cell apoptosis as CD8 T cells lacking Bim survived longer and accumulated within the liver.[74,75] Alloreactive T cells activated by liver-resident bone marrow-derived phagocytic cells, presumably KCs, also underwent Bim-dependent apoptosis.[76]

Non-apoptotic T-cell clearance in hepatocytes

In addition to their early apoptosis, we have recently demonstrated that naïve allogeneic CD8 T cells activated within the liver rapidly invaded hepatocytes, and were subsequently degraded inside lysosomal compartments.[77] This non-apoptotic process, coined 'suicidal emperipolesis' (SE), accounted for the removal of at least 75% of alloantigen-specific CD8 T cells in the liver within the first 24 h of antigen encounter,[77] suggesting that it had a key role in early control of alloreactive CD8 T cells. CD8 T cells need to be activated and metabolically functional to enter hepatocytes,[77] suggesting that T-cell entry is not mediated by passive phagocytosis, but is instead the result of an active process. This process is independent of macropinocytosis or endocytosis;[78] the underlying molecular pathways are yet to be identified. Emperipolesis has been associated with several disease conditions, notably as a histological feature of Rosai-Dorfmann disease, Creutzfeld-Jakob, Crohn's disease and a host of other inflammatory diseases, including autoimmune hepatitis and chronic hepatitis C.[78] Although associated with the severity of liver damage in the later conditions, the physiological significance of emperipolesis has never really been understood. Association of emperipolesis with autoreactive T-cell clearance suggests a critical role in regulating immune tolerance, and partially explains why cell-in-cell structures have been observed in human liver pathology.

PD-1-mediated CD8 T-cell exhaustion

Although most CD8 T cells activated in the liver are deleted by apoptosis and SE, some CD8 T cells survive this process, as they are detected in the liver several weeks after their activation. Although located in the tissue, these cells are unable to degranulate or secrete IFN-γ when restimulated *in vitro* with cognate antigen. This silenced state is known as functional exhaustion[70] and is the result of a specific programme of CD8 T-cell differentiation that promotes their functional silencing. Exhaustion is generally associated with the expression of inhibitory molecules, such as programmed death-1 (PD-1) and T-cell immunoglobulin and mucin-3 (Tim-3).

PD-1 is expressed on the surface of recently activated T cells.[79] By interacting with its ligands, PD-1 ligand 1 (PD-L1) and ligand 2 (PD-L2), expressed on cells presenting cognate antigen, PD-1 suppresses T-cell activation and proliferation and dampens the function of effector T cells.[80] PD-1 is also highly expressed by CD8 T cells that become unresponsive or 'exhausted' after chronic antigen stimulation,[80] and is thus commonly used to identify exhausted CD8 T cells. Restoration of exhausted T cells by blocking antibodies that inhibit PD-1/PD-L1 interaction was first reported in mice persistently infected with lymphocytic choriomeningitis virus.[81] This strategy has been successfully translated to the clinic as cancer immunotherapy.[82]

Several resident liver cell populations express PD-1 ligands. PD-L1 has been identified on hepatocytes,[83] Kupffer cells, LSECs[84] and HSCs.[85,86] Although it is expressed at low levels in the steady state, PD-L1 expression is upregulated during inflammation, hepatotropic viral infection or after interaction with antigen-specific CD8 T cells.[83,85–89] PD-1/PD-L1 interactions between CD8 T cells and LSECs promotes poor CD8 T-cell activation,[89] whereas interactions between T cells and PD-1-expressing HSCs leads to early T-cell apoptosis.[83,85,86] PD-L1 constitutively expressed by KCs has been shown to suppress T-cell proliferation.[84]

Transgenic CD8 T cells detected in the liver several weeks after intrahepatic activation express high levels of PD-1 and Tim-3,[70] a result consistent with their functional exhaustion. These results suggest that although most CD8 T cells activated in the liver are rapidly cleared by SE and apoptosis, T cells continuously stimulated by a high intrahepatic antigen load will eventually become exhausted.

Importance of these findings for liver transplantation

Information obtained from studies performed in intact animals are important, as they help us to predict that following liver transplantation, alloreactive naïve CD8 T cells would not only be activated in SLOs by PLs (direct presentation pathway) but also via cross-presentation of alloantigen by recipient DCs (indirect presentation pathway), as in most solid organ allografts. They would also be activated by liver cells via the direct presentation pathway within the liver graft itself (Figure 2). Using a mouse model of liver transplantation, we recently confirmed parallel activation of adoptively transferred graft-reactive naïve CD8 T cells via the direct presentation pathway in both liver and lymph nodes.[90] T-cell activation in both compartments

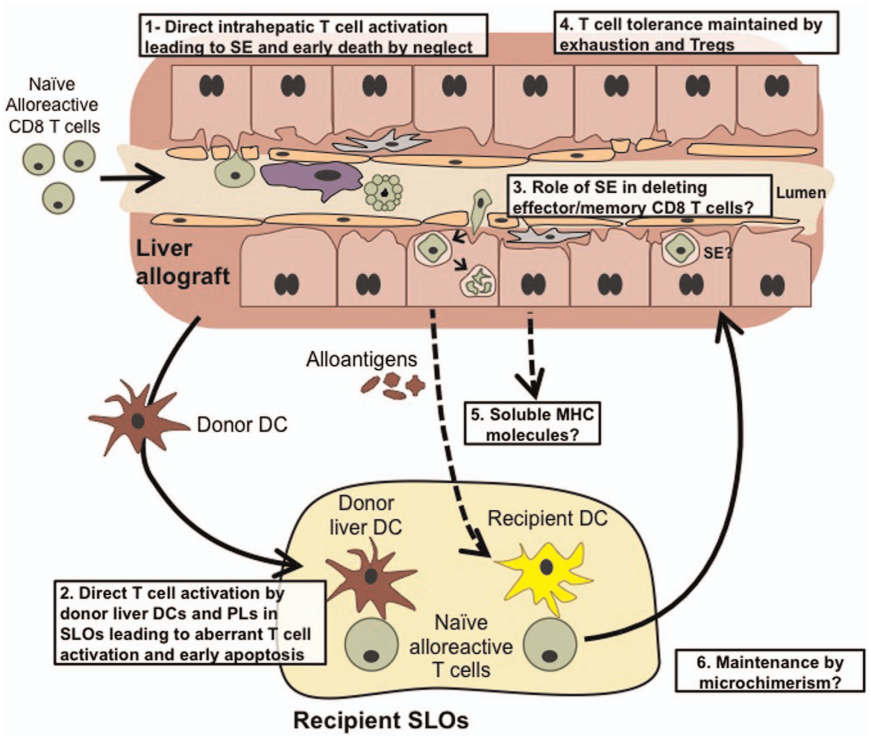

Figure 3 Hypothetical mechanisms of tolerance after liver transplantation. Several mechanisms have been reported to contribute to the induction and maintenance of tolerance after liver transplantation. 1. Naïve alloreactive CD8 T cell recruited to the liver allograft and activated in the setting of a high alloantigen load undergo rapid cell death mediated by both SE and death by neglect. 2. Most naïve alloreactive CD8 T cells activated by PLs in SLOs via the direct antigen presentation pathway undergo early apoptosis. 3. Alloreactive CD8 T cells recirculating to the liver from SLOs might be cleared by SE. 4. Effector T cells or pre-existing memory T cells might be subjected to chronic alloantigen stimulation leading to T-cell exhaustion or negative regulation by host Treg. Finally, the release of high amounts of soluble MHC molecules by the liver allograft (5) or the development of microchimerism (6) have been proposed to be critical to the silencing of alloreactive CD8 T cells and the maintenance of tolerance after liver transplantation.

occurred as soon as 5 h following transplantation.[90] This activation pathway within the allograft is likely to be unique to liver allografts, as most other solid organs have not been described to support activation of naïve CD8 T cells.

The fate of alloreactive CD8 T cells activated in the liver graft has not yet been elucidated. However, as this activation occurs in the presence of a high alloantigen load, we predict that T cells would be silenced by similar mechanisms as those described in an intact animal. The fate of liver-activated CD8 T cells and the relative contributions to tolerance of direct activation in the liver and direct and indirect activation in SLOs will be discussed in the next section of this review.

TOLERANCE DURING LIVER TRANSPLANTATION

In the clinical setting, hyperacute rejection mediated by preformed antibodies is seldom seen in liver transplantation, even when donor and recipient are positively cross-matched.[63] Similary, as operative and anaesthetic techniques have improved, IRI in liver transplantation has generally become less severe. However, as liver transplant waiting lists grow this progress is being counterbalanced by a tendency to use increasingly marginal donors. Thus, although innate immune cells would be activated by IRI after transplantation, the spontaneous acceptance of liver allografts in animal experimental models suggests that these mechanisms are opposed and dominated by the tolerogenic mechanisms operating in this organ. The section below will mostly focus on very early events that ameliorate the development of acute rejection, which is likely predominately mediated by the direct presentation pathway.

Several mechanisms have been proposed to explain why liver transplants are spontaneously accepted and induce donor-specific tolerance. The literature suggests that both PLs and liver tissue contribute to this process via multiple pathways. These include the large size of the liver tissue and/or higher number of donor PLs, the different nature of these PLs (in particular hepatic DCs), the unique structure of the hepatic tissue that allows direct activation of alloreactive naïve CD8 T cells within the liver graft itself, long-term donor chimerism, T_{regs} and soluble allo-MHC molecules secreted by the liver allograft. The main mechanisms have been reviewed in detail elsewhere[63] and are summarised in Figure 3. These mechanisms can be grouped in three main categories: early mechanisms of tolerance induction in SLOs, early mechanisms of tolerance induction in the liver graft, and regulatory mechanisms that maintain tolerance in the long term. In this review, we will list the more significant mechanisms in these three categories. The role of microchimerism and soluble MHC molecules in liver transplantation tolerance remains controversial. As these mechanisms have been extensively reviewed elsewhere, they will be mentioned very briefly in this review.[63]

Early mechanisms of tolerance induction in SLOs

Role of donor PLs. Following the early observation made by Starzl *et al.*[91] that acceptance of human liver transplants is strongly associated with the presence of a low frequency of donor cell chimerism in recipient tissues, it was hypothesised that migration of PLs into SLOs and their long-term persistence (also known as microchimerism) was the key mechanism responsible for the spontaneous acceptance of liver grafts. Although the role of this phenomenon remains uncertain,

many subsequent studies have focused on identifying the nature and the role of donor PLs in liver transplantation. A significant number of donor PLs are detected in recipient SLOs after liver transplantation in rat and mouse models.[4,92] Donor PLs were also detected in the blood of human liver transplant recipients.[93] Using a mouse model of liver transplantation, we have recently shown that PLs interacted with alloreactive CD8 T cells in SLOs as soon as 24 h after liver transplantation.[90] Importantly, PLs are an early contributor to spontaneous tolerance induction.[94] Depletion of PLs by irradiation of donor rat livers before transplantation promoted acute graft rejection.[94,95] Conversely, if liver allografts from irradiated donors were firstly transplanted into primary recipients syngeneic to the donors to allow repopulation of PLs, such grafts were accepted in the secondary allogeneic recipients.[94] Similar tolerogenic effects could be achieved by transferring liver or splenic leucocytes into recipients immediately after transplantation of irradiated liver grafts.[96] Moreover, liver PLs, as well as parenchymal cells, both mediate liver allograft-induced tolerance to subsequent donor strain allogeneic skin grafts.[97] The mechanisms of PL induction of liver graft tolerance in SLOs have been investigated. Increased interleukin-2 (IL-2) and IFN-γ messenger RNA levels early after transplantation were detected in SLOs of recipient rats in which liver allografts were accepted compared with those in which rejection resulted.[92] In addition, many apoptotic T cells were found in both SLOs and liver grafts in tolerant rats.[98] These results have been hypothesised to suggest a role for 'death by neglect' as a mechanism tolerizing T-cell response induced by PLs within SLOs.[99]

Although donor splenic leucocytes prevented the rejection of rat kidney allografts,[100] neither donor leucocytes from liver nor spleen alone could prevent rejection of allogeneic rat cardiac transplants.[96] The survival of heart allografts was, however, prolonged when recipients were treated with donor splenocytes in combination with immunosuppressive therapy.[101] These studies suggest that although transfer of donor leucocytes dampens the alloresponse and helps to induce acceptance of some organ allografts such as the liver or kidney, additional mechanisms are required to promote long-term survival of most organ allografts.

One possible hypothesis to explain the role of PLs in promoting acceptance of liver allografts is that the quantity or quality of hepatic PLs is different to those of other organs. Liver PLs include large numbers of B, T, NKT and NK cells and various myeloid subsets including DCs. Many rapidly leave the liver after transplantation and enter SLOs with varying kinetics.[90] The role of the different PLs in liver transplant tolerance has been reviewed elsewhere.[63] In this review, we will limit our description of the main findings to liver DCs, as these are the main mediators of T-cell activation, and for this reason, we have received the most attention in the literature.

Role of DCs. Some studies have suggested that donor hepatic DCs have an important role in inducing CD8 T-cell tolerance after liver transplantation.[102] As most hepatic DCs are located in portal areas where they cannot interact with circulating naïve T cells, it is unlikely these cells are major contributors to intrahepatic T-cell activation. However, there is now strong evidence that they are the main PLs responsible for activating alloreactive CD8 T cells in SLOs. In animal models, donor liver DCs are clearly detected in recipient SLOs, where they form clusters with proliferating CD8 T cells.[103,104] Donor DCs are also detected in the peripheral blood of human liver transplant recipients.[105]

Several studies have shown that in contrast to DCs derived from the spleen or bone marrow, liver DCs propagated *in vitro* promote acceptance of islet[106] and cardiac allografts.[107] Furthermore, livers isolated from mice lacking DCs transplanted into allogeneic wild-type recipients were rapidly rejected.[108,109] Although these results suggest that hepatic DCs are capable of inducing *in vivo* tolerance, other findings contradict this view. Expanding the number of liver DCs in the graft before transplantation using the haematopoietic growth factor FMS-like tyrosine kinase 3 (Flt3) ligand failed to favour tolerance. Flt3 ligand treatment enhanced the number of DCs in SLOs[110] and in the liver;[111] however, surprisingly liver allografts from donors receiving this treatment were acutely rejected.[111] The stimulatory activity of hepatic DCs from Flt3 ligand-treated mice was associated with upregulated expression of costimulatory molecules.[112] Enhanced expression of IL-12 in the grafts and increased cytotoxic T-cell activity were detected within the transplanted Flt3 ligand-treated livers,[113] resulting in the grafts being acutely rejected.[111,112] This suggests that T-cell tolerance is influenced by the quality and immature state of hepatic DCs rather than by their numbers.

Early mechanisms of tolerance induction within the liver graft

Role of hepatocytes in clearing allogeneic T cells. We predict that just after liver transplantation, circulating alloreactive T cells recruited into the liver graft would be rapidly activated in the hepatic sinusoids, prompting them to cross the endothelial barrier, invade hepatocytes and die by SE (Figure 3). From findings in non-transplant settings, we expect that this process will result in the rapid clearance of alloreactive CD8 T cells able to reach the graft. Consistent with this prediction, allogeneic graft-reactive naïve CD8 T cells were rapidly activated and depleted from recipient mice within the first day after transplantation.[90] This depletion occurred within a similar time frame as would be expected if T cells were cleared by SE. Furthermore, alloreactive CD8 T-cell depletion was not observed in recipients receiving a syngeneic liver graft, suggesting that it was dependent on donor-specific T-cell activation. We are currently examining whether such rapid T-cell loss in liver transplantation is caused by SE, and assessing the respective contributions of PLs and the liver tissue to this clearance.

Early death by neglect of intrahepatic activated T cells. Although SE cleared a large proportion of alloreactive CD8 T cells activated in the liver, some of these T cells survived and proliferated in the liver for 2–3 days, before undergoing apoptosis *in situ*.[69,74,75] Consistent with these findings in a non-transplant setting, rat liver allograft acceptance is often associated with large number of apoptotic T cells within rat liver grafts.[98] The rate of apoptosis among infiltrating T cells in mouse liver allografts has also been reported to increase over time, whereas alloreactive cytotoxic activity within the grafts decreases.[114]

KCs in liver transplantation tolerance. Although KCs are thought to be tolerogenic, their role in promoting liver graft acceptance remains controversial. KCs can cause Fas-mediated death of alloreactive T cells, and selective KC depletion with gadolinium chloride in donor rat liver allografts before transplantation resulted in rapid rejection.[115] However, other studies have found a beneficial outcome on liver allograft survival after gadolinium chloride treatment in other animal models.[116,117] In mice, deletion of KCs with clodronate liposomes in donor liver allografts does not affect their acceptance.[118]

Immunoregulatory mechanisms to maintain long-term tolerance in the liver

Mechanisms that establish tolerance immediately after transplantation are sufficient to prevent early graft rejection, but may not

prevent rejection of liver transplants in the longer term. Regulatory mechanisms that silence alloreactive T cells would be required to maintain long-term tolerance.

Role of Tregs and other regulatory mechanisms. T_{regs}, mainly $CD4^+$ $CD25^+FoxP3^+$ T cells, are responsible for the maintenance of allograft tolerance following transplantation of various organs[119] (Figure 3); indeed, one of the first characterisations of this population was in the context of exploration of transferable tolerance to rat heart allografts.[120] In human liver transplant patients, a transient reduction of $CD4^+CD25^+$ T_{regs} was observed in the peripheral blood early after transplantation.[121] The T_{reg} population recovered over time to normal level in patients who did not have rejection, but remained low in patients who developed acute rejection,[121] suggesting an association between T_{regs} and liver allograft fate. Rodent models of liver transplantation have also confirmed the role of regulatory cells, including $Foxp3^+CD25^+CD4^+$ T_{regs} in liver transplantation tolerance. T_{reg} frequency was increased in liver grafts and recipient spleens after transplantation.[122] These cells expressed cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and were linked to the production of the immunoregulatory cytokines, tumour growth factor-β and IL-4[122]. Rat splenocytes isolated from a liver transplant recipient and adoptively transferred into another host prolonged the survival of skin, heart or irradiated liver allografts that would normally have been rejected.[63] Interestingly, most studies suggest that T_{regs} able to efficiently suppress the function of alloreactive T cells require several weeks to develop. One of the earliest studies by Kamada and co-workers[123] on this topic identified two distinct phases of suppression in the spleen mediated by different cells. An early first immunosuppressive phase occurred between 5 and 28 days after liver transplantation. This first phase was transient, could not transfer long-term tolerance and was mediated by macrophage prostaglandins. A second phase mediated by allospecific regulatory T cells was observed after 20 weeks. This process was donor-specific and provided long-lasting suppression.[123] Other studies have confirmed that splenocytes isolated at day 60 after liver transplantation were more effective at providing allograft protection compared with those isolated at day 30.[124] Early studies have also suggested that donor T cells, presumably Tregs, may contribute to the maintenance of allograft tolerance.[96,125] More recently, studies involving depletion of recipient $CD4^+CD25^+$ T_{regs} just before transplantation resulted in liver rejection by preventing apoptosis of graft-infiltrating T cells.[122,126] Interestingly, depletion of $CD4^+CD25^+$ cells at 3 weeks after transplantation did not affect the spontaneous acceptance of liver allografts,[126] suggesting that other regulatory mechanisms are also involved in maintaining long-term tolerance.

Thus, although recipient $CD4^+CD25^+$ T_{regs} contribute to liver transplantation tolerance,[122,126] their slow development and generation in the late phase of tolerance induction suggest that they might reinforce the effects of early tolerance-inducing mechanisms and provide additional late regulatory mechanisms contributing to the maintenance of long-term tolerance.

Role of coinhibitory pathways. The PD-1/PD-L1 coinhibitory signalling pathway have a critical role in the survival of several different organ allografts.[127–130] Enhanced PD-L1 expression has been detected along hepatic sinusoids and in leucocyte-infiltrated areas in mouse liver allografts.[131] Human liver allografts also expressed PD-L1 on hepatocytes and leucocytes along the sinusoids and bile ducts,[132] whereas PD-1 was highly expressed by infiltrating CD8 T cells.[132] Morita *et al.*[131] used two similar mouse models to examine the role of PD-1/PD-L1 interactions in liver graft acceptance. Transplantation of PD-L1-deficient livers into wild-type allogeneic recipients led to rapid graft rejection,[131] while treating recipients with anti-PD-1- or anti-PD-L1-blocking antibodies starting immediately after transplantation also resulted in early liver allograft rejection.[131] Blockade of PD-1/PD-L1 interactions led to enhanced CD8 T-cell infiltration and increased production of proinflammatory cytokines within allografts.[131] Non-bone marrow-derived liver-resident cells seemed to be involved in mediating liver graft acceptance via the PD-1/PD-L1 pathway. This was linked to their ability to upregulate PD-L1 expression via the IFN-γ signalling pathway.[118] Although these studies suggest that LSECs or HSCs, but not hepatocytes, are involved in this process,[118] the role of hepatocytes in this process has not been formally examined.

In an acute liver injury model induced by alloreactive CD8 T cells, blocking PD-1/PD-L1 interactions at the beginning of or commencing 20 days after initial antigen encounter increased the survival of alloreactive CD8 T cells, but both settings failed to influence their functionality.[75] This suggests that although the PD-1/PD-L1 pathway might control alloreactive T-cell populations during chronic antigen encounter associated with transplantation, additional mechanisms maintaining functional T-cell exhaustion in the liver contribute to promoting allograft survival.

One of the major determinants of the development of impaired alloreactive CD8 T-cell function, characterised by the expression of coinhibitory receptors, is likely to be persisting high-level expression of alloantigen by the liver graft. Consistent with this, transfer of Bim-deficient alloreactive CD8 T cells into mice expressing the cognate alloantigen intrahepatically led to expansion of these cells within the liver.[74,75] However, they remained non-functional and failed to exacerbate liver damage,[74,75] implying that alloreactive T cells rapidly lose function in the presence of persisting high antigen load, independent of apoptosis. Similarly, in the presence of high-level expression of exogenous antigen by hepatocytes, liver-reactive CD8 T cells capable of clearing antigen expression at lower levels were rendered functionally inactive and expressed coinhibitory receptors, including PD-1.[70]

PD-1 is not the only coinhibitory receptor that may regulate CD8 T-cell function. CTLA-4 is a coinhibitory receptor expressed by activated T cells that binds to the costimulatory molecules CD80 and CD86.[80] Rather than inducing T-cell activation, this interaction results in reduced T-cell activation.[80] Blockade of the CTLA-4 signalling pathway has been shown to promote accelerated rejection of cardiac[133] and islet allografts,[134] and also resulted in rapid rejection of liver allografts.[135] Although anti-CTLA-4 treatment did not significantly affect the number of intrahepatic CD8 T cells, it decreased the number of apoptotic T cells and increased both alloreactive cytotoxic activity and the number of alloreactive T cells secreting IL-2 and IFN-γ in liver allografts and recipient SLOs.[135]

Influence of viral infection in dictating immunological outcome following liver transplantation

Viral infection may be associated with significant complications in clinical transplantation. However, the influence of viral infections on the outcome of antiallograft immune responses is not yet fully understood. It has been suggested that liver transplant patients with primary cytomegalovirus (CMV) infection developed a reduced donor-specific CD8 T-cell responses associated with fewer episodes of late acute rejection.[136] However, despite this potential initally beneficial effect, CMV infection has been demonstrated to have a detrimental long-term effect on liver allograft survival, as it is

associated with increased chronic allograft rejection and mortality.[137] Although it is not entirely clear how CMV influences allograft survival, a recent study suggested that this could be mediated by its direct effect on LSECs.[138] LSECs were found to be CMV targets, and CMV-infected LSECs expressed increased levels of T-cell adhesion molecules and promoted enhanced reactivation of IFN-γ-producing CD4 T cells *in vitro*.[138] These findings suggest a model in which CMV causes allograft rejection by enhancing immune activation and inflammation, following increased T-cell recruitment to the liver via infected LSEC.

Another major viral liver pathogen is the hepatitis C virus. The effect of hepatitis C virus on liver allograft rejection is not clear. Although recurrent hepatitis C virus infection in post-transplant patients seemed to lead to increased liver allograft damage, resulting in more rapid graft loss,[139] a recent study suggests that hepatitis C virus infection promoted operational tolerance via altered immunoregulation within liver allografts and increased T-cell exhaustion.[140]

POTENTIAL APPLICATION OF THE KNOWLEDGE GAINED FROM LIVER TRANSPLANTATION

The ability of liver transplants to induce ineffective activation of alloreactive T cells has inspired some investigators to artificially induce antigen expression in the liver to induce tolerance, and ultimately prevent autoimmunity or rejection of other allografts.

Expressing a neural autoantigen in the liver resulted in the induction of antigen-specific T_{regs}.[141] These T_{regs} suppressed the onset of experimental autoimmune encephalomyelitis in susceptible mice after being challenged with the triggering autoantigen.[141] Similarly, expression of high levels of allo-MHC-I molecules in hepatocytes using hepatotropic recombinant adeno-associated viral vectors promoted long-term acceptance of skin grafts carrying the same allo-MHC-I and even prolonged skin allograft survival in presensitised mice,[142] suggesting that this strategy promoted tolerance induction in both naïve and memory alloreactive CD8 T cells.

A similar strategy has also been used to induce tolerance in gene therapy.[143] Clinical studies have confirmed that transgenes can be stably expressed in the liver when hepatocytes were targeted in human patients.[144,145] Our recent studies showing that the percentage of transgene-expressing hepatocytes dictates the outcome of transgene-specific responses[70] would predict that low transduction efficiency promoting expression of the transgene in a low frequency of hepatocytes would result in the clearance of transduced hepatocytes by functional CD8 T cells, whereas high transduction efficiency (promoting expression of the transgene in a high proportion of hepatocytes) would lead to CD8 T-cell exhaustion and would allow persistent transgene expression. Our study predicts that in order to achieve optimal results in gene therapy, maximum numbers of hepatocytes should be transduced. This not only enhances transgene production but also prevents subsequent destruction of transgene-expressing hepatocytes by CTLs, promoting long-term transgene expression.

A further implication includes the issue of the use of early high-level immunosuppression in human liver transplantation. As we have shown, the induction of liver transplant tolerance is an abortive process of CD8 T-cell activation leading largely to deletion of high-affinity allograft-responsive cells. This process is likely to be inhibited by T-cell depletion therapies such as antithymocyte globulin and use of early high-dose calcineurin inhibitors. To maximise tolerance in humans, an approach to allow this early ineffective CD8 T-cell activation phase to occur may be required. This would require studies to detect this in humans in the immediate phase post-transplant, and then the instigation of adequate immune suppression immediately thereafter.

CONCLUSIONS

The liver is a unique organ. Hepatic allografts can be spontaneously accepted even across multiple MHC mismatches in many animal models, with alloreactive T-cell tolerance developing in recipients. Although the pathways underlying this outcome remain to be fully determined, multiple mechanisms have been indicated to be involved in promoting T-cell tolerance in this setting. T cells are ineffectively activated within the liver, rendering them poorly functional; many undergo rapid deletion via SE, with a significant proportion of remaining intrahepatically activated alloreactive cells subsequently undergoing apoptosis. PLs from the liver grafts also promote T-cell tolerance and early apoptosis of graft-reactive cells within SLOs. At later time points, remaining graft-reactive CD8 T cells are likely under the negative influence of the coinhibitory pathways, and additionally subject to regulation by T_{regs}. In combination, these mechanisms result in liver transplant tolerance without the need for immunosuppression in a variety of animal models, and likely contribute to the ability of some liver transplant recipients to cease immunosuppressive medications in the longer term, and maintain normal allograft function in a state of operational tolerance. It is hoped that further exploration of these pathways will eventually lead to the institution of protocols to enhance the development of spontaneous liver transplant tolerance in the clinic, and of clinically relevant methods to harness liver-induced tolerance to improve outcomes in transplantation of other solid organs and in autoimmunity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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