Immune Mechanisms Contributing to Spontaneous Acceptance of Liver Transplants in Rodents and Their Potential for Clinical Transplantation

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Abstract. The fate of organ transplants between unrelated individuals of the same species is almost always rejection unless the recipient receives immunosuppressive drugs. Liver transplants are an exception, as in a number of animal models they are often accepted without requiring any treatment. Several mechanisms have been proposed for liver transplant acceptance, including: the vascular structure of the liver, which allows interaction between naive T cells and liver parenchymal cells; the atypical leukocyte populations of the liver—particularly immature dendritic cells; neutralization of rejection by donor soluble MHC antigen; establishment of microchimerism by donor hematopoietic stem cells; and death by “neglect” of recipient T cells in response to inappropriate activation by donor liver leukocytes. Although all these mechanisms may contribute to liver acceptance to some degree, an important finding is that liver acceptance appears to be mainly due to donor leukocytes transplanted with the liver. In combination with the observation of rapid T cell activation followed by their death after liver transplantation, these findings have identified a prominent role for donor leukocyte-induced deletion of liver-reactive T cells. These findings suggest novel ways to explore improved treatment for transplant patients, including the administration of donor leukocytes at the time of transplantation and the delay of some components of immunosuppressive-drug induction therapy.

Key words: transplantation tolerance; liver transplantation; graft rejection; graft survival; immunosuppressive agents.
barrier, even though the recipient does not receive immunosuppression. The first report of acceptance of liver transplants in unrelated individuals was in a pig model, where the MHC relationship between donor and recipient was not known\textsuperscript{20}. Subsequently, examination of inbred miniature swine with a defined swine lymphocyte antigen mismatch showed that mismatched livers were rejected\textsuperscript{25}, suggesting that the original observation was partly due to genetic similarities between the pigs used.

In rodent models, where the genetic differences between strains is well-defined, the situation is straightforward. Liver allografts between completely MHC-unrelated rats are accepted in low-responder strains such as PVG and DA\textsuperscript{44}, while in high-responder strains, such as Lewis, Brown-Norway or AUG, liver transplants are rejected\textsuperscript{143}. In inbred mice, all strains tested accept a liver transplant across a complete MHC barrier\textsuperscript{85}. Few primate liver transplants have been performed and of 4 rhesus monkey liver transplants without immunosuppression, one animal survived more than 6 months\textsuperscript{20}.

**Acceptance of Non-Liver Transplants in Mismatched Recipients**

Although liver transplantation in rats and mice has been the most studied of the spontaneous acceptance models, it is not the only example amongst transplanted organs. Spleen allografts between completely unrelated strains of rats are also accepted without requiring immunosuppression and induce tolerance to skin or pancreas grafts of the spleen donor but not a third-party strain\textsuperscript{12, 13}. Kidney allografts are also spontaneously accepted between unrelated individuals in a number of species. This is well established in mouse models where kidney transplantation across complete MHC mismatches often results in long-term survival and induction of donor-specific hypo-responsiveness to skin grafts\textsuperscript{94, 103} without requiring immunosuppression, similar to liver allografts in rats or mice\textsuperscript{10}. In contrast to the mouse, rat kidney allografts across a complete MHC barrier are rejected in all strain combinations tested; however, class I-mismatched, class II-matched kidneys are accepted in low-responder rat strains\textsuperscript{39}. In an inbred miniature swine model in which there are defined class I and class II differences, recipients of class II-matched kidneys accept the kidney long-term provided that there is also one class I allele matched\textsuperscript{38}. These long-term acceptors are hypo-responsive to kidney donor strain skin grafts (reviewed in \textsuperscript{38}). Heart transplants are rejected in all MHC-mismatched donor-recipient strain combinations tested in mice, rats and pigs. There is, consequently, an overall gradation in response between species and type of organ transplanted. Livers are accepted in all mouse strains, low-responder rats across a complete MHC barrier and in unrelated but not MHC-mismatched pigs. Kidneys are accepted in some completely mismatched mice, but not in any completely mismatched rats, while mismatched hearts are not accepted in any species.

**Concordance between Clinical Transplantation and Animal Models**

These findings of a spectrum of responses to different transplanted organs would predict that liver transplant patients should be less likely to suffer from acute rejection and more likely to survive long-term without chronic rejection than kidney or heart transplants. It is difficult to compare clinical liver, kidney or heart transplants for the incidence of acute or chronic rejection directly, as this is influenced by many factors, including HLA match, the type and dosage of immunosuppression, cadaver versus living donor, and differences between organs in the relative rates of infection or recurrence of underlying disease. Even so, the incidence of chronic rejection in liver transplant recipients appears to be lower than in kidney or heart transplant recipients. The rate of chronic rejection in long-term liver recipients is between 4–12\textsuperscript{29, 137}, while in renal transplantation chronic rejection remains more of a problem, with a rate of 10–80\%, depending on the time of follow-up\textsuperscript{26}.

Transplant registry data, which has analyzed tens of thousands of patients, show that there was approximately 79\% survival of liver grafts at one year, which decreased to 51\% at 10 years, a long-term loss of 28\% of these HLA-unmatched grafts\textsuperscript{104}. For cadaver kidney grafts that were MHC-mismatched, graft survival was 87\% at one year\textsuperscript{21} and 37\% at 10 years\textsuperscript{123}, a loss of 50\%. For MHC-matched kidneys the loss was 35\% over the same period. These results show that the attrition rate of kidney grafts between 1 year and 10 years after transplantation was almost double that of a comparable liver transplant patient group, although there was a greater loss of liver than kidney grafts during the first year. This higher early liver transplant loss possibly reflects the greater complications of the liver transplant operation rather than increased levels of acute rejection. Comparison of clinical heart transplants with liver or kidney transplants reveals that patient survival
at 10 years is approximately 45%\textsuperscript{46}, while for liver transplants the corresponding figure is 59%\textsuperscript{104}.

Consequently, the overall clinical data are consistent with the animal models showing that liver transplants tend to have a better outcome than kidney or heart transplants, although it is not clear whether there is a difference between kidney and heart transplant patients in their susceptibility to rejection. A further difference between liver transplant patients and those with other grafts is that recipients of livers with no evidence of rejection, unlike recipients of other organs, can be weaned from all immunosuppression\textsuperscript{76, 92, 122}. Although there are reports of renal transplant patients who have retained their grafts in spite of non-compliance with their immunosuppressive treatment, weaning renal transplant patients from immunosuppression is generally considered too dangerous to attempt in the clinic.

**Mechanism of Spontaneous Acceptance of Liver Transplants**

Acceptance of liver allografts in rodents is a particularly powerful means to induce transplant tolerance. Not only is the liver accepted without requiring immunosuppression or treatment of any kind, but it also rapidly induces tolerance to subsequent grafts of liver donor origin\textsuperscript{54}. Furthermore, a liver transplant can act similarly to an immunosuppressive drug, as it is able to reverse ongoing rejection of a rat heart\textsuperscript{57} or pancreas\textsuperscript{55} transplant. In this setting, it is more effective than cyclosporine immunosuppression in its ability to reverse cardiac allograft rejection\textsuperscript{57}. A further demonstration of the tolerogenic ability of the liver is its ability to be spontaneously accepted in presensitized recipients\textsuperscript{54}. As liver transplants in rodents provide the most powerful model of peripheral tolerance to transplanted organs, understanding the immune mechanism should identify novel means to promote acceptance of transplanted organs. This has the potential to reduce or eliminate the requirement of treating transplant patients with immunosuppressive drugs, which cause many problems, especially when used long-term.

Immune mechanisms to induce tolerance to transplanted organs center around reducing the T cell response, as T cell-deficient animals are unable to reject organ grafts, even from genetically widely disparate donors\textsuperscript{73}. Means to inactivate graft-reactive T cells include: deletion, where the T cells are destroyed; anergy, where they are stimulated to become non-reactive with alloantigen; or suppression, where a subset of T cells develops the ability to prevent potentially graft-reactive T cells from becoming activated. Immune deviation is another means by which T cells are not able to mount a rejection response. It arises from the polarity of immune responses, either cell-mediated, driven by T cell production of T helper (Th) 1-type cytokines, or antibody-mediated, driven by T cell production of Th2 cytokines, which provide help to B cells. These two responses are thought to be mutually exclusive to some extent, due to the cross-inhibition of Th1 responses by Th2 cytokines and vice versa. Immune deviation has been proposed to lead to abrogation of rejection responses, as rejection is associated with a Th1 response involving cytokines such as interleukin (IL)-2, interferon (IFN)-\(\gamma\), IL-12 and lymphotoxin, and the production of Th2 cytokines, such as IL-10 and IL-13, can inhibit this response\textsuperscript{63, 79}.

Studies by Davies and Kamada in the 1980s established that there was clonal deletion of alloreactive cells early after liver transplantation\textsuperscript{24, 55, 56}. They sub-lethally irradiated recipients to delay rejection and showed that adoptive transfer of thoracic duct lymphocytes, a leukocyte population enriched in T cells, from normal rats accelerated rejection. In contrast, adoptive transfer from tolerant strain recipients that had received a liver transplant 30 days previously neither delayed nor accelerated rejection. This is evidence that liver donor-reactive T cells of the host are functionally deleted, although it is interesting that this deletion is misleading in some respects. The lack of responsiveness of liver graft recipients to subsequent grafts of liver donor strain is consistent with this deletion, however, a paradoxical finding is that these recipients, who are unable to reject grafts, can still respond to liver donor antigen. Donor-reactive T cells persist in liver-tolerant animals, as detected by in vitro studies\textsuperscript{52} and by their ability to mount a graft-versus-host reaction to donor antigen in F1 recipients\textsuperscript{52}. Taken together, the above findings are consistent with an early clonal deletion of donor-reactive T cells as a consequence of liver transplantation. This deletion is not complete, however, and donor-reactive T cells persist, as defined by rigorous assays of alloreactivity.

A number of properties of the liver have been proposed to contribute to its ability to delete, or otherwise tolerate, recipient alloreactive T cells. It is a large organ with a dual blood supply, consisting of arterial blood from the hepatic artery and venous blood from the gut delivered via the portal vein. In addition, the liver provides a site where naive T cells, which are excluded from entering other parenchymal compartments, are able to interact with liver parenchymal cells, principally hepatocytes. Moreover, the liver has a large
resident population of leukocytes, including many atypical populations that are uncommon in other sites, as well as many recirculating T and B lymphocytes and considerable numbers of hematopoietic stem cells. The following sections describe characteristics of the liver that have been proposed to contribute to its ability to neutralize a rejection response.

The role of the liver vascular endothelial structure in tolerance

The liver has a unique vascular endothelial structure that influences its ability to attract and activate naïve T lymphocytes. Naïve T cells can interact directly with the liver, but are unable to access other organs randomly, as the endothelial cells of non-lymphoid organs form an effective barrier which prevents any contact between naïve T cells and parenchymal cells. Specific adhesion molecules are a prerequisite for transendothelial migration and naïve T cells obtain these adhesion molecules during activation, which is usually initiated in the spleen or lymph nodes (reviewed in 7). In the spleen and lymph nodes, naïve lymphocytes encounter professional antigen-presenting cells (APC) presenting a combination of specific peptide with the MHC. After activation, proliferation and differentiation, T cells are capable of transendothelial migration. The structure of the hepatic sinusoids distinguishes the liver from other organs and allows interaction directly between naïve T cells and hepatic parenchymal cells, primarily hepatocytes (Fig. 1A). T lymphocytes interact with hepatocytes in the sinusoids, where there is a low-velocity blood flow. In contrast to other endothelial cells, no junctions exist between liver sinusoidal endothelial cells (LSEC) and adjacent endothelial cells, and these LSEC are perforated by numerous 120 nm-diameter holes, termed fenestrae. Liver sinusoids lack a basal membrane, and between LSEC and hepatocytes is a perisinusoidal space, the space of Disse, in which stellate cells can be found. In contrast, Kupffer cells and intrahepatic lymphocytes are located in the lumen of the hepatic sinusoids. There is, consequently, an opportunity for direct interaction between hepatocytes, which can extrude their cell membrane through the fenestrae, and circulating naïve T cells (reviewed in 7).

Hepatocytes express MHC class I molecules, CD1 and intercellular adhesion molecule (ICAM), whereas these cells normally do not have MHC class II antigens, CD40 ligand (CD40L) and costimulatory molecules such as CD80. Those hepatocytes that do not express

Fig. 1. Proposed mechanisms of liver transplant tolerance. Cells marked with a cross are apoptotic recipient T cells. A – liver vascular structure, B – immature liver dendritic cells (DC), C – soluble class I antigen, D – microchimerism.

Soluble MHC class I antigen, released from the liver, neutralises anti-donor antibodies and kills cytotoxic T cells specific for donor class I.
MHC class II may only act as APC for MHC class I-restricted T cells. In an in vitro study, the ability of purified hepatocytes to activate T cell receptor (TCR)-transgenic CD8⁺ T cells was demonstrated. Hepatocytes that lack expression of CD80 and CD86 costimulatory molecules could induce activation and proliferation of specific naïve CD8⁺ T cells without requiring added cytokines. No difference was found between proliferation induced by hepatocytes and that induced by dendritic cells (DC), the predominant APC for both MHC class I-restricted (CD8) and MHC class II-restricted (CD4) T cells. However, after 3 days of co-culture, T cells activated by hepatocytes lost their cytolytic function, whereas T cells activated by splenocytes maintained this property. This suggests that, although hepatocyte-stimulated T cells are effectively activated, proliferate and have cytotoxic T lymphocyte (CTL) activity, they do not survive. A comparison of T cells activated by hepatocytes with those activated by splenocytes revealed that hepatocyte-activated T cells expressed lower levels of the Bcl-xL survival gene and 30 times less IL-2 mRNA. Adding IL-2 or splenic cells prevented apoptosis. Both IL-2 and Bcl-xL expression could be augmented by CD28 costimulation, and cross-linking of CD28 prevented premature death in the leukocytes. This suggests that naïve T lymphocytes can be activated within the liver by hepatocytes, but are unable to promote survival of these CD8⁺ T cells.

In addition to its vascular structure, other features of the liver that influence its immunological function relate to its dual blood supply from both the hepatic artery and the portal vein, to the distinct lymphocyte populations found in the liver, and to the role of the liver as a graveyard for dying cells. The portal vein, which carries nutrients, toxins and antigens from the gut to the liver, is the main supplier of blood. This portal vein, which carries nutrients, toxins and antigens from the gut to the liver, is the main supplier of blood. The combination of the low-velocity blood flow and the unique architecture of the liver may be the explanation for the liver’s permeability to naïve CD8⁺ cells and its ability to activate these cells. In liver transplantation, alloreactive T cells might be activated by hepatocytes, but die by neglect. Deletion of these cells by the liver is one mechanism that can contribute to the immune tolerance of liver transplants.

Liver dendritic cells as regulators of immune reactivity

Another means by which the liver might induce its own acceptance across complete MHC barriers is through its large population of immature DC. DC are APC that are important in initiating and regulating an immune response. DC originate from CD34⁺ bone marrow progenitors and express MHC class II molecules. Mature DC are considered to be the most potent APC and effectively induce proliferation of naïve allo-generic T cells. These cells have a high surface expression of MHC products and many accessory molecules for intercellular adhesion (CD54, CD58) and costimulation (CD40, CD86). However, evidence exists that DC do not only initiate immune responses, but also play a role in central (intrathymic) and peripheral tolerance (reviewed in 125). Several in vivo studies support the existence of tolerogenic DC activity. Immature DC that express surface MHC class II, but are deficient in surface costimulatory molecules, can induce T cell hyporesponsiveness and inhibit immune reactivity. Tolerogenic DC might play a role in liver tolerance. Immature DC that express surface MHC class II, but are deficient in surface costimulatory molecules, can induce T cell hyporesponsiveness and inhibit immune reactivity. In a normal mouse liver, costimulatory molecules CD40, CD80, and CD86 could not be detected using immunohistochemical staining techniques. This means that normal liver DC normally do not express detectable amounts of these molecules in situ. In a study in which these liver-derived DC of donor origin were used to pretreat pancreatic allograft islet recipients 7 days before transplantation, a significantly extended survival was found. Donor bone marrow-derived immature DC administered 7 days before a heart allograft prolonged heart survival in mice.
In contrast, treatment of liver-transplanted mice with Flt3 ligand, a hematopoietic growth factor which stimulates proliferation and maturation of hepatic DC in situ, resulted in acute rejection in normally tolerated livers. Consequently, mature DC seem to be involved in priming a rejection response, whereas immature or liver-derived DC are more tolerogenic.

A number of modes of action of DC in liver transplant tolerance have been proposed. One mechanism described above is that immature DC lack costimulatory molecules such as CD40, CD80 or CD86. Costimulatory molecule-deficient DC have been shown to be tolerogenic in allograft models. In this case, DC which express high levels of MHC class I and II have a high affinity for the TCR (signal 1), but lack the costimulatory signals (signal 2) that can promote survival of the activated T cells. One means by which the liver might maintain DC in a state that lacks costimulatory molecules is that transforming growth factor-β, produced in the liver by hepatocytes and other liver types after alloantigen stimulation, ischemia or regeneration, enhances IL-10 production by hepatocytes, which in turn prevents expression of costimulatory molecules by DC (reviewed in). Using CTLA4-Ig to block costimulatory molecules on propagated DC resulted in apoptosis of alloreactive T cells. DC expressing Fas ligand (FasL) were effective inducers of apoptosis in T cells. However, there may have been an additional Fas-independent pathway to induce T cell death. Another mechanism by which DC might tolerate T cells is by production of nitric oxide (NO). NO is generated by nitric oxide synthase, which is induced in cells such as macrophages and hepatocytes. NO is produced by bone marrow DC after stimulation with IFN-γ or coculture with purified naïve allogeneic T cells. Addition of a NO donor resulted in apoptosis of DC. NO is also associated with suppression of lymphocyte proliferation and apoptosis of T cells. In liver transplants, interaction of allogeneic T cells and DC might result in NO production by the DC with subsequent inhibition of T cell proliferation and apoptosis of DC, which in turn decreases alloantigenic stimulation, resulting in tolerance.

**Soluble MHC class I antigen**

Soluble MHC class I antigen was first described in human serum in 1970. Its presence has also been found in different animal species and in culture supernatants of cell lines. Soluble MHC class I antigen is about 5 kDa smaller than that of the classic class I molecule, which is a membrane-bound glycoprotein. Soluble class I antigen has been isolated in quantity from rat liver extracts and the liver appears to be a major source of this antigen.

It has been shown that the main source of soluble class I antigen in the serum of liver transplant recipients is the donor liver. In rat liver transplant acceptance, free class I antigen of liver donor type is detectable in the recipient’s serum shortly after liver grafting and persists for the duration of the graft. This has led to the suggestion that these soluble antigens may prevent rejection by blocking the binding of graft-specific antibodies or by inhibiting alloreactive cytotoxic T cells. In contrast with this hypothesis, some investigators demonstrated that increasing levels of donor soluble antigen in human recipients of liver transplants were associated with episodes of acute rejection.

The role of soluble class I antigen in transplant tolerance has been investigated by administering it to the recipient after transplantation of non-liver organs. Prevention of rejection has not been very successful, as shown in a rat heart allograft model in which daily injections of DA serum in a PVG recipient had no effect on heart survival. In the same study, continuous infusion only slightly, but statistically significantly, delayed rejection. Other groups have been unable to show any effect, and found that intravenously administered soluble class I liver antigen did not prolong graft survival in a heart transplant model. In a study in mice, antigen-specific CTL activity to a membrane-bound antigen could not be blocked by large amounts of the free form of this antigen in the circulation. Also concluded that soluble classical class I molecules are incapable of prolonging allograft survival and unable to influence cytotoxic T cell responses, although relatively high doses of donor antigen were used. Further evidence that soluble MHC class I antigen is not essential for liver tolerance is that livers transplanted from MHC class I-deficient mouse donors were accepted in a situation where no soluble class I antigens could be produced by the liver.

**Microchimerism in liver transplant tolerance**

It is a long established observation that transplanted livers rapidly become chimeric, as most of the resident donor-derived leukocytes, such as Kupffer cells, disappear from long-surviving human liver allografts and are replaced by recipient cells. The fate of the original donor leukocytes was, however, unknown and, more recently, the presence of small, but detectable, numbers
of donor migratory cells, sometimes with the morphology of dendritic cells, were observed in the recipient and termed microchimerism (reviewed in 109). It was also observed that this microchimeric state could persist for many years after transplantation and it was consequently proposed that establishment of microchimerism was an indicator of a stable state of graft acceptance107,108. Microchimerism has been demonstrated in the recipient after lung, heart and kidney, as well as liver transplantation62,98,110,111, although, due to its large population of resident leukocytes, the liver was thought to be better able to establish a microchimeric state. As a result, a clinical trial was undertaken to examine the effect of increasing the level of allogeneic chimerism by giving donor bone marrow at the time of transplantation. This did not, however, lead to a detectable improvement66. In a recent, large, non-blinded clinical trial, it was observed that augmentation of microchimerism with bone marrow transfusion resulted in an improvement of some parameters of graft function65. The long-term outcome of enhancing microchimerism remains unknown, as there was a relatively short follow-up in these studies.

Different hypotheses exist to explain the mechanism of tolerance induction by microchimerism. Initially, the mechanism proposed was that the host-versus-graft (rejection) response was partially antagonised by leukocytes of the donor, which mounted a limited graft-versus-host reaction (Fig. 1D)80. Under cover of immunosuppression, these donor leukocytes promoted graft survival by destroying recipient alloreactive T cells and eventually persisted to establish the microchimeric state (reviewed in 113). Another mechanism to explain tolerance in microchimerism is by veto cells present in the transplanted organ. When the TCR of a cytotoxic T cell of the recipient recognizes a specific MHC/peptide complex expressed by a donor veto cell, the recipient T cell is killed or inactivated79. This “reverse killing” of recipient cytotoxic T cells by donor veto cells has been proposed to promote transplant acceptance by neutralizing recipient cytotoxic T cells (reviewed in 51). This mechanism has also been shown in a study in which donor bone marrow cells, which contained veto cells, inhibited graft rejection of second-set skin allografts86. The mechanism of inactivation is unknown, and might involve clonal anergy, clonal deletion, or both51.

Despite the early enthusiasm for microchimerism as a method to promote graft acceptance, recent findings question the relationship between microchimerism and tolerance. A correlation between long-term tolerance and the level of microchimerism after transplant could not be found77. A study evaluating 15 patients with kidney allograft survival of more than 20 years showed that microchimerism was detectable in only 33%118. In another study, 11 of 12 (92%) liver transplant recipients with an episode of rejection had donor specific microchimerism102. Moreover, in a rat heart transplant model, depletion of donor specific microchimerism, using a monoclonal antibody to donor leukocytes several weeks after transplantation, did not prevent long-term acceptance. When these cells were removed immediately after transplantation, the heart allograft was chronically rejected160, which shows that long-term microchimerism is not essential for organ allograft acceptance. Consequently, the presence of donor leukocytes at the time of transplantation appears to be important in prolonging transplant survival, an observation that will be extended in the following section. The role of microchimerism in tolerance remains controversial.

**Role of Donor Leukocytes in Liver Transplant Tolerance**

Evidence was presented independently by two groups in 1995 that leukocytes from the donor that had been transferred to the recipient with the transplanted liver were responsible for liver-induced tolerance. One study used “parking” of a DA-strain transplanted liver in an intermediate PVG-strain recipient to replace the mobile DA passenger leukocytes in the liver with those of the recipient PVG-strain. This chimeric liver, containing DA parenchyma and PVG leukocytes, was retransplanted to a fresh PVG strain recipient. The liver was not rejected, although its ability to induce tolerance to DA-strain skin grafts was significantly reduced, showing that donor leukocytes contributed to liver acceptance160.

The second study, from our group, showed that depletion of liver leukocytes by irradiation of the liver donor led to rejection of leukocyte-depleted livers in a strain combination that normally accepts the liver20. In this study we confirmed that radiation damage to the liver was not responsible for this failure, as parking of the irradiated liver in a donor strain intermediate host, which reconstituted its population of passenger leukocytes, led to its acceptance. The role of donor leukocytes in liver transplant acceptance was subsequently confirmed by our own and several other groups when it was shown that purified populations of leukocytes could reconstitute acceptance of irradiated livers22,101,121. Although it has been suggested that the characteristics of liver leukocytes might be responsible for liver trans-
plant acceptance, we found that leukocytes prepared from the spleen were at least as effective as those from liver in reconstituting acceptance of livers from irradiated donors\textsuperscript{121}.

These studies show that liver transplant tolerance depends on donor leukocytes and in this respect it resembles the blood transfusion effect. For many years it has been known that transfusion with donor blood prior to transplantation results in improved survival of the transplanted organ. This has been termed the blood transfusion effect and its efficacy when administered a week or more prior to transplantation has been demonstrated both in animal models and in clinical transplantation (reviewed in\textsuperscript{15, 78}). Liver transplant acceptance differs slightly from the blood transfusion effect because in liver transplants the leukocytes are present at the time of transplantation, while for the blood transfusion effect they are only considered to be effective when delivered a week or more prior to transplantation\textsuperscript{16, 81}.

This difference could be due to the combination of the tolerogenic effects of the liver itself, in combination with donor leukocytes. While this is a likely possibility, it is noteworthy that rat kidney transplants in a low-responder (PVG to DA) strain combination behave in a similar fashion to liver transplants in low-responder strains when donor leukocytes are administered at the time of transplantation\textsuperscript{140}. Both are accepted without requiring immunosuppression and both induce tolerance to subsequent donor-strain skin grafts. Even so, the liver remains more tolerogenic than the kidney, as additional donor leukocytes injected at the time of liver grafting overcome rejection in the high-responder Lewis strain\textsuperscript{140}, while this treatment is unable to promote acceptance of kidney transplants in Lewis recipients (unpublished data). This property of the transplanted liver is most likely due to some of the liver-specific tolerance mechanisms discussed earlier. These findings show that a combination of donor leukocytes injected at the time of transplantation together with a liver or a kidney graft, but not a heart or skin graft\textsuperscript{16, 121}, can induce long-term acceptance and donor-specific tolerance. In some circumstances, donor leukocytes administered at the time of transplantation can significantly prolong survival of heart allografts, but the heart is ultimately rejected\textsuperscript{129}.

Several groups have investigated the nature of the donor leukocyte population that is responsible for liver transplant tolerance. A number of groups have examined the ability of DC to prolong graft survival, as discussed above. In the liver transplant model, studies from our group concentrated on identifying the subset of donor cells that was responsible for reconstitution of acceptance of livers from irradiated donors. This involved using positive selection of populations of T cells, B cells or monocytes/macrophages to deplete these subsets from the reconstituting inoculum. This showed that depletion of B cells or monocytes/macrophages did not affect the ability of the donor cells to reconstitute acceptance; however, when T cells were depleted, the livers were chronically rejected. The role of T cells in promoting acceptance was confirmed in the liver transplant model\textsuperscript{121} and in a model of heart transplantation\textsuperscript{129}. It was, consequently, of some surprise when we found that the donor B cell population most effectively promoted acceptance of rat kidney allografts\textsuperscript{141}. It is not clear why the donor leukocyte population that is most effective for promotion of survival of liver and heart grafts might be different from those that promote kidney grafts. In this respect, donor leukocyte-induced acceptance may resemble the blood transfusion effect, where a large number of different donor cell types are effective in promoting acceptance\textsuperscript{45, 71, 138}.

**Role of Recipient T Cell Activation**

Studies of the pathology of liver transplants in tolerant strain combinations showed that there was an extensive infiltrate early after transplantation and some evidence of graft damage\textsuperscript{52, 59}. Nevertheless, this early rejection response resolved and the grafts went on to survive long-term and, as described above, could reverse ongoing rejection in other organs. This immunosuppressive property of liver transplants, in spite of their own rejection-resolving response, could have been due to immune deviation, where the cytokine response is inappropriate and leads to inhibition of the appropriate response. In the context of transplant tolerance it was postulated that early after transplantation there might be an excess of the Th2 cytokines such as IL-4, IL-10 and IL-13 that would inhibit rejection through inhibition of the Th1 cytokines IL-2, IL-12 and IFN-\gamma\textsuperscript{51}. We tested this possibility in the liver transplant model by comparing the cytokine profiles in tolerance and rejection of grafts. This showed that there was no clear difference between them, as there were rapid increases in both Th1 and Th2 cytokines, which confirmed that there was indeed immune activation that occurred during tolerance that was not detectably different from the activation that occurred during rejection.

While this was evidence that immune deviation did not occur in the graft, and that there was no evidence for a Th2 response in the tolerant liver, the possibility
was investigated that immune deviation might occur at the site of induction of rejection, the spleen and draining lymph nodes of the recipient. The surprising finding was that when the cytokine profiles of these tissues were examined, there was greater expression of the Th1 cytokines IL-2 and IFN-γ in the draining lymph nodes of tolerant than rejecting liver transplant recipients. This finding runs counter to the Th1/Th2 paradigm, especially considering that the cells that produced the bulk of the IL-2 and IFN-γ in recipient lymphoid tissues during tolerance induction were recipient CD4 T cells, the cells that are central to the rejection response.

**Activation-Induced Cell Death or Death by "Neglect" Link Recipient T Cell Activation and Death**

How then is it possible to resolve the activation of recipient CD4 T cells in liver transplant tolerance with acceptance of the liver? This question can be resolved by the observation that the activated T cells die by apoptosis during liver transplant tolerance, but survive during allograft rejection. Apoptosis is a process of programmed cell death that was originally observed in areas of hepatocyte damage in liver transplants during episodes of acute rejection. It was also observed in infiltrating leukocytes as well as hepatocytes, which raised the possibility of liver-induced tolerance as a result of its ability to induce death of infiltrating leukocytes. This observation was consistent with the findings that the liver was a "graveyard" for activated T cells (reviewed in ). While the liver may have properties that promote apoptosis, death of infiltrating leukocytes during transplant tolerance is not confined to the liver, and apoptosis of infiltrating leukocytes has been observed in heart allografts that have not been treated to promote tolerance, and in donor leukocyte-induced kidney transplant tolerance.

The association between apoptosis of cells in areas of infiltrate and subsequent acceptance of the liver was demonstrated by the reduction of these cells when the recipient was treated to promote rejection or in rejecting compared with tolerant grafts. It was subsequently confirmed by purification of leukocytes from tolerant and rejecting grafts that the apoptotic cells in these tolerant transplants were indeed leukocytes and that apoptosis was concentrated in the activated CD4 T cell population. Apoptosis was not confined to the graft and apoptosis of large numbers of cells in the T cell areas of recipient lymphoid organs has been observed in conjunction with transplant tolerance.

The pattern of infiltration of tolerant grafts was consistent with activation of T cells followed by their death, as there was an early, rapid increase in T cells and IL-2 receptor-expressing cells in tolerant liver grafts, which reached a peak on day 3. At the same time, there was extensive apoptosis of activated T cells in the infiltrate and in recipient lymphoid tissues and a subsequent decline in activated cells in the tolerant liver grafts. In contrast, there was a slower accumulation of activated T cells during rejection, but this ultimately resulted in greater levels of activation and eventual graft failure.

There are two main immune mechanisms that link activation with death in T cells. One is passive cell death, also termed death, by "neglect", where an activated T cell, expressing receptors for IL-2, IL-4, IL-7, IL-9 or IL-15, is deprived of these cytokines and rapidly dies by apoptosis. Death by neglect can result from lack of costimulation, with consequent lack of production of T cell survival cytokines, such as IL-2, or it can result from T cell activation and proliferation in the presence of cytokines, followed by cytokine withdrawal. Direct evidence for T cell death by neglect in liver transplant tolerance comes from the finding that tolerance is broken by injection of exogenous IL-2 early after transplantation. Rapid rejection of the liver resulted from IL-2 treatment, consistent with IL-2-induced survival of the large number of IL-2 receptor-expressing cells observed on day 3 in tolerant livers. This resulted in rejection and a massive infiltrate of IL-2 receptor-expressing cells and CD4 and CD8 cells. These findings were confirmed in mouse liver transplants, where IL-2 administration reduced survival from >100 days to 5–7 days and led to a massive infiltrate of T cells.

The other pathway that links T cell activation with death is activation-induced cell death (AICD), which is dependent on IL-2 to increase FasL expression on activated T cells, which then induces death of surrounding T cells by binding to Fas expressed on their surface. In addition, IL-2 reduces expression of the anti-apoptotic molecule flice-like inhibitory protein (FLIP), whose expression in resting T cells protects them from apoptosis. There is some evidence that this pathway might be operating in liver transplant tolerance, as transplantation of livers from FasL-deficient donors or from Fas-deficient recipients abrogates acceptance of the transplanted liver. Although this is not conclusive, it shows that donor expression of FasL on the liver and/or passenger leuko-
cytes within the liver, in addition to Fas expression on recipient leukocytes, is necessary for liver acceptance.

There is, consequently, some experimental support for both AICD and death by neglect as mechanisms of reduction of the numbers of activated alloreactive T cells in liver transplant tolerance. The observation that IL-2 can break tolerance is evidence that death by neglect might be the main mechanism by which liver transplants induce recipient T cell death. As AICD is dependent on IL-2, additional IL-2 would not be assumed to alter the outcome and the recipient T cells would be expected to die. While recipient T cell death by neglect appears to be the main mechanism of liver transplant acceptance, the issue is by no means resolved and further experiments are required to determine the relative contributions of AICD and death by neglect. The roles of T cell survival cytokines other than IL-2 and of Fas and FasL expression on the graft and on donor and recipient leukocytes need to be established.

**Implications of Death by Neglect for Immunosuppressive Drug Treatment of Transplant Patients**

The above studies have shown that there is rapid activation, followed by recipient T cell death, that occurs during liver allograft acceptance. These findings soon led to experiments by our group to investigate the effect of immunosuppressive drugs on liver transplant tolerance. These showed that a short course of methylprednisolone treatment ablated the ability of liver transplants to induce development of tolerance to subsequent skin grafts from the liver donor strain\(^\text{11}\). Furthermore, methylprednisolone treatment of liver allograft recipients led to chronic rejection of livers in a low-responder DA-strain combination, where the liver is normally accepted\(^\text{136}\). Not all immunosuppressive drugs appear to inhibit liver transplant acceptance and a short course of cyclosporine that could prevent rejection of livers in a high-responder Lewis-strain recipient did not inhibit spontaneous acceptance in a low-responder DA-strain\(^\text{49}\).

These experiments show that it might be useful to screen immunosuppressive drugs in models of liver transplant tolerance to identify those that are least likely to interfere with the active processes that lead to liver transplant acceptance.

A consequence of early activation and death of recipient T cells is that delay of administration of immunosuppressive drugs to avoid their inhibition of tolerance-associated activation, might improve their effectiveness. Furthermore, if T cell death by neglect is responsible for liver transplant tolerance, then delay of immunosuppression, to allow T cells to become activated and express cytokine receptors, followed by treatment with agents such as cyclosporine which effectively inhibit cytokine expression, should also lead to increased T cell death. In contrast, if cells do not become activated to the stage where they are dependent on cytokines then they will not be deleted, even in the...
presence of high levels of the drugs that inhibit cytokine expression. Consequently, when the dose of immunosuppression is reduced to maintenance levels, these T cells remain potentially able to mount a rejection response.

There is some support for this, as delay of methylprednisolone immunosuppression for 3 days prolonged liver transplant survival in a high-responder Lewis-strain recipient, while delay of 7 days gave the longest median survival time. Similarly, delay of cyclosporine immunosuppression gave a slight, but non-significant, improvement in outcome after liver transplantation. More convincing evidence for the effectiveness of delay of cyclosporine administration comes from a heart transplant model, where delay for 6 days led to long-term survival, while rejection rapidly ensued after immediate commencement of therapy. A similar result was obtained when FK506 was given as a single dose at the time of heart transplantation, followed by a drug-free “window” of three days and recommencement of treatment at day 4. This pattern of administration of FK506 followed the recommendations of the “WOFIE” hypothesis (window of opportunity for immunological engagement), although the function of the initial dose of immunosuppressive drug has not been established. It is possible that this initial dose might interfere with the immune activation required to optimally induce death by neglect. Not all drugs are more effective if their administration is delayed, and mycophenolate mofetil, which does not markedly inhibit production of cytokines such as IL-2, cannot inhibit rejection when it is commenced three days after transplantation.

It is interesting that in the two examples above of the effectiveness of delayed administration of the calcineurin-inhibitor drugs cyclosporine and FK506, there was synergy between drug treatment and peri-transplant administration of donor leukocytes. As donor leukocyte administration induces rapid immune activation of recipient T cells, similar to the activation observed in liver transplant acceptance, this is consistent with a requirement of T cell activation followed by inhibition of cytokine expression. There are few reports of delay of immunosuppression in human transplantation; however, it is interesting that in a study of a large number of patients treated with OKT3, an antibody that activates T cells, delay of cyclosporine immunosuppression gave a highly significant improvement in graft survival. Hence there is a need to investigate the optimum immunosuppressive regimen in the clinic, with treatments such as donor leukocyte infusion or OKT3 therapy to activate alloreactive T cells combined with delayed administration of selected immunosuppressive drugs.

Conclusions

There are several findings from the model of spontaneous acceptance of liver allografts that are of interest both as a means to understand the mechanism of tolerance of transplanted organs and as potential improvements to clinical management of transplant patients. There are a number of mechanisms that have been proposed to account for liver transplant acceptance, some of which have considerable support from experimental models. One important aspect that unites a number of these mechanisms is that donor passenger leukocytes transferred with the transplanted liver are central to induction of tolerance. The donor-cell population that is primarily involved in tolerance induction has yet to be established, as is the role of the liver vasculature and parenchymal cells. Another important aspect of liver transplant tolerance is that recipient alloreactive T cells are activated and die. Current immunosuppressive therapies are likely to interfere with this process and modifications to these therapies, including administration of donor leukocytes and delay of immunosuppressive drug administration, should be tested in pre-clinical models.

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