

Organ transplantation—how much of the promise has been realized?

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Since the introduction of organ transplantation into medical practice, progress and optimism have been abundant. Improvements in immunosuppressive drugs and ancillary care have led to outstanding short-term (1–3-year) patient and graft survival rates. This success is mitigated by several problems, including poor long-term (>5-year) graft survival rates, the need for continual immunosuppressive medication and the discrepancy between the demand for organs and the supply. Developing methods to induce transplant tolerance, as a means to improve graft outcomes and eliminate the requirement for immunosuppression, and expanding the pool of organs for transplantation are the major challenges of the field.

Historical overview

Burns suffered by victims of aerial bombardment in World War II inspired Sir Peter Medawar's now famous studies in transplantation immunology. Seeking to understand skin graft rejection, he observed that rabbits rejected foreign skin grafts more quickly if the animals were re-transplanted with a graft from the same donor¹. At the same time, however, Ray Owen noted that nonidentical Freemartin cattle that share a common placenta fail to reject skin grafts from littermates but not from other cattle². Collectively, these findings showed that the immune system could respond to transplantation antigens by the induction of memory or, alternatively, by the induction of tolerance. In 1953, Billingham, Brent and Medawar, in what is probably the most famous single paper in transplantation immunology, described the creation of acquired immunological tolerance to alloantigens in mice³. The following year, Joseph Murray's team at the Brigham and Women's Hospital, building on roughly fifty years of progress in vascular surgical techniques, performed a successful renal transplant between identical twins⁴.

Current status of organ transplantation

Since the birth of the field, progress in transplantation medicine has been rapid. Chemical immunosuppression with corticosteroids and 6-mercaptopurine was first used to enable transplantation between nonidentical individuals in the early 1960s. The introduction of newer immunosuppressive agents and improvements in surgical techniques and ancillary

care have made transplantation a routine and preferred therapy for treatment of end-stage renal, cardiac, hepatic and pulmonary failure; pancreatic transplantation provides similar benefits for diabetic patients.

In the case of end-stage liver, lung and heart disease, transplantation is generally the only available therapeutic option. In the case of renal and pancreatic transplantation, hemodialysis and insulin therapy provide alternatives to transplantation. At least in the case of renal failure, studies indicate that patients who undergo transplantation have lower morbidity and mortality rates and a higher functional status than appropriately matched nontransplanted control patients⁵. **Table 1** provides a synopsis of the most current data from US registries showing both numbers of transplants for various organs and the size of the waiting lists, as well as 1-year and 5-year graft survival rates. It can be readily seen that currently available immunosuppressive medications provide outstanding short-term results (1-year graft survival in the 80–95% range) in renal, cardiac, liver, lung and pancreatic transplantation.

Also evident from **Table 1**, in conjunction with published literature, are the two major problems in the field. First, improvements in short-term graft survival rates have not been accompanied by improved long-term outcomes among the organs that are not rejected in the initial post-transplant period⁶. Over the past 40 years, 12-month renal allograft survival for first-time recipients of cadaveric kidneys has improved from approximately 45% to almost 95%, but, of the grafts that function after 1 year, the half-life (defined as the point in time at which 50% of the grafts have ceased to function) has changed little. Accompanying the problem of late graft loss are the complications of continual immunosuppressive therapy, which include markedly increased risks for cardiovascular disease, opportunistic infections and malignancy. The second major problem is the organ shortage. For those without living donors, waiting times for renal transplants can exceed 5 years, although they can be maintained with dialysis. There being no such chronic substitute for heart, lung or liver function, in the year 2003 over 2,700 patients in the US died waiting for transplants.

Given these circumstances, the scientific discussions at most transplantation immunology meetings are dominated by designing effective ways to induce transplantation tolerance (which would improve graft

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Table 1 2003 organ transplant data for the United States^a

Organ or tissue	Transplants	Patients on waiting list ^b	Patients added to list ^c	1-year graft survival	5-year graft survival
Kidney	13,765	57,211	24,493	94.3% (Live donor) 88.7% (Cadaver donor)	78.6% (Live donor) 65.7% (Cadaver donor)
Pancreas	527	1,390	926	78.8%	45.4%
Kidney + pancreas	1,236	2,472	1,653	92.0% (Kidney) 85.1% (Pancreas)	74.2% (Kidney) 69.8% (Pancreas)
Liver	2,239	17,515	10,331	80.6%	64.2%
Heart	2,065	3,529	2,942	85.3%	70.6%
Lung	1,110	3,874	1,954	77.0%	43.6%

^aData were obtained from the US Transplant website (www.ustransplant.org). ^bThe number of total patients on the waiting list on December 31, 2003. ^cThe number of new patients added to the list between January 1, 2003 and December 31, 2003.

outcome and obviate the need for immunosuppression) and expanding the supply of organs. Here we consider why these problems have been so difficult to solve, despite 50 years of research, and assess the prospects for the future.

Molecular basis of rejection

The term 'allograft' refers to a cell, tissue, or organ transplant between two genetically distinct (*i.e.*, 'allogeneic') members of the same species. The primary basis for allograft rejection is the ability of T cells to recognize (through their antigen receptors) polymorphic versions of a variety of proteins, which in this context, are often referred to as alloantigens. By far, the most important series of alloantigens are those encoded within the major histocompatibility complex (MHC). Although the physiologic role of MHC molecules is to present peptide determinants to T cells as part of normal immune responses, they were first discovered (and named) because of their dominant role as a target for rejection. A very high proportion of T cells (~0.1–10%) are able to directly bind and respond to allogeneic MHC molecules⁷. Moreover, the genes encoding the MHC are among the most polymorphic known, with hundreds of different alleles so far identified at the five major human MHC (HLA) loci (HLA-A, HLA-B, HLA-C, HLA-DR and HLA-DQ). Consequently, the chance of finding a truly matched, unrelated organ graft donor is minimal. HLA matching was once clearly advantageous with respect to long-term graft survival⁸, and as a result, most organ distribution algorithms favored transplantation of well-matched grafts, even if that meant long-distance shipping of the organ with a consequent increase in cold ischemic time. The magnitude of this 'matching benefit' has diminished as more powerful immunosuppressants have been developed, and the potentially deleterious effect of cold ischemia on graft survival has become increasingly apparent⁹. As a result, the benefit of efforts to match cadaveric donors and recipients is controversial at present.

Immunosuppression is required even when donors and recipients are completely matched for MHC antigens. This is because 'minor' histocompatibility antigens (mH) result from polymorphisms in proteins not encoded in the MHC. There are probably hundreds of mH in mice and humans, and the combination of multiple 'minor' histoincompatibilities (mismatches) may collectively induce as vigorous a response as an MHC mismatch, and rapidly lead to rejection. The importance of specific minor histocompatibility antigen mismatches in causing graft-versus-host disease (GVHD) and graft rejection in humans has been shown in bone marrow transplantation between HLA-identical siblings¹⁰.

Graft rejection and GVHD involve multiple cell populations, but with few exceptions, CD4⁺ and/or CD8⁺ T lymphocytes have a requisite role (**Fig. 1**). Although some rodent models involving limited immunogenicity show crucial requirements for both subsets of T cells, it is probable that either subset is sufficient to cause rejection in most

clinically relevant settings. The lack of a strict requirement for CD4 help in CD8 cell-mediated rejection processes probably reflects the potency of the alloresponse against MHC, the intense inflammatory state created by organ grafting and the contribution of pre-existing memory CD8⁺ T cells to the alloimmune response. Optimal activation of naive CD4⁺ and CD8⁺ T cells is accomplished by both antigen recognition through the T-cell receptor (TCR) and the delivery of costimulatory signals through the binding of T cell–surface receptors such as CD28 to its ligands CD80 or CD86, expressed on antigen-presenting cells (APCs)^{11–13}. Once activated and recruited by chemokines expressed in the graft, T cells can directly kill target cells by two mechanisms. The first is direct lysis of target cells through perforin and granzyme B. This cytotoxic T lymphocyte (CTL) effect requires cell–cell contact and is generally mediated by CD8⁺ T cells; however, CD4⁺ cells can also kill targets on contact through FasL–Fas interactions. Alternatively, T cells can secrete cytokines that recruit and activate cells of the innate immune system (*e.g.*, interferon- γ and macrophages; interleukin (IL)-4 and eosinophils), which in turn cause graft destruction. Cytokines (*e.g.*, tumor necrosis factor- α , lymphotoxin) or other mediators (*e.g.*, nitric oxide) secreted by T cells and recruited macrophages may also directly cause tissue destruction. In some models, antibody- and complement-mediated mechanisms have a major role in graft rejection.

THE WORLD OF IMMUNOSUPPRESSION

Currently used immunosuppressive agents

The current immunosuppressive armamentarium is comprised of some agents that are products of fortuitous discovery (*e.g.*, cyclosporine or tacrolimus) and others that are the result of rational design (*e.g.*, mycophenolate mofetil or antibody to the IL-2 receptor α chain). In either case, they target the cascade of events leading from antigen recognition, processing and presentation to clonal proliferation of immune effector cells. Common to all of these agents is the ability to (ultimately) inhibit T-cell responses, either directly, or through actions on other cell types such as APCs (**Table 2**). These effects include inhibition of APC development and maturation (corticosteroids, rapamycin), blockade of production of IL-2 and other cytokines by T cells (cyclosporine, tacrolimus), blockade of responses of leukocytes to growth factors (rapamycin, IL-2 receptor α chain-specific antibody), and inhibition of DNA synthesis (azathioprine, mycophenolate mofetil). In general, calcineurin inhibitors are the most efficacious immunosuppressive agents, whereas drugs such as rapamycin and DNA synthesis inhibitors have somewhat weaker effects on T cell–mediated immune responses.

Why immunosuppression is not satisfactory

Although these agents have dramatically improved the life of transplant recipients over the past 30 years, two notable problems remain. First, these drugs are associated with significant toxicities. These include both the toxic-

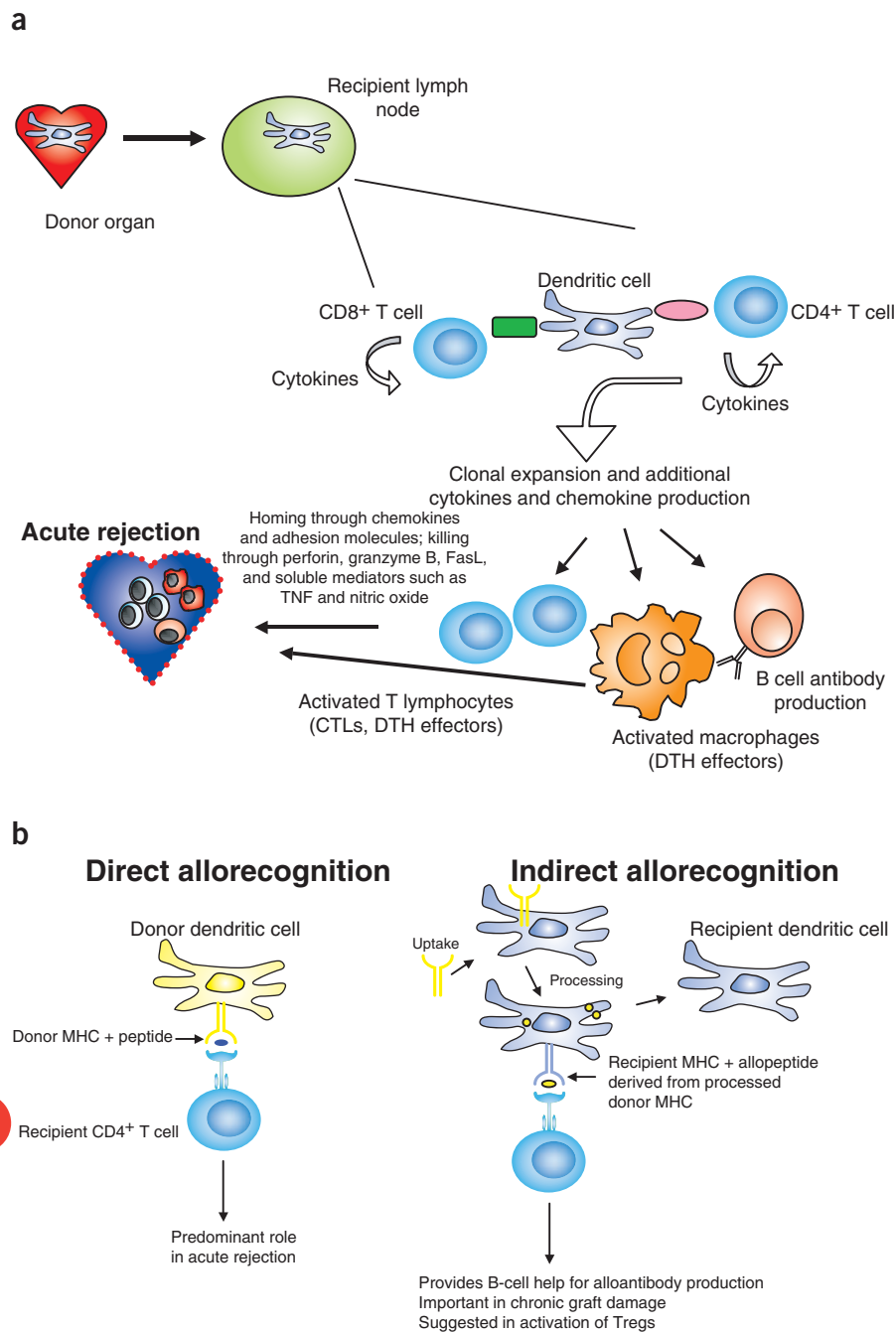


Figure 1 Mechanisms of graft rejection.

(a) Within 24–48 h after engraftment, dendritic cells that normally reside within the donor organ migrate to regional recipient lymphoid tissue. In the lymph node, they stimulate alloreactive CD4⁺ and CD8⁺ T cells. Activated T cells, particularly CD4⁺ cells, produce cytokines (e.g., IL-2, IL-4, IFN- γ), and both populations respond by proliferating and differentiating. Activated T lymphocytes can cause graft destruction by direct lysis (cytotoxic T lymphocytes, CTLs) or by local production of cytokines, a delayed-type hypersensitivity (DTH) reaction. Cytokines also promote macrophage and eosinophil activation and recruitment, and these cells can also secrete soluble inflammatory mediators that kill their targets. Lastly, activated T cells provide help for alloantibody production by B cells. (b) There are two types of allorecognition. Direct allorecognition occurs when T cells recognize intact foreign MHC molecules (as depicted in a). This is thought to be the dominant initiator of acute graft rejection. T cells may also recognize peptide fragments derived from processing of donor antigens presented on self-MHC molecules. This is termed indirect allorecognition, and it is believed to be important in chronic graft dysfunction, in part perhaps because of its role in providing T cell help for alloantibody production by B cells. Indirect allorecognition is also implicated in activating regulatory T cells, which may act to limit graft damage and promote tolerance.

of chronic rejection, in which the vascular lesions are thought to develop through repeated inflammatory cell-mediated endothelial cell activation or injury followed by repair, proliferation of smooth muscle cell and fibroblasts, and net deposition of extracellular matrix, all of which lead to vascular occlusion. Accumulating evidence suggests that both alloantigen-dependent and alloantigen-independent mechanisms are involved in the initiation and perpetuation of chronic rejection. These include ischemia-reperfusion injury, and cytokines and growth factors that drive tissue remodeling. It has been difficult to pinpoint more specific targets for intervention, and this has been a major limitation to progress in prevention and treatment of chronic rejection. Rapamycin and mycophenolate mofetil have shown promise for the inhibition of chronic rejection in preclinical

ties of immunosuppression itself (enhanced risk of opportunistic infections and selected malignancies), and side effects unrelated to immunosuppression (e.g., nephrotoxicity of calcineurin inhibitors, hypertension and cardiovascular disease resulting from use of corticosteroids, etc.). Second, as noted above, although the array of currently available drugs and biologic agents has proven very successful in the prevention and treatment of acute rejection, similar success has not been achieved in preventing chronic graft dysfunction (often termed chronic rejection) and extending long-term graft survival¹⁴. In the case of renal and cardiac transplants, the target of chronic rejection is the vasculature, whereas in lung transplantation it is the bronchial tree (chronic rejection is rarely seen in liver allografts, and indeed the liver is a relatively immunoprivileged organ). Alloantibody has long been considered to be an important effector mechanism in the pathophysiology

models, presumably because of their abilities to suppress cytokine-driven cell proliferation and intercellular adhesion molecule expression, respectively. Combination of these drugs with agents that block costimulation may be particularly worthy of testing for improved long-term clinical transplant outcome. Also important is the fact that selected immunosuppressive agents, in particular calcineurin inhibitors, may in fact pose a barrier to achieving the longer-term goal of tolerance in certain settings, a point that has important implications for the design of some clinical trials.

The promise of new agents

Various new agents are available or are currently being investigated as adjunctive immunosuppression or as a part of true tolerance-inducing protocols (Box 1). In the former case, the goals are to improve long-term

Table 2 Sites of action of antirejection drugs in clinical use

Category	Molecular target
Inhibitors of DNA synthesis	
Azathioprine	Inhibitor of purine synthesis; blocks phosphoribosyl pyrophosphate synthase, inosinate-monophosphate dehydrogenase and inosine monophosphate dehydrogenase
Mycophenolate mofetil	Inhibitor of purine synthesis; blocks inosine monophosphate dehydrogenase
Mizoribine ^a	Same as mycophenolate mofetil
Inhibitors of cytokine production	
Cyclosporine	Binds to cyclophilin and blocks calcineurin, thus preventing activation of the transcription factor NFAT
Tacrolimus	Binds to FKBP-12 and blocks calcineurin
Inhibitors of cytokine binding	
IL-2 receptor-specific monoclonal antibody	Binds the IL-2 receptor α chain and blocks IL-2 from binding to the receptor
Inhibitors of cytokine receptor signal transduction	
Rapamycin	Blocks mammalian target of rapamycin (mTOR)
Inhibitor of antigen presenting cell differentiation or function	
Deoxyspergualin ^a	Blocks activation of NF- κ B; may have other as yet unidentified actions

^aUsed clinically in renal transplantation, in Japan. Note: corticosteroids have multiple different anti-inflammatory effects and are omitted from this table.

graft survival, to develop either steroid- or calcineurin inhibitor-free drug regimens (these classes of drugs are generally agreed to have the worst side effects) and to treat patients who have rejection episodes that do not respond to conventional agents. Animal data and very preliminary clinical studies suggest reasons for optimism, although more definitive conclusions await longer follow up. In the instance of tolerance induction, the goals are to both improve long-term graft survival and to obviate the need for maintenance drug therapy.

Transplantation without immunosuppression

'Transplantation tolerance' describes a state in which a donor organ is 'accepted' without chronic immunosuppressive therapy, while the remainder of the immune system is left intact. Thus, lack of a pathogenic response to the alloantigen is specific, and the recipient is capable of responding to potentially pathogenic microorganisms and malignan-

cies. Tolerance does not imply the absence of an immune response. Indeed, there is abundant evidence for active immunoregulatory mechanisms, which may operate to maintain transplant tolerance.

The problems of immunosuppressive drug toxicity could clearly be alleviated by induction of immune tolerance. It is almost an axiom of faith that chronic rejection would be prevented as well. This is based on studies showing that chronic rejection does not occur in the absence of histocompatibility differences¹⁵ and that transplant tolerance in large animal models is associated with freedom from graft scarring¹⁶⁻¹⁸.

Over the last 25 years, several strategies have been used successfully to induce transplantation tolerance. Each of these has been validated in at least one rodent model, with varying degrees of success upon extension to large animals, nonhuman primates and humans. Varying tissues and organs have been used in these studies, and it should be kept in mind when evaluating them that there is a rough hierarchy of 'tolerability': liver >> heart > kidney > islet >> skin (easiest to hardest).

Tolerance through mixed hematopoietic chimerism. Over 20 years ago, Ildstad and Sachs showed that mice whose hematopoietic system was ablated by lethal whole-body irradiation and then reconstituted with a mixture of syngeneic and allogeneic bone marrow exhibited donor-specific tolerance to skin allografts¹⁹ (Fig. 2a). The approach was later rendered much less toxic by using specific, nonmyeloablative conditioning involving T cell-depleting antibody treatment. In this strategy, initial rejection of the donor bone marrow is prevented by depletion of peripheral and intrathymic T cells with T cell-specific antibodies, with or without local thymic irradiation and without myeloablation²⁰. Once the immune compartment has begun to reconstitute itself, tolerance is induced and maintained by intrathymic deletion of potential donor-reactive T cells. This occurs when the immature T cell encounters donor antigen expressed on donor-derived APCs that reside in the thymus, and this process is analogous to the deletion of potentially autoreactive T cells whose antigen receptors have a high affinity for antigen expressed by self-APCs. Establishment of this tolerant state then permits organ graft acceptance without immunosuppression.

Subsequent studies in miniature swine and nonhuman primates²¹ have clearly established the principle that nonmyeloablative mixed chimerism is a highly effective means to induce tolerance. The approach has also been extended to pilot clinical trials in which tolerance has been achieved in some patients without GVHD or other toxicities from the bone marrow transplant^{22,23}. This is a key point, as the high morbidity of GVHD (and the consequent requirement for prolonged immunosuppressive treatment) render it an unacceptable complication of hematopoietic cell transplantation if the procedure is being used for the purpose of tolerance induction to solid organ grafts. Thus, these results provide important proof of principle that mixed chimerism can be used to induce tolerance in humans, and several ongoing trials are testing this approach in the HLA-identical and HLA-mismatched setting. Notably, long-term tolerance in some of the above patients and in nonhuman primates²¹ may not be purely deletional, as it has been achieved in association with only transient mixed chimerism. Recently, nonmyeloablative and nonmyeloablative conditioning regimens using

BOX 1 PROMISING AGENTS FOR CLINICAL DEVELOPMENT

- T-cell depletion or TCR signal transduction
 - Anti-CD3 immunotoxin
 - Campath-1H (CD52-specific monoclonal antibody)
 - CD45RB-specific monoclonal antibody
- Costimulatory blockade
 - CD154:CD40 pathway (CD40-specific monoclonal antibody)
 - CD28:B7 pathway (B7-specific monoclonal antibodies, CTLA4Ig, LEA29Y)
 - Cytokine receptor signaling (JAK 3 kinase inhibitors)
- B-cell depletion
 - CD20-specific monoclonal antibody
- Lymphocyte trafficking
 - LFA-1-specific monoclonal antibody
 - FTY720 (sphingosine-1-phosphate receptor modulator)
 - causes lymphocyte sequestration in lymphoid tissue
 - CXCR3/CCR1/5 antagonists
 - inhibits lymphocyte trafficking to rejection site

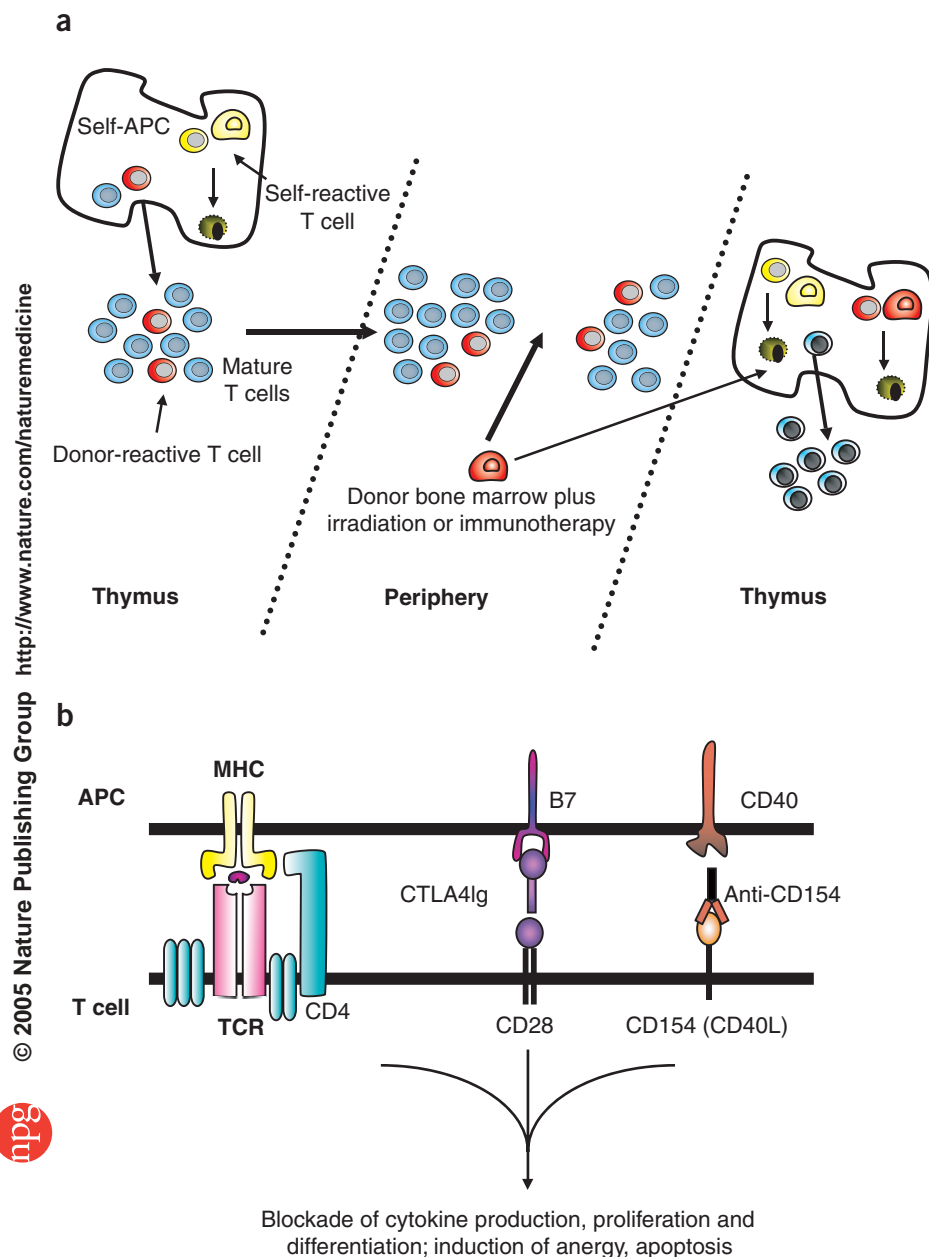


Figure 2 Selected strategies for tolerance induction now in clinical trials. **(a)** Induction of mixed hematopoietic chimerism. Autoreactive T cells (yellow) are normally eliminated in the thymus following interactions with self-APCs; however, alloreactive T cells (red) along with other T cells (blue) mature and are released into the periphery (left). In mixed chimerism, the recipient is given T cell-depleted donor bone marrow along with a 'conditioning' regimen that prevents rejection of the marrow (center). APCs present in the donor marrow migrate to the thymus and delete donor-reactive T cells (right). **(b)** Costimulatory blockade. Optimal activation of T cells by APCs requires costimulatory signals delivered through CD28-B7 and CD154-CD40 interactions. Blockade of either pathway leads to incomplete T-cell responses and promotes tolerance.

CTLA4Ig, but neither agent alone nor the combination has been shown to induce tolerance^{32,33}. Both agents also have been used in clinical trials. Although there was suggestive evidence for the efficacy of CD154-specific monoclonal antibody in the treatment of autoimmunity, marked toxicity in the form of thromboembolic events led to a halt of all clinical trials of this agent. It seems possible that this side effect is a result of CD154 expression on platelets^{34,35} and not a nonspecific effect of the particular monoclonal antibody used. Current efforts at targeting the CD154-CD40 pathway focus therefore on blocking CD40. In contrast, CTLA4Ig has been studied in psoriasis, rheumatoid arthritis and renal transplantation with no obvious limiting toxicity^{36,37} and is currently undergoing regulatory review in the US. A second-generation agent called LEA29Y, with a higher affinity for CD80 and CD86 than CTLA4Ig, seems to be more effective than CTLA4Ig in initial primate studies³⁸, and is currently being used in combination with immunosuppressive drugs in phase 3 clinical trials in renal transplantation.

either T cell costimulatory blockade or high doses of donor marrow have been developed in rodent models^{24,25}. These have not yet been tested in primates.

Costimulatory blockade. In addition to specific antigen, naive T cells require costimulatory signals for optimal activation. The best-characterized T-cell costimulatory receptor is CD28, whose two ligands, CD80 (B7-1) and CD86 (B7-2), are expressed on APCs²⁶. Transiently blocking CD28-B7 costimulation using either monoclonal antibodies or CTLA4Ig²⁷ (a soluble receptor-immunoglobulin fusion protein that binds CD80 and CD86 with higher affinity than CD28) prolongs allograft survival in rodent models and in some instances induces tolerance²⁸ (Fig. 2b). Similar results are seen using CD154-specific antibodies to block the CD154-CD40 costimulatory pathway^{29,30}.

Blockade of both pathways has been well-studied in primates^{16,31}. Here, monoclonal antibodies to CD154 have been more effective than

costimulatory pathways have been identified and suggested as targets for therapeutic interventions, most prominently ICOS and CD134, both of which are expressed on T cells³⁹. Nondepleting antibodies to CD4 have also been used with success^{40,41}. In most instances, studies in these systems have not yet progressed beyond rodent models involving primarily vascularized grafts or MHC-identical skin grafts and have not yet been tested in more stringent rodent systems or large animals. Nonetheless, these results are very promising, and this approach may prove fruitful in the clinic.

Another set of important related targets are the members of the CD28 family, which are negative regulators of T-cell function, most prominently CTLA-4 (ref. 42) and PD-1 (ref. 43). Both of these molecules are expressed on activated T cells, and studies using blocking monoclonal antibodies or targeted genetic deletion show their important role in preventing autoimmunity. Strategies designed to directly stimulate these negative pathways are theoretically attractive, although they have been very limited to date because of a lack of appropriate reagents.

Nonetheless, preliminary data from rodent models indicate that this avenue is worth pursuing further⁴⁴.

Studies in both mice and nonhuman primates suggest that tolerance induction through costimulatory blockade (using CTLA4Ig and/or antibodies to CD154) may be abrogated by immunosuppressive drugs such as cyclosporine and tacrolimus, which interfere with the initial phases of T cell activation^{16,45–47}. As noted above, an extremely high percentage of T cells react to any given set of MHC alloantigens⁷. Experimental evidence suggests that costimulatory blockade works in part by promoting apoptosis in activated T cells, and that cyclosporine and tacrolimus interfere with induction of apoptosis^{45,48}. In contrast, rapamycin, which targets signal transduction through cytokine receptors, does not block apoptosis susceptibility and is permissive for tolerance induction by costimulatory blockade⁴⁵, a consideration that has come into play in the design of clinical trials. It should be noted, however, that the ability of calcineurin inhibitors to block apoptosis and T-cell tolerance induced by costimulatory blockade may not be a universal principle, but may vary by model. For example, in a mouse system, the use of CD154-specific monoclonal antibodies to promote bone marrow engraftment induces CD4⁺ T-cell tolerance that is not blocked by cyclosporine and occurs in conjunction with apoptosis of peripheral donor-reactive CD4⁺ cells⁴⁹. Therefore, different mechanisms seem to be involved when the tolerizing antigen source is bone marrow compared to other types of grafts. It should also be borne in mind that the one approach (combined renal and bone marrow transplantation) that has successfully been used to intentionally induce clinical tolerance includes a short post-transplant course of cyclosporine^{22,23}.

Peripheral T-cell depletion in combination with ‘tolerance-permissive’ drugs. A variety of strategies have focused on T-cell depletion as a means to eliminate alloreactive T cells and ‘reset’ the immune system. This approach was first tested in animal models to show that depleting CD4- plus CD8-specific monoclonal antibodies could be used to achieve tolerance to foreign proteins⁵⁰. Transplantation tolerance (to MHC-matched skin) could not be achieved with depletion alone, but in fact was induced using nondepleting CD4- plus CD8-specific monoclonal antibodies⁴¹. The mechanism for their efficacy is not completely defined, but they appear to alter the response to antigen, and also selectively promote the development of regulatory T cells⁵¹.

In primates, varying results have been reported, perhaps because of the use of different models. For example, in a cardiac allograft model using T-cell depletion (by irradiation and reconstitution with T cell-purged autologous marrow) rejection was delayed, but tolerance was not observed⁵². In contrast, using the combination of diphtheria toxin conjugated to a CD3-specific monoclonal antibody (to create a T cell-depleting agent for primates) plus the immunosuppressive drug deoxyspergualin (which has been suggested to act by inhibiting NF- κ B⁵³) long-term renal allograft survival was achieved in Rhesus monkeys¹⁷. Chronic allograft nephropathy was not prevented unless the animals were treated several days in advance of transplantation, suggesting that tolerance was not achieved in a setting that is clinically relevant for the use of cadaveric donors⁵⁴.

Three T cell-depleting agents are currently available for use in humans: a CD3-specific monoclonal antibody (not toxin conjugated), polyclonal anti-lymphocyte sera, and the CD52-specific monoclonal antibody Campath-1H. Antibody to CD3 effectively clears T cells from the peripheral blood, but is not believed to provide effective removal of T cells from lymph nodes and spleen. Therefore, although effective for prophylaxis or treatment of graft rejection, it cannot be considered a deletional agent in the context of tolerance induction. Polyclonal anti-lymphocyte sera provide potent depletion of T cells from blood, but data on its effect in lymphoid tissue are lacking. A number of clinical trials are currently underway using these agents, in some instances combined with donor

bone marrow, as part of tolerance trials. In one report, an uncontrolled series of transplant recipients treated with anti-lymphocyte serum plus tacrolimus were weaned to low-dose alternate-day (or even less frequent) tacrolimus⁵⁵. Such results are promising, but given the use of ongoing immunosuppression and uncertainty about the future incidence of chronic allograft nephropathy, these patients cannot be considered truly tolerant. Although CD52 is broadly expressed on cells of hematopoietic origin, Campath has somewhat selective effects and causes peripheral T-cell depletion for up to a year following a short course of therapy⁵⁶. There are no data on the effects of Campath on central lymphoid compartments, but given the prolonged effects on peripheral T-cell counts it is generally assumed that this agent depletes cells from lymph node and spleen as well. Originally approved for treatment of malignancy, it has been used off-label in studies to minimize or avoid immunosuppression. Puzzlingly, renal transplant patients treated with Campath alone or in combination with rapamycin have frequently had acute rejection characterized by monocytic infiltration of their grafts^{34,57}. More recent studies successfully combine Campath with tacrolimus to prevent this early rejection, but it is too soon to know whether tolerance has been achieved⁵⁶. This suggests that either calcineurin inhibition is having a direct effect on monocytes or that residual T cells not eliminated by Campath were an important component of the rejection process seen in patients treated with that drug alone. Notably, a recent report indicates that memory T cells (which may be a particular barrier to tolerance induction) are relatively resistant to depletion by Campath⁵⁸.

In vivo induction or ex vivo expansion of regulatory T cells. In most rodent models of transplantation tolerance (with the exception of mixed chimerism), there is strong evidence for the importance of regulatory T cells⁴¹. The lack of these cells in mixed chimeras may be the result of the deletion of donor-reactive cells, leading to a failure to maintain and/or activate regulatory cells⁵⁹. This has prompted the development of regimens to promote regulatory T-cell development. One promising strategy is *ex vivo* expansion of regulatory T cells (Treg) using a mixture of antibodies to CD3 plus CD28 in combination with IL-2. Although not yet tested in the transplantation setting, this approach has been successfully used to generate large numbers of regulatory cells capable of preventing autoimmunity in a mouse model of diabetes⁶⁰. Strategies to promote Treg development *in vivo* are under investigation as well. Studies have identified a reciprocal dialogue between dendritic cells (DCs) and Tregs^{61,62}, and show that tolerogenic DCs can be used to drive generation of Treg and promote graft acceptance⁶³. To date, this work on regulatory cells has been confined to rodents, and will need to be validated in primates or other clinical settings before being applied to solid organ transplantation. At least one clinical trial in bone marrow transplantation using *ex vivo*-expanded polyclonal regulatory T cells to prevent GVHD will be enrolling patients shortly (B. Blazar, personal communication). This should provide important information regarding the safety and efficacy of this approach.

Common themes and lessons derived from them. Successful strategies to induce transplantation tolerance mimic normal mechanisms of self-tolerance (*i.e.*, deletion and regulation). Targeted elimination of alloantigen-specific T cells through the induction of mixed chimerism is an obvious demonstration of the efficacy of deletion. Studies showing that T cells from a tolerant host (in which tolerance is induced by blocking costimulatory pathways) can adoptively transfer tolerance to an otherwise unmanipulated naive animal clearly establish the power of Tregs. These observations have prompted the development of strategies that promote the deletion of alloreactive effector cells while sparing regulatory cells. One such approach is a combination of IL-15 blockade (to inhibit effector cell proliferation), long-acting agonistic IL-2Fc (which promotes apoptosis of effector but not regulatory T cells) plus rapamycin⁶⁴. This

has been tested in both mouse and primate islet transplant models and is likely to be moved to clinical trials in the near future.

The special problem of T- and B-cell memory

T-cell memory. Compared with naive T cells, memory T cells respond more rapidly to antigen stimulation, require only low doses of antigen and have relatively diminished requirements for costimulation. For these reasons, it is generally accepted that they are a barrier to tolerance, as has been shown in animal models. In addition, there are ample clinical data to associate immunologic memory with poor transplant survival⁶⁵.

Memory T cells reactive to a given alloantigen could arise in one of three ways. First, a recipient might have prior exposure to that antigen, as a result of transfusion, pregnancy or previous transplantation. Second, a T cell may have more than one antigen specificity. Because of cross-reactivity, a given TCR may recognize both alloantigen and a foreign peptide plus self-MHC. Alternatively, because of inefficient allelic exclusion during thymic development at the locus encoding TCR α , as many as 30% of human T cells can express two distinct antigen receptor pairings (TCR α 1 β and TCR α 2 β)⁶⁶. T cells that have two antigen specificities may be able to respond to alloantigen in addition to foreign peptide plus self-MHC. Indeed, it has been formally shown that approximately 50% of the alloresponse of adult humans is provided by pre-existing memory cells⁶⁷. This phenomenon, termed heterologous immunity, may in part explain why primates and humans are so difficult to tolerize compared to rodents, as the latter are typically kept in housing that minimizes exposure to pathogens. A third way in which memory cells can arise is as a consequence of homeostatic proliferation⁶⁸ (also termed lymphopenia-induced proliferation), which refers to the ability of self-antigen-MHC complexes plus cytokines and chemokines to induce the expansion of T cells in a lymphopenic environment, even in the absence of foreign antigen. During homeostatic proliferation, T cells develop effector function. Even after homeostatic proliferation has ceased, T cells retain memory function⁶⁹. Although normally these cells are ultimately replaced by new thymic emigrants⁷⁰, this may not occur in older hosts in whom the thymus has involuted. This phenomenon may be of particular relevance to trials of tolerance induction that rely on peripheral lymphodepletion, and could in part explain the occurrence of rejection in patients treated with Campath as part of a lymphodepleting strategy in renal transplantation.

B-cell memory. Pathogenic B-cell memory is most commonly manifest as preformed complement-fixing cytotoxic IgG antibodies directed against MHC class I specificities. They are generated as a result of prior sensitization from blood transfusions, pregnancies or previous transplants. These preformed antibodies cause irreversible hyperacute rejection, except in the case of liver allografts, which for unknown reasons are resistant to antibody-mediated damage. Also of importance are 'naturally occurring' IgM-specific antibodies against ABO blood group antigens or against the Gal α 1-3 Gal sugar moiety widely expressed on pig tissues. Both types of antibodies arise as a result of the expression of cross-reactive carbohydrate epitopes on intestinal bacterial flora and cause hyperacute graft rejection. As with HLA-specific antibodies, transplantation is prohibited by preformed ABO-specific antibodies; however, ABO-mismatched heart transplants have been successfully performed in neonates before the development of antibodies, and in this case tolerance seems to develop to donor blood group antigens⁷¹. Although renal allografting can be successfully performed in the presence of donor-specific antibodies by combining antibody absorption by plasmapheresis with strong B-cell immunosuppressive regimens and other modalities such as plasmapheresis and intravenous immune globulin, long-term outcomes are generally inferior to those obtained without positive donor-specific crossmatches⁷². There is currently strong interest in the depletion of preexisting B cells with a

CD20-specific monoclonal antibody (rituximab) in such patients. Many long-lived IgG-producing plasma cells are CD20 negative, so the efficacy of this approach might be expected to be partial at best. In mouse models, the responses of both natural antibody-producing and presensitized B cells can both be overcome by the induction of mixed hematopoietic chimerism^{73,74}, which may have important therapeutic implications for sensitized patients or in the case of xenotransplantation.

Issues in clinical tolerance trials

Although transplantation tolerance continues to be a highly desirable goal, there are two practical difficulties with conducting clinical tolerance trials, both created by the excellent rates of short-term success. First, it can be difficult to design a trial in which a strategy to induce tolerance does not carry with it the risk of compromising short-term success rates, as many of these trials call for new and/or reduced immunosuppressive approaches. This raises the ethical dilemma in which patients may be asked to potentially sacrifice good short-term results for the hope of better long-term outcomes. Second, it is impossible to use short-term graft survival or acute rejection as end points in trials. Surrogate markers of clinical tolerance are urgently needed. These could be used to determine the safety of withdrawing whatever immunosuppressive drugs are combined with a tolerance-promoting therapy.

The approach that has been adopted within Europe and the United States is that of assembling a cohort of 'tolerant' transplant patients (*i.e.*, patients who have discontinued their medications and maintained clinically acceptable, stable transplant function). These patients are extremely rare, because in most cases, discontinuation of drugs leads to transplant rejection. Occasionally, patients do fall into this category, and are being tracked down by two consortia, one funded by the US National Institutes of Health Immune Tolerance Network (www.immunetolerance.org) and the other by the European Union (www.transplant-tolerance.org.uk). Blood samples from these patients will be subjected to a wide number of tests of antigen-specific T-cell responses, patterns of cytokine release and gene expression analysis in the hope of defining a 'fingerprint' of clinical transplantation tolerance.

Up to 20% of liver transplant recipients can have their immunosuppressive medication withdrawn gradually without experiencing rejection (*i.e.*, they are truly tolerant)⁷⁵. Notably, the 80% who reject upon withdrawal can be treated successfully without compromising graft survival^{75,76}. This presents a unique opportunity to deliberately withdraw liver transplant recipients from their medications and to determine which tests might discriminate between those who are tolerant from those who are not. A multicenter trial for this purpose is currently being conducted with funding from the Immune Tolerance Network.

BEYOND TOLERANCE: THE ORGAN SHORTAGE

Overview of the problem

Organ transplantation is a victim of its own success. As results have improved, so has demand, and the gap between the number of transplant recipients and the number on transplant waiting lists is becoming ever wider. Amongst the approaches that have been adopted to address this problem are the increased use of organs from 'marginal' donors (*e.g.*, donors whose hearts do not beat or older donors). Although this increases the available pool of organs, the long-term outcomes in recipients of such organs are notably poorer, and these patients return to the waiting list for retransplantation prematurely. Indeed, it is noteworthy that in the US, almost 20% of patients on the cadaveric renal transplant waiting list are patients whose first grafts have failed and are seeking retransplantation⁷⁷. A second approach is to use living donors for kidney, liver and even lung donation. This has certainly helped to increase the rates of transplantation, but will never be sufficient to meet demand.

Two potential solutions

There are formidable obstacles to be overcome if xenotransplantation is ever to become a viable solution to the organ shortage (reviewed in ref. 78). As mentioned previously, human serum contains abundant amounts of 'naturally occurring' IgM-specific antibodies against the Gal α 1-3 Gal sugar moiety expressed on pig tissues. These antibodies cause hyperacute rejection dependent on complement activation. A step forward was taken when a human gene encoding a complement regulatory protein, decay accelerating factor (CD55), was first introduced into pigs⁷⁹. Survival of vascularized organs rose from hours to weeks, but graft loss eventually occurred, probably due to activation of coagulation and inflammatory cascades as a result of antibody deposition, even in the absence of complement activation. In the wake of these studies, many of the major sources of funding withdrew from the xenotransplantation field, resulting perhaps from unrealistic expectations for rapid translation. The field has continued to advance, and pigs have been produced in which the gene encoding the enzyme that creates the Gal α 1-3 Gal carbohydrate epitope has been inactivated using nuclear transfer or cloning⁸⁰⁻⁸². Preliminary pig-to-primate transplant studies using these donors are encouraging, particularly when a tolerance-inducing strategy involving thymic xenotransplantation is used^{83,84}. Nonetheless, the central question that remains to be answered is whether the combination of Gal knockout, complement and coagulation regulation by transgenesis, and an imaginative tolerance strategy and immunosuppression will lead to long-term survival of porcine tissues in nonhuman primates.

Concern regarding the risk posed by porcine endogenous retroviruses (PERV) was highlighted by the finding that human cells could be infected during coculture with porcine cells *in vitro*⁸⁵. No human subjects exposed to porcine cells and tissues *in vivo* have shown evidence of PERV transmission⁸⁶. Furthermore, pig lines lacking replication-competent PERVs have been described⁸⁷. Nonetheless, there is still the risk of introducing new infections into humans from xenogeneic source animals, and the International Xenotransplantation Association has recommended that national health authorities institute measures to regulate xenotransplantation appropriately.

The possibility of inducing embryonic or hematopoietic stem cells to differentiate into a variety of cell types *in vivo* or *in vitro* has attracted much attention, as a means of repairing damaged tissues^{88,89}. It also offers an exciting alternative to islet allo- or xenotransplantation as a treatment for diabetes. Despite the hype that has surrounded this field, there remain disappointingly few reproducible examples of tissue repair using stem cells in humans, and at present, the future of this technology in the realm of transplantation remains largely theoretical. Nonetheless, as knowledge of the genetic programs that control cell differentiation down different pathways is acquired, there are grounds for hope that stem cell therapy and regenerative medicine will have a prominent role in organ repair within the next decade.

Conclusion

While many lessons have been learned during the past 50 years of transplant immunology, progress has been slower than expected. But advances have been made—tolerance has been intentionally induced through nonmyeloablative mixed chimerism induction in a small number of patients^{22,23}. Moreover, there has been a recent explosion of information in the field of immunology and in the number of molecular targets to explore. Newly available tools should allow for a greatly improved understanding of human allograft responses. But given the redundancy and vigor of the MHC-specific alloresponse, the complexities of human immunology, and the problems of immune memory, there is unlikely to be a single 'magic bullet' drug for tolerance. A multifaceted approach will

probably be needed, and a growing understanding of tolerance mechanisms in stringent rodent models, in nonhuman primates and in humans will lead us to such approaches. Moreover, large-scale genetic studies are needed to understand the influence of genetics on tolerance and rejection mechanisms as well as drug responses. Although interesting data have been produced in small groups of patients⁹⁰, it seems unlikely that there will be a single 'molecular fingerprint' of tolerance. Tolerance probably can develop in different ways that may relate to underlying disease and treatment used. The first challenge is to understand these mechanisms, whereas the second will be to use this knowledge to define which approaches are best tailored to individual patients. This may represent a 'paradigm adjustment' from a one-protocol-will-fit-all model to a more flexible approach. Large studies combined with a systems-based approach to data interpretation may be needed to address this complex issue, with an ultimate goal to match therapies with the intricate interplay of genetic and environmental factors unique to each individual.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests

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