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Tolerance-Inducing Immunosuppressive Strategies in Clinical Transplantation

An Overview

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Contents

Abstract

The significant development of immunosuppressive drug therapies within the past 20 years has had a major impact on the outcome of clinical solid organ transplantation, mainly by decreasing the incidence of acute rejection episodes and improving short-term patient and graft survival. However, long-term results remain relatively disappointing because of chronic allograft dysfunction and patient morbidity or mortality, which is often related to the adverse effects of

immunosuppressive treatment. Thus, the induction of specific immunological tolerance of the recipient towards the allograft remains an important objective in transplantation. In this article, we first briefly describe the mechanisms of allograft rejection and immune tolerance. We then review in detail current tolerogenic strategies that could promote central or peripheral tolerance, highlighting the promises as well as the remaining challenges in clinical transplantation. The induction of haematopoietic mixed chimerism could be an approach to induce robust central tolerance, and we describe recent encouraging reports of end-stage kidney disease patients, without concomitant malignancy, who have undergone combined bone marrow and kidney transplantation. We discuss current studies suggesting that, while promoting peripheral transplantation tolerance in preclinical models, induction protocols based on lymphocyte depletion (polyclonal antithymocyte globulins, alemtuzumab) or co-stimulatory blockade (belatacept) should, at the current stage, be considered more as drug-minimization rather than tolerance-inducing strategies. Thus, a better understanding of the mechanisms that promote peripheral tolerance has led to newer approaches and the investigation of individualized donor-specific cellular therapies based on manipulated recipient regulatory T cells.

During the past two decades, solid organ transplantation has become the therapy of choice for many end-stage organ diseases. On the basis of extensive successful data obtained in rodents and large-animal experimental transplantation models, newer immunosuppressive strategies have progressively been developed and transposed to routine clinical practice.[1] Following the use of newer immunosuppressive drug combinations, short-term outcomes, such as patient and allograft survival, as well as rates of acute allograft rejection episodes in the first year after transplantation, have steadily improved. However, optimal long-term allograft survival remains a problem because, despite potent therapies and successful prevention or treatment of acute rejection, there is still an inexorable loss of transplanted organs due to chronic allograft dysfunction – a process involving immunological and non-immunological factors. In addition, the chronic use of nonspecifically targeted immunosuppressive drugs is associated with unwanted adverse effects and increased morbidity and mortality.[2-4]

Because of the ever increasing number of potential transplant candidates, including recipients who have to return to the transplantation waiting list because of chronic allograft dysfunction, and the shortage of donor organs, long-term outcome of clinical transplantation must be optimized. Selective short-term tolerogenic therapies that would eliminate allogeneic responses against the graft while preserving normal immune function with limited adverse effects could be the ultimate solution. Transplantation tolerance indeed refers to a state of sustained specific non-responsiveness of the recipient's immune system to donor alloantigens, allowing long-term allograft survival in the absence of potential harmful chronic immunosuppressive therapy. Since the pioneering work of Billingham, Brent and Medawar more than half a century ago,[1,5] many experimental animal models as well as some clinical data in specific settings support the concept that transplantation tolerance could be an achievable target for selected patients. In this article, we briefly describe the mechanisms leading to allograft rejection and immunological tolerance, and we then review current knowledge on the strategies that may lead to clinical transplantation tolerance.

1. Allorecognition and Mechanisms of Acute Allograft Rejection

The recognition by recipient T cells of the allogeneic major histocompatibility complex (MHC)-

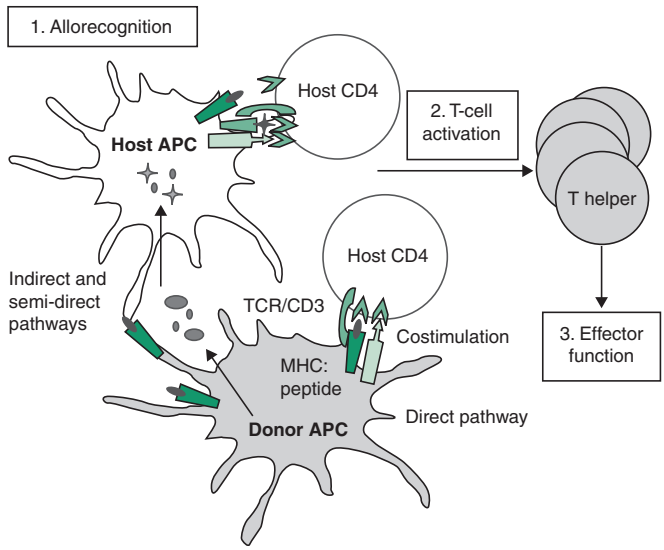

Fig. 1. Pathways of allorecognition and T-cell activation. **APC** = antigen-presenting cell; **MHC** = major histocompatibility complex; **TCR** = T-cell receptor.

mismatched antigens is the primary event that ultimately leads to graft rejection. In the early stages after transplantation, tissue-resident antigen-loaded donor professional antigen-presenting cells (APCs), mainly immature dendritic cells (DCs), migrate out of the allograft towards secondary lymphoid organs, where they mature and encounter recipient alloreactive naive T cells and resting/central memory T cells (direct pathway of allorecognition). Once activated, alloantigen-specific T cells will home to the allograft and induce an inflammatory response leading to graft dysfunction. The trafficking and maturation of donor DCs is triggered by pro-inflammatory signals produced as a result of tissue injury in the early post-transplant period, and it is the cornerstone for the initiation of effective adaptive immune responses.[6,7] At later stages, once donor DCs are depleted, the immune response against an allograft is maintained by recipient APCs (DCs and B cells) that process and present allogeneic MHC molecules shed from the graft (indirect and semi-direct pathway).[8-10]

CD4+ T cells play a central role in the alloresponse because they can initiate rejection through direct recognition of allogeneic MHC class II antigens displayed at the surface of donor APC, as well as indirect and semi-direct recognition of allogeneic MHC peptides presented by self-APC. These pathways were shown to help cytotoxic CD8+ T cells, and induce macrophage responses and allospecific antibody production by B cells (figure 1).[8-10] The relative contributions of these pathways to graft rejection are not completely defined as yet. The high frequency of T cells with direct allospecificity and the relative low frequency of T cells with indirect allospecificity in the normal T-cell repertoire has led to the concept that the direct alloresponse dominates the early post-transplant period and is mainly involved in acute transplant rejection, while the indirect pathway plays a major role in later forms of alloresponses and in chronic transplant rejection.[11-14] Thus, the indirect pathway might be the one to target to achieve long-term graft survival, particularly because experimental models have shown the importance of indirect allorecognition in the induction of transplantation tolerance.[15,16]

2. Chronic Allograft Dysfunction

The first use of the calcineurin inhibitor (CNI) ciclosporin in kidney transplantation in the 1980s allowed a spectacular improvement of 1-year allograft survival from approximately 60% to 85%

when compared with the previous combination of azathioprine and corticosteroids.[17] The progressive introduction of more intensive immunosuppressive protocols together with the systematic prophylaxis of infectious complications resulted in a further decrease of the incidence of acute rejection (to <20% within the first year after transplantation) and a better 1-year patient and graft survival (about 95% and 90%, respectively, in the US for recipients of a first cadaveric kidney, and even better outcomes for a first living donor kidney).[1,3,4]

In clinical transplantation, acute allograft rejection can be successfully prevented or treated in most patients with current immunosuppressive regimens. However, despite initial enthusiasm, the lower rates of acute rejection in the first year have not resulted in a significant improvement in long-term (i.e. 5–10 years) allograft survival.[1,18,19] Thus, even if grafts display a good function in the early years after transplantation, progressive tissue damage generally develops, leading to slow and progressive late organ dysfunction, also referred to as chronic allograft dysfunction. Chronic allograft dysfunction can affect all transplanted organs and is the most common cause of graft loss >1 year after transplantation. The late loss of allografts results from immunological and alloantigen-independent factors. Immunological factors consist of inadequate immunosuppression or noncompliance in the context of poor HLA matching or previous hyperimmunization, often with a history of acute rejection episodes. Non-immunological factors include the quality of the graft at the time of implantation (donor age and co-morbidities), graft injuries (organ preservation and perioperative ischaemia leading to delayed graft function), drug toxicities such as vasculopathy and nephropathy related to the use of CNIs.[2-4]

Despite a better control of the rejection process, the chronic use of nonspecifically targeted immunosuppressive drugs is associated with unwanted adverse effects and increased morbidity and mortality (table I and table II). Besides the increased susceptibility to infections and development of *de novo* malignancies due to nonspecific chronic immunosuppression, many of theses drugs have been associated with non-immunological complications – mainly an increase in cardiovascular risk factors such as hypertension, hyperlipidaemia and diabetes mellitus. Indeed, death from cardiovascular diseases, with a functioning graft, is becoming a major

Table I. Induction therapies used in clinical solid organ transplantation

Drugs	Mechanisms	Adverse effects
Thymoglobulin®; ATG-Fresenius®; ATGAM®	Polyclonal horse or rabbit antithymocyte globulins; T-cell depletion	Cytokine-release syndrome, thrombocytopenia, leukopenia, serum sickness
Basiliximab, daclizumab	Chimeric (basiliximab) or humanized (daclizumab) mAb, with high affinity for the α subunit of the IL-2 receptor (CD25); depletion of activated T cells and inhibition of IL-2-induced T-cell activation	Rare hypersensitivity reactions
Alemtuzumab (CAMPTH-1H)	Humanized anti-CD52 mAb; depletion of T and B cells, most monocytes/macrophages, NK cells	Mild cytokine-release syndrome, neutropenia, anaemia, autoimmune thrombocytopenia, thyroid disease
Belatacept (LEA29Y)	CTLA-4 Ig fusion protein; binds to CD80/86 and blocks signal 2	Not known
Fingolimod (FTY720)	Lymphocyte sphingosine-1-phosphate-receptor antagonist; enhances homing to lymphoid tissues and prevents egress	First-dose bradycardia, gastrointestinal symptoms, liver toxicity, macular oedema
Muromonab-CD3 (OKT3), teplizumab, otelixizumab, visilizumab	Mouse (muromonab-CD3) or humanized Fc-receptor-non-binding (teplizumab, otelixizumab, visilizumab); depletion of T cells after initial activation and cytokine release	Severe cytokine-release syndrome, pulmonary oedema, acute renal failure, gastrointestinal symptoms; all effects described for muromonab-CD3. Humanized forms should be less toxic.
Rituximab	Chimeric anti-CD20 mAb; depletion of B cells	Rare hypersensitivity reactions

CTLA-4 Ig = cytotoxic T-lymphocyte associated antigen 4 immunoglobulin; **IL** = interleukin; **mAb** = monoclonal antibodies; **NK** = natural killer.

Table II. Maintenance immunosuppressive therapies used in clinical solid organ transplantation

Drugs	Mechanisms	Adverse effects
Ciclosporin	Binds to cyclophilin, inhibits calcineurin-phosphatase, blocks T-cell activation	Hypertension, hyperlipidaemia, nephrotoxicity, thrombotic microangiopathy, neurotoxicity, tremor, gingival hyperplasia, hirsutism
Tacrolimus	Binds to FKBP12, inhibits calcineurin-phosphatase, blocks T-cell activation	Post-transplantation diabetes mellitus, nephrotoxicity, thrombotic microangiopathy, neurotoxicity
Azathioprine	Interferes with DNA synthesis, blocks cell proliferation	Bone marrow depression, macrocytosis, liver toxicity
Mycophenolic acid (mycophenolate mofetil, enteric-coated mycophenolate)	Inhibit inosine-monophosphate-dehydrogenase and block purine synthesis, block cell proliferation	Gastrointestinal symptoms (mainly diarrhoea), bone marrow depression
Sirolimus, everolimus	Bind to FKBP12, inhibit mTOR, block IL-2-driven T-cell proliferation	Delayed graft function, delayed wound healing, mouth ulcers, pneumonitis, increased proteinuria, peripheral oedema, hyperlipidaemia

IL = interleukin; **mTOR** = mammalian target of rapamycin.

cause for late allograft loss. If the existing experimental models can be successfully applied to clinical transplantation, the induction of donor-specific tolerance could effectively prevent the development of chronic allograft dysfunction and preserve the host from drug-related adverse effects.

Thus, the main objective in clinical transplantation remains to safely achieve long-term allograft acceptance, which implies sustained donor-specific T- and B-cell non-responsiveness with preserved allograft function, in the absence of (operational tolerance) or with minimal (near tolerance) chronic immunosuppression. A number of clinical reports of occasional 'tolerant' transplant recipients, characterized by prolonged allograft survival with minimal or no immunosuppression, suggest that immunological tolerance may indeed be achievable in some patients.[20] Spontaneous operational tolerance can take place after years of immunosuppression. It is rare in kidney transplant recipients, but has been more frequently reported in liver transplant recipients, suggesting that various mechanisms may be involved, such as the induction of mixed chimerism[21-23] or the promotion of peripheral regulatory mechanisms.[24-26] Collaborative international studies are currently underway to pool data on these tolerant transplant recipients in order to determine more precisely how this state arises and to develop reliable tests to predict tolerance.[20,27-29]

3. Transplantation Tolerance

Over recent years, experimental models have shown that it is possible to exploit the mechanisms that normally maintain immune homeostasis and tolerance to self-antigens to induce tolerance to alloantigens. The regulation of the immune response and the induction of immunological tolerance involve central and peripheral mechanisms. Self-tolerance is partly achieved by intrathymic deletion of self-reactive lymphocytes from the immune repertoire (clonal selection). As not all self-antigens are expressed in the thymus, other mechanisms exist in the peripheral immune system to maintain a safe T-cell repertoire: ignorance, clonal deletion, anergy and regulation/suppression.

4. Central Tolerance and Mixed Chimerism Approaches

The thymus plays an important role in the maintenance of tolerance to self-antigens, and many experimental data support its role in the induction of sustained robust tolerance to alloantigens.[21,30] During the physiological maturation process in the thymus, central tolerance is achieved by intrathymic deletion of T cells with high avidity for thymic expressed self-antigens (negative selection), so that potentially deleterious antigen-reactive T cells will not reach the periphery. In the transplantation setting, this process can be exploited by the delivery of donor alloantigens to the thymus prior to solid organ

transplantation. This can be achieved either experimentally by direct intrathymic injections of donor-derived allopeptides or by the induction of a state of haematopoietic mixed chimerism in the recipient's repertoire (presence of cells from both recipient and donor origin) allowing donor APCs to migrate to the recipient's thymus and induce negative selection of donor-reactive T cells.[21,30-32] For mixed chimerism to occur in the recipient, the infused bone marrow cells from donor and recipient must engraft, implying the need for pre-conditioning treatments that allow the creation of 'space' for engraftment combined to deletion of pre-existing cross-reactive peripheral T cells that could reject the donor bone marrow. In experimental animal models, high-intensity myeloablative pre-conditioning protocols (total body irradiation and high-dose chemotherapeutic agents) could be successfully replaced by other less toxic approaches (high-dose bone marrow combined to co-stimulatory blockade and thymic irradiation). Extensive work performed in small- and large-animal models have since demonstrated that sustained donor-specific transplantation tolerance can be induced through the generation of a state of haematopoietic mixed chimerism.[33-37] Based in part on these experimental data, a small number of highly selected patients (e.g. end-stage renal failure secondary to refractory multiple myeloma) underwent HLA-matched combined bone marrow and kidney transplantation (either sequentially or concomitantly) from the same living donor, and had long-term acceptance of their renal allograft in the absence of ongoing immunosuppression.[38-43]

The proof of concept of the clinical applicability of mixed chimerism approaches to induce transplantation tolerance was thus first established in patients with haematopoietic malignant disorders, and more recently these protocols have been adapted to patients without concomitant malignancy. While aggressive myeloablative regimens must be applied for classic bone marrow transplantation in the context of haematopoietic malignancy, this potentially toxic approach cannot be justified to achieve tolerance in regular solid organ allograft recipients. Following on their previous protocols developed in animal models and clinical studies, the Stanford group recently described the successful case of a patient undergoing combined HLA-matched kidney and haematopoietic cells transplantation, using a low-intensity conditioning regimen (total lymphoid irradiation and antithymocyte globulin [ATG]).[44] The first transplant recipient, in this small study of patients with end-stage renal disease without malignancy, had persistent mixed chimerism and normal kidney allograft function >2 years after discontinuation of all immunosuppressive drugs. Of interest, the same group had attempted a few years ago a similar protocol for the induction of allograft tolerance in HLA-mismatched recipients,[42] but rejection developed when immunosuppression withdrawal was attempted. However, the recent report of the Massachusetts General Hospital group brings new hope to the possibility of extending these protocols to HLA-mismatched patients.[45] In their study, five patients with end-stage renal disease (and no malignancy) received combined bone marrow and one-haplotype HLA-mismatched living-donor kidney transplantation, with the use of a non-myeloablative pre-conditioning regimen. Except for one patient who developed non-reversible humoral rejection, immunosuppression was withdrawn in the remaining patients at 9–14 months after transplantation, with stable kidney function and donor-specific T-cell unresponsiveness. Interestingly, all of the recipients displayed only transient chimerism post-transplantation, suggesting that, while the induction of tolerance is dependent on central deletion of donor-reactive T cells, peripheral mechanisms may be involved in the long-term maintenance of transplantation tolerance.

5. Peripheral Transplantation Tolerance

In the transplant setting, circulating alloreactive T cells are crucial in the initiation and coordination of the rejection response and to promote peripheral tolerance, the alloreactive effector T-cell pool must be minimized, while enhancing regulatory mechanisms.[46] Various strategies have been explored to achieve peripheral tolerance to alloantigens in experimental transplantation models: (i) deletion of

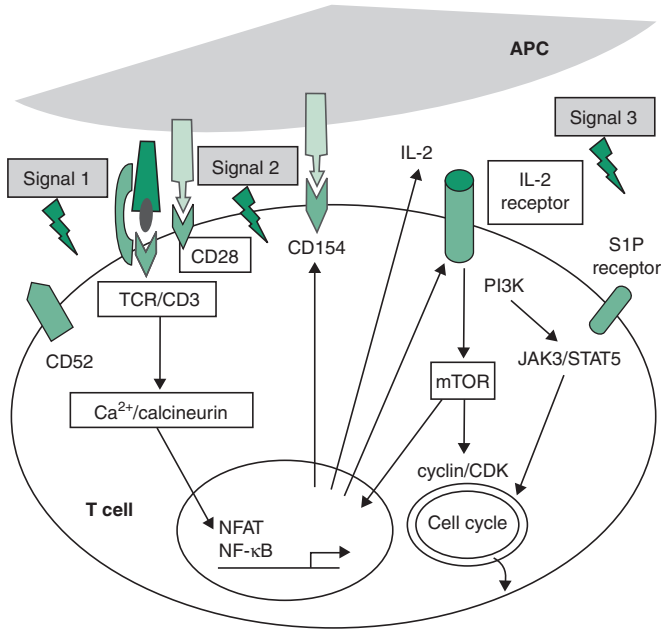

Fig. 2. Cellular targets of immunosuppressive drugs. **APC** = antigen-presenting cell; **CDK** = cyclin dependent kinase; **IL** = interleukin; **JAK3** = Janus kinase 3; **mTOR** = mammalian target of rapamycin; **NFAT** = nuclear factor of activated T cells; **NF-κB** = nuclear factor κB; **PI3K** = phosphoinositide-3-kinase; **S1P** = sphingosine-1-phosphate; **STAT5** = signal transducer and activator of transcription 5; **TCR** = T-cell receptor.

peripheral effector T cells (lymphocyte-depleting protocols); (ii) inhibition of T-cell activation by blocking or modifying co-stimulatory signals (co-stimulatory blockade, manipulation of DCs); (iii) interference with the effector function or homing of activated T cells (anti-cytokines, anti-chemokines); and (iv) active regulation of effector T cells by antigen-specific CD4+CD25+Foxp3+ regulatory T (T_{reg}) cells. Most of these approaches have been extensively studied in experimental models and are now being progressively transposed to the clinic, as is discussed in sections 6–9 and section 11.

Three distinct signals are required for the activation and differentiation of alloreactive T cells into proliferating effector T cells (figure 2). The first signal (signal 1) is delivered through the T-cell receptor (TCR)/CD3 complex by the recognition of peptide antigens presented in the context of MHC molecules on the surface of APCs. In addition to antigen recognition, full T-cell activation requires a second distinct co-stimulatory signal (signal 2) provided by the APC. Co-stimulatory signals are delivered via constitutive or inducible receptors on the responding T-cell surface interacting with their ligands constitutively expressed or up-regulated on the activated APC. If partial activation occurs, e.g. in the presence of altered TCR ligands or in the absence of co-stimulation, T cells become unresponsive to proliferative signals – a state referred to as anergy.[47,48] The activation of signals 1 and 2 initiates a cascade of downstream signalling pathways and the induction of transcription factors, leading to the expression of new surface molecules such as inducible co-stimulatory molecules and cytokine receptors. Interleukin (IL)-2 and other cytokines can then trigger T-cell proliferation and differentiation via their receptors up-regulated on the recently activated T cell (signal 3). Current experimental and clinical data suggest that the interruption of these signalling pathways at specific steps prevents acute allograft rejection and may promote immune tolerance in some circumstances.[49,50]

6. Targeting Signal 1

The primary mediators of the immune response are T and B cells. T and B cells have antigen-specific receptors that recognize foreign antigens and induce donor-specific cellular and humoral responses. With the development of polyclonal and monoclonal antibodies (mAb), newer therapeutic strategies have been developed based on powerful cell-depletion at the time of transplantation (induction therapy), when immune activation is most intense. In various experimental rodent and non-human primate (NHP) models, anti-T-cell antibodies have been used either alone or in combination with other strategies that aim to limit clonal expansion of effector T cells such as co-stimulatory blockade or transfusion of donor-derived peptides.[51] By depleting T cells, and for some agents also B cells (anti-CD52, anti-CD45RB mAb) and monocytes (deoxyspergualin), cell-depleting approaches result in a profound reduction of circulating leukocytes capable of mounting an alloresponse at a time when the allograft is already susceptible to inflammatory damage following the ischaemia/reperfusion injury. Lymphocytes will gradually repopulate the host weeks to months later when the innate immune response has resumed and the allograft is more quiescent.[52,53] Depletion strategies have been extensively studied in NHP transplantation models and encouraging results have led to clinical trials using polyclonal ATGs, a humanized anti-CD52 mAb (alemtuzumab, CAMPATH-1H), or anti-CD3 mAb.

6.1 Polyclonal Antithymocyte Globulins and Alemtuzumab

Polyclonal ATGs are produced in horses or rabbits after immunizing these animals with human leukocytes. By binding to T-cell surface receptors, these ATG opsonize the cells for complement-mediated lysis and phagocytosis. Initially approved for the treatment of corticosteroid-resistant acute cellular rejection, they are also used as induction agents in high immunological risk recipients (pre-sensitized, high donor-recipient MHC-mismatches, African Americans) and in the setting of peri-transplant acute tubular necrosis to avoid CNIs. They have broad T-cell specificity and are among the most potent induction agents used in solid organ transplantation. Rabbit preparations such as Thymoglobulin® [1] and ATG-Fresenius® have appeared to be slightly more potent than horse preparations (ATGAM®).[54] Alemtuzumab, or CAMPATH-1H, is a humanized anti-CD52 mAb that was first developed for use in lymphoproliferative diseases. CD52 is expressed on T cells, B cells, monocytes and macrophages, natural killer (NK) cells and granulocytes.[55] ATG and alemtuzumab induce profound and durable lymphopenia that can be associated with immunodeficiency complications such as infections (mainly viral such as cytomegalovirus or Epstein-Barr virus) or, as reported for ATG, *de novo* post-transplant lymphoproliferative disorders (PTLD).[56] Other toxic adverse effects include thrombocytopenia, a cytokine-release syndrome or allergic reactions. The benefits and adverse effects of these induction therapies in the transplant setting are better known for preparations of ATG, which have been widely used in the clinic for more than 20 years, compared with alemtuzumab, which is still used off-label in relatively small clinical trials.

Calne et al.[57] first published interesting clinical data in deceased donor kidney transplantation, using lymphocyte-depletion with alemtuzumab, which provided long-term rejection-free allograft survival with minimal maintenance therapy (low-dose ciclosporin) in most patients. Because these recipients did not exhibit true operational tolerance, Calne and colleagues coined the term 'prope tolerance' (near-tolerance) to describe their immunological state. Other groups have extended this approach of drug minimization by combining anti-T-cell antibodies together with deoxyspergualin, sirolimus or tacrolimus in NHP transplantation models as well as in clinical trials. In animal models, despite profound peri-transplant T-cell depletion, consistent transplantation tolerance could not be achieved when

1 The use of trade names is for product identification purposes only and does not imply endorsement.

anti-T-cell antibodies were used in monotherapy, as most treated animals lost their grafts through subacute or chronic rejection. However, the addition of deoxyspergualin or sirolimus allowed the induction of transplantation tolerance in NHP models.[58-60] Similarly, subsequent clinical studies confirmed that, although T-cell-depleting antibodies greatly reduced the requirements for maintenance immunosuppression, rarely could immunosuppressive therapy be completely withdrawn.[61-63] It should be noted that the use of alemtuzumab alone or in combination with sirolimus or deoxyspergualin was associated with an unusually high rate of early cellular rejection episodes characterized by predominant monocytic infiltrates, as well as antibody-mediated rejection episodes, so that CNI had to be added for maintenance immunosuppression in later studies.[53,61,64-66]

Thus, current data suggest that, although T-cell depletion combined with deoxyspergualin can induce tolerance in NHP models, some form of maintenance immunosuppression is required with these protocols in humans. How can these discrepancies be explained? Although anti-T-cell antibodies can deplete nearly all circulating peripheral T cells (>99% depletion), they are less efficient on cells that have homed to peripheral tissues and lymph nodes – mainly effector memory T cells. These memory T cells differ from naive T cells by their activation requirements and could undergo homeostatic proliferation in a cell-depleted host, thus contributing to the rejection process associated with monocytes and repopulating B cells that are less efficiently depleted using ATGs or alemtuzumab.[67,68] Furthermore, in adult humans, even at the time of a first transplant, pre-existing cross-reactive memory T cells account for a larger proportion of the T-cell pool than in most laboratory animals that have not been exposed to as many environmental antigens and pathogens.[69-72] These pre-existing memory T cells are now considered to be a major hurdle to the induction of tolerance in human transplant recipients, and newer strategies have to be developed to target this population more efficiently.

At present, ATGs and alemtuzumab must be considered rather as potentially 'drug-minimizing' than as 'tolerance-inducing' agents. Larger, well designed, prospective clinical trials are needed to define the optimal maintenance drug regimen to be used following induction with lymphocyte-depleting antibodies. Further investigation is also needed to compare the different anti-T-cell antibodies with other induction agents, such as anti-IL-2 receptor mAb or co-stimulatory blockade, looking at their efficacy (rate of acute rejection, graft survival), safety, costs and drug administration modalities (number of doses, route of administration), in kidney and other solid organ transplantation. For example, one study has compared Thymoglobulin®, alemtuzumab and daclizumab (a humanized anti-IL-2 receptor mAb) in deceased donor kidney transplant recipients. Maintenance immunosuppression included tacrolimus, mycophenolate mofetil and corticosteroids, except for the alemtuzumab group, which remained corticosteroid-free. There was no difference in patient and graft survival, or in acute rejection rates, between the three groups.[73]

6.2 Anti-CD3 Monoclonal Antibody (mAb)

Muromonab-CD3 (OKT3), a mouse mAb binding to the CD3 component of the TCR signal-transduction complex, has been successfully used for the past 20 years as an induction agent for high-risk patients or for the treatment of corticosteroid-resistant acute rejection episodes.[74] Because of the important adverse effects of muromonab-CD3, including a cytokine-release syndrome, ATGs are now usually preferably used. Non-activating, thus less toxic, humanized Fc-receptor-non-binding anti-CD3 mAbs (i.e. teplizumab, otelixizumab and visilizumab) are currently being tested in phase I and II clinical trials in autoimmune diseases settings (type 1 diabetes, Crohn's disease, arthritis) as well as in renal and pancreatic islet transplantation.[75]

6.3 Rituximab (Anti-CD20 mAb)

Rituximab is an anti-CD20 chimeric mAb that eliminates most B cells from the circulation. It was first approved for the treatment of B-cell lympho-

proliferative diseases in non-transplant patients, and has been used to treat PTLD after solid organ transplantation. It is now also used for the treatment of antibody-mediated rejection and for the suppression of preformed alloantibodies in sensitized patients (retransplant, ABO-incompatible), together with maintenance immunosuppressive drugs, plasmapheresis and/or intravenous immunoglobulin (Ig), and splenectomy in some protocols.[76] However, depletion of antibody-producing cells may be incomplete because rituximab cannot target CD20− plasmocytes (a relatively important part of the cell population in the plasma). It remains to be determined whether rituximab may also be useful in T-cell-mediated rejection because, by depleting recipient's B cells that can act as efficient APCs, it may limit indirect pathway T-cell responses.

7. Targeting Signal 2 (Co-Stimulatory Blockade)

There is a growing number of characterized co-stimulatory receptor:ligand molecules that are key for T-cell stimulation and regulation, including the CD28:B7(CD80/CD86) and CD154(CD40L):CD40 pathways, which have been extensively studied in transplantation models. These positive activating co-stimulatory signals are balanced by inhibitory inducible signals, such as CTLA-4:B7 and PD1:PDL, allowing a down-regulation of the response after initial T-cell activation.[77] In the absence of appropriate co-stimulation, partially activated T cells either become hyporesponsive to subsequent antigen-specific TCR signals (donor-specific anergy) or die by apoptosis.[47,48] By inhibiting T-cell activation rather than eliminating all T cells as in cell-depleting protocols, the strategy of co-stimulatory blockade might more selectively target effector T cells and spare beneficial T_{reg} cells, promoting the induction of tolerance. In many experimental rodent and NHP transplantation models, dual blockade of the CD154:CD40 and CD28:B7 pathways was shown to act synergistically to prevent rejection or induce tolerance.[78]

7.1 CD154(CD40L):CD40 Blockade

The CD154(CD40L):CD40 pathway plays a central role in effective antigen presentation because CD40 is constitutively expressed on DCs, B cells and macrophages, and its ligation up-regulates the expression of B7, MHC and adhesion molecules on APCs, potentiating their immunogenicity. The ligand of CD40 (CD154) is induced on T cells (mainly CD4+ T cells) following TCR:antigen engagement.[79] Of the various co-stimulatory molecules that have been targeted in rodent models, the most successful results were obtained using BMS-202448 (MR1), an anti-CD40L mAb. A humanized anti-CD40L mAb (clone hu5C8) also promoted long-term allograft survival in NHP models, but true tolerance was not achieved when used alone.[80] Of note, the administration of a humanized anti-CD40L mAb (hu5C8) in transplant recipients resulted in unexpected thromboembolic complications. This was later shown to be possibly due to the expression of CD154(CD40L) on platelets and all clinical trials with this agent were therefore terminated.[81] To circumvent these complications, while still targeting the CD154(CD40L):CD40 pathway, anti-CD40 mAbs are currently being developed.[82]

7.2 CD28:B7(CD80/CD86) Blockade

CD28 is constitutively expressed on CD4+ T cells and up to 50% of CD8+ T cells, and its ligation by B7 molecules (CD80, CD86) synergizes with TCR signalling to lower the activation threshold of T cells. CD28 may also deliver a reverse signal to DCs through CD80 and CD86, inducing the production of IL-6.[83,84] Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is induced on T cells upon activation, binds to the same ligands as CD28 but with a higher affinity, and delivers an inhibitory signal for the T cell. The ligation of CTLA-4 also induces the expression of indoleamine 2,3 dioxygenase on DCs, contributing to the regulation of T-cell responses.[83]

Blocking antibodies against CD80 and CD86 have been evaluated in preclinical studies; however, in monotherapy protocols, they have not significantly prolonged renal allograft survival. In comparison,

combined blockade of CD80 and CD86 led to prolonged renal allograft survival in NHP, but without resulting in tolerance.[85,86] CTLA-4 Ig, a fusion protein with specificity for CD80 and CD86, was first used in small-animal models with excellent outcomes. In NHP models, CTLA-4 Ig prolonged pancreatic islet survival and, in combination with CD154:CD40 pathway blockade, induced indefinite acceptance of renal and heart allografts, while allowing prolonged skin graft survival.[82,87-89] Following encouraging preclinical results, clinical trials were initiated with belatacept (LEA29Y), a promising agent in NHP models. Belatacept is a fusion protein combining the extracellular binding domain of CTLA-4 with the Fc portion of IgG_1, with specificity for CD80/86 expressed on APCs. Compared with the original CTLA-4 Ig compound used in animal models, belatacept has two amino acid substitutions resulting in a higher affinity and a slower dissociation rate from human B7 molecules, thus achieving a more complete blockade *in vivo*.[90] In a recent phase II renal transplantation clinical trial, after induction with basiliximab, belatacept was administered every 4 weeks together with mycophenolate mofetil and corticosteroids. The reported short-term results were found to be promising in terms of safety and efficacy (rate of biopsy-proven acute rejection) within the first year, when compared with a 'classical' ciclosporin-based maintenance regimen.[91] Longer follow-up data will have to establish whether this drug can prevent chronic allograft dysfunction and/or favour the induction of transplantation tolerance, as was suggested in animal models.

Compared with animal models, co-stimulatory blockade has been used in the clinic more as a selective immunosuppressive agent (and an alternative to CNI) for preventing acute allograft rejection than an agent for inducing donor-specific tolerance. In a similar way to lymphocyte-depleting protocols, the translation of co-stimulation blockade strategies to the clinic has to overcome hurdles related to the complexity of the human immune system. It has been shown that memory T cells and CD8+ T cells have different co-stimulation requirements for full T-cell activation when compared with CD4+ naive T cells, and may therefore be more resistant to some tolerance induction strategies. As not all T-cell subsets are as susceptible to blockade of co-stimulatory signals, other additional strategies were needed in some models to induce long-term graft acceptance.[92,93]

Co-stimulatory blockade may also result in differential effects on pathogenic effector T cells and T_{reg} cells. Indeed, the CD28:B7 pathway has been shown to be important for the expansion and survival of T_{reg} cells. Furthermore, one must consider that the combined blockade of CD80 and CD86 by B7 antagonists simultaneously prevents the ligation of CTLA-4 on T cells, and this negative signalling may contribute to tolerance induction and the function of T_{reg} cells.[94-96] Thus, blocking a particular co-stimulatory pathway may give opposite results depending on the model. The CD28:B7 pathway could be preferentially targeted by selectively blocking CD28, as suggested by *in vitro* data as well as in rodent transplantation models.[97,98]

8. Targeting Signal 3

8.1 Anti-Interleukin-2 Receptor mAbs

Activated T cells produce cytokines such as IL-2 that trigger their proliferation via signalling through up-regulated surface receptors (signal 3 – see figure 2). The IL-2 receptor α-chain, also called CD25, is not expressed on most resting T cells, but is up-regulated after T-cell activation following antigen encounter. Thus, anti-IL-2 receptor mAbs preferentially target alloreactive T cells activated in the early post-transplantation period, and inhibit the IL-2-mediated proliferation and effector function. Daclizumab and basiliximab are now commonly used in renal transplantation as induction agents in low-risk recipients (e.g. first allograft, living-related donor, no delayed graft function).[99,100] As these drugs specifically target activated T cells, they do not cause significant lymphocyte depletion and have not been associated with major adverse effects. So far, *in vitro* and *in vivo* data have not shown a deleterious effect of anti-IL-2 receptor mAb on the T_{reg}

subset of CD4+ T cells, which constitutively express CD25.[101,102]

9. Targeting Leukocyte Trafficking

9.1 Fingolimod

For the immune response to be deleterious to the allograft, primed alloreactive T cells and accessory cells must migrate and infiltrate the graft. Sphingosine-1-phosphate (S1P) is expressed on lymphocytes and bone marrow-derived DCs, and regulates cell migration and survival by interacting with S1P receptors expressed in lymphoid tissues. Fingolimod (FTY720) is a S1P analogue that engages lymphocyte S1P receptors, resulting in altered leukocyte trafficking. Fingolimod traps T cells into lymphoid tissues and prevents them from homing to the allograft after T-cell priming (lymphocyte sequestration). This drug is under clinical investigation in autoimmune diseases, such as multiple sclerosis, but clinical trials have recently been discontinued in kidney transplantation because of a higher incidence of adverse events compared with current standard immunosuppression.[103,104]

9.2 Chemokines and Adhesion Molecules

The trafficking of activated T cells is also mediated by chemokines released within the transplanted tissue and adhesion molecules up-regulated on endothelial cells following the inflammatory process induced by initial ischaemia/reperfusion injury. Recent reports using rodent models as well as clinical data suggest that interference with chemotaxis or blockade of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) or lymphocyte function-associated antigen-1 (LFA-1) could be another promising approach of immunosuppression and/or tolerance induction.[105] Clinical trials are underway.[106,107]

10. Minimal Maintenance Immunosuppression

The use of powerful induction therapies may promote regulatory mechanisms and the induction of peripheral transplantation tolerance by depleting or interfering with the activation and/or effector function of alloreactive T cells at the time of transplantation. In most patients, maintenance immunosuppression can be tapered safely without acute rejection within months after transplantation. However, unlike in animal models, only a small proportion of patients can completely discontinue therapy (operational tolerance) or be kept on minimal immunosuppression (near tolerance) safely, i.e. in the absence of donor-specific alloresponses and with long-term normal graft function. Thus, in the absence of the induction of robust tolerogenic mechanisms (as may occur following a state of donor-type mixed chimerism), some degree of immunosuppression must be maintained as long as the allograft is in place. Maintenance regimens generally consist of CNIs, which block alloantigen-dependent T-cell activation, and/or antiproliferative agents.[3,4]

10.1 Calcineurin Inhibitors

The engagement of the TCR/CD3 complex (signal 1) in the presence of signal 2 activates the intracellular calcium-calcineurin signalling pathway and the induction of transcription factors, leading to the expression of new surface molecules such as inducible co-stimulatory molecules and cytokine receptors (figure 2). The use of a CNI remains the cornerstone of current maintenance immunosuppression.[108] Ciclosporin engages cyclophilin and tacrolimus engages FK506-binding protein 12 (FKBP12), both forming a complex that blocks calcineurin (hence the term CNI). Tacrolimus has become the CNI of choice for maintenance regimens in many centres because it inhibits calcineurin with slightly greater efficacy, and most trials suggest that there is slightly less acute rejection with tacrolimus than with ciclosporin. Both substances can result in nephrotoxicity and, rarely, thrombotic microangiopathy. Tacrolimus is less likely than ciclosporin to cause hypertension, hyperlipidaemia and cosmetic problems, but its use is associated with slightly more post-transplantation diabetes, mainly in elderly and obese recipients. Many of the adverse effects of CNIs are related to the concentration of the drug,

and careful monitoring of blood concentrations is therefore critical.

10.2 Antiproliferative Agents

Once T cells have been specifically activated through their TCR, the activation of the calcium-calcineurin pathway leads to the expression of cytokines and their receptors. The engagement of the IL-2 receptor delivers growth and proliferation signals via the down-stream phophoinositide-3-kinase and the mammalian target of rapamycin (mTOR) pathways, initiating the cell cycle.

Because of their great efficacy, inhibitors of nucleotide synthesis, such as mycophenolate mofetil or enteric-coated mycophenolic acid, are now included in most maintenance regimens. Protocols combining mycophenolic acid (MPA) agents and CNIs have improved patient and graft survival with reduced early and late allograft rejection.[109] However, the use of MPA agents can be limited by their adverse effects (mainly gastrointestinal and haematological) and higher costs, compared with azathioprine, which was previously used routinely in combination with a CNI. Furthermore, recent prospective randomized studies in recipients of cadaver kidney transplants suggest that, when used in combination with the new ciclosporin microemulsion formulation, MPA agents appear to offer no advantages over azathioprine in preventing acute rejection or chronic allograft dysfunction.[110,111]

Sirolimus and everolimus engage FKBP12, forming a complex that blocks mTOR (but does not inhibit calcineurin), resulting in inhibition of the cell cycle. By blocking cell proliferation, mTOR inhibitors have also been shown to have antineoplastic and arterial protective effects. *In vitro* assays as well as experimental transplantation models suggest that long-term immunosuppression regimens based on mTOR inhibitors may favour the induction of peripheral tolerance. Indeed, sirolimus was shown experimentally to facilitate peripheral deletion of effector alloreactive T cells by promoting activation-induced cell death, leaving a small pool of residual alloreactive T cells that could be regulated by T_{reg} cells.[46,112,113] Furthermore, recent data have demonstrated that sirolimus could selectively expand T_{reg} cells *in vitro* and *in vivo*, while CNIs appear to have an inhibitory effect.[114-116] Therefore, mTOR inhibitors may be interesting drugs to use in protocols that are aimed at inducing clinical tolerance. However, the potential beneficial effects of this class of drugs are balanced by known adverse effects such as impaired wound healing and skin lesions, hyperlipidaemia, delayed recovery from acute tubular necrosis and aggravation of proteinuria. Furthermore, the association of mTOR inhibitors with a CNI at normal dosages should be avoided as it results in synergistic nephrotoxicity.

11. Potential New Tools

In the past two decades, the use of immunosuppressive drugs has changed the outcome of organ transplantation mainly by preventing acute rejection and significantly improving survival. However, as discussed in the previous sections, most of the currently used drugs are associated with deleterious immune and non-immune adverse effects. Many of the immune-related complications are due to non-specific inhibition of T-cell effector functions, which impair appropriate responses against invading pathogens or tumours. Furthermore, as yet, none of the current clinical protocols have allowed the development of widely reproducible robust donor-specific transplantation tolerance. Thus, transplant immunobiologists are now exploring newer individualized therapies based on manipulated donor or recipient cells that specifically target donor alloantigens.[117]

11.1 Regulatory T Cells

CD4+CD25+Foxp3+ T_{reg} cells are a naturally occurring small population of CD4+ T cells that constitutively co-express the IL-2 receptor α chain (CD25). To date, because CD25 is also up-regulated on the surface of other T cells upon activation, the best marker for T_{reg} cells is the transcription factor Foxp3. These cells were shown to be crucial for the control of autoreactive T cells in autoimmune disease models, as well as being potent suppressors of the effector function of alloreactive T cells in trans-

plant models *in vivo*.[118-120] There is increasing evidence that in many experimental protocols where robust peripheral transplantation tolerance is achieved (via lymphocyte depletion or co-stimulatory blockade), immunoregulatory mechanisms dependent on donor-specific T_{reg} cells are critical in the induction and maintenance of the tolerant state.[121,122] On the basis of these observations, strategies exploiting antigen-specific T_{reg} cells in the induction of transplantation tolerance are currently being explored.

A better understanding of the mechanisms that promote immune regulation after transplantation has led to investigation of various approaches in experimental settings. A first strategy to promote peripheral tolerance would be to manipulate host immune responses in order to delete peripheral effector alloreactive T cells and at the same time favour the development and expansion of allospecific T_{reg} cells.[46,112,113] In accordance with previous experimental results, induction regimens based on co-stimulatory blockade or lymphocyte-depleting antibodies, as well as the use of mTOR inhibitors appear to be promising protocols in clinical transplantation. Another strategy that is currently under investigation is to achieve regulation of *in vivo* alloresponses by the transfer to the recipient of tailored donor-specific T_{reg} cells selected and expanded *ex vivo*. Preliminary data suggest that donor-specific T_{reg} cells could be used as an immunotherapeutic tool to promote transplantation tolerance.[117,123]

11.2 Manipulation of Dendritic Cells

DCs can modulate the differentiation of naive T cells into polarized T helper cells and thus are a major component in the regulation of T-cell responsiveness.[6] Agents that block DC maturation have been shown to induce tolerance in various experimental models.[7,124] Because of their reduced expression of co-stimulatory molecules and, to some extent, MHC and adhesion molecules, immature DCs are less efficient in activating allospecific T cells and thus induce donor-specific anergy. Furthermore, immature DCs have been shown to favour the generation of T cells with regulatory properties *in vitro*[125] and, by continuously acquiring antigens from the engrafted organ *in vivo*, could promote a state of donor-specific tolerance.[126]

12. Conclusion

Encouraging results from rodent and NHP experimental transplantation models have led to preliminary clinical trials with combination therapies aimed at promoting the induction of operational tolerance. However, the translation of these successful experimental protocols across species and into the clinic has proven to be a major challenge. Detailed characterization of rare spontaneous 'tolerant' transplant recipients, who are drug-free or only minimally immunosuppressed, may help in designing new tolerance-inducing strategies.[20,27-29] For the time being, while pursuing experimental and clinical research on defining the mechanisms involved in transplantation tolerance and investigating new tools for immune monitoring, it might be more realistic and safer for the majority of our patients to aim for efficacious maintenance immunosuppressive regimens with low toxicities, rather than trying to achieve true tolerance.

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