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THE ROLE OF THE GRAFT IN ESTABLISHING TOLERANCE

Mahzuz Karim, Ulrich Steger, Andrew R. Bushell, Kathryn J. Wood

Nuffield Department of Surgery, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK

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1. ABSTRACT

At the present time, clinical solid organ transplantation continues to rely on the use of non-specific immunosuppressive protocols in order to prevent graft rejection. However, these regimens bring with them complications related both to the global immunosuppression that they cause, and to toxicity related to individual drugs. The pursuit of protocols that will allow graft-specific tolerance thus remains a major goal of research both in animal models and in clinical practice. There is evidence that the graft itself may play an active part in establishing and maintaining donor-specific hyporesponsiveness and ultimately tolerance; the aim of this review is to analyze this role in more detail.

2. INTRODUCTION

In clinical practice, solid organ transplantation represents a mainstay of therapy for end stage organ failure. However, for the process to be successful, rejection of the graft by the recipient's immune system must be prevented. Historically, transplantation only became a realistic option following the development of effective immunosuppressive drugs. However, the long term use of these agents is far from optimal. The non-specific nature of the immunosuppression that they provide carries with it an increased risk of infection (1) and malignancy (2, 3). The individual drugs used also possess their own side effects; for example, calcineurin inhibitors such as cyclosporin and tacrolimus are nephrotoxic and may thus contribute to the failure of renal allografts or cause renal failure in recipients who receive other solid organ grafts (4).

The majority of drugs in current clinical use act by preventing leukocyte activation and / or proliferation, and thereby prevent graft rejection. However, the eventual goal in transplantation is to achieve donor-specific unresponsiveness or tolerance, and it is now recognized that some of the approaches to achieving this may require activation of the recipient's immune system. Most of the immunosuppressive agents in current clinical use inhibit lymphocyte activation in a non-specific manner, and may therefore paradoxically inhibit the development of tolerance in some situations (5-7). There is thus intense interest in trying to unravel the mechanisms by which such tolerance may be achieved; this involves factors within not only the recipient immune system but also the graft itself. The aim of this review is to examine the role of the graft in more detail; figure 1 provides an overview of some of the proposed mechanisms.

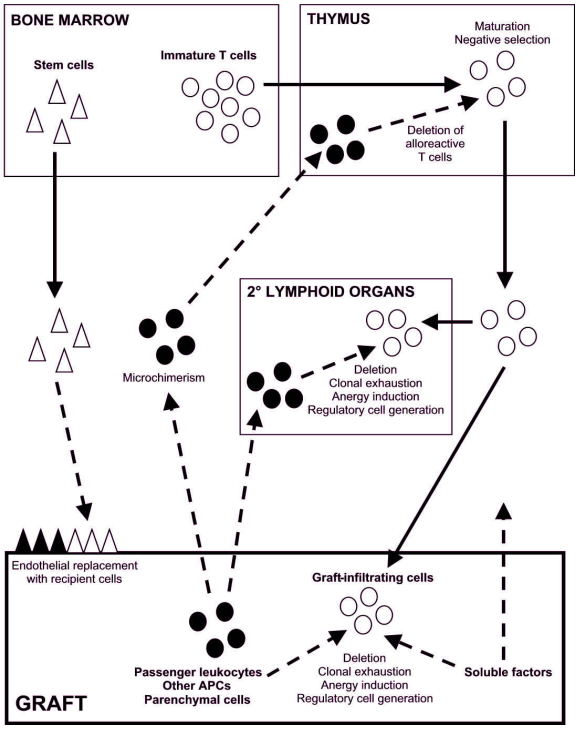

Figure 1. Proposed mechanisms by which the graft may contribute to the induction of tolerance. Open symbols represent recipient cells, closed symbols donor cells. Dotted arrows represent processes potentially contributing to tolerance induction.

3. ACTIVE ROLE OF THE GRAFT

A number of experimental studies have demonstrated that solid organ grafts, rather than simply provoking an immunological rejection response, may also play an active role in establishing and maintaining tolerance. In various protocols it has been shown that if primary grafts are performed under the cover of tolerance induction therapy and are then removed, tolerance, assessed by the acceptance of a second donor-type graft, is lost (8-11). Olausson *et al*. demonstrated this phenomenon in a rat cardiac allograft model, where anti-thymocyte globulin pre-treatment of the recipient led to long term survival of primary grafts. These were then removed, and secondary donor-type grafts performed after varying intervals. If the time between removal of the first graft and placement of the second graft was greater than 25 days, the second graft was rejected (8). Similar findings were also reported in a mouse model, where acceptance of primary cardiac allografts was induced by treatment with anti-CD4. Second cardiac allografts performed in animals with a functioning primary graft were accepted, but if the primary graft was removed at day 50 then tolerance to donor alloantigens was eventually lost, so that secondary grafts performed 200 days later were rejected. In this study, polymerase chain reaction analysis of tolerant mice failed to demonstrate the peripheral presence of donor class I MHC positive cells, suggesting that tolerance was due to ongoing presence of the graft itself rather than to donor microchimerism (9).

The duration of donor-specific unresponsiveness after removal of the primary graft is somewhat variable between models. In the rat, when tolerance was induced by leukocyte depletion, 17 to 25 days in the absence of the primary graft resulted in the loss of operational tolerance (8, 10), whereas in mice the tolerant state appeared to persist for longer in the absence of alloantigen (9, 11)

The requirement for antigen persistence to maintain tolerance is not unique to transplantation, as it is a common feature of the maintenance of tolerance to self antigens. For example Garza *et al.*, measuring responses to ovarian peptide, found that female mice were hyporesponsive to the peptide compared to males, requiring 100 fold more peptide antigen in order to elicit a response *in vivo*. However, if the ovaries were removed either in the neonatal period or more than seven days prior to antigen challenge, tolerance was abrogated. This again suggests that ongoing antigen presence is required to maintain tolerance, and that neonatal exposure alone, in this case to a self antigen, is insufficient (12).

The role of the graft in establishing tolerance appears to require activation of the recipient immune system, since several studies have shown that if immune recognition and activation do not take place, operational tolerance to donor alloantigens is not induced or maintained (13-15). Thus the acceptance of an allograft *per se*, if it occurs in the absence of appropriate immune activation, may not be sufficient to achieve systemic tolerance (16). Rat pancreatic islet allografts cultured for 14 days under high oxygen tension in order to deplete passenger leukocytes were permanently accepted when transplanted under the cover of cyclosporin. Subsequent injection of recipient lymphocytes previously sensitized to the donor alloantigens led to rejection of all the cultured islet grafts while, in contrast, islet grafts that had not been cultured under high oxygen tension were not rejected by the sensitized leukocytes, suggesting that activation of the recipient immune system by the transplanted tissue was required for tolerance to develop (13). Taking a different approach, Tullius and colleagues showed that replacement of primary rat renal allografts that had undergone chronic rejection by a second graft from the same donor strain between two and twelve weeks after the initial transplant resulted in the second graft showing good function with lower levels of proteinuria and little cellular infiltration (14). This effect was not tissue-specific, as donor type cardiac allografts were also accepted, but it was donor-specific as third party renal and cardiac allografts were rejected. Similar data have also been obtained in a miniature swine model. Renal allografts transplanted across a single haplotype class I MHC mismatch developed acute rejection; however, in 30 per cent of these animals this rejection resolved spontaneously, and graft function remained stable thereafter. In addition, the animals with long term functioning grafts showed prolonged survival of donor-type skin grafts (15). The data obtained in each of these model systems can be interpreted as suggesting that activation of the immune system by the graft can lead to specific unresponsiveness to a subsequent challenge. In clinical transplantation, analysis of reactivity to donor

alloantigens in patients who have rejected a renal transplant also suggests that some exhibit donor-specific hyporesponsiveness *in vitro* (17).

There are numerous examples in the literature, particularly in rodent models, where graft survival can be achieved by the administration of immunomodulatory agents for a short period around the time of transplantation without the need for ongoing therapy. In these models, the immunosuppressive effect achieved in the longer term is graft-specific and does not affect the entire immune repertoire. Peri-operative administration of cyclosporin has been shown to prolong the survival of rat cardiac (18) and renal (13, 19, 20) grafts as well as rabbit (21-23) and porcine (24-26) renal allografts. Similarly, transplantation under the cover of anti-CD4 antibody therapy allows the acceptance of cardiac (27-32) and pancreatic islet (33) allografts when used alone, and mouse skin allografts when used in conjunction with anti-CD8 therapy (34-36). CD4 is not the only target that can lead to this type of unresponsiveness – manipulation of the CD40-CD40L and B7-CD28 costimulatory pathways at the time of transplantation provides a further potential method of promoting graft acceptance. Blockade of the CD40-CD40L pathway using antibodies directed against CD40L has been shown to prolong the survival of mouse skin (37, 38) and cardiac (39) as well as primate pancreatic islet (40, 41) and renal (42) allografts, while blockade of the B7-CD28 pathway with CTLA-4-Ig has been used successfully in mouse (43, 44) and rat (45) cardiac and primate pancreatic islet (46) allograft and human to mouse pancreatic islet xenograft (47) systems. Administration of antibodies directed against LFA-1 and ICAM-1 also allows the acceptance of allografts such as mouse cardiac transplants (48). These observations suggest that, under appropriate conditions, antigen presentation by allografts may favor the induction of operational tolerance rather than rejection.

If the graft is playing a key role in inducing and maintaining tolerance, one would expect that only leukocytes that had previous experience of the donor antigens would be responsible. Baker *et al.* examined donor-specific responses in different $CD4^+$ populations present in patients with a functioning renal allograft. They observed that hyporesponsiveness resided within the $CD4^+CD45RO^+$ subset of previously activated cells rather than the $CD4^+CD45RA^+$ subset. $CD4^+CD45RO^+$ antigen-experienced T cells are capable of circulating through the graft, whereas naïve $CD4^+CD45RA^+$ T cells are only able to circulate between blood and lymphoid tissues. The authors concluded that trafficking of antigen-experienced leukocytes through the graft was important for the development of donor-specific hyporesponsiveness (49).

4. GRAFT ADAPTATION

Various theories have been proposed to explain the mechanisms by which allograft tolerance may occur. Broadly, these hypothesize that the graft may induce a change in the recipient's immune system, or that the graft itself may undergo changes after transplantation (such as altered levels of MHC expression, replacement of graft endothelium by recipient-derived cells, or loss of passenger leukocytes) that render it less susceptible to rejection, a phenomenon known as graft adaptation. The subject of passenger leukocytes is discussed in more detail later.

In the rat, long term surviving renal allografts transplanted into naïve secondary recipients are not accepted in all strain combinations (50). In cases where second grafts were accepted, no evidence could be found for replacement of the graft with recipient endothelium, or for down-regulation of MHC antigens. Hart and colleagues therefore postulated that successful graft transfer was due to the loss of donor passenger leukocytes. This view is supported by the observation by Lechler and Batchelor that, in a model where long term surviving renal allografts were accepted when re-transplanted into secondary recipients, administration of donor strain dendritic cells to these recipients resulted in acute graft rejection (51). Analysis of the role of altered graft MHC expression is complicated by the fact that class II molecules are expressed by vascular endothelial cells in humans and large animals but not in rodents (52-56). Examination of mouse liver (57) and aortic (58) allografts and human liver (57), renal (59, 60), and heart (61) transplants has confirmed that replacement of graft endothelium by recipient cells may indeed occur following transplantation. Furthermore, analysis of mouse (62) and human (59, 62) renal allografts and human cardiac transplants (61) has demonstrated that recipient bone marrow-derived cells may also repopulate the graft parenchyma. However, importantly, some of these studies have shown that such replacement only occurs following graft damage (for example as a result of acute or chronic rejection), suggesting that this phenomenon may be a response to injury rather than a normal process of graft adaptation (59, 60, 62). Given the data cited above, that activation of the recipient's immune system by the graft is essential for the induction of tolerance, at this stage in the analysis it is not clear whether replacement of donor by recipient cells in the graft is linked.

Evidence against graft adaptation being solely responsible for the development of tolerance is supported by data from many animal models where a second donor-specific graft is accepted without the need for further immunosuppression (25, 31, 63-72). This does not rule out the possibility that graft adaptation still occurs in conjunction with changes in the recipient immune system but a number of studies have suggested that graft adaptation does not play a significant role in establishing tolerance in some settings. In their rat cardiac allograft model, where tolerance was induced by anti-CD4 antibody therapy, Onodera *et al.* demonstrated that long term surviving grafts were rejected when transplanted into naïve recipients (10). Coulombe *et al.* have described a mouse pancreatic islet allograft model where rejection could be elicited in the post-transplant period by immunizing recipients with donor antigen presenting cells. However, when the grafts were left in place for a longer period they became resistant to rejection after the immunization. In this model, resistance to rejection was shown to be due to a change in the recipient immune system rather than adaptation of the graft (73). When long term surviving

renal allografts were removed from MHC class I-mismatched miniature swine and replaced with fresh grafts of donor type, all were accepted, while re-transplantation of the long term surviving grafts into naïve recipients resulted in rejection (74, 75). Taken together, these studies suggest that graft adaptation does not play a major role in establishing tolerance.

5. MICROCHIMERISM

In addition to parenchymal cells, solid organ grafts normally also carry circulating bone marrow-derived cells, so-called passenger leukocytes. Some of these cells are able to engraft within the recipient leading to the state of microchimerism, where donor and recipient cells coexist. Since the initial reports by the Pittsburgh group describing the presence of bone marrow lineage cells of donor origin in patients with long term surviving liver (76) and kidney (77) allografts, Starzl and colleagues have postulated that the establishment of such systemic microchimerism may play a beneficial role in the promotion of graft survival (78, 79). However, the presence of microchimerism does not in itself imply an active role in the induction or maintenance of tolerance and, moreover, the absence of microchimerism does not preclude the development of operational tolerance. Examples of this latter situation from experimental studies include the following (9, 80-83). In the clinical setting, a number of studies have failed either to detect microchimerism or, in situations where it has been detected, to demonstrate a correlation with graft survival in the settings of kidney (84-88), heart (89), lung (90), and liver (87, 91-96) transplantation.

In contrast, other studies have demonstrated a beneficial effect of microchimerism; however, importantly, most of these studies involve administration of donor bone marrow under the cover of other therapy such as lymphoid irradiation. In a mouse pancreatic islet allograft model where such pre-treatment was administered prior to transplantation, all recipients that had more than 1% donor cells detectable in the peripheral blood accepted their grafts (97). In a study using a mouse skin allograft model, recipient pre-treatment with total lymphoid irradiation or non-myeloablative irradiation combined with anti-CD4 and anti-CD8 therapy and donor bone marrow infusion led to the development of chimerism, reduction in donor-specific cytotoxic T lymphocyte precursor frequency, and acceptance of donor-specific skin grafts (98, 99). When cells from these chimeric recipients were transferred into irradiated hosts, the reduction in donor-reactive cell frequency was only maintained if the adoptive transfer recipient was of donor type, again suggesting that maintenance of tolerance required the continued presence of antigen (98). In a primate model, pre-treatment of cynomolgus monkeys with non-myeloablative therapy (including anti-thymocyte globulin, total body irradiation, thymic irradiation) together with donor bone marrow infusion allowed the long term acceptance of renal allografts, associated with microchimerism, acceptance of donor-specific skin grafts, and donor-specific hyporesponsiveness *in vitro* (100).

The suggestion that chimerism may lead to the deletion of donor-reactive cells is supported by experimental observations in mice that are mixed chimeras. In this setting of macrochimerism, donor-derived cells locate to the thymus resulting in deletion of donor-reactive thymocytes (101, 102); it has also been postulated that chimerism leads to clonal exhaustion of mature donor-reactive lymphocytes in the periphery (79, 103). The same mechanisms may be operating in recipients with detectable microchimerism, although in this setting the number of donor cells present may not be sufficient to achieve tolerance. Another potential explanation may be that the use of immunosuppressive therapy facilitates the engraftment of donor-derived cells leading to microchimerism, but if this were the case microchimerism would be an epiphenomenon rather than a causal step in establishing tolerance.

A further insight into the role of microchimerism was provided by a study reported by Anderson *et al.* Nude mice that received skin allografts developed donor microchimerism derived from passenger leukocytes within the graft. Reconstitution of these microchimeric recipients with recipient type fetal thymic tissue led to ongoing donor-specific tolerance, whereas reconstitution with mature recipient type cells led to priming of donor-reactive cells (104). These results suggest that donor microchimerism is able to induce donor-specific tolerance of developing thymocytes, but potentially leads to priming of mature T cells.

Take together, these observations suggest that strategies attempting to achieve tolerance by means of donor microchimerism also need to target the mature peripheral T cell pool in order to prevent graft rejection (figure 2).

6. PASSENGER LEUKOCYTES

In addition to their potential role in the development of microchimerism, passenger leukocytes may also have other, more immediate, effects that play a role in influencing graft survival. In some, but not all, settings depletion of passenger leukocytes can have a negative impact on graft survival. Depletion of passenger leukocytes from donor livers or heart grafts prior to transplantation prevented spontaneous graft acceptance (82, 105-110), but the survival of liver grafts could be restored by the administration of donor leukocytes to the recipient at the time of transplantation (105-110). Likewise, removal of passenger leukocytes by graft irradiation led to an increased risk of chronic rejection of small bowel grafts, but this could be reversed by the administration of donor bone marrow to the recipient (111). In a clinical series of lung transplant recipients analyzed by O'Connell *et al.*, the mean number of donor passenger leukocytes present within grafts for the first 200 days post-transplantation correlated with clinical outcome, with lower levels being associated with acute or chronic rejection (112).

These observations suggesting a beneficial role for passenger leukocytes must, however, be balanced by studies indicating a possible detrimental effect. Removal of

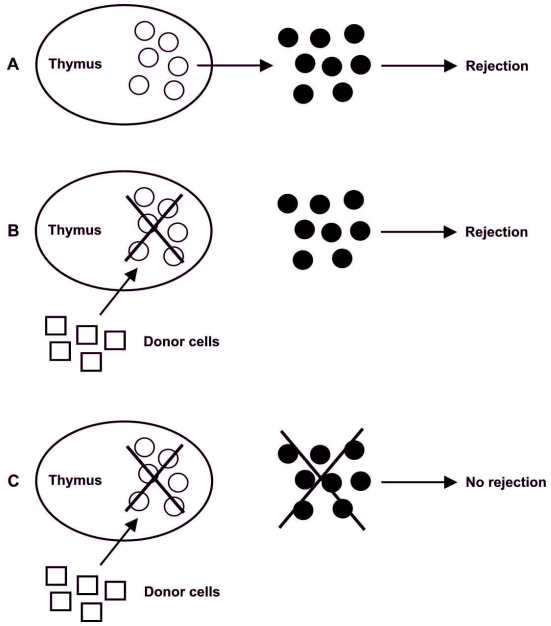

Figure 2. Potential role of microchimerism. (A) Graft rejection is mediated by mature peripheral alloreactive T cells (solid circles); this population is continuously replenished by the development of new T cells (open circles) within the thymus. (B) Donor microchimerism may allow the deletion of developing donor-reactive T cells within the thymus, but rejection will still occur unless the peripheral T cell pool is also targeted (C).

passenger leukocytes from grafts has led to prolonged allograft survival in rat cardiac (113), renal (114), and lung (115), and mouse pancreatic islet (73, 116-118) and thyroid (119-123) models. In corneal allografts, which normally contain few antigen presenting cells (124-126), the presence of such cells is associated with increased rejection rates (127, 128).

In studies where prolonged survival of thyroid and pancreatic islet allografts was achieved by depletion of passenger leukocytes using *in vitro* culture under high oxygen tension, subsequent recipient challenge with cells of donor origin led to graft rejection when given in the early post-transplant phase, whereas grafts that had been resident for over 100 days were more resistant to rejection. Recipients with long term surviving grafts were able to accept second donor-specific grafts *in vivo*, although they showed normal anti-donor reactivity *in vitro* (73, 118, 120-122). A similar potentially deleterious effect of passenger leukocytes has been demonstrated by Larsen *et al.*, who found donor leukocytes present in the spleens of mice that had rejected cardiac allografts (129). Experiments where long term surviving rat renal allografts were re-transplanted into secondary naïve recipients have shown that, in some strain combinations, these grafts are accepted (50, 130, 131), but that rejection can be restored by infusion of donor strain dendritic cells at the time of re-transplantation (51); the authors thus proposed that graft survival in the secondary recipients was due to loss of donor passenger leukocytes. Attempts have been made to exploit this mechanism in a clinical renal transplantation study (132-134). Prior to transplantation grafts were perfused with antibodies directed against CD45, leading to coating of passenger leukocytes. This pre-treatment was associated with a lower rate of acute rejection, suggesting that neutralization of passenger leukocytes was preventing recipient sensitization.

From these observations it is apparent that there is a complex relationship between the presence of passenger leukocytes and graft outcome and that, depending on other factors, including the microenvironment present in the graft or draining lymphoid tissue, passenger leukocytes may either facilitate tolerance or promote rejection. Bishop *et al.* have proposed that when antigenic stimulation (in part via passenger leukocytes) provided by grafts is compared to the rejection response there is a bell-shaped relationship. Thus at lower levels of antigenic stimulation (such as in heart or kidney allografts) reduction in stimulation by removal of passenger leukocytes leads to diminished rejection, whereas in the case of the liver (where there is a greater level of antigenic stimulation) passenger leukocyte depletion results in augmented rejection (135). This view is consistent with the hypothesis by Starzl and Zinkernagel (79, 103) that the beneficial role of passenger leukocytes on graft survival may be, at least in part, the result of recipient-reactive passenger leukocytes causing stimulation and clonal exhaustion of donor-reactive recipient cells and *vice versa*. If the balance between these two processes is correct, the risks of graft rejection and graft versus host disease respectively are reduced. In support of this view, in rodent skin allograft models recipient total lymphoid irradiation is insufficient to prevent graft rejection but the addition of donor bone marrow infusion leads to graft survival (136, 137), whereas in rat cardiac (137) and primate liver and kidney (138) allograft models, recipient total lymphoid irradiation alone is sufficient to allow graft survival. These observations suggest that in situations where the graft itself contains low numbers of passenger leukocytes, administration of additional donor cells promotes graft survival. A fuller understanding of these effects may allow the manipulation of donors or recipients in such a way as to harness the role of passenger leukocytes in achieving tolerance.

7. THE ROLE OF THE THYMUS

One theoretical mechanism through which allograft tolerance may be established is through the migration of graft-derived cells bearing donor alloantigen to the thymus resulting in the deletion of donor-reactive T cells centrally during the process of thymic development. Although the thymus undergoes significant reduction in size during adult life, there is evidence in humans that functional thymic tissue exists even in old age (139).

Direct evidence supporting the hypothesis that introduction of donor antigen into the thymus can modulate immune responsiveness comes from experimental systems where intrathymic administration of donor alloantigen in the form of peptides, whole cells, or tissues has been shown

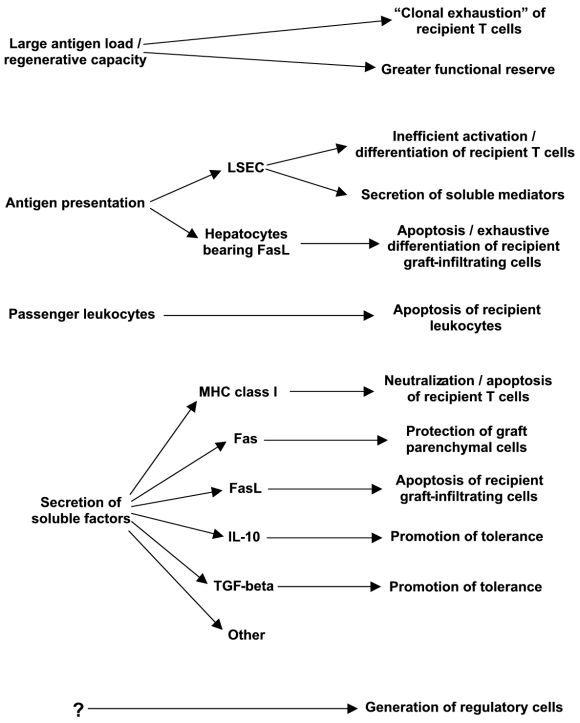

Figure 3. Possible factors contributing to the "liver effect". LSEC: liver sinusoidal endothelial cells.

to lead to prolonged survival of mouse cardiac (140-143), skin (65), and pancreatic islet (144), and rat cardiac (66, 80, 145-148), pancreatic islet (149, 150), liver (151), small bowel (152), and renal (153-155) allografts. However, even when graft survival is prolonged, chronic rejection may still occur (80). Similarly, graft protection may not extend to all organs, as demonstrated by a rat study where intrathymic administration of donor splenocytes led to the acceptance of cardiac but not skin or renal allografts (145). Rat cardiac allograft models where intrathymic donor splenocyte administration led to long term graft survival have suggested that donor microchimerism *per se* is not responsible for operational tolerance in the long term (80, 147).

The implication from these data is that if a sufficient dose of alloantigen from the graft were to enter the thymus this might reshape the repertoire and thereby prevent rejection. The mechanisms responsible could include anergy (155), immunoregulation (143), or deletion (140-142, 144) of alloreactive cells, all of which have been shown to occur after intrathymic injection of alloantigen, although it has also been shown that deletion is not necessarily required for the induction (141) or maintenance (140, 142) of tolerance in this setting.

The thymus may also play a role in the development of tolerance in systems that do not involve direct intrathymic antigen administration. In a rat cardiac allograft system, Onodera *et al.* demonstrated that an intact thymus was required for tolerance induced by anti-CD4 therapy (10). In the miniature swine model described by Sachs and colleagues, it has been shown that tolerance induction to cardiac (156) and renal (156-158) allografts is prevented by recipient thymectomy. Similarly in this model, concomitant transplantation of vascularized donor thymus tissue prevents the rejection of renal allografts (158). Thus, at least in some situations, the thymus may play a significant role in the development of allograft tolerance.

8. THE LIVER EFFECT

Since the seminal observations by Calne, it has been recognized that hepatic allografts possess distinct immunological properties compared to other organs. Transplanted livers are spontaneously accepted in mouse (159, 160), rat (161-167), and pig (168, 169) models, and there have been numerous reports of human liver transplant recipients who have been successfully weaned off immunosuppressive therapy without compromising graft function (96, 170-172). In addition to their own enhanced survival, liver allografts may also have protective effects on other organs transplanted into the same recipients. In one rat study, liver transplantation performed prior to or at the same time as pancreas allografts prevented rejection of the pancreas; moreover, rejection of lone pancreatic allografts could be arrested and even reversed by transplantation of a donor type liver performed up to six days later (164). In similar rodent models, liver co-transplantation has been shown to prevent the rejection of skin allografts (159, 173) and to reverse acute rejection of cardiac allografts (174). The protective effect of liver allografts may not require transplantation of the whole organ, as demonstrated by a rat study in which infusion of donor hepatocytes, but not hepatic leukocytes, into the portal vein prevented the rejection of cardiac allografts (175). In the clinical setting, liver co-transplantation has been shown to have a protective effect on small bowel (176), heart (177), and combined heart and lung (178) transplantation. Studies of human liver transplant recipients have also demonstrated donor-specific hyporeactivity *in vitro.* In a series by Reinsmoen *et al*, such donor-specific hyporeactivity of recipient lymphocytes was demonstrated in 40% of patients, a state that correlated with lower rates of acute rejection (179). A similar study comparing kidney and liver transplant patients with good graft function two years post transplantation found donor-specific hyporesponsiveness in the majority of liver patients but only a minority of kidney recipients (180).

Several potential mechanisms have been proposed for the so-called "liver effect"; these are summarized in figure 3. Some of these phenomena may provide approaches that could be harnessed to promote tolerance to other organ allografts.

8.1. Secretion of soluble MHC class I and other mediators

Given that one of the major functions of the liver is to synthesize a wide range of proteins and other products, it has been suggested that it may secrete soluble factors that have immunomodulatory effects, one such factor being soluble MHC class I. It has been postulated that donor MHC molecules secreted into the recipient circulation by

hepatic allografts may bind to alloantigen-specific T cells, neutralizing them and thus preventing graft damage. In addition, soluble class I has also been shown to induce apoptosis of alloreactive cytotoxic T lymphocytes (181, 182). Such donor MHC class I secretion by liver grafts has indeed been identified in the rat (183-185) and in humans (186-188); however, the presence of circulating soluble MHC does not necessarily imply a causal role in graft survival. In a study in which donor MHC class I was administered to rat renal allograft recipients, no effect on graft survival could be demonstrated (189). However, in this protocol, donor MHC was administered as a single bolus injection or as twice weekly injections; this schedule may be suboptimal since the half life of soluble MHC is around two hours. Indeed, in a further rat model, administration of donor class I MHC by continuous intravenous infusion did lead to prolonged cardiac allograft survival, even when commenced four days after transplantation (190), suggesting that soluble MHC may contribute to the liver effect. Nevertheless, class I secretion by grafts may not be an absolute requirement for liver acceptance since liver allografts from mice deficient in class I still show indefinite survival (160).

In addition to MHC class I, other soluble factors produced by the liver graft have also been identified that may play a role in hepatic allograft acceptance. In the rat, soluble Fas (CD95) and Fas ligand (FasL, CD95L) have been detected within grafts; it has been proposed that these serve to prevent apoptosis of parenchymal cells and promote apoptosis of recipient graft-infiltrating lymphocytes respectively (167). Other novel proteins have also been isolated from the serum of tolerant rats (but not recipients of syngeneic grafts, from strain combinations where livers are rejected, or from recipients of other solid organ grafts) that are able to suppress donor-specific mixed lymphocyte responses *in vitro* (191, 192). Finally, induction of tolerance by alloantigen administration into the portal vein has been demonstrated to lead to the secretion of soluble specific immunosuppressive factors (193, 194).

8.2. Antigen load

Spontaneous liver allograft survival does not appear to be due simply to the inability of recipient cells to react against the graft. For example, in rat models, liver acceptance is often preceded by self-limiting acute rejection episodes (161, 167). Similarly, in a mouse transgenic model where a large proportion of recipient T cells possess donor-specific T cell receptors, liver allografts were still spontaneously accepted and, moreover, donor-reactive T cells divided more vigorously than in recipients that rejected donor type cardiac allografts (U. Steger and K.J. Wood, unpublished observations). One explanation that has been proposed is that the large antigen load provided by the liver causes “overstimulation” and “clonal exhaustion” of alloreactive recipient cells (135). In support of this hypothesis, Sun *et al.* demonstrated that when two hearts and a kidney were transplanted into a single rat recipient all three organs were accepted, whereas transplantation of each organ separately resulted in rejection (195). Similarly, in a miniature swine model, solitary cardiac allografts were rejected, but double cardiac or combined cardiac and renal allografts were accepted (196, 197). The protective effect of the large mass of the liver may not, however, simply be due to immunological effects: it also provides a greater degree of functional reserve, allowing the organ to withstand a greater degree of damage before dysfunction ensues.

8.3. Antigen presentation

In addition to the quantitative differences outlined above, there are also qualitative differences in antigen presentation within the liver. Many antigens, both dietary and microbial, are encountered via the gut, and inappropriate immune responses to these may result in undesirable consequences such as food allergy. The immune system has thus developed mechanisms by which oral antigen encounter often leads to tolerance rather than sensitization, the well-recognized phenomenon of “oral tolerance” (198-201). The liver is known to play an important role in this process (the majority of blood draining the gut passes through the portal vein); this was first demonstrated over thirty years ago by Cantor and Dumont, who observed that portal-systemic bypass (which leads to drainage of blood from the gut directly into the systemic circulation without passing through the liver) prevented the development of oral tolerance in dogs (198). Conversely, direct administration of antigen into the portal vein, which mimics blood drainage from the gut, can result in the development of tolerance in some rodent models (175, 193, 194, 202-204).

The importance of active antigen presentation within the liver in establishing tolerance is demonstrated by the observation that inhibition of the phagocytic function of intrahepatic antigen presenting cells prevents tolerance induction (203, 204). Although macrophage lineage Kupffer cells reside within the liver and are able to present antigen, non-bone-marrow-derived liver sinusoidal endothelial cells (LSEC) have also been shown to be capable of antigen presentation (205-209). However, antigen presentation by these LSEC is inefficient in activating naïve T cells (205) and instead favors tolerance, with reduced T cell production of IL-2 and IFN-gamma (202, 209) and impaired deviation towards a Th1 phenotype (207). As described above, intrahepatic antigen presentation has also been shown to lead to the secretion of humoral immunosuppressive factors, allowing the adoptive transfer of tolerance into naïve recipients using serum in a mouse model (193, 194). Finally, the hepatic microenvironment is known to be rich in IL-10 and TGF-beta (210, 211), substances that have been shown to promote the development of tolerance in certain settings (212-224); in the case of IL-10, cytokine is not only present at significant levels within the liver, but release into the systemic circulation has also been demonstrated following reperfusion of hepatic allografts (225). There are thus several mechanisms by which the unique pattern of antigen presentation within the liver may contribute to the development of tolerance rather than priming of the immune response.

8.4. Passenger leukocytes, microchimerism, and the thymus

The liver is rich in passenger leukocytes, and

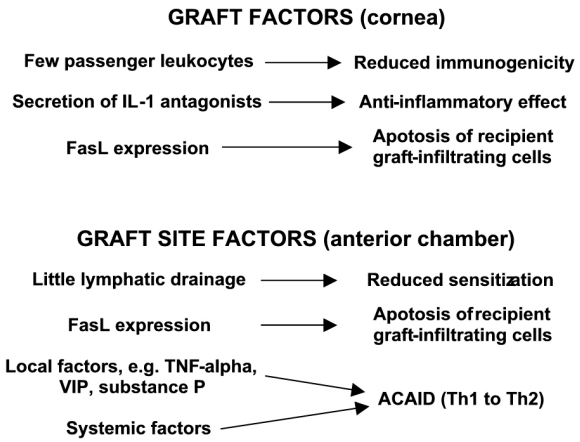

Figure 4. Possible factors contributing to the immunological privilege of orthotopic corneal allografts. ACAID: anterior chamber-associated immune deviation; VIP: vasoactive intestinal peptide.

these cells do appear to play an important role in the development of spontaneous tolerance to hepatic allografts. In rats, graft depletion of passenger leukocytes prior to transplantation prevents spontaneous acceptance, but survival can be restored by reconstitution with donor leukocytes (105-110). However, as discussed above, in clinical practice the establishment of full microchimerism, whereby donor cells are detectable in the recipient circulation, does not appear to be either necessary or sufficient for the development of tolerance since some patients may show long term graft survival in the absence of microchimerism (94) while others in whom microchimerism is present may undergo graft rejection (87, 91). Thus the development of microchimerism does not appear to be the dominant method by which passenger leukocytes contribute to the development of tolerance to liver grafts, and other mechanisms are likely to be involved. One such alternative role for passenger leukocytes was demonstrated by Bishop *et al.*, where migration of graft passenger leukocytes to the spleen and other lymphoid organs was observed following rat liver transplantation; this was associated with early upregulation of IL-2 and IFN-gamma mRNA expression in these tissues (162, 163) and apoptosis of recipient spleen cells (166). In contrast to this migration of passenger leukocytes to secondary lymphoid tissues, few such cells migrate to the thymus following liver transplantation (162, 226), and recipient thymectomy prior to transplantation does not prevent graft survival (227). Thus the role of hepatic passenger leukocytes in establishing tolerance does not appear to involve central deletion of donor-reactive T cells (with or without the development of microchimerism), but is more likely to involve processes within secondary lymphoid tissues.

8.5. Alloreactive recipient T cells

While intrathymic deletion of alloreactive recipient T cells does not seem to be required for spontaneous liver acceptance, such deletion does appear to occur elsewhere (228). In addition to the death of recipient cells within secondary lymphoid tissues following migration of passenger leukocytes, apoptosis of recipient graft-infiltrating cells has also been demonstrated in rodent models (165, 229, 230); the expression of FasL by graft hepatocytes may contribute to this process (167). This deletion of recipient cells is not confined to small animal models, but also appears to occur in clinical practice: a study by de Hann *et al.* of patients with good graft function two years after liver transplantation revealed a reduction in donor-specific cytotoxic T lymphocyte precursor frequency without a concomitant reduction in helper T lymphocyte precursor frequency, suggesting deletion of alloreactive $CD8^+$ cells (231).

In recent years there has been increasing interest in the role of "regulatory" or "suppressor" T cells in the maintenance of self-tolerance and in models of autoimmune disease and transplantation; this area is discussed in more detail below. The role of such donor-specific regulatory T cells in spontaneous liver allograft acceptance has, to date, not been well studied. However, preliminary data from our own laboratory suggest that in a mouse model where liver allografts are spontaneously accepted, $CD4^+CD25^+$ cells can be isolated from recipient spleens ten days post transplantation that are able to suppress donor-specific responses *in vivo* (U. Steger, C.I. Kingsley, M. Karim, and K.J. Wood, unpublished observations). Similarly, in a recent study by Zhang *et al.* using a mouse oral tolerance model, oral antigen feeding led to the relative expansion of an antigen-specific $CD4^+CD25^+$ population possessing regulatory properties both *in vitro* and *in vivo* (232); Thorstensen *et al.* have also demonstrated the generation of $CD4^+CD25^+$ cells with antigen-specific regulatory properties following oral antigen administration (233).

9. IMMUNOLOGICAL PRIVILEGE

In addition to the liver, a number of other organs exhibit immune privilege, whereby they are protected from rejection. The best characterized of these is the eye. In clinical practice, the one year survival rate for corneal allografts is as high as 90% despite the use of only limited topical immunosuppression (234, 235). The privilege of corneal grafts is related to a combination of factors, summarized in figure 4, involving both the graft itself and the graft site, the anterior chamber of the eye. The importance of the graft site is exemplified by the fact that, in experimental models, other allogeneic tissues and tumor grafts transplanted to the anterior chamber also show enhanced survival; this may partly be a result of limited lymphatic drainage of the anterior chamber and due to the local cytokine milieu (236, 237). Within the anterior chamber of the eye, Th2 cytokines tend to predominate over Th1 cytokines – so-called "anterior chamber-associated immune deviation" (ACAID) (236-238); TGF-beta is also present at significant levels (239). This deviation promotes graft survival, since corneal allograft rejection is associated with a Th1 type response, whereas Th2 cytokines tend to favor acceptance (240-243). The phenomenon of ACAID appears to be a systemic phenomenon rather than a purely local one, since it fails to develop in asplenic mice (240), and systemic IL-10

blockade inhibits ACAID, whereas IFN-gamma blockade promotes the process (238). Manipulation of the local cytokine profile has the potential to affect the outcome of corneal allografts: transfection of grafts leading to increased expression of IL-10 prolonged survival in a study by Klebe *et al.* (244), although Pleyer *et al.* failed to demonstrate any benefit when IL-4 was overexpressed (245). Further soluble factors that appear to play a role in ACAID are the neuropeptides vasoactive intestinal peptide (VIP) and substance P (SP): increased levels of the former can occur in response to TNF-alpha or exposure to light and lead to the promotion of ACAID, whereas the latter is predominantly expressed under conditions of darkness and appears to inhibit ACAID (246, 247). Consistent with these observations, TNF-alpha blockade has been shown to inhibit the development of ACAID (248). However, the role of TNF-alpha is not clearcut, since in a study by Qian *et al.*, blockade achieved by topical administration of soluble TNF-alpha receptor type I promoted the survival of corneal allografts; this effect was associated with a reduction in expression of the chemokines RANTES and MIP-1-beta (249).

The importance of the graft site in corneal allograft acceptance is underlined by the fact that corneal allografts transplanted to heterotopic sites in mouse models are rejected, demonstrating that the grafts themselves are indeed capable of eliciting a rejection response (250, 251). Nevertheless, graft factors do play a role in influencing survival. Firstly, passenger leukocytes appear to have a negative influence on graft outcome. The cornea normally carries few Langerhans cells (124-126), and an increase in the number of these cells is associated with a higher rate of rejection (127), whereas their depletion promotes graft survival (128). MHC expression by corneal grafts also has a bearing on outcome. The cornea expresses class I MHC (252, 253) at levels sufficient to elicit cytotoxic T lymphocyte responses *in vitro* (254), whereas class II is expressed at lower levels (253). While class I matching has been shown to improve the outcome of clinical corneal allografts (255, 256), the effect of class II matching is less clear in that some studies have demonstrated an improved outcome with better matched grafts (257), whereas others have suggested the converse (256, 258).

In addition to the local cytokine environment within the anterior chamber, the cornea itself may attenuate the effects of cytokines such as IL-1, a pro-inflammatory and chemotactic cytokine that is able to recruit Langerhans cells into the cornea (259). In a mouse system, IL-1 blockade has a beneficial effect on corneal allograft outcome (260). Furthermore, the importance of IL-1 attenuation may be more than experimental artifact, since human corneal tissue has been shown to produce soluble IL-1 receptor antagonist proteins (261). Alterations in the IL-1 pathway may thus play a genuine role in promoting corneal allograft survival.

A further important factor that appears to play a significant role in maintaining immunological privilege within the eye is the Fas-FasL system. FasL is constitutively expressed within the anterior chamber of the eye, including the cornea, leading to Fas-FasL-mediated apoptosis of infiltrating inflammatory cells and thus preventing tissue damage (262-265). The consequences of the loss of this mechanism are demonstrated by *gld*-deficient mice, which lack FasL. These animals develop uncontrolled inflammation within the anterior chamber in response to herpes simplex virus infection, whereas this does not occur in normal mice (262, 263). Similarly, corneal allografts from FasL-deficient mice show a significantly higher rate of rejection than grafts from normal mice when transplanted into allogeneic recipients (264, 265).

From these observations it is likely that the immunological privilege enjoyed by corneal allografts is the result of a combination of effects involving both the graft itself and the transplant site, and that no one factor alone is responsible for the effect.

A further tissue that exhibits immunological privilege is the testis. In rodents, rat pancreatic islets are not rejected when transplanted at low doses into mouse testis (266). Similarly in mice, testicular allografts transplanted beneath the renal capsule are accepted whereas pancreatic islet and thyroid allografts are rejected (267). Further investigation has revealed that this property appears, at least in part, to be contained within the Sertoli cell component of the testis: in mice, Sertoli cells are not rejected when transplanted alone (267), and in rats, co-transplantation of Sertoli cells promotes the survival of pancreatic islet allografts (268). As with the eye, FasL appears to play a role in this immune privilege. Mouse Sertoli cells have been shown to express FasL constitutively, and testicular or Sertoli cell allografts from FasL-deficient *gld* mice do not show immunological privilege and instead undergo rejection (267). However, care must be taken in extending these observations to man, since there is evidence in humans that FasL is not expressed constitutively in the testis (269).

10. FASL EXPRESSION AND OTHER TISSUES

In general, the Fas-FasL system plays an important role in controlling lymphocyte homeostasis, as exemplified by the fact that both Fas-deficient *lpr* and FasL-deficient *gld* mice show defective activation-induced death of both T and B lymphocytes (270, 271) and develop splenomegaly, lymphadenopathy, and fatal autoimmune disease (272). Similarly, this system has also been implicated in the evasion of immune surveillance by tumors, since FasL expression by tumors has been shown to cause apoptosis of infiltrating cytotoxic T lymphocytes, thus promoting tumor survival: such FasL expression has been demonstrated in examples of human hepatocellular carcinoma (273), colonic carcinoma (274), and melanoma (275). While the evidence discussed above from studies on the liver, eye, and testis suggest that FasL expression by transplanted tissues may be beneficial in promoting graft survival, the situation is not entirely straightforward, since FasL expression may also have adverse consequences. Kang *et al.* and Allison *et al.* have both observed that induction of expression of FasL by pancreatic allografts

fails to lead to survival when transplanted into allogeneic hosts, but rather leads to accelerated rejection; moreover, transgenic animals that constitutively express FasL on pancreatic islets develop inflammatory infiltrates within the pancreas and diabetes at a young age (276, 277). Similar results have been demonstrated in a mouse cardiac allograft model, where Takeuchi *et al.* found that transplantation of transgenic grafts expressing FasL into allogeneic or even syngeneic recipients resulted in the development of severe hemorrhage, edema, and neutrophil infiltration within the graft; in the allogeneic recipients, these histological changes and the tempo of graft rejection were more severe than when using grafts from wild type donors (278). Even in a study by Judge *et al.* where adenovirally-mediated induction of FasL expression on pancreatic islet allografts led to apoptosis of Fas^+ cells and the FasL-bearing adenoviral vector was able to suppress mixed lymphocyte responses *in vitro*, these benefits failed to translate to prolongation of islet allografts *in vivo* (279). Taken together, these studies suggest that FasL may have other effects such as a pro-inflammatory role, particularly in the setting of ischemia-reperfusion injury.

To balance these negative observations, there have also been studies suggesting that FasL expression may play a useful role in promoting graft survival. Lau *et al.* found that survival of mouse pancreatic islet allografts could be prolonged by co-transplantation within composite grafts also containing myoblasts transfected to express FasL, whereas myoblasts that were either untransfected or transfected to express Fas did not offer this protection (280). However, other groups have been unable to reproduce these findings (281, 282). In a rat renal allograft model, Swenson *et al.* showed that transient transfection of donor kidneys using a FasL-expressing adenoviral vector led to detectable FasL expression within the graft for two weeks, and prolongation of graft survival to 27.8 days compared to 11.6 days in control animals (283). A potentially beneficial role for FasL expression has also been demonstrated in a clinical study of renal transplant recipients by Porter *et al*, who.observed that FasL expression within the graft prior to transplantation was associated with a reduced risk of acute rejection (284). Protocols aimed at inducing FasL expression by grafts may thus provide a useful strategy in attempts to achieve operational tolerance, but must be evaluated carefully in view of the potential adverse consequences.

11. IMMUNOREGULATION, INFECTIOUS TOLERANCE, AND LINKED EPITOPE SUPPRESSION

Several mechanisms contribute to the maintenance of self tolerance by the immune system. These include central deletion of autoreactive T cells during thymic development (285, 286), peripheral deletion of autoreactive T cells (287, 288), ignorance of autoantigens to which the immune system is not normally exposed (289, 290), induction of anergy of autoreactive T cells (291, 292), and active suppression by antigen-specific regulatory T cell populations (293-296). It may equally be possible to harness these mechanisms for the induction of tolerance to allografts. The potential roles of central and peripheral deletion have already been discussed. Immunological ignorance of the graft has been shown to promote survival, as exemplified by a study by Lakkis *et al.* where mice lacking secondary lymphoid organs permanently accepted cardiac allografts (297). This mechanism may also be exploited in the clinical setting: the novel immunosuppressive agent FTY720 partly exerts its effects by altering lymphocyte trafficking leading to sequestration within secondary lymphoid organs and thus potentially maintaining immunological ignorance by preventing lymphocyte circulation through the graft (298, 299). Anergy has been shown to contribute to the induction of tolerance in a number of experimental systems: for example, in a rat renal allograft model, Sayegh *et al.* have demonstrated that anergy may contribute to tolerance induced by intrathymic alloantigen administration (155), and Gao *et al.* have shown that administration of allogeneic splenocytes to mice in the neonatal period leads to anergy of alloreactive $CD8^+$ cells and the acceptance of donor-specific skin grafts (300).

Following the increased attention in recent years focused on the identification and characterization of suppressor or regulatory T cells, it has become apparent that they may play a useful role in achieving allograft tolerance. This is exemplified by the ability of the adoptive transfer of spleen cells, $CD4^+$ cells, or $CD4^+$ cell subsets from tolerant animals to suppress donor-specific graft rejection in secondary recipients in rat (301-304) and mouse (222, 305-307) models. In a clinical study of patients who had long term surviving liver and kidney allografts but were off immunosuppressive therapy, van Buskirk *et al.* demonstrated the presence of cells capable of suppressing donor-specific responses *in vitro* (308). Further evidence for the existence of active regulatory mechanisms in transplantation settings is provided by the phenomenon of infectious tolerance, first described by Waldmann's group, whereby regulatory cells are not only able to suppress alloantigen-specific responses by naïve recipient lymphocytes, but also to convert these recipient cells to a regulatory phenotype (307, 309, 310).

In common with other T cell populations, regulatory cells express antigen-specific T cell receptors and require activation through these receptors in order to exert their suppressive activity. However, prevention of graft rejection does not necessarily require the presence different regulatory cell populations specific for the whole range of alloantigens expressed by the graft: in certain experimental models, induction of tolerance to a single alloantigen is sufficient to allow the acceptance of grafts also expressing other alloantigens, the phenomenon of so-called "linked epitope suppression" (70, 307, 311). This may be explicable by the observation that *in vitro*, although regulatory cells require antigen-specific activation through their T cell receptor in order to act, once activated in this way they are able to suppress responses in a non-antigen-specific manner (312, 313). Thus, activation *in vivo* of regulatory cells specific for a single graft alloantigen (or a subset of such antigens) may be sufficient to suppress rejection of the graft by regulating the activity of effector cells present in the local microenvironment that are specific

Table 1. Examples of studies using gene therapy to modify grafts

Model	Modification	Outcome	Reference
Rat hepatic allografts	FasL expression	Graft survival prolonged	322
Rat renal allografts	FasL expression	Graft survival prolonged	283
Mouse cardiac allografts	FasL expression	Graft rejection accelerated	278
Mouse pancreatic islet allografts	FasL expression	Grafts rejected	276
Mouse pancreatic islet allografts	FasL expression	Grafts rejected	277
Mouse pancreatic islet allografts	FasL expression	Grafts rejected	279
Mouse pancreatic islet allografts	Co-transplantation of myoblasts expressing FasL	Graft survival prolonged	280
Mouse pancreatic islet allografts	Co-transplantation of myoblasts expressing FasL	Graft rejection accelerated	281, 282
Rat to mouse pancreatic islet xenografts	FasL expression	Graft survival prolonged	323
Rat hepatic allografts	CTLA-4-Ig expression	Graft survival prolonged	324
Rat pancreatic islet allografts	CTLA-4-Ig expression	Graft survival prolonged	325
Rat pancreaticoduodenal allografts	CTLA-4-Ig expression	Graft survival prolonged	326
Mouse cardiac allografts	CTLA-4-Ig expression	Graft survival prolonged	327
Mouse pancreatic islet allografts	CTLA-4-Ig expression	Graft survival prolonged	328
Rat to mouse pancreatic islet xenografts	CTLA-4-Ig expression	Graft survival prolonged	323
Rat cardiac allografts	IL-4 expression	No benefit	329
Rat corneal allografts	IL-4 expression	No benefit	245
Mouse cardiac allografts	IL-10 expression	Graft survival prolonged	330-332
Sheep corneal allografts	IL-10 expression	Graft survival prolonged	244
Mouse cardiac allografts	TGF-beta expression	Graft survival prolonged	213, 330
Rat cardiac allografts	Inducible nitric oxide synthase expression	Reduced graft vasculopathy	333
Rat cardiac allografts	ICAM-1 antisense oligonucleotide expression	Reduced graft vasculopathy	334

for other graft antigens. Linked epitope suppression may help to explain the clinical observation that cadaveric renal transplant recipients who have received blood transfusions prior to transplantation show enhanced overall graft survival, and that this beneficial effect is augmented as the number of transfusions is increased, the so-called "transfusion effect" (314-318).

In common with autoimmune disease models, regulatory cells capable of suppressing allograft rejection have been shown to lie predominantly within the $CD4^+$ population (301, 302, 307, 309, 310), with the $CD45RB^{low}$ (in the mouse) (222, 319) and $CD25^+$ (222, 303, 320) subfractions being further enriched for regulatory activity. While these studies isolated regulatory cells from animals that had previously received grafts, data from our own laboratory have demonstrated that treatment of mice with allogeneic blood transfusion under the cover of anti-CD4 antibody generates $CD4^+CD25^+$ regulatory cells that are able to suppress the rejection of donor-specific skin allografts (321); similarly, other studies have shown that $CD4^+CD25^+$ regulatory cells may be generated following oral antigen administration (232, 233). It is thus likely that strategies will be developed that promote the development of regulatory cells in clinical transplant recipients that will contribute to enhanced graft survival, either alone or as an adjunct to other therapies.

12. GRAFT MODIFICATION

Given that there are mechanisms by which allografts can play a role in establishing tolerance, attempts have not surprisingly been made to exploit these mechanisms. One such strategy is the use of gene therapy to modify grafts to increase the efficiency of tolerance induction. Examples of some approaches that have been attempted to date are shown in table 1. These studies have resulted in mixed outcomes: expression of CTLA-4-Ig, IL-10, and TGF-beta has been successful in promoting graft survival, while a beneficial role of IL-4 expression has yet to be demonstrated. As discussed above, expression of FasL has led to an improvement in graft outcome in some settings but a deterioration in others. Despite these mixed results, these observations, together with improvements in technology for gene delivery and expression, suggest that graft modification by means of gene therapy may provide a promising strategy for the future.

13. PERSPECTIVE

Attempts to reduce the reliance of clinical organ transplantation on non-specific immunosuppressive therapy are likely to require a shift away from the paradigm of the use of drugs to prevent T cell activation and towards strategies where the active acquisition of tolerance is facilitated. There is now mounting evidence that the graft itself may make a major contribution to this process, for example by presenting donor antigen under specific conditions throughout the post-transplant course. A fuller understanding of the mechanisms involved may allow the development of strategies to promote the induction of tolerance rather than graft rejection. It is likely that no

single such strategy will be sufficient to achieve this, but that a combination of approaches may allow the field of transplantation research to advance one step closer towards the holy grail of allograft-specific immunosuppression.

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Abbreviations: ACAID: anterior chamber-associated immune deviation, LSEC: liver sinusoidal endothelial cells.

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Send correspondence to: Dr. Mahzuz Karim and Prof. Kathryn J. Wood, Nuffield Department of Surgery, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK. Tel: (44)-1865-221300, Fax: (44)-1865-763545, E-mail: mkarim@doctors.org.uk, E-mail: kathryn.wood@nds.ox.ac.uk