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Permanent survival of organ transplants without immunosuppression: Experimental approaches and possibilities for tolerance induction in clinical transplantation

Andrew Bushell and Kathryn J. Wood

During the past 30 years, organ transplantation has developed from a highly experimental procedure into an important part of routine clinical practice. This is reflected by the fact that graft survival times, which were once considered in terms of days or weeks, are now measured in terms of years or decades, with enormous corresponding benefits for the patient. Much of this improvement is due to the development of sophisticated immunosuppressive drugs that inhibit the rejection response mediated by the immune system of the recipient. However, almost without exception, all of the grafts that are transplanted from one genetically disparate individual to another are eventually rejected. The Holy Grail in transplantation is the development of protocols that lead to transplantation tolerance and provide stable graft function without long-term drug therapy. In this article, we have discussed the need for alternatives to current immunosuppression and reviewed the results of animal models, which suggest that long-term stable tolerance is an achievable goal.

Transplantation is the treatment of choice for most patients with end-stage kidney failure, and in many cases is the only option for patients with progressive heart or liver disease. In addition, transplantation is now seen as a viable treatment for patients with insulin-dependent diabetes mellitus and is a developing possibility for paediatric patients with deficiencies in small-bowel function. The overall success of transplantation is reflected by the number of procedures performed throughout the world. By the end of 1997, almost 416 000 kidney, 46 300 heart and 62 500 liver transplants had been carried out worldwide (Ref. 1).

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Figures for graft survival have improved steadily with time, such that current one-year survival for kidney, heart and liver transplants is 70–90% at most transplant centres (Ref. 1). Such survival depends on a number of factors but the most significant of these is the administration of powerful immunosuppressive drugs. Transplantation between genetically disparate individuals (transplantation of an allograft or allotransplantation) evokes a rapid and potentially destructive alloreactive immune response that, if left unchecked, can lead to almost complete destruction of the transplanted organ within a matter of days. Administration of immunosuppressive drugs (such as the purine analogue azathioprine, the steroid methylprednisolone, the cyclic peptide cyclosporin A and a variety of anti-lymphocyte antibody preparations) attenuates this response and thus prevents acute graft rejection. However, continued graft survival depends on life-long immunosuppression because, except for a very small number of cases (the majority of which involve liver transplantation and should probably be regarded as a special case), withdrawal of immunosuppression results in re-activation of the rejection response, leading to rapid graft destruction.

Limitations of current immunosuppression

Although the immunosuppressive drugs that are currently available are very effective in the short term, substantial problems in four specific areas indicate a pressing need to develop alternative and more sophisticated ways of preventing graft rejection. These areas can be summarised as follows: (1) chronic graft rejection, (2) transplant-associated vasculopathy, (3) infection and (4) cancer.

Chronic graft rejection

During the earliest days of clinical kidney transplantation, graft survival rates after one year were often as low as 30% (Ref. 2), but improvements in immunosuppression, matching of the major histocompatibility complex (MHC) of donors and recipients, and improved patient management have led to current one-year survival figures of 80–90%. However, this remarkable improvement has not resulted in a corresponding increase in long-term graft survival. For example, recent US registry data indicate that although the one-year graft survival rate for first kidney transplants from cadaveric donors performed since 1989 was 82%, half of these grafts fail within 8 years. In fact, the data show that, despite improvements in early graft survival, the rate of delayed graft loss has not changed significantly over the past 20 years (Ref. 3). Chronic rejection is poorly understood and almost certainly involves a number of interrelated factors (see below). One of the most important of these is acute rejection and several studies have shown that increased rates of acute rejection are closely associated with an increased incidence of delayed graft loss (Refs 4, 5). Thus, even though acute rejection episodes can be treated successfully, the graft almost certainly sustains some damage from which it cannot recover.

Transplant-associated vasculopathy

Heart transplantation is now considered to be a realistic therapy for patients with coronary artery disease (CAD), cardiomyopathy and other end-stage heart disease. One-year graft survival figures at most centres approach those of kidney transplants but, as with the kidney, despite good early graft function there is a continual loss of grafts and 10-year survival rates are only 40–50%. Biopsies from some of the earliest successful heart transplants showed evidence of a progressive form of occlusive CAD, and routine biopsy of all transplanted hearts has shown that transplant-associated CAD is an unexpected, yet widespread, complication of heart transplantation (Ref. 6). Of greatest concern is the fact that such disease is seen in young hearts that are transplanted into patients with no previous history of CAD, and that because of its progressive nature the only available treatment is re-transplantation.

The process of transplant-associated CAD is poorly understood but it seems likely that it is simply one manifestation of the wider process of chronic rejection. Immune-mediated damage to the endothelial cells of the coronary arteries shortly after transplant appears to initiate a cycle of damage and repair, leading to cellular proliferation within the blood vessels (intimal thickening), which results in arterial narrowing and ischaemic injury (Refs 7, 8). However, it should also be noted that the accelerated appearance of a form of CAD in patients who have received **only** a kidney transplant suggests that non-immunological factors are also involved. Immunosuppression itself has been implicated in the development of transplant-associated CAD

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because some immunosuppressive drugs are thought to interfere with key aspects of lipid metabolism. Taken together, the data suggest that transplant-associated vasculopathy arises from the immunosuppression itself and from its inability to completely prevent immune-mediated damage.

Infection

The immunosuppressive agents currently used in clinical transplantation are non-specific in nature and cannot distinguish between beneficial immune responses against infectious pathogens and destructive immune responses against the graft. Thus, the administration of immunosuppressive drugs necessary to prevent graft rejection can lead to an increased risk of opportunistic infection. In kidney transplantation, infection is in fact less of a problem than might be expected, probably because routine clinical monitoring allows immunosuppression to be adjusted on the basis of an assessment of graft function and the severity of rejection. The situation is rather different in cardiac transplantation, because graft function is less easy to monitor and the doses of immunosuppressive drugs tend to be higher. During the development of heart transplantation programmes, infection rapidly emerged as a significant problem and, as recently as 1991, infection was the major cause of death in heart transplant patients (Ref. 6). Improvements in the management of patients and changes in immunosuppressive regimens continue to reduce this problem, but according to recent multi-centre data, infection remains a major cause of hospitalisation following heart transplantation (Ref. 9).

Cancer

Immune surveillance against both oncogenic (cancer-causing) viruses and spontaneously arising malignant cells probably plays an important role in restricting the development of tumours. Thus, it might be expected that non-specific immunosuppression would lead to an increase in the incidence of cancer in transplant patients. Several studies have shown that this is indeed the case. For example, registry data from >6000 transplant patients in Australia and New Zealand indicate that the incidence of skin malignancies and non-skin malignancies increases in an almost linear fashion as a function of time following transplantation (Ref. 10). Although the majority of these cases were skin cancers (which can often be treated surgically), the data also showed that after 20 years of continuous immunosuppression there was a 20% incidence of non-skin malignancy. Current regimens to control graft rejection involve lower doses of immunosuppressive agents and rely much more on cyclosporin than in the past, but there is no indication that cancer rates are any lower in what is regarded as the 'cyclosporin era'. Recent registry data support the earlier observations and show that the predicted incidence of non-skin cancer, skin cancer and all cancers after 30 years of continuous immunosuppression is 33%, 75% and 80%, respectively (Ref. 11). The fact that such figures are not restricted to Australasia demonstrates that cancer is a general problem in immunosuppressed patients (Ref. 12). Of even greater potential concern is the fact that a recent report indicates that cyclosporin might actually enhance tumour growth, in a manner that is unrelated to its effect on the immune system (Ref. 13).

The need for transplantation tolerance

All of the problems described above are related (directly or indirectly) to the fact that current immunosuppressive agents are non-selective in their action. Chronic rejection could probably be prevented by administration of very high doses of immunosuppression, but this would inevitably lead to unacceptable increases in transplant-associated CAD, infection and cancer. The **full** potential of transplantation will be realised only when alternatives to non-specific immunosuppression are found. The major aim of transplantation immunology is to develop protocols that prevent immune responses towards the graft but leave the rest of the immune system intact. Transplantation tolerance appears to offer the best hope of achieving this degree of effectiveness and specificity.

What is transplantation tolerance?

In immunological terms, the word tolerance has a very specific meaning, particularly in relation to the development and maturation of the mammalian immune system; however, in 'transplantation', the same word is used far more broadly and is thus rather poorly defined. For the purposes of this discussion, the most straightforward definition will be used. Transplantation tolerance is thus simply defined

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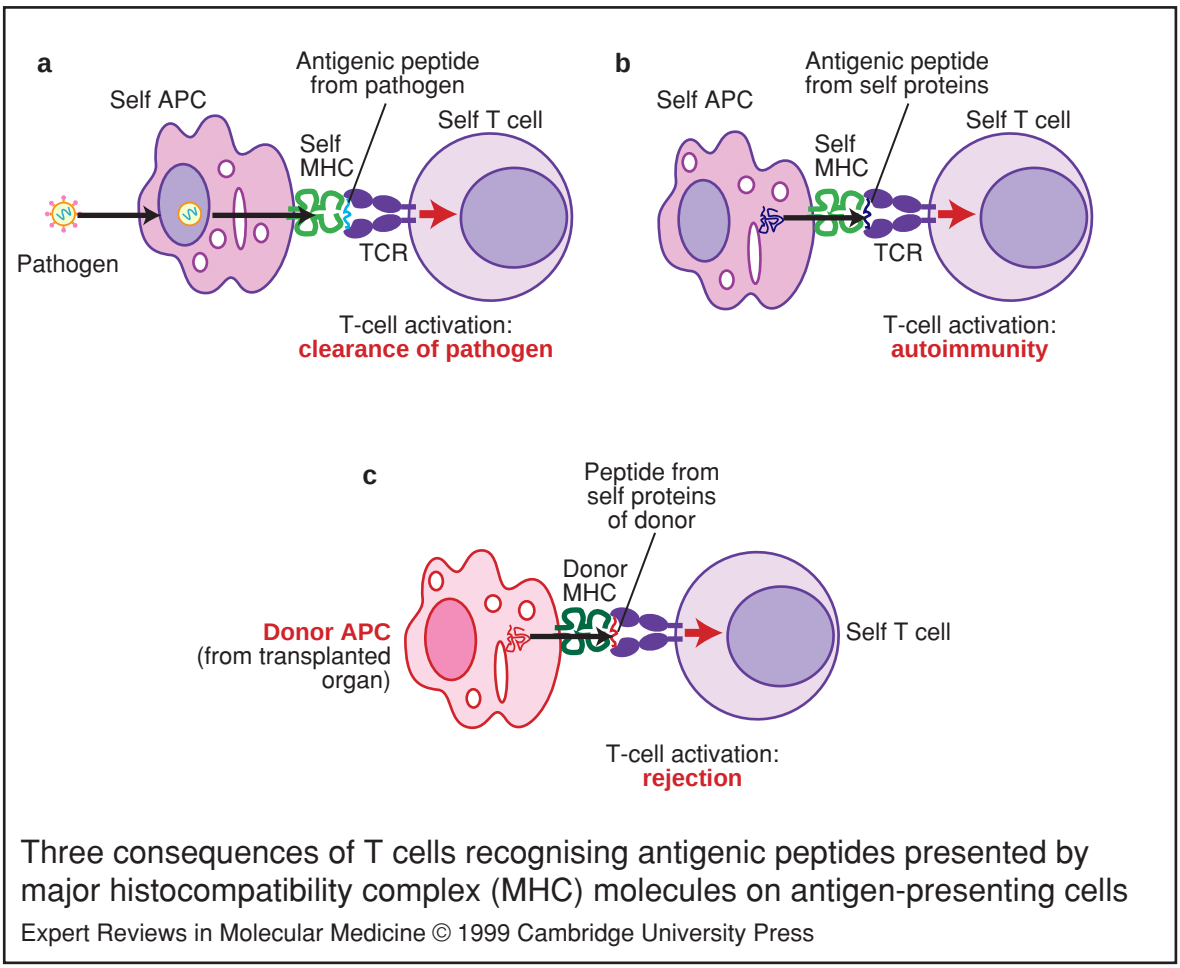

Figure 1. Three consequences of T cells recognising antigenic peptides presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells. (a) During an infection, T cells recognise antigenic peptide fragments of the pathogen that are presented on the surface of MHC molecules. This recognition is mediated by the T-cell receptor (TCR) and stabilised by additional cell-surface molecules (see also Fig. 9). (b) The nature of T-cell development means that potentially self-reactive T cells are produced that can respond to self MHC molecules, even in the absence of infection. (c) In transplantation, T-cell activation in response to foreign (allogeneic) MHC molecules on the surface of donor antigen-presenting cells (APCs) induces destructive immune responses **(fig001abo)**.

as 'the continued survival and function of an allograft without long-term immunosuppression'.

Strategies for transplantation tolerance must target T cells

The final destruction of an allograft might involve most, or perhaps all, of the cellular and non-cellular components of the immune system, but many studies have shown that T lymphocytes (T cells) and particularly $CD4^+$ T cells play an essential, pivotal role in graft rejection (Refs 14, 15, 16, 17). Therefore, the majority of protocols aimed at providing true transplantation tolerance are designed to tolerise T cells. In order to assess the possibilities for the induction of T-cell tolerance to transplanted organs, it is necessary to consider some fundamental aspects of T-cell function and examine the basis of tolerance to self.

The normal function of T cells is to recognise peptide fragments derived from invading viruses, bacteria or other pathogens. As shown in Figure 1, this recognition involves interaction of the T-cell receptor (TCR) with antigenic fragments presented by MHC molecules on the surface of antigen-presenting cells (APCs). This interaction leads to T-cell activation and mobilisation of other cells of the immune system, such as

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cytotoxic T lymphocytes (CTLs), macrophages and antibody-producing B lymphocytes (B cells), which eventually clear the infection.

MHC molecules (class I and class II) are cell-surface proteins encoded within the MHC. They are highly polymorphic, that is many different class I and class II molecules exist in the population. MHC mismatches between donor and recipient activate rejection responses, leading to immune-mediated graft destruction. Matching donors and recipients for MHC molecules reduces the risk of rejection but **complete** matching between unrelated individuals is almost impossible to achieve.

In general terms, each T cell has only one type of TCR on its cell surface, and so in order to be able to respond to a myriad of potential pathogens in the environment, the immune system has evolved mechanisms to generate huge numbers of diverse TCRs by random rearrangement of TCR-gene fragments. It has been estimated that the mammalian immune system has the potential to produce $>10^{12}$ different TCRs and, therefore, a vast number of different T cells. An unavoidable consequence of diversity based on random TCR rearrangements is that many T cells in an individual might be capable of interacting with MHC molecules on the individual's own (self) cells occupied with peptides derived from their own cellular proteins, as well as with proteins derived from pathogens. In the absence of infection, almost all MHC molecules are complexed with self peptides, so an uncontrolled maturation of self-reactive T cells would inevitably lead to systemic autoimmunity and death. Thus, the immune system has evolved at least two distinct mechanisms to prevent autoimmune destruction of self tissues. These are referred to as central T-cell tolerance and peripheral T-cell tolerance.

In transplantation, destructive immune responses are mobilised when recipient T cells recognise genetically distinct MHC molecules on the surface of donor tissue (Fig. 1c). One of the central questions for transplant immunologists is whether the mechanisms of central or peripheral T-cell tolerance, which prevent the development of self-reactive T cells, could also be engaged to deliver tolerance to transplanted organs or tissues.

Central tolerance

During fetal development and the very early neonatal period, haematopoietic stem cells migrate from the bone marrow to the thymus and develop into mature T cells. Surprisingly, studies of the kinetics of T-cell maturation revealed that >95% of all T-cell precursors entering the thymus die before they have an opportunity to leave (Refs 18, 19, 20). This suggested that one potential mechanism for preventing the development of self-reactive T cells was the deletion of T cells in the thymus. Evidence for such deletion was subsequently provided using monoclonal antibodies to follow the fate of potentially auto-reactive T cells (Ref. 21), and deletion was later shown to involve apoptosis followed by macrophage-mediated clearance (Ref. 22). Although the precise detail of events shaping the T-cell repertoire in the thymus is outside the scope of this review, an overall scheme containing the relevant points is shown in Figure 2. Given that T cells have to recognise peptide fragments presented by MHC molecules, those with TCRs that are unable to bind MHC at all are allowed to die because they are of no use to the immune system. The remaining T cells (those that have been **positively selected** for further development) are then tested for their ability to bind to MHC in a complex with self peptides. Those T cells that bind with an avidity above a certain threshold (and that might, therefore, be self-reactive) are deleted by apoptosis (**negative selection**). The remainder, which by definition have a relatively low avidity for self MHC, are allowed to mature fully and then migrate to peripheral lymphoid tissues, where they are able to respond to pathogenic infections (for reviews, see Refs 20, 23, 24, 25).

Generally speaking, the incidence of autoimmune disease in the population is relatively low, which suggests that central tolerance is a very effective way of removing specific T cells. Could the powerful mechanisms of central tolerance be exploited in transplantation?

Direct delivery of donor antigen into the thymus causes specific T-cell deletion

Shortly after it became clear that specific T-cell deletion took place in the thymus, attempts were made to induce donor-specific tolerance in experimental animals by the intrathymic injection of donor-strain cells (Refs 26, 27). Posselt and colleagues injected allogeneic

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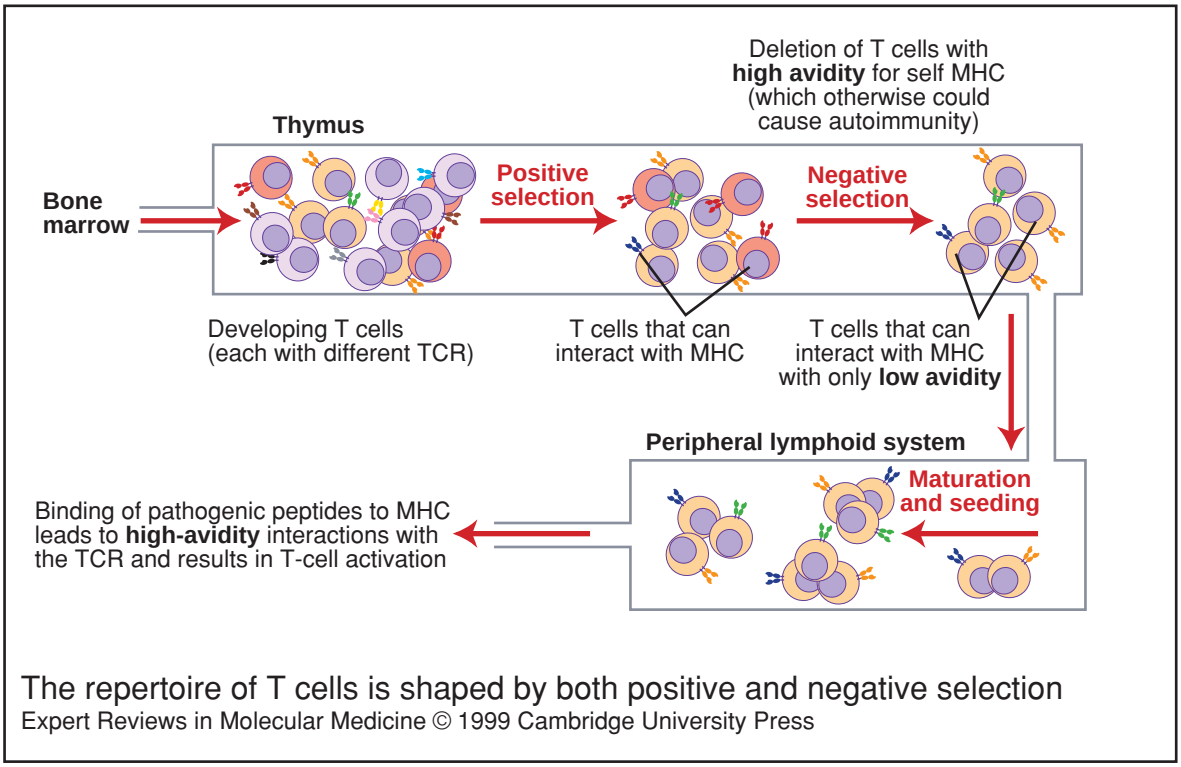

Figure 2. The repertoire of T cells is shaped by both positive and negative selection. T-cell receptors (TCRs) are generated by random gene rearrangements. In the thymus, those T cells with TCRs that are unable to recognise self major histocompatibility complex (MHC) molecules are allowed to die, because they will not play a useful role in the immune system. The T cells that remain are 'positively selected' for further development. Many of these positively selected T cells recognise self MHC molecules with such a high avidity that they would be autoreactive and capable of causing autoimmune disease. These T cells are deleted (i.e. they are killed) in the thymus by negative selection. The remainder (probably <5% of those that originally entered the thymus from the bone marrow) are allowed to mature and enter the peripheral immune system **(fig002abo)**.

(MHC-incompatible) pancreatic islets into both lobes of the thymus of rats that had been made diabetic by the drug streptozotocin. This procedure reversed diabetes but, furthermore, led to true allograft tolerance, because the rats subsequently accepted donor-type islets that were transplanted under the kidney capsule, but rejected islets from a genetically unrelated 'third-party' strain (Ref. 28). The authors speculated that intrathymic injection had led to the elimination of specific alloreactive T cells, but in their model were unable to look directly for clonal deletion. Recently, however, the first clear evidence that intrathymic injection of alloantigen causes specific clonal deletion was obtained by Jones and colleagues, using transgenic mice and a specific anti-TCR monoclonal antibody to follow the fate of alloreactive T cells (Refs 29, 30). An outline of their approach is shown in Figure 3.

In a normal mouse, the fate of alloreactive T cells after their encounter with antigen cannot be studied easily because of their relatively low frequency in the total T-cell population. By using BM3-TCR-transgenic mice, in which practically all of the T cells express the same TCR (which is reactive against the mouse class I MHC alloantigen H-2K^b), such problems are overcome. Using this system, it was possible to ask whether intrathymic injection of specific alloantigen (cells expressing H-2K^b) leads to specific T-cell deletion. Injection of H-2K^b-negative cells, which are ignored by the BM3 T cells, led to a profound deletion of BM3-positive T cells; however, this deletion is almost certainly non-specific because it is followed by a rapid and almost total recovery of T-cell numbers within a few days. In sharp contrast, intrathymic injection of H-2K^b-positive cells not only led to the abrupt initial fall but, more

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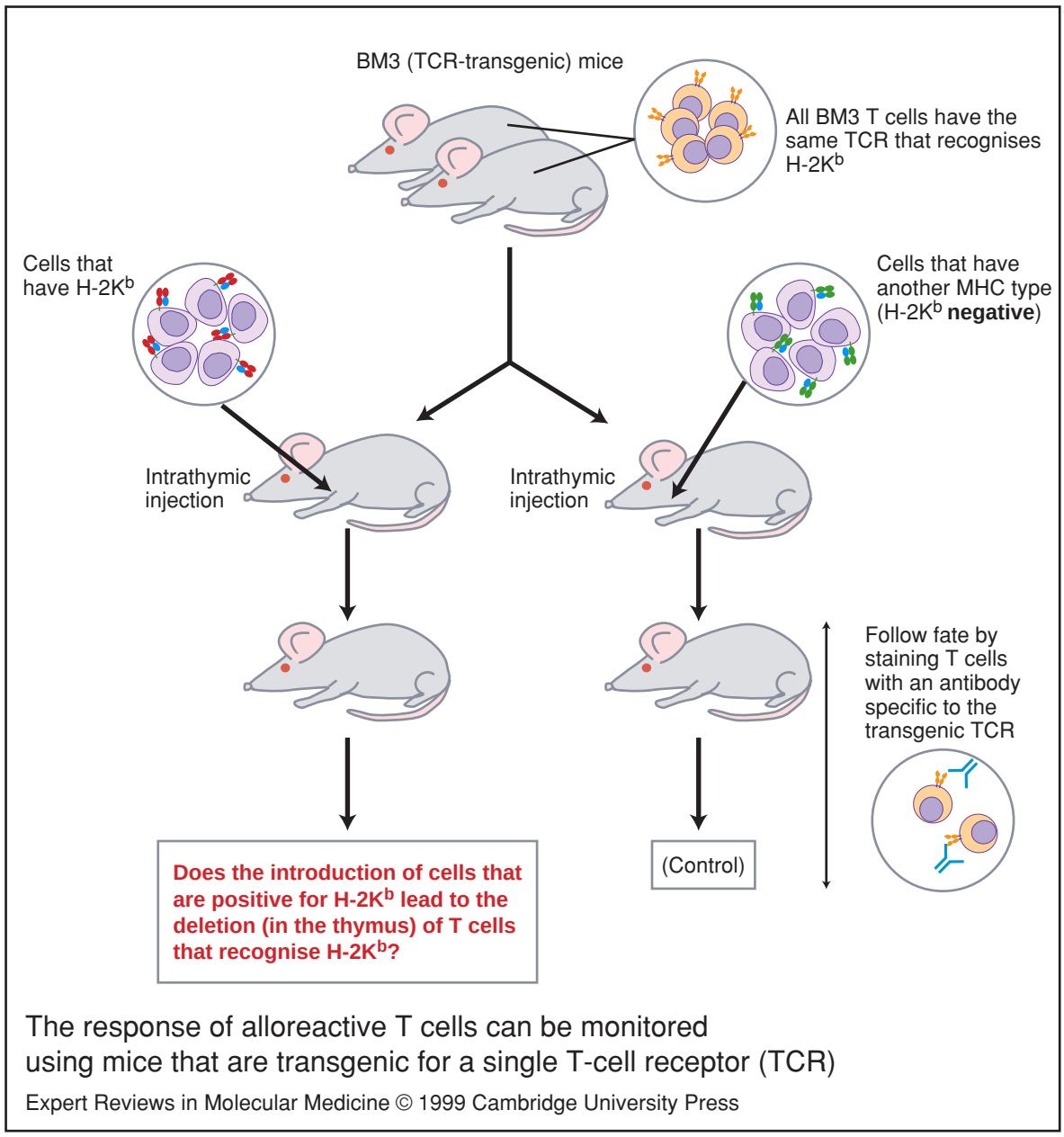

Figure 3. The response of alloreactive T cells can be monitored using mice that are transgenic for a single T-cell receptor (TCR). In a given individual (experimental animal or human), T cells that respond to a genetically different transplanted organ (allograft) might be only a small fraction (perhaps ≤10%) of the total T-cell population, and examining the responses of these relatively rare cells is difficult. This problem (which is referred to as low precursor frequency) can be overcome using a transgenic animal that expresses the same TCR on all of its T cells. In this example, all the T cells are specific for the mouse histocompatibility complex (MHC) molecule H-2k^b. In an attempt to induce transplantation tolerance by negative selection of T cells, this approach has been used to examine the fate of alloreactive T cells, following injection of donor cells directly into the thymus (not shown). Transgenic T cells can be monitored in the animal by staining samples of peripheral blood or other tissue with antibodies that have been previously raised to the TCR **(fig003abo)**.

importantly, resulted in a sustained reduction in the numbers of H-2K^b-reactive T cells, such that 28 days after injection specific deletion remained at ~60% (Ref. 29). Thus, intrathymic injection of specific alloantigen leads to a sustained and specific deletion of donor-reactive T cells.

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Does intrathymic injection of alloantigen lead to transplantation tolerance?

To test this possibility, BM3 mice received an intrathymic injection of H-2K^b-positive cells or H-2K^b-negative cells, combined with an intravenous injection of anti-CD4 and anti-CD8 antibodies to deplete mature peripheral T cells. Twenty-eight days after intrathymic injection, the mice were transplanted with an H-2K^b-positive, fully vascularised cardiac allograft. Intrathymic injection of H-2K^b-positive cells combined with peripheral T-cell depletion led to the indefinite survival of cardiac allografts [median survival time (MST) >100 days], compared with acute rejection in untreated controls (MST of 8 days). The effect was specific, in that neither peripheral depletion alone nor peripheral depletion plus intrathymic injection of H-2K^b-negative cells resulted in more than modest graft survival (Ref. 29). Thus, an intrathymic protocol that has been shown to lead to specific T-cell deletion results in graft survival without further therapy, and therefore to transplantation tolerance. Once established, continued graft survival in this model seems to involve sustained deletion of donor-reactive cells in the periphery (i.e. outside the thymus), a phenomenon that might play an important part in many models of transplantation tolerance (see below).

Peripheral depletion of mature T cells with anti-T-cell antibodies was necessary in the model of Jones and colleagues (Refs 29, 30) because modification of the thymic environment is thought to have an effect only on newly developing thymocytes. However, other work has shown that in some experimental models peripheral depletion is not required for intrathymic injection to be effective (Refs 31, 32). If this were shown to be a general phenomenon, the implications could be significant because there will almost certainly be some clinical reluctance to accept the idea of non-specific T-cell depletion, owing to concerns that such depletion in humans could lead to prolonged immunodeficiency (Ref. 33).

Possible limitations of intrathymic tolerance in clinical transplantation

Studies in intrathymic models have provided important information on the mechanisms of acquired transplantation tolerance, but intrathymic injection might not be the ideal route to tolerance in clinical transplantation for two reasons. First, because the majority of T-cell differentiation takes place during fetal development and in the very early neonatal period, it is not at all clear whether the introduction of allogeneic tissue into the adult human thymus would shape the T-cell repertoire in the same way as it does in rodent models. Second, both lobes of the thymus must be exposed to guarantee efficient injection of alloantigen; this involves opening the chest via a sternotomy. Although intrathymic injection of alloantigen is certainly possible during routine cardiac transplantation, and has been carried out in at least two patients (Ref. 34), it is unlikely that the risks of such a procedure would be considered worthwhile for other organs. However, as discussed below, it might be possible to engage the mechanisms of central tolerance without resorting to direct intrathymic injection.

Chimerism as a route to transplantation tolerance

Most of the clonal deletion of T cells in the thymus is driven by bone-marrow-derived cells (probably dendritic cells; Ref. 35), which present self antigens to developing T cells. If a male mouse of the B.10 strain is crossed with a female of the genetically distinct CBA strain, bone-marrow cells of the developing B.10 x CBA fetus migrate to the thymus and present B.10 **and** CBA antigens to maturing T cells. The resulting F_1 mouse is, therefore, tolerant of both B.10 and CBA self antigens. Would it be possible to engage the powerful mechanisms of central tolerance if donor bone marrow could be introduced into the **peripheral** circulation?

For many years, it has been possible to generate bone-marrow chimeras in rodent models and, indeed, such models provided some of the earliest evidence for positive and negative selection of maturing T cells (Refs 36, 37). Similar approaches were later applied to transplantation models in which bone-marrow-depleted recipient-strain mice were reconstituted with a mixture of both donor and recipient bone marrow (Refs 38, 39). These mixed allogeneic chimeras (which are genetically unaltered by the bone-marrow infusion) accept donor skin grafts without immunosuppression, but are capable of normal B-cell and T-cell responses to other challenges. Although they therefore exhibit true transplantation tolerance, most of these models required whole-body irradiation to allow the

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injected bone marrow to become established. Such an approach is probably unacceptable in routine clinical transplantation. However, recent work has shown that it is possible to achieve a state of mixed chimerism and transplantation tolerance with a much less aggressive conditioning regimen in both mouse (Refs 40, 41) and primate (Ref. 42) transplant models. These protocols involve transient inhibition of T-cell function and low-dose irradiation followed by intravenous infusion with donor bone marrow. Using TCR-transgenic mice analogous to those discussed above (Refs 29, 30), Manilay and colleagues have been able to demonstrate that administration of donor bone marrow into the peripheral circulation leads to tolerance through specific deletion of donor-reactive T cells, indicating that central deletion is possible without recourse to intrathymic injection (Ref. 43). Furthermore, it has also been demonstrated recently that administration of bone marrow without **any** form of peripheral T-cell modification can lead to long-term transplantation tolerance in a mouse model, again probably through the development of mixed allogeneic chimerism (Ref. 44).

Success in experimental models led to several attempts in clinical transplantation to augment conventional immunosuppressive protocols with the peripheral administration of donor bone marrow (Refs 45, 46, 47, 48). Some of the results have been encouraging but, overall, the rates of rejection have not been reduced significantly. Several reasons could account for this including: (1) the inability of the bone-marrow cells to migrate efficiently to the thymus, (2) the deficient maturation and thymic export of T cells in the adult or (3) the disruption of delicate tolerance mechanisms by the co-administration of immunosuppression. Nonetheless, the enormous potential of central tolerance means that this area will continue to be one of intense research.

Peripheral tolerance as a route to long-term graft survival

From a conceptual standpoint, it is clear that central tolerance cannot be the only mechanism in the normal immune system to prevent T-cell-mediated autoreactivity. Although deletion in the thymus removes T cells that respond to the majority of self proteins, there must be proteins that (for a variety of reasons) have little access to the thymus. These might include those expressed in the brain and central nervous system or those that are expressed only after selection of the T-cell repertoire in the thymus has occurred, such as proteins involved in reproduction and pregnancy. Self-reactive T cells **can** be found in the systemic circulation (Ref. 49), yet in the normal course of events they do not lead to autoimmune disease. Understanding the mechanisms of peripheral tolerance might hold the key to permanent allograft survival without long-term immunosuppression.

Pre-transplant blood transfusion

During the earliest days of clinical kidney transplantation, there was great concern that pre-transplant administration of multiple blood transfusions to overcome the anaemia caused by dialysis would immunise potential recipients to donor antigens, and thus lead to accelerated graft rejection. However, it soon became clear that far from increasing rejection rates, pre-transplant blood transfusions appeared to enhance graft survival (Refs 50, 51). Such observations led many transplant centres to adopt a policy of deliberate transfusion of dialysis patients before transplant, and this resulted in a significant increase in graft survival. For example, in one study of >2500 renal transplants, one-year graft survival was 41% in non-transfused patients, compared with 75% in patients given >20 units of blood (Ref. 52). Before the introduction of cyclosporin, pre-operative blood transfusion was the most important factor in determining graft outcome, and it seems highly likely that such transfusions were in fact activating mechanisms of peripheral tolerance.

The blood-transfusion effect can be reproduced in both rat (Refs 53, 54, 55) and mouse models (Refs 56, 57) by donor-specific transfusion (DST), and in many cases leads to indefinite allograft survival without further therapy, in situations where untreated recipients reject their grafts within a few days. Three general mechanisms could potentially explain this phenomenon: (1) peripheral deletion of donor-reactive cells, (2) inactivation of donor-reactive cells and (3) regulation or suppression of donor-reactive cells.

Peripheral deletion of donor-reactive cells

Experiments examining responses to specific antigens, rather than to transplanted allografts, have demonstrated that peripheral deletion of T cells can sometimes explain T-cell tolerance,

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usually when rapid T-cell proliferation leads to activation-induced cell death (Refs 58, 59, 60, 61). It is highly likely that similar events play a role in allograft tolerance. For example, in their intrathymic tolerance model, Jones and colleagues showed that, following tolerance induction by intrathymic delivery of alloantigen (which led to transient **central** T-cell deletion), long-term tolerance was subsequently maintained by the deletion of donor-reactive cells in the periphery, probably owing to the presence of the graft itself (Ref. 30). Although peripheral deletion appears to be operating in these systems and can be very efficient, it is unlikely to explain the blood-transfusion effect. Several studies have shown that tolerance induced by donor-specific pre-treatment can be adoptively transferred to second recipients, which will then accept a donor-strain graft (Refs 62, 63, 64, 65). Adoptive transfer of tolerance seems to be inconsistent with just a deletion of donor-specific T cells.

Inactivation of donor-reactive cells

Under certain conditions, stimulation of T cells with antigen leads to a state of T-cell inactivation (also known as T-cell anergy), in which the T cells are not deleted but instead remain functionally inert (Refs 66, 67). Often this anergy can be reversed by the addition of the T-cell growth factor interleukin 2 (IL-2), suggesting that functional inactivation could be a consequence of defects in IL-2 production or IL-2-dependent responsiveness (Refs 68, 69, 70). Such a process might be involved in the blood-transfusion effect. In an experimental rat transplant model in which kidneys from the DA strain are transplanted into PVG recipients, pre-operative transfusion of donor blood leads to indefinite graft survival, whereas untreated rats reject their grafts within ~8 days. In a series of experiments, Dallman and colleagues removed DA-derived kidney grafts that had been transplanted into either untreated or blood-transfused rats, and examined the cells infiltrating the graft for the ability to kill donor-strain target cells in vitro, as a measure of the rejection response. As expected, cells isolated from rejecting grafts displayed specific cytotoxicity towards donor-strain targets. Surprisingly, however, cytotoxic cells were also readily recovered from blood-transfused rats, and these showed equivalent levels of cytotoxicity towards donor-cell targets, despite the fact that these rats would go on to accept their grafts long term (Ref. 71). Although differences in cytotoxicity were not seen, subsequent experiments in this model of allograft tolerance implicated specific changes in the IL-2 pathway of T-cell activation. Graft-infiltrating cells were isolated from blood-transfused and untreated rats, and examined for critical features of IL-2-driven T-cell responses (Ref. 72). Cells from tolerant animals were characterised by: (1) reduced expression of the interleukin-2-receptor α chain (IL-2Rα), (2) reduced expression of the high-affinity IL-2R receptor, (3) a reduced ability to respond to exogenous IL-2 and (4) an inability to make biologically active IL-2. Taken together, these data indicated that an early defect in the IL-2 pathway of T-cell activation was, at least partly, involved in the tolerance induced by blood transfusion. To test this possibility, rats were blood transfused then given systemic IL-2 for 5 days from the time of transplant. This treatment reversed the blood-transfusion effect, with most rats rejecting their grafts by approximately day 30 (Ref. 72).

Deficiencies in IL-2 production and/or responsiveness have been shown to be a feature of other models of tolerance to alloantigens in vivo. For example, transgenic mice that have been engineered to express the class I alloantigen H-2K^b on insulin-producing β cells of the pancreas are tolerant of H-2K^b, and the β cells are not destroyed (Ref. 73). However, when these mice were crossed with transgenic mice expressing IL-2 under a β-cell-specific promoter (such that the offspring expressed both the alloantigen H-2K^b and IL-2 on the β cells), the mice subsequently developed diabetes because of T-cell activation driven by antigen recognition in the presence of locally produced IL-2 (Ref. 74).

There is no doubt that some models of tolerance involve critical deficiencies in IL-2-driven T-cell activation; however, a wealth of data suggests that tolerance to alloantigen involves far more than simply a collection of passive processes.

Tolerance in many models involves antigen-specific T-cell regulation

Shortly after models of transplantation tolerance were established, many groups demonstrated that tolerance could be transferred to secondary recipients by the adoptive transfer of cells from long-term tolerant animals (Refs 62, 63, 64, 65). Such observations gave rise to the concept of

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donor-specific suppression, and for some time it was suggested that the phenomenon was mediated by a specific subtype of T cells, which were referred to as suppressor T cells. Although there was no doubt that as a phenomenon suppression did exist, attempts to identify and characterise specific suppressor cells were unsuccessful. However, specific suppression continues to be an area of considerable research interest, and it is quite possible that understanding these processes offers the best opportunity for providing stable tolerance in clinical transplantation.

A demonstration of the potential of T-cell regulation (or suppression) was provided by the work of Qin and colleagues (Ref. 75), whose experiments are summarised in Figures 4 and 5. Thymectomised CBA mice were treated with non-depleting antibodies against CD4 and CD8 to cause transient impairment of T-cell function; then they were transplanted with an allogeneic skin graft (Fig. 4a). Such mice accepted their grafts indefinitely, whereas responses to unrelated third-party antigens rapidly recovered to normal levels. Infusion of naive CBA (recipient-strain) T cells into mice with long-term accepted grafts failed to reconstitute rejection, a phenomenon referred to as resistance. Such resistance was shown to be mediated by $CD4^+$ T cells in the tolerant animals. The phenomenon of resistance was investigated further using an adoptive transfer system, summarised in Figure 4b. Spleen cells from both tolerant and naive CBA mice were combined in so-called test-tube CBA mice (i.e. mice depleted of their own T cells), which were then grafted with a donor-specific skin graft. These reconstituted mice were unable to reject their skin grafts, despite having naive CBA cells that in other situations can reject skin grafts acutely. As predicted from the previous experiments, resistance in these reconstituted animals was mediated by $CD4^+$ T cells from the tolerant mice.

Although similar phenomena had been shown previously in other systems, the experimental outline (shown in Fig. 5) provided a unique and revealing approach to the examination and understanding of this process. CBA mice were available that had been made transgenic for the human leukocyte molecule CD2. Such mice express human CD2 (hCD2) on the surface of all of their T cells, but in every other respect are identical to normal CBA mice. The availability of an antibody to hCD2 allowed the specific removal of these cells in vivo without affecting the responses of any non-transgenic T cells introduced into these animals. CBA-hCD2 mice were made tolerant (using thymectomy followed by treatment with non-depleting anti-CD4 plus anti-CD8 antibodies) and skin grafted as in previous experiments. Spleen cells from naive CBA mice were then transferred to these tolerant recipients of CBA-hCD2 skin grafts. At either 7 days or 14 days after transfer, the tolerant hCD2 cells were depleted using anti-hCD2 antibody, and the mice were transplanted with a fresh donor-specific skin graft. Animals depleted of the tolerant hCD2 cells at 7 days after naive cell transfer rejected both their second and original skin grafts (MST ~22 days for both groups); however, in clear contrast, mice depleted of the tolerant hCD2 cells after 14 days accepted both the fresh skin grafts and the original skin grafts for >60 days. The most straightforward interpretation of these data is that during 14 days (but not 7 days) of coexistence the tolerant hCD2 cells had conferred tolerance upon the infused naive CBA cells, such that they acquired a tolerant phenotype. They were thus tolerised by an 'infective' process.

These experiments were subsequently extended by Chen and colleagues, using a similar approach in the high-responder (i.e. difficult to tolerise) BALB/c to CBA mouse-strain combination (Ref. 76). They induced tolerance to BALB/c heart grafts using non-depleting anti-CD4 and anti-CD8 antibodies. Adoptive transfer of spleen cells from tolerant animals transferred tolerance to naive CBA mice, such that without any immunosuppression these secondary recipients accepted BALB/c hearts indefinitely (MST >100 days). In a remarkable demonstration of the potential of T-cell regulation in this system, they then showed that tolerance could be transferred from these secondary recipients to another set of naive mice, and subsequently repeated in a serial fashion up to nine times. Tolerance transfer in this system required the infusion of 50×10^6 spleen cells. Because it was calculated that after nine serial transfers mice in the last group would have contained a maximum of only 190 cells from the original tolerant mice, Chen and colleagues concluded that the transfer of tolerance could be explained only by expansion of the tolerant cells or by recruitment of naive recipient cells into a regulatory T-cell population. The data of Qin and colleagues discussed above

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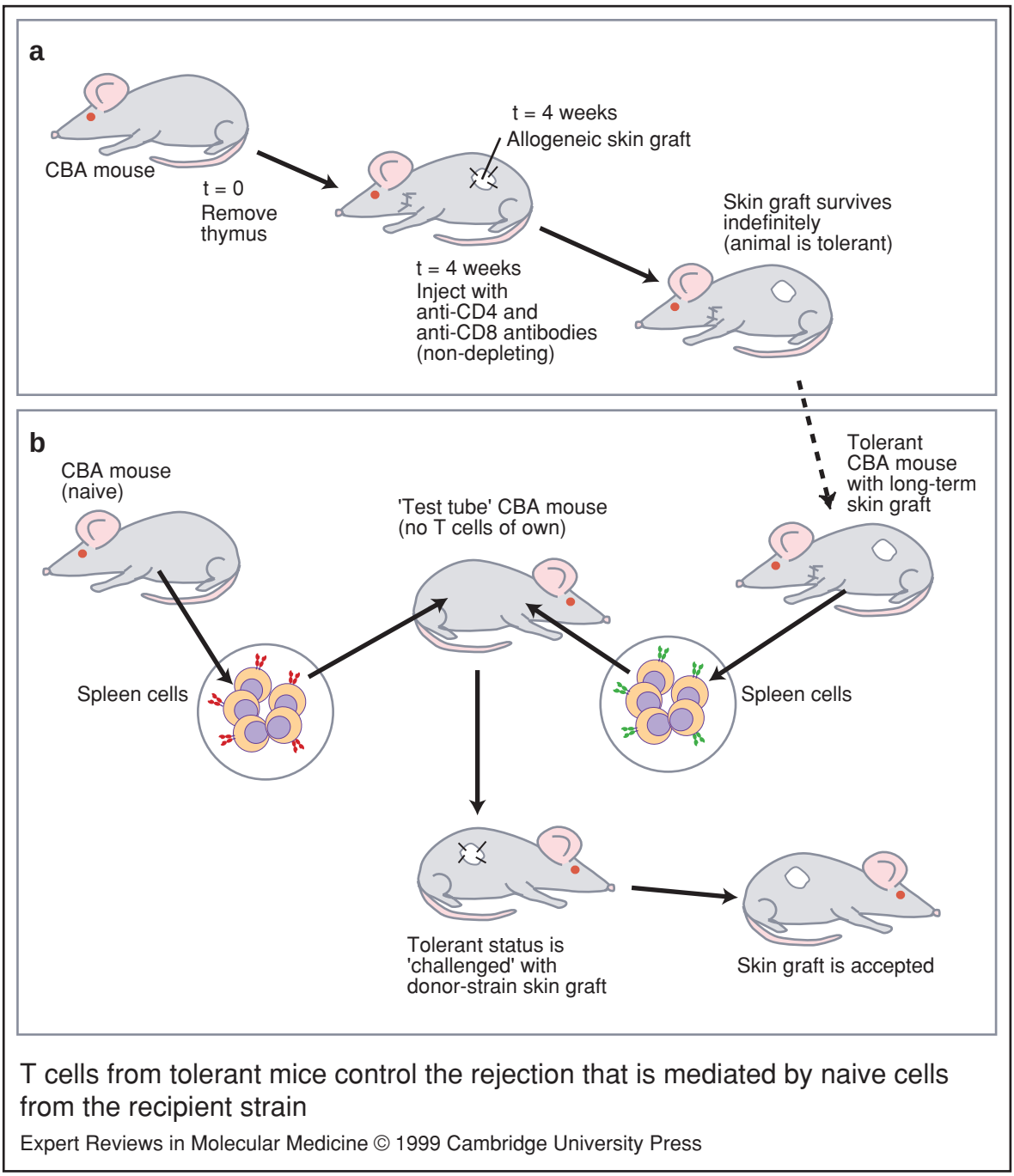

Figure 4. T cells from tolerant mice control the rejection that is mediated by naive cells from the recipient strain. (a) Tolerance to a skin graft (which was mismatched with the recipient for multiple minor transplantation antigens) can be induced by removal of the thymus (thymectomy), followed by administration of non-depleting anti-CD4 antibodies plus anti-CD8 antibodies. (b) T cells that were harvested from mice with long-term surviving grafts and then transferred to 'test-tube' mice (i.e. mice with no T cells of their own) prevent naive cells from rejecting a fresh skin graft (naive cells are those that have no 'experience' of the antigens in question). Such results suggest that the tolerant mice contain regulatory cells that are capable of suppressing naive T-cell responses **(fig004abo)**.

(Ref. 75) appear to rule out an expansion of the **original** tolerant cells, because administration of the anti-hCD2 antibody would have removed both the original cells and any that had arisen

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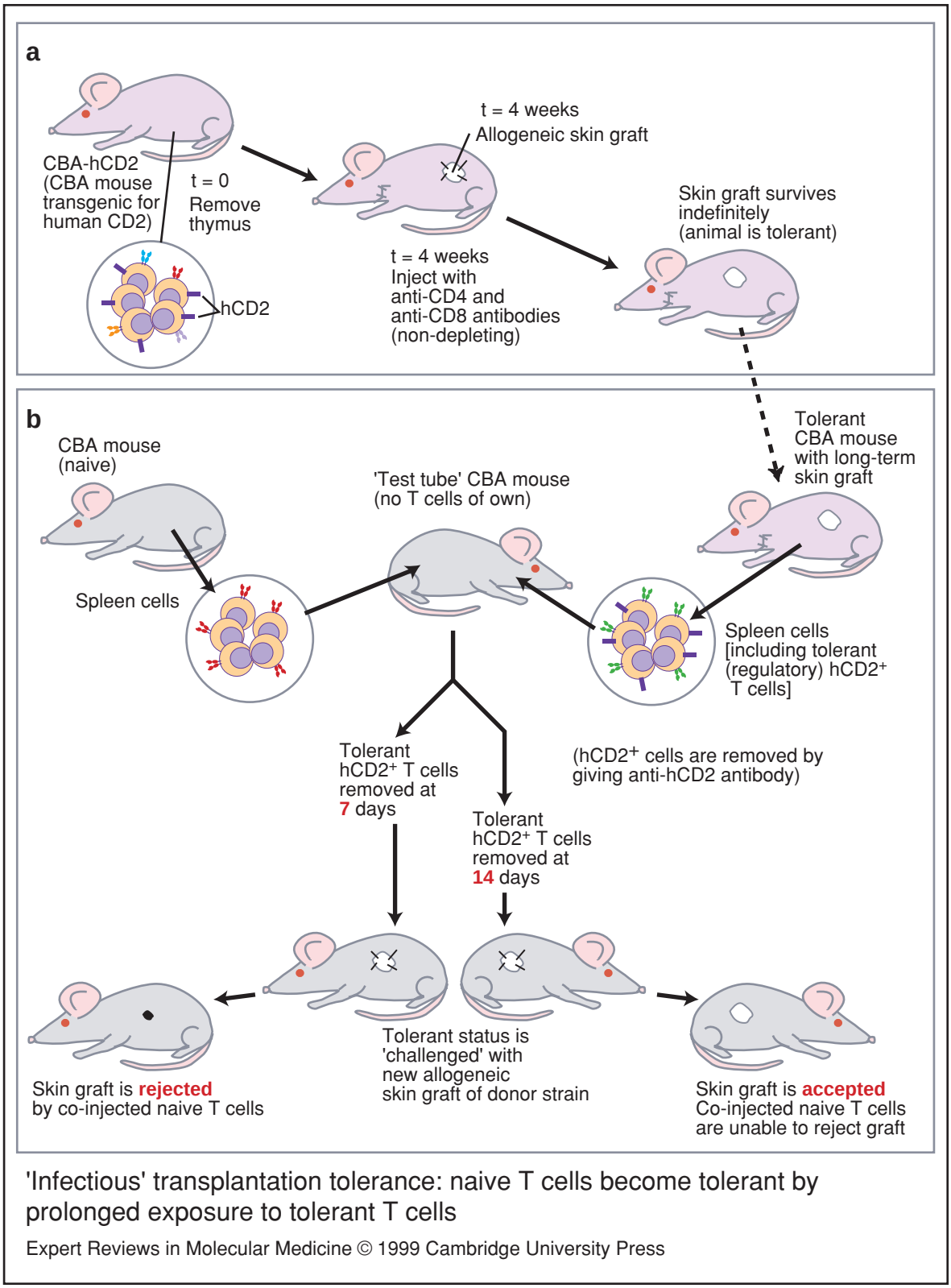

'Infectious' transplantation tolerance: naive T cells become tolerant by prolonged exposure to tolerant T cells

Figure 5. 'Infectious' transplantation tolerance: naive T cells become tolerant by prolonged exposure to tolerant T cells (*see next page for legend*) **(fig005abo).**

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Figure 5. 'Infectious' transplantation tolerance: naive T cells become tolerant by prolonged exposure to tolerant T cells. The same tolerance induction protocol (as in Fig. 4) was applied to transgenic mice that expressed human CD2 (hCD2) on all of their T cells. (This model allowed the selective removal of the tolerant T cells at different times after injection of naive recipient-strain cells.) When the tolerant and naive cells were allowed to co-exist in the recipients for 7 days before removal of the tolerant $hCD2^+$ cells, fresh skin grafts were rejected. However, when the cells were allowed to co-exist for 14 days, removal of the tolerant T cells did not lead to rejection. These experiments suggest that the naive T cells become tolerant as a result of prolonged exposure to tolerant T cells **(fig005abo)**.

by cell division. Conversion of naive cells into regulatory cells thus seems to be the most likely, but surprising, explanation.

Regulation depends on T-cell recognition of donor MHC molecules

As shown by the above examples, perioperative (at the time of transplant) treatment with anti-T-cell antibodies is a powerful way of inducing long-term graft survival and tolerance. The antigen specificity of tolerance based on T-cell regulation in such models suggests that regulatory T cells develop as a direct consequence of contact between recipient T cells and donor MHC molecules, and that this contact is prevented from activating a destructive response only by the presence of the antibodies themselves. Although the contact that is necessary for the development of a regulatory T-cell population leads to tolerance in the long term, it is likely that it causes some initial graft damage, which eventually leads to a decline in the long-term graft function. For example, in a mouse heart-transplant model, perioperative administration of two doses of a depleting anti-CD4 antibody leads to the indefinite survival of B.10 cardiac allografts transplanted into C3H recipients (Ref. 77). In this system, mice with long-term surviving allografts are tolerant, because second donor-specific heart grafts transplanted after removal of the primary graft are accepted indefinitely (Ref. 78). Significantly, the function of these secondary hearts (as assessed by palpation and electrocardiography) was far superior to that of the primary grafts, suggesting that although the recipients were indeed tolerant, this was achieved at the cost of some damage to the primary graft itself. Clearly, in the clinical situation, the induction of tolerance in a patient at the cost of irreversible graft damage is unlikely to be acceptable.

Are there ways of inducing donor-specific tolerance that do not rely on the graft making the first contact with the recipient immune system?

Donor antigen combined with anti-CD4 antibody leads to tolerance based on specific T-cell regulation

The potential of anti-CD4-antibody therapy and the historical impact of pre-transplant blood transfusion in clinical transplantation led our group to examine a combined treatment approach in which blood transfusion was given under the cover of anti-CD4 antibody. In this protocol, recipient mice are pre-treated with two small doses (typically 25–50 μg) of a depleting anti-CD4 antibody to partially inhibit T-cell function, and this is combined with a single DST to provide a source of donor MHC alloantigen. An important feature of this protocol is that the animals are then rested without further treatment (usually for 28 days) to allow their immune systems to recover from the non-specific effects of the antibody therapy, and are transplanted only when their general immune responses have otherwise returned to normal. Recipients pre-treated in this way are specifically unresponsive to donor alloantigens, in that donor-specific hearts are accepted indefinitely (MST >100 days), whereas those of an unrelated third-party strain are rejected. Indefinite graft survival is entirely dependent on the combined anti-CD4/DST treatment, because mice pre-treated with either DST alone or anti-CD4 alone reject their grafts (MST ~20 days; Refs 77, 79).

In this model, mice with long-term surviving grafts are tolerant, because continued graft survival is independent of any further immunosuppressive treatment and the adoptive transfer of spleen cells transfers tolerance to untreated secondary recipients. Tolerance in this model appears to involve $CD4^+$ regulatory T cells, as shown by the fact that depletion of $CD4^+$ cells at the time of transplant allows naive recipient cells to reconstitute rejection (Ref. 80). The ability to transfer tolerance from recipients with long-term surviving grafts is not a new observation, but we have recently shown that regulatory cells in this model arise as a direct consequence of anti-CD4/DST pre-treatment alone (Ref. 81).

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Spleen cells harvested from pre-treated mice on day 0 (the time at which they would normally be transplanted) transferred tolerance to naive, unmodified recipients, which accepted donor-specific grafts indefinitely without additional therapy (MST >100 days).

This type of approach has two advantages over other experimental protocols. First, the initial contact between the recipient immune system and donor MHC antigen, which is essential for the development of regulatory cells, is made with cells in the blood transfusion rather than with cells of the allograft itself. Second, several experimental studies have shown that the development of tolerance is a time-dependent process, which suggests that a regulatory population must reach a certain maturation and/or size to be effective (Refs 82, 83). In our model, the delay of 28 days between pre-treatment and transplant provides enough time for this cell maturation or cell expansion to occur, so that the graft is afforded some protection from the outset. We believe that early protection from immune-mediated damage will be an important factor in the development of tolerogenic protocols for clinical transplantation.

Conceptual difficulties of donor-specific pre-treatment

Donor-specific pre-treatment in experimental transplant models is straightforward because of the availability of inbred mouse and rat strains, and would certainly be a possibility in living-related or living-unrelated kidney transplantation. However, the majority of transplants use organs obtained from cadaver donors, such that donor-specific pre-treatment would be virtually impossible. Furthermore, because the availability of a cadaveric donor organ cannot be predicted in advance, pre-treatment at a specified time before transplant would also be practically impossible. However, there might be solutions to these two problems.

Specificity

The blood-transfusion protocols that led to spectacular improvements in early graft outcome in the 1970s and early 1980s (Ref. 52) used transfusions of random (in terms of MHC alloantigens) blood drawn from blood banks. Although still far from clear, it seems likely that by exposing the recipient's immune system to many different MHC molecules, a state of partial non-responsiveness was established in a large number of **different** clones of recipient T cells. If some of the MHC molecules expressed on the graft were the same or very similar to those previously encountered in the random transfusions (which is very likely because of the conserved expression of MHC molecules in geographical regions and ethnic groups), it is probable that the magnitude of the immune response towards the graft itself would be reduced. It is, therefore, possible that random blood transfusions could be combined with transient immunotherapy to provide a realistic means of inducing unresponsiveness in cadaveric transplantation. In fact, in the mouse heart model described above, random blood transfusion was shown to be as effective as DST when given in combination with anti-CD4 antibody (Ref. 79).

Timing of pre-treatment

Many models of tolerance that are based on active regulation indicate that this regulation is a time-dependent phenomenon. For example, in the anti-CD4/DST model discussed above, transplantation 28 days after pre-treatment leads to indefinite graft survival, whereas transplantation 42 days after pre-treatment leads to graft rejection (Ref. 77). Furthermore, it has been demonstrated in several models that, in the absence of the tolerising antigen, tolerance decays with time and virtually normal immune responses are eventually restored (Refs 82, 84, 85). Overall, the data indicate that after any type of pre-treatment protocol that leads to tolerance induction, transplantation must be carried out within a relatively narrow time window. This is clearly possible in experimental models, and might be possible in living-related or living-unrelated transplantation; however, pre-treatment at a specified time before cadaveric transplantation would be a problem.

A potential solution to the problem of pre-treatment timing was suggested by Benjamin and Waldmann (Ref. 86), who demonstrated that mice pre-treated with the protein antigen human gamma globulin and anti-CD4 antibody induced stable tolerance to human gamma globulin, which could be maintained by repeated exposure to the antigen alone. In an attempt to extend this observation to a transplant situation, we modified the anti-CD4/DST model described above, such that the recipient mice were pre-treated with DST under the cover of anti-CD4 antibody and then

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re-challenged with DST alone at 28 days, 42 days and 56 days later. The mice were then transplanted 14 days after the final DST, 70 days after the initial pre-treatment. These mice accepted their grafts, with an MST of >100 days compared with 15 days for the controls (Ref. 79). These data demonstrated that, once established, tolerance to an allograft can be maintained by repeated re-exposure to the initial tolerising antigens. Such observations suggest at least a theoretical solution to the problem of timing of tolerance induction in clinical transplantation. For example, patients awaiting a kidney transplant are maintained on dialysis until a suitable organ becomes available. Pre-treatments designed to establish a state of unresponsiveness (e.g. random blood transfusion combined with targeted immunotherapy) could be carried out during this time, and the unresponsive state maintained by repeated challenge with random blood transfusions before transplant. With suitable modifications, this approach could also probably be extended to patients awaiting other organs.

Immunodominance and linked-epitope suppression in transplantation tolerance

Rejection responses can be directed towards any MHC molecules that differ between the donor and recipient, but it is clear that in a specified donor–recipient combination some MHC molecules stimulate more vigorous responses than others. They are thus said to be immunodominant. If tolerance can be induced towards these immunodominant molecules, it might not be necessary to make an individual tolerant to **all** of the MHC molecules that are likely to be encountered on the donor organ. For example, C3H (H-2^k) mice reject B.10 (H-2^b) cardiac allografts within ~8 days because they recognise at least four H-2^b MHC molecules as foreign. However, when C3H mice were pre-treated with their own cells that had been genetically modified to express just one of these four donor MHC molecules (the immunodominant molecule H-$2K^b$), graft survival was increased fivefold despite the presence of the other three foreign MHC molecules on the graft (Ref. 87).

How can exposure to only one donor MHC molecule lead to the prolonged survival of a graft that is mismatched for at least three (and probably more) MHC molecules, each of which can induce powerful immune responses in their own right? The answer might lie in linked-epitope suppression.

Linked-epitope suppression is a phenomenon by which tolerant or regulatory T cells are able to suppress the responses of T cells recognising other MHC molecules. This effect is summarised in Figure 6. In this schematic, a recipient has been made tolerant of MHC molecule 'a' (e.g. by pre-treatment with cells bearing MHC 'a' plus anti-CD4 antibody), which results in a population of T cells that are tolerant of MHC molecule 'a'. If the recipient then receives a transplant expressing other MHC molecules but not MHC 'a' (Fig. 6c), the result is a normal T-cell response, leading to prompt graft rejection. However, transplant of a graft expressing MHC 'a' as well as MHC 'b' and MHC 'c' (probably on the same or adjacent cells) activates the tolerant cells, which then suppress the responses towards MHC 'b' and MHC 'c', resulting in diminished responses towards the graft (Fig. 6b).

Linked-epitope suppression has been demonstrated in vivo in a number of transplant models. For example, Wong and colleagues used a CBK–CBA mouse model to explore the consequences of tolerance to the mouse MHC class I molecule H-$2K^b$ (Ref. 88). As shown in Figure 7, the cells of CBK mice express H-$2K^b$ in addition to the normal CBA MHC molecules (H-$2K^k$, L^k, D^k, I-A^k and I-E^k). CBA mice pre-treated with CBK bone marrow in combination with anti-CD4 antibody develop tolerance to H-$2K^b$, such that B.10 hearts expressing H-$2D^b$ and I-A^b in addition to H-$2K^b$ are accepted indefinitely. Thus, tolerance to H-$2K^b$ results in tolerance to additional MHC molecules, probably through linked-epitope suppression. An even more dramatic demonstration of this effect was seen when mice were tolerised in the same way but were then transplanted with hearts from F_1 donors. BALB/c x **CBA** F_1 hearts express CBA H-2^k MHC molecules **plus** BALB/c H-2^d MHC molecules. These grafts were rejected acutely because the H-2^d molecules were recognised as foreign (Fig. 7d, lower right). However, when pre-treated CBA recipients were transplanted with BALB/c x **CBK** F_1 hearts (the only difference is the presence of H-$2K^b$ in addition to the other MHC molecules), the grafts were accepted indefinitely. Thus, the presence of H-$2K^b$ (to which the mice had been made tolerant) resulted in diminished rejection responses towards the BALB/c MHC molecules and long-term graft

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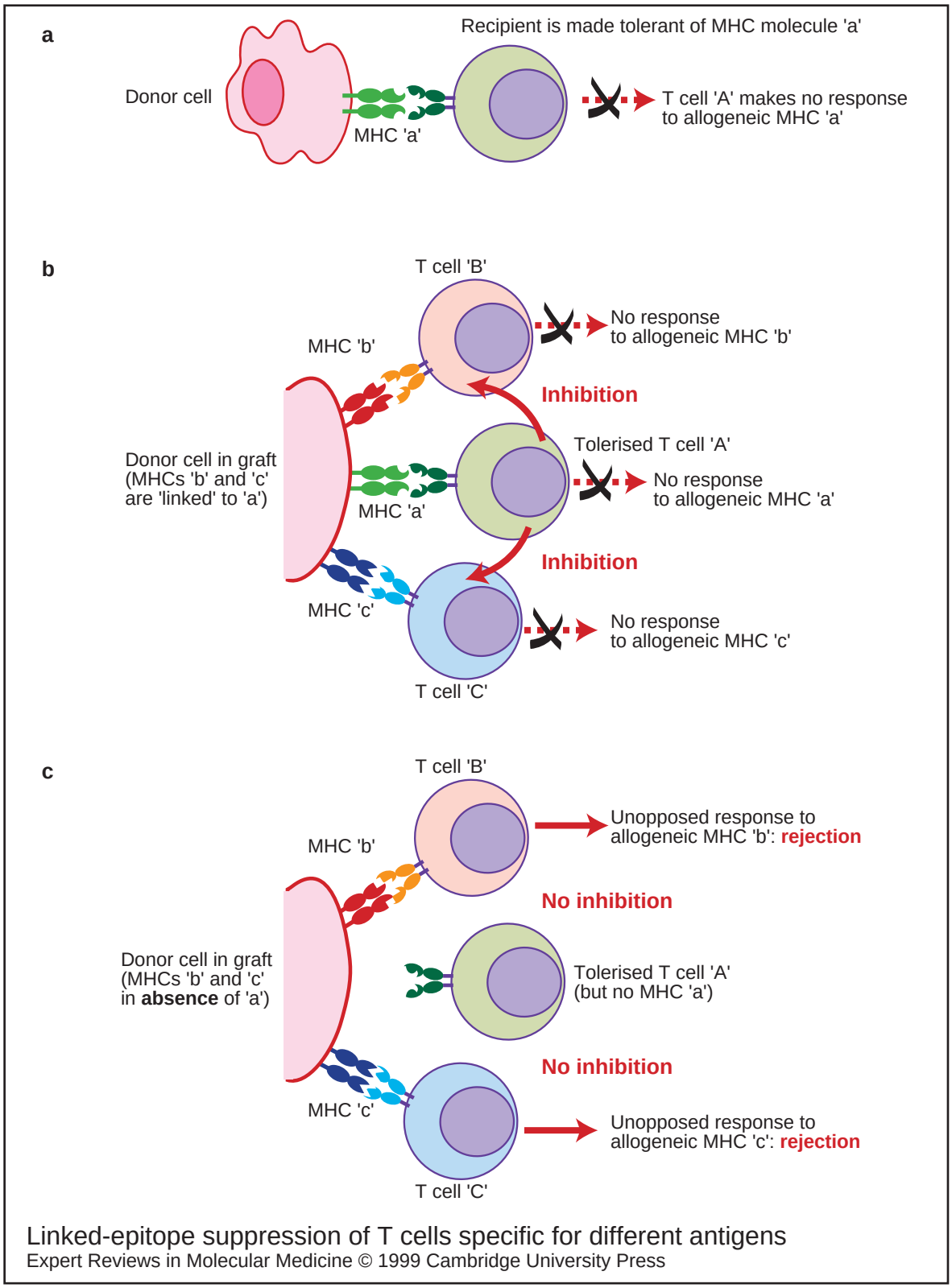

Figure 6. Linked-epitope suppression of T cells specific for different antigens (*see next page for legend*) **(fig006abo).**

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Figure 6. Linked-epitope suppression of T cells specific for different antigens. Experimental evidence indicates that if T cells are made tolerant to a given antigen, they can subsequently suppress the responses of other T cells, providing that the tolerising antigen is presented to the T cells by the same antigen-presenting cell. This phenomenon is of considerable interest in transplantation where, for example, T cells that are tolerant of the (hypothetical) major histocompatibility (MHC) molecule 'a' might be capable of suppressing T-cell responses to the other MHC molecules 'b' and 'c'. The existence of such linked-epitope suppression offers the hope that it might not be necessary to tolerise human transplant recipients to all of the MHC antigens that might be encountered on an allograft **(fig006abo)**.

survival (Fig. 7c). Similar results have been reported in other models of tolerance across full MHC-mismatch barriers (Ref. 76) and in models of tolerance to multiple minor transplantation antigens (Ref. 89), indicating that linked-epitope suppression is a relatively robust phenomenon.

The underlying mechanisms of linked-epitope suppression are not fully understood. Recent in vitro evidence suggests that regulatory cells might compete with other allo-reactive T cells for co-stimulatory molecules on the surface of APCs (Refs 90, 91, 92), but other data indicate that an inhibition of IL-2 production (Ref. 93) or the local production of interleukin 4 (IL-4; Ref. 94) might also be involved. What is clear, however, is that in the design of protocols for potential clinical use, exposure of the recipient to all of the MHC molecules that are likely to be encountered on the donor organ might not be necessary. Thus, pre-treatments based on random blood transfusion combined with transient T-cell inactivation remain of considerable interest.

The Th1–Th2 paradigm in transplantation

One of the primary roles of activated T cells is the production and secretion of cytokines, which act on other cells including those of the immune system. Most cytokines are produced by $CD4^+$ T cells, which can be subdivided into Th1 cells and Th2 cells on the basis of their pattern of cytokine secretion (Refs 95, 96). Th1 and Th2 cells produce many cytokines in common, but some (the so-called signature cytokines) are restricted to a particular subtype. For example, the signature cytokines for Th1 cells are IL-2 and interferon gamma (IFN-γ), whereas for Th2 cells they are IL-4, interleukin 10 (IL-10) and interleukin 13 (IL-13). In vitro, Th1 and Th2 cells have the capacity for the reciprocal regulation of differentiation and expansion, which is mediated by the cytokines they secrete (see Fig. 8). Significantly, such regulation has also been demonstrated in vivo in animal models of parasitic infection and autoimmune disease (Refs 97, 98, 99, 100).

In both animal models and clinical transplantation, rejection has often appeared to correlate with the detection of Th1 cytokines rather than Th2 cytokines (Refs 55, 101, 102, 103). Such observations are the basis of the Th1–Th2 paradigm in transplantation. In its simplest form, this model predicts that if rejection correlates with a Th1 T-cell bias, then the opposite situation (tolerance) might involve a dominant Th2 response (for reviews, see Refs 104, 105). Could enforced deviation towards a Th2-dominated response (perhaps by the manipulation of the cytokine environment) lead directly to tolerance? This idea is certainly attractive and although there are ways of enhancing Th2 responses and diminishing Th1 responses (Refs 106, 107, 108), the available data do not support a simple correlation either between a Th1 bias and rejection or between a Th2 bias and stable engraftment. In a recent survey of 15 immune-activation genes in clinical kidney transplantation, no direct evidence could be found in support of the Th1–Th2 paradigm for either rejection or stable graft function (Ref. 109). A similar breakdown of the paradigm is also seen in many experimental transplant models. For example, the acute rejection of (1) allogeneic islets in IL-2-deficient (i.e. IL-2 knockout) mice (Ref. 110), (2) hearts in IFN-γ knockout mice (Refs 31, 111) and (3) hearts in IL-2–IFN-γ double-knockout mice (Y. Li and T. Strom, pers. commun.) appears to rule out a strict requirement for either of these Th1 cytokines in graft destruction. As far as IL-4 is concerned, the prolonged survival of allogeneic islets (Ref. 112) and hearts (Refs 113, 114) in IL-4-deficient (i.e. IL-4 knockout) mice does not support an absolute role for this Th2 cytokine in long-term engraftment.

However, other data in the literature suggest that although Th2 cytokines are not essential for prolonged graft survival, they might at least be involved. For example, in a perioperative anti-CD4 model, Mottram and colleagues have shown that long-term surviving heart allografts contained infiltrating T cells that were positive for

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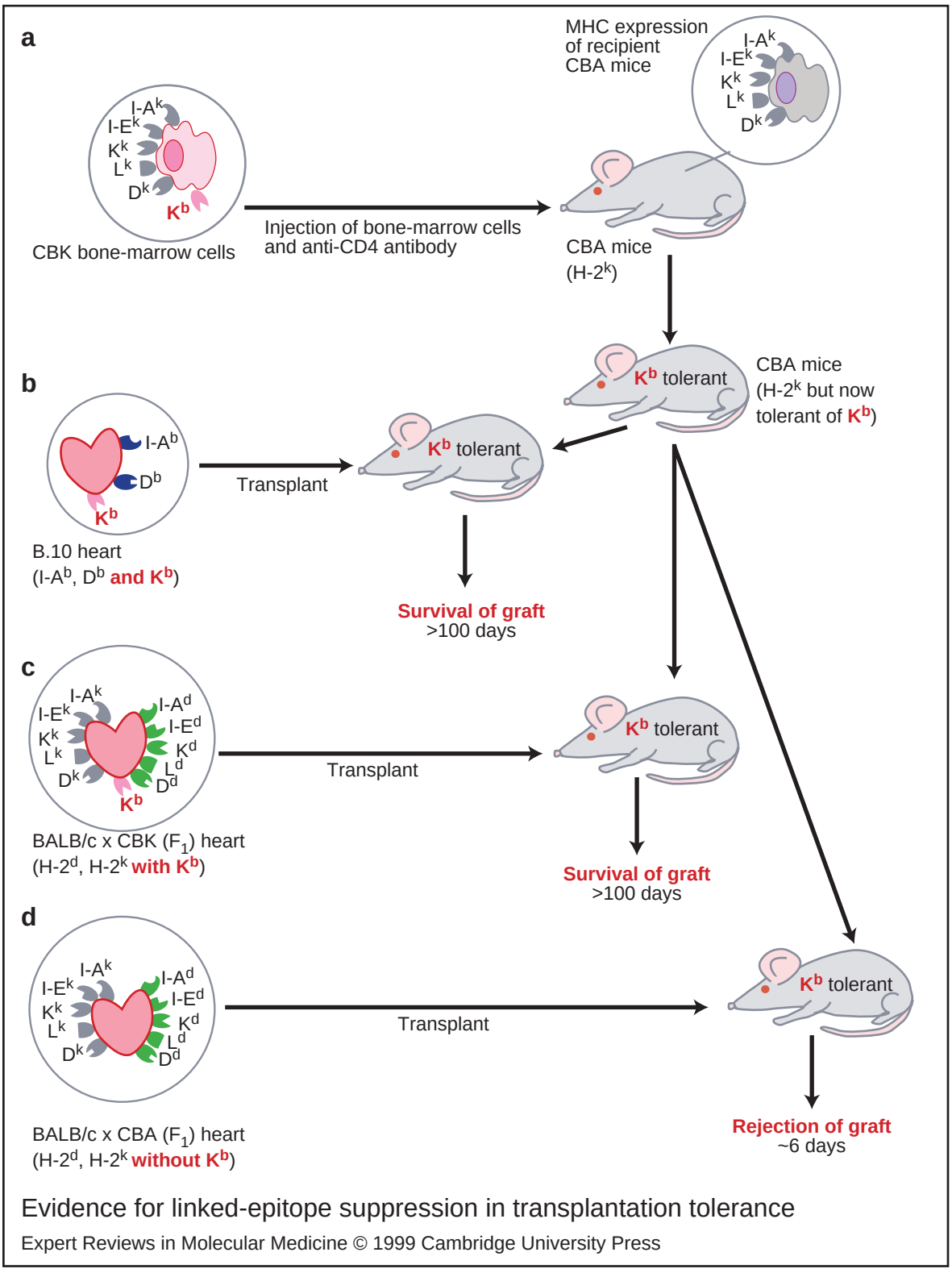

Figure 7. Evidence for linked-epitope suppression in transplantation tolerance (*see next page for legend*) **(fig007abo).**

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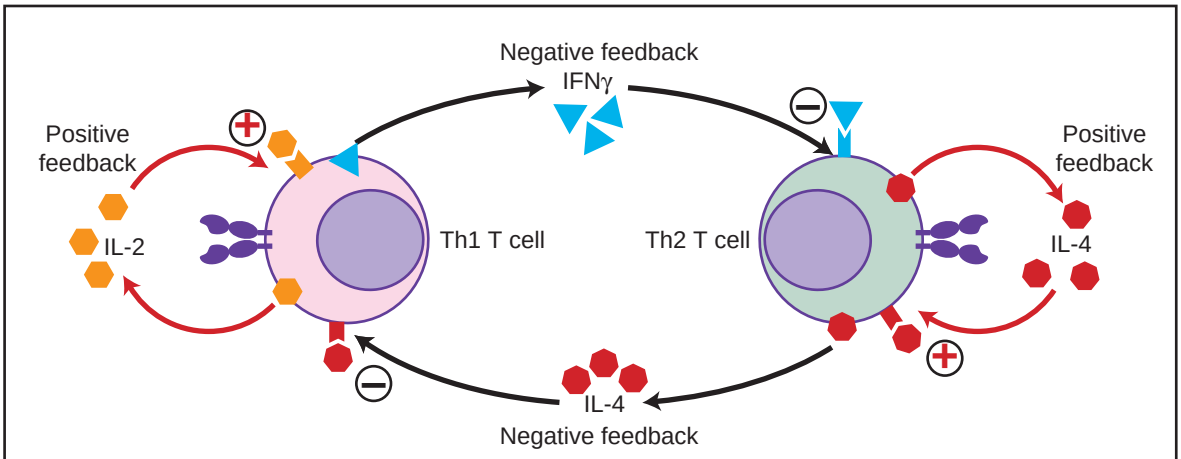
Figure 7. Evidence for linked-epitope suppression in transplantation tolerance. (a) To induce tolerance to H-2K^b, CBA mice were pre-treated with bone-marrow cells from donor CBK transgenic mice under the cover of tolerising anti-CD4 monoclonal antibody. CBK mice are identical to CBA mice, but also express the major histocompatibility complex (MHC) class I molecule K^b. Thus, the recipient CBA mice recognise K^b as an alloantigen, but become tolerant to it because of the effects of the anti-CD4 antibody. (b) The pre-treated (tolerised) mice subsequently accept a heart transplant from B.10 mice, even though, in addition to the tolerising K^b antigen, the heart cells express D^b and I-A^b (which the recipient has never been exposed to before). This tolerance to more than one MHC antigen is probably the result of linked-epitope suppression. This effect was even more dramatic when tolerant mice were transplanted with hearts from BALB/c x CBK or BALB/c x CBA F_1 mice. BALB/c x CBK hearts express the same five H-2^k antigens but also five H-2^d MHC antigens; however, they also express the tolerising antigen K^b (from the transgenic CBK mouse parent). The K^b tolerance imparts tolerance to the five H-2K^d antigens, because they are present on the same antigen-presenting cells, and these hearts were accepted for >100 days (c). BALB/c x CBA hearts, in contrast, express at least five H-2^d MHC antigens but, most importantly, do not express K^b; thus, the hearts from BALB/c x CBA mice were rejected (d) **(fig007abo)**.

IL-4 and IL-10, whereas this was not seen in rejecting grafts (Ref. 101). In addition, it has been shown recently that neutralisation of either IL-4 or IL-10 prevented long-term survival of heart allografts in mice that were treated with anti-LFA-1 (leukocyte function antigen 1) plus anti-ICAM-1 (intracellular adhesion molecule 1) antibodies (Ref. 102). Sirak and colleagues have shown recently that whereas wild-type C57BL/6 mice treated with either the immunosuppressive compound gallium nitrate or anti-CD4 antibody show prolonged survival of DBA/2 hearts, the same protocols are much less effective in IL-4-deficient C57BL/6 recipients (Ref. 115). In another model using anti-CD4 antibody, Onodera and colleagues have demonstrated that tolerance could be transferred from rats with long-term heart allografts to lightly irradiated syngeneic secondary recipients (Ref. 116). Although there was no evidence for the involvement of Th2 cytokines in the **primary**-graft recipients, there was a selective up-regulation of IL-4 and IL-10 in



Th1 and Th2 T cells can regulate each other (reciprocal regulation)

Figure 8. Th1 and Th2 T cells can regulate each other (reciprocal regulation). Th1 and Th2 T cells secrete a number of the same cytokines, but some (signature cytokines) are unique to either T-cell subset. The Th1 cytokine interferon gamma (IFN-γ) and the Th2 cytokine interleukin 4 (IL-4) have been shown in vitro to inhibit the differentiation and/or expansion of Th2 and Th1 subsets, respectively. Thus, IFN-γ inhibits the development of Th2 cells, and IL-4 inhibits the development of Th1 cells. Such observations, combined with the fact that Th1 cytokines are often (though not always) implicated in graft rejection, have led to the suggestion that immune deviation to a Th2-dominant T-cell response might be responsible for tolerance in experimental transplant models. This is the basis of the so-called Th1–Th2 paradigm **(fig008abo)**.

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secondary recipients, following adoptive transfer and donor-specific transplantation. In a direct examination of the effect of IL-10 on engraftment, Qin and colleagues have demonstrated that retrovirus-mediated delivery of viral IL-10 at the time of transplantation can extend allograft survival in a non-vascularised mouse heart model (Ref. 117). Based on our adoptive-transfer studies, we have recently suggested that individual Th2 signature cytokines, such as IL-4, might normally play a redundant role in T-cell tolerance and are essential only under the most stringent of situations (Ref. 81). Such a possibility is consistent with at least one report in the literature (Ref. 118). If this is indeed the case, then manipulation of cytokine environments (e.g. at the time of initial exposure to alloantigen) might be a useful addition to other tolerance-induction protocols but is unlikely to lead to tolerance in its own right.

Blockade of T-cell functions as a route to transplantation tolerance

As indicated in Figure 1, T cells recognise donor tissue as foreign via the TCR, but this recognition is stabilised and enhanced by a number of adhesion molecules and co-stimulatory cell-surface molecules. Some of these are shown for a $CD4^+$ T cell in Figure 9. The interaction between the TCR and MHC itself is of relatively low affinity (i.e. ~1 x 10^{-5} M; Refs 119, 120), which indicates that productive T-cell interactions with APCs (i.e. self APCs in the case of normal responses against pathogens or APCs from the donor graft in the case of transplantation) are absolutely dependent on these additional interactions. The disruption of such contacts is potentially an attractive way of inhibiting responses towards the graft, and might provide an effective route to long-term tolerance.

Anti-CD4 antibodies and T-cell blockade

As discussed above, anti-CD4 antibodies have proved to be very effective in experimental models; furthermore, there has been a limited introduction of their use in clinical transplantation (Refs 121, 122, 123, 124). Their exact mode of action in vivo is not clear. Inhibition of close contact between the TCR and the MHC by steric hindrance seems likely, but it is also possible that antibody binding delivers negative signals to the T cells (Refs 125, 126, 127). The fact that anti-CD4 antibodies target those cells known to play an essential role in rejection and can lead to the development of regulatory T cells suggests that such antibodies are an effective way of limiting graft rejection. However, their effectiveness in clinical transplantation is yet to be established.

Adhesion molecules as targets for immunotherapy

Adhesion molecules play an essential role in the interactions between T cells and APCs (for reviews, see Refs 128, 129). Prominent among many such interactions are those between LFA-1 and members of the ICAM family. In a striking example of the importance of adhesion molecules in allograft rejection, Isobe and colleagues transplanted BALB/c hearts into C3H mice given anti-LFA-1 antibody plus anti-ICAM-1 antibody for the first 6 days post-transplant (Ref. 130). In this donor–recipient strain combination, untreated recipients rejected their grafts within 8–10 days, but 9/9 of the mice in the antibody-treated group accepted their grafts indefinitely, and showed no histological evidence of rejection. More importantly, the protocol led to specific tolerance, because animals with long-term surviving hearts accepted donor-strain skin grafts but rejected those from an unrelated third-party strain. These experiments have recently been extended to a number of different strain combinations in the mouse with similar encouraging results (Ref. 102). Other adhesion molecules that might be important candidates for blockade include members of the selectin family, which mediate the initial binding of leukocytes to the surfaces of endothelial cells (Refs 131, 132). Inhibition of such interactions might prevent accumulation of T cells within the graft.

Co-stimulation molecules as targets for T-cell blockade

In order to become fully activated, T cells not only need to bind to the MHC but also require additional co-stimulatory signals (e.g. recognition of the MHC alone in planar lipid membranes fails to activate T cells, and can lead to T-cell inactivation or anergy; Ref. 133). A number of such signals are probably provided to T cells, but almost certainly the most important are those delivered through CD28, by interaction with its ligands CD80 and CD86 (B7.1 and B7.2, respectively; Refs 134, 135, 136, 137). The importance of these signals has suggested that blockade of CD28–B7 interactions could be an

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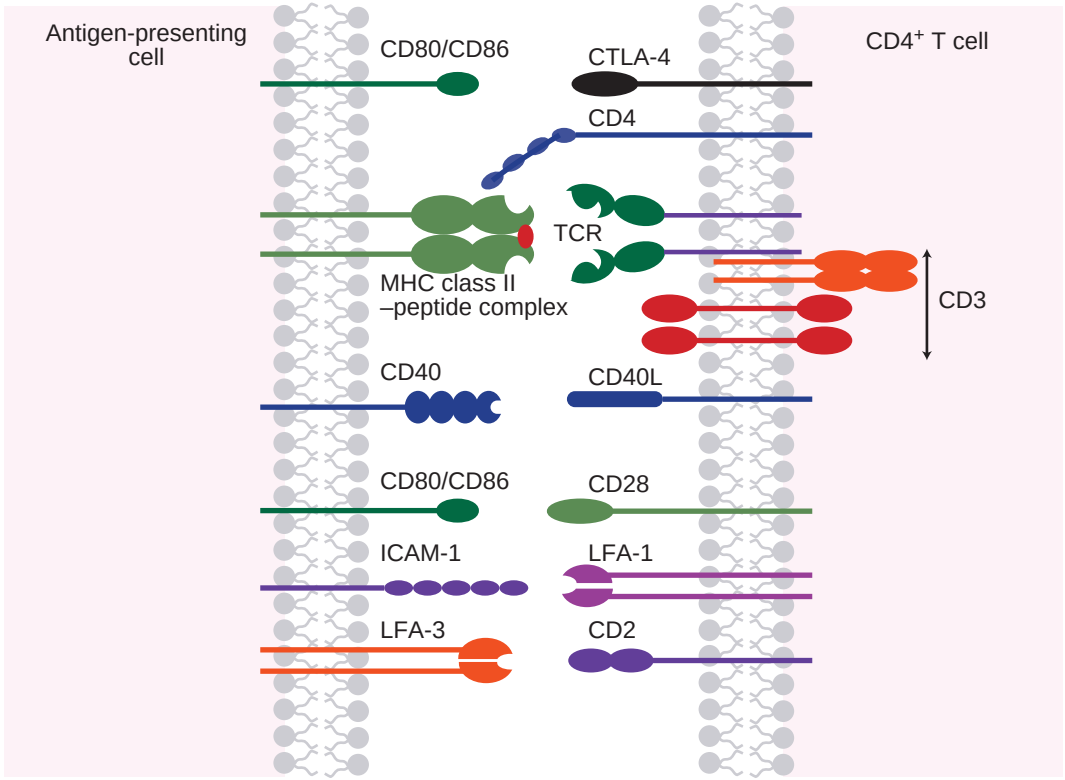

Some of the cell-surface molecules involved in T-cell activation that are potential targets for immunotherapy

Figure 9. Some of the cell-surface molecules involved in T-cell activation that are potential targets for immunotherapy. The interactions between the T-cell receptor (TCR) and major histocompatibility complex (MHC) class II-peptide complexes (also shown in Fig. 1) are fundamental to T-cell activation, but additional interactions between T cells and antigen-presenting cells (APCs) are essential for effective T-cell function. Some of these are summarised here for a $CD4^+$ T cell. Among the interactions that have attracted particular interest in transplantation tolerance are those between: (1) CD80 (or CD86) and CD28, (2) CD40L (also known as CD154) and CD40 and (3) ICAM-1 (intracellular adhesion molecule 1) and LFA-1 (leukocyte function antigen 1) **(fig009abo)**.

effective method of inhibiting T-cell responses towards an allograft.

In addition to interacting with CD28, B7.1 and B7.2 bind CTLA-4, a second molecule on T cells that is homologous to CD28 but has a significantly higher affinity for B7.1 and B7.2. This higher affinity is thought to be important in the role of CTLA-4 as a negative regulator of T-cell responses in vivo (Refs 137, 138, 139, 140), and has been exploited in attempts to deliver transplantation tolerance. Recombinant DNA technology has been used to produce the fusion protein CTLA4-Ig, which comprises an Fc portion of an antibody (i.e. immunoglobulin; Ig) joined to the C-terminal end of CTLA-4. This fusion protein retains its ability to bind members of the B7 family, and can be used as a soluble reagent to block B7 family members binding to CD28. In animal models, CTLA4-Ig has been shown to be very effective at inhibiting the rejection of both xenogeneic and allogeneic grafts, and in some circumstances can lead to tolerance (Refs 141, 142, 143, 144, 145). Significantly, CTLA4-Ig seems to be most effective when combined with donor antigen (usually DST), which probably activates donor-reactive T cells, such that they are more susceptible to subsequent blockade.

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Although blocking CD28–B7 signals is effective in these experimental models, the redundancy of signalling interactions between T cells and APCs suggests that blockade of additional signals might be required for reliable T-cell tolerance. Interactions between CD40 on APCs and CD40L (gp39 or CD154) on T cells (Fig. 9) seem to be particularly important, not only for antibody production by B cells but also for T-cell activation (Ref. 146). The ability of CD40 or CD40L blockade to inhibit the development of autoimmune disease (Ref. 147) has led a number of groups to explore similar possibilities in transplant models. The enormous potential of such an approach has recently been demonstrated by Larsen and colleagues, who examined the effects of CTLA4-Ig and MR1 (an anti-CD40L antibody), administered either separately or in combination (Ref. 148). In a BALB/c to C3H mouse heart-transplant model, either reagent given alone led to prolonged graft survival, with MSTs of 50 days and 70 days for CTLA4-Ig and anti-CD40L, respectively. However, histological examination of the transplanted hearts in both groups showed extensive tissue necrosis and coronary artery vasculopathy, indicating that a rejection response had occurred. In marked contrast, combined administration of CTLA4-Ig plus anti-CD40L led to indefinite graft survival in 100% of recipients, but more impressively this protocol appeared to protect the grafts from either acute or chronic rejection because, on histological examination, they were almost identical to normal hearts.

A further demonstration of the potential for this type of therapy was shown in the same paper in a skin-graft model (Ref. 148). The acceptance of MHC-mismatched skin grafts, unlike that of vascularised grafts, is difficult to achieve, and few protocols provide more than a modest prolongation of skin-graft survival. This difficulty is emphasised in these experiments by the fact that, unlike in the heart model, C3H mice treated with either CTLA4-Ig or anti-CD40L antibody rejected BALB/c skin grafts at similar rates to untreated controls (MST 13 days). However, perioperative administration of CTLA4-Ig plus anti-CD40L antibody led to indefinite skin-graft survival, with good hair growth on the graft and essentially normal histology. Such results have led to a successful extension of this approach to xenograft animal models (Ref. 149) and also to a pre-clinical primate kidney model, in which 2/2 animals remained healthy and rejection free 150 days post-transplant (Ref. 150). However, neither of these recipients was truly tolerant, because both monkeys eventually rejected their grafts. Based on preliminary data, which suggested that long-term graft survival could be achieved using anti-CD40L antibody alone, Kirk and colleagues then developed a regimen in which rhesus monkeys were treated with the anti-CD40L antibody hu5C8 twice on the day of transplant, followed by additional doses on days 3, 10, 18 and 28, and then once each month for 5 months (Ref. 151). This extended hu5C8 monotherapy protocol led to impressive long-term graft survival in 8/9 recipients, with three of the animals remaining rejection free for >510 days. More importantly, at least five of the animals continued to accept their grafts long after the final dose of antibody (some for >6 months). Similar observations using hu5C8-induction therapy have been reported recently in a pre-clinical pancreatic-islet transplant model in the rhesus monkey (Ref. 152). Islet engraftment, long-term function and insulin independence were achieved in 6/6 animals for up to 476 days post-transplant. Most significantly, when hu5C8 therapy was discontinued in three animals ~12 months post-transplant, all three remained normoglycaemic without further immunosuppression.

The results of these two important pre-clinical trials demonstrate that anti-CD40L monotherapy can lead to operational transplantation tolerance in non-human primates and pave the way for the introduction of such strategies in clinical transplantation. However, as discussed below, the introduction of such protocols into clinical transplantation will not be straightforward.

Application of tolerance protocols in clinical transplantation: substantial problems remain unresolved

Blockade of signals through CD28 and CD40L either alone or in combination is clearly a very successful way to prevent graft rejection, but additional observations in experimental models have highlighted two significant problems. The first of these might be restricted only to CD28 and CD40L blockade, whereas the second is a general problem that will have to be addressed when any potentially tolerogenic protocol is considered for clinical transplantation.

Although blockade of the CD28 and CD40L pathways of T-cell co-stimulation leads to long-

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term engraftment in both mouse heart and skin models and in a primate model, the protocols do not appear to lead to true tolerance (Ref. 151; C. Larsen and T. Pearson, pers. commun.) It is clear that such protocols appear to meet the criteria of transplantation tolerance, which have been described in the introduction of this review article; however, the data of Kirk and colleagues (Ref. 151) suggest that long-term graft acceptance in their model depends largely on T-cell **ignorance** or T-cell **inactivation**. This might be because CTLA4-Ig and anti-CD40L antibody are so effective at preventing T-cell–APC contact that, as well as preventing the activation of destructive responses, the development of regulatory (beneficial) cells is also inhibited. If **active** T-cell regulation is seen as the most promising approach to delivering life-long tolerance to an allograft, complete inhibition of such T-cell contacts might in fact be counter-productive.

The second and much more challenging problem concerns the way in which potentially tolerogenic protocols are introduced into clinical transplantation. Early graft survival is now so good that it would be unethical to introduce any tolerogenic protocol in the absence of the best immunosuppressive protocols that are currently available. Thus, any new treatment would have to be introduced in addition to conventional therapy. The major uncertainty then is whether tolerance would develop in the presence of such immunosuppression. A clear example of the potential problem is shown in protocols using CTLA4-Ig plus anti-CD40L antibody. These two reagents in combination can lead to indefinite graft survival; however, the addition of the immunosuppressive drug cyclosporin A to such protocols has resulted in acute graft failure in at least two transplant models (Refs 148, 153). In a mouse heart model using anti-CD4 antibody as the primary immunosuppressive agent, a similar detrimental effect of cyclosporin A has also been reported, although the inhibitory effect varied with the type of protocol used (Ref. 154). Of perhaps even greater concern is the finding that, in the pre-clinical rhesus-monkey model described above (Ref. 151), the addition of conventional immunosuppression to the anti-CD40L-antibody protocol reduced graft survival significantly.

The question of the introduction of tolerance protocols in clinical transplantation has recently been the subject of a working party in the USA, under the auspices of the US National Institutes of Health (Ref. 155). The panel acknowledged the growing body of evidence that suggests that conventional immunosuppressive drugs might prevent the induction of true transplantation tolerance and also accepted that tolerogenic protocols can be evaluated in humans only if standard immunosuppression is withheld. They concluded that the most appropriate setting for the introduction of tolerogenic protocols would be in either kidney or pancreatic-islet transplantation in adults, because in both situations rescue immunosuppressive therapies are well established, and in the event of irreversible graft failure, patients could be returned to dialysis or treated with insulin. The guidelines that were drawn up by the expert panel should form the basis for the evaluation of tolerance protocols in clinical transplantation.

Concluding remarks

Animal models have shown conclusively that the induction of transplantation tolerance in an adult immune system is an achievable goal. There is a wealth of encouraging data to suggest that under the right circumstances intrinsic immune regulatory mechanisms can be activated, and that once established, tolerance is a self-sustaining process, which requires no further therapy. The challenge for the early years of the new millennium is to understand the mechanisms involved in these processes and use this understanding to develop equally effective tolerance protocols in humans.

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Further reading, resources and contacts

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Features associated with this article

Figures

Figure 1. Three consequences of T cells recognising antigenic peptides presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells (fig001abo).
Figure 2. The repertoire of T cells is shaped by both positive and negative selection (fig002abo).
Figure 3. The response of alloreactive T cells can be monitored using mice that are transgenic for a single T-cell receptor (TCR) (fig003abo).
Figure 4. T cells from tolerant mice control the rejection that is mediated by naive cells from the recipient strain (fig004abo).
Figure 5. 'Infectious' transplantation tolerance: naive T cells become tolerant by prolonged exposure to tolerant T cells (fig005abo).
Figure 6. Linked-epitope suppression of T cells specific for different antigens (fig006abo).
Figure 7. Evidence for linked-epitope suppression in transplantation tolerance (fig007abo).
Figure 8. Th1 and Th2 T cells can regulate each other (reciprocal regulation) (fig008abo).
Figure 9. Some of the cell-surface molecules involved in T-cell activation that are potential targets for immunotherapy (fig009abo).

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