Transplantation Tolerance

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Summary: Tolerance following organ transplantation was first described in experimental models over 50 years ago. Reports of tolerance in clinical transplantation have appeared in the literature sporadically for decades. Despite this long-standing fascination with transplantation tolerance, the ability to reproducibly induce tolerance in humans undergoing organ transplantation has remained elusive. Recent advances in our knowledge of the mechanisms that contribute to the induction and maintenance of tolerance as well as those factors that oppose tolerance may allow the design of clinical trials aimed at introducing tolerance-inducing strategies into clinical transplantation.

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Although the work of work of Billingham et al[1] often is cited as providing the conceptual basis for tolerance, it should be noted that these experiments arose in large part from the earlier work of Owen,[2] who showed that the exchange of blood cells that occurred in utero in dizygotic cattle twins resulted in a state of persistent hematopoietic chimerism in each twin. Although Medawar et al first proposed using skin grafting as a means of distinguishing between monozygotic and dizygotic cattle twins, their repeated observation that dizygotic freemartin cattle accepted skin grafts from their dizygotic twin caused them to reformulate their hypothesis to postulate that the exchange of fetal blood in utero would promote tolerance to transplanted tissues in adult cattle. This hypothesis was directly tested in their seminal experiments in mice.

Briefly, a crude cell/tissue mixture from an allogeneic adult mouse was injected into 6 fetuses borne by a CBA female. Five healthy pups were born and 8 weeks later each underwent skin grafting with skin from the same allogeneic mouse strain used for the cell inoculation. Two of the 5 skin allografts were destroyed promptly (likely acutely rejected), 1 underwent a prolonged involution (likely chronic rejection), however, the final 2 allografts appeared perfectly healthy for 77 and 101 days. At this time, these 2 mice were challenged by implanting fragments of lymph nodes from mice immunized with donor antigen. This lead to the acute rejection of the 2 long-term surviving skin allografts. Attempts to reproduce this effect by inoculating neonatal mice with various tissues from mice of different strains were largely unsuccessful, with only 9 of 96 mice experiencing prolonged skin graft survival. Perhaps because of an incomplete appreciation of the details of these experiments, many in the field of transplantation took these results to indicate that a brief intervention before transplantation fundamentally could reset the immune system to promote the routine and nearly indefinite survival of organ allografts. However, as pointed out by Billingham et al,[1] the effect of this treatment was a continuum, with prolonged graft survivals ranging from a few days to indefinitely. Second, the regimen was largely ineffective in neonatal and adult mice and was consistently overcome by memory cells resulting from previous exposure to donor antigens.

The first functionally tolerant human transplant recipients were reported in 1975.[3] The term *functional tolerance* is used to distinguish the persistence of normal allograft function in

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the absence of immunosuppression in human beings from the more rigorous definition of *tolerance* used in experimental transplantation. In this series 6 patients who had been off immunosuppression for an average of 27 months were reported. Interestingly, only 2 rejections were noted. Owens et al went so far as to propose that in the absence of rejection, serious consideration should be given to not resuming immunosuppression. Similar to a subsequent report,[4] rejection, when it did occur, often was delayed for weeks or months. This group observed that the successful cessation of immunosuppression was more likely in the setting of human leukocyte antigen identical transplantation as identified by serotyping and mixed lymphocyte culture; the first use of an immunologic assay to predict tolerance.

Perhaps in response to the rarity of spontaneous tolerance and the limited effectiveness of available immunosuppressive agents and their toxicities, investigators began to explore strategies aimed at inducing tolerance in adults after transplantation. As early as 1955 Main and Prehn[5] created a state of full hematopoietic chimerism by injecting bone marrow into lethally irradiated allogeneic mice. To avoid some of the defects in protective immunity associated with the state of full hematopoietic chimerism, Ildstad and Sachs[6] devised an experimental strategy that resulted in a state of mixed hematopoietic chimerism in which both donor and recipient hematopoietic cells persisted long term. These mice displayed tolerance to donor-strain skin grafts. However, concerns about the toxicity associated with the conditioning regimens necessary to attain a state of stable hematopoietic chimerism has limited the enthusiasm for the routine clinical application of these approaches.

The next major advance toward achieving tolerance was based on the observation that signals resulting from engagement of the T-cell receptor alone were insufficient to promote complete activation of T lymphocytes. The additional or costimulatory signals necessary for full activation subsequently were shown to result from the engagement of receptors on T cells by their ligands, expressed largely by professional antigen-presenting cells. Two of the most widely studied costimulatory pathways and among the first described were the CD28/B7 (CD80 and CD86) and CD154/CD40 pathways. For a recent in-depth review of the ever-expanding known costimulatory pathways see Clarkson and Sayegh.[7] In 1992 it was reported that as a single intervention the short-term blockade of the CD28/B7 costimulatory pathway using cytolytic T lymphocyte-associated antigen immunoglobulin (CTLA4Ig) prolonged the survival of transplanted xenogeneic islets in mice and allogeneic hearts in rats.[8,9] Subsequently, it was reported that although blockade of either the CD28/B7 or CD154/CD40 pathways alone prolonged survival of heart allografts in mice, brief treatment with a combination of agents that blocked these 2 pathways prevented acute rejection and resulted in long-term allograft survival.[10] Although allografts from treated mice subsequently were shown to develop progressive damage suggestive of a chronic immunologic injury, combined costimulation blockade was viewed as the most immediate and feasible approach toward attaining tolerance to transplanted organs clinically.

TRANSLATION TO TRANSPLANTATION TOLERANCE IN HUMAN BEINGS: NONHUMAN PRIMATE STUDIES

Increasingly when possible the development of new therapeutic strategies in human beings is predicated on safety and efficacy data in preclinical models. Consequently, over the past decade many strategies aimed at inducing tolerance to transplanted organs and tissues have been investigated using nonhuman primate (NHP) models. Despite the promise and initial optimism surrounding numerous methods of inducing tolerance in rodent transplant models, subsequent experience has shown the sobering reality of how difficult it is to routinely induce tolerance in preclinical models and in clinical transplantation. Four major strategies for inducing transplantation tolerance have been investigated in-depth in NHP models including (1) blockade of costimulatory pathways, (2) bone marrow infusion/mixed chimerism, (3) pro-

found T-cell depletion, and (4) induction or transfer of regulatory T cells.

Tolerance Induction by Blockade of Costimulatory Molecules

Of those approaches targeting costimulatory molecules, only the CD28/B7 and CD154/CD40 pathways have been studied in-depth in NHP transplant models. Several antibodies specific for CD154 have been shown to prolong the survival of renal, heart, and islet allografts when administered as monotherapy, although none of these agents alone prevented the development of alloantibodies or created a state of tolerance.[11] Extension of these studies to human beings was halted because of the unexpected problem of thrombosis. Additional studies in NHP models confirmed the prothrombotic effects of anti-CD154 antibodies, which likely is a result of the expression of CD154 by platelets. In an attempt to avoid this complication, approaches to blocking CD40, which is not expressed by platelets, have been investigated and shown to prolong the survival of islet and kidney allografts in NHPs, although again virtually all recipients experienced rejection.[12]

In addition to CTLA4Ig antibodies to B7.1 and B7.2 (CD80 and CD86) have been used to block the CD28/B7 pathway in NHPs. Simultaneous treatment with anti-B7.1 and anti-B7.2 prolonged renal allograft survival in NHPs.[13] Importantly, blockade of either pathway alone failed to affect allograft survival. This may explain the limited protective effect of CTLA4Ig monotherapy initially observed in a NHP model of renal transplantation because CTLA4Ig has a relatively low affinity for CD86.[14] Consistent with this possibility, LEA29Y, a mutated form of CTLA4Ig with a 10-fold higher affinity for CD86, has been shown to inhibit rejection significantly when administered as monotherapy in NHP models of islet and renal transplantation.[14] Based on the synergy observed in rodent transplant models when blockade of CD154 was combined with the blockade of CD28, studies in NHP transplant models have been performed using CTLA4Ig or anti-CD86 and anti-CD154 or anti-CD40. The most promising results were obtained using either anti-CD86 or LEA29Y in combination with anti-CD40,[12] although again neither approach was associated with tolerance or prolonged drug-free survival.

Mixed Chimerism as a Means of Inducing Tolerance

As discussed earlier, in rodents various methods of recipient conditioning allow the engraftment of infused bone marrow cells, which in turn creates stable mixed hematopoietic chimerism and a state of donor-specific tolerance to organ allografts. Based on this powerful effect similar studies have been performed in NHP transplant models with interesting results. Similar to the results of the rodent studies, this strategy has been associated with the long-term, drug-free acceptance of renal allografts and donor-specific skin allograft acceptance in some NHP recipients.[15] However, this phenomenon was associated with only transient chimerism, raising questions about the mechanism of tolerance in this system. Also, chronic rejection has been reported in a heart transplant model in NHPs treated with this regimen, raising questions about the duration and robustness of tolerance induced by this approach.[16] Because of persistent concerns about the toxicities of early conditioning regimens, much work has focused on developing less-toxic conditioning regimens to make this therapy more clinically applicable.

Induction of Tolerance by T Cell Depletion

The magnitude of the alloimmune T-cell response is several log-fold greater than that to nominal antigens. From this standpoint, profound depletion of T cells is conceptually appealing as a means to prevent the early alloimmune injury of transplanted organs. It has been postulated that T cells re-emerging in the absence of the intense inflammation associated with ischemia/reperfusion injury and in the presence of donor antigen may be less likely to injure a transplanted organ. The most effective approach to profoundly deplete T cells in NHPs has been treatment with a monoclonal antibody specific for CD3 coupled to a modified diphtheria toxin. Used as monotherapy, anti-CD3/immunotoxin results in prolonged survival of renal allografts in a significant percentage of

recipients.[17] Despite the therapeutic appeal of this approach most recipients treated with this agent develop chronic rejection and eventually lose the allograft.[18] Unfortunately, because of the poor binding of alemtuzimab and many of the antibodies that comprise Thymoglobulin (Genzyme Corporation, Cambridge, MA) to NHP T cells, the effect of profound T-cell depletion mediated by these agents has not been explored in NHPs.

Regulatory Cells as a Means of Promoting Tolerance

Adoptive transfer of various populations of regulatory cells has been shown to induce tolerance effectively in a number of rodent models of autoimmunity and transplantation. In large part because of technical limitations, the adoptive transfer of regulatory T cells as a means to inhibit rejection or induce transplantation tolerance in NHPs has only begun to be investigated. The report that allografts surviving long term after the cessation of various forms of immunosuppression were infiltrated by CD4+transforming growth factor (TGF)-β1+ regulatory cells suggests that regulation may be an important and common mechanism responsible for allograft acceptance.[19] The recent demonstration that recipient T cells expanded in vitro by culture with apoptotic donor splenocytes under conditions of CD80 and CD86 blockade become anergic and when transferred into NHPs induced a state of donor-specific tolerance after renal transplantation in a subset of recipients illustrates the importance of regulatory cells as agents to promote transplantation tolerance.[20]

TOLERANCE STUDIES IN HUMAN BEINGS

Similar to studies in NHPs, it has proven frustratingly difficult to induce transplant tolerance in human beings. However, a number of isolated reports have documented the spontaneous occurrence of tolerance in human beings after transplantation. A review of the PubMed data base identified approximately 30 tolerant kidney transplant recipients reported in the literature worldwide, suggesting that spontaneous tolerance after kidney transplantation is exceedingly rare. In contrast, single-center reports suggest that approximately 20% of selected liver transplant recipients eventually may be weaned completely from immunosuppression, albeit with an approximately equal risk of developing acute rejection.[21] The largest single cohort of tolerant kidney transplant recipients reported included 10 patients who displayed spontaneous tolerance.[22] Interestingly, 9 of these 10 patients received kidneys from deceased donors, 7 were treated with calcineurin inhibitors, and 5 experienced at least 1 episode of acute rejection. The mean duration to complete drug withdrawal was 7.8 years, with a mean duration of tolerance when studied of 9.4 years. Phenotypic comparisons between peripheral blood mononuclear cell from a subset of these tolerant patients and patients receiving immunosuppression who had stable function or with healthy volunteers showed no significant differences.[23,24] However, in the peripheral blood of immunosuppressed patients with chronic allograft nephropathy, the frequency of $CD4^+CD25^+$ regulatory-type cells was decreased and the frequency of $CD8^+CD28^-$ effector-type cells increased relative to tolerant patients. To address the possibility that tolerant patients are globally immunosuppressed relative to other individuals, the response of tolerant patients to influenza vaccination was compared with that of transplant recipients receiving chronic immunosuppression and healthy volunteers. Importantly, although the antibody response of patients receiving immunosuppression was reduced dramatically compared with healthy volunteers, there was no significant difference between the humoral responses of tolerant patients and healthy volunteers.[25] All groups displayed similar T-cell responses to vaccination against influenza as assessed by immunologic assay-enzyme-linked immunospot (ELISPOT). As part of a study sponsored by the Immune Tolerance Network, we also have identified a cohort of spontaneously tolerant kidney transplant recipients. A preliminary analysis of the first 16 tolerant patients was presented at the World Transplant Congress in 2006. Currently, 21 tolerant recipients have been enrolled and mechanistic studies are underway. Taken together, these reports

show that although apparently infrequent, tolerance to transplanted organs does occur and can be long-lived.

Although reports of spontaneous tolerance bolster our conviction that tolerance is achievable in at least a subset of transplant recipients, the true aim is to be able to prospectively induce tolerance in at least a subset of transplant recipients. One approach toward achieving tolerance in human beings after transplantation is to use T-cell–depleting agents in an attempt to decrease the large size of the T-cell pool capable of responding to alloantigens. Although by no means an established principle, it has been postulated that by reducing the magnitude of the initial alloimmune T-cell response, early alloimmune injury is prevented, allowing time for the development of adaptive responses by the allograft and/or protective immune responses by the recipient immune system. Alemtuzumab [Campath-1H (Genzyme Corporation, Cambridge, MA)] is a monoclonal antibody specific for CD52. A short course of therapy with alemtuzumab results in transient but profound depletion of T cells and a lesser but still significant depletion of B cells, natural killer cells, dendritic cells, and monocytes. When used as an induction agent, alemtuzumab was shown to result in excellent graft and patient survival in patients maintained on low-dose cyclosporine monotherapy; a state termed *prope tolerance*. Subsequent groups attempted to exploit the profound immunosuppressive properties of alemtuzumab to induce transplantation tolerance. Kirk et al[26] reported a series of 7 kidney transplant recipients treated with a brief course of alemtuzumab in the absence of maintenance immunosuppression. All 7 patients developed acute rejection within the first month despite profound depletion of lymphocytes. Histologic assessment of allografts at the time of clinical rejection showed a paucity of lymphocytes but intense infiltration by monocytes and macrophages, suggesting that components of the immune system other than lymphocytes are sufficient to mediate acute rejection. Subsequent studies by this group also showed the relative resistance of T cells with a memory phenotype to depletion by alemtuzumab, suggesting that memory T cells may contribute to rejection after treatment with alemtuzumab.[27] In an attempt to increase the efficacy of alemtuzumab monotherapy, this group treated 5 renal transplant recipients with a combination of alemtuzumab and deoxyspergualin, an agent known to have inhibitory effects on monocytes and macrophages.[28] However, in all cases acute, but reversible, rejection developed. A variant of this approach has been used by other groups of investigators who have combined induction therapy with alemtuzumab with maintenance therapy with a variety of agents with the ultimate aim of withdrawing all immunosuppression gradually over time as mechanisms supporting tolerance develop. Knechtle et al[29] combined induction with alemtuzumab with sirolimus as a maintenance agent in 29 patients undergoing renal transplantation. Although graft and patient survival rates were excellent and 15 patients were able to be maintained on a single immunosuppressive agent, relatively high rates of early rejection caused the investigators to recommend changes in the initial maintenance regimen to include the addition of a calcineurin inhibitor or mycophenolate mofetil. Similar to this experience, Tan et al[30] recently reported the use of alemtuzumab induction in combination with tacrolimus monotherapy in recipients of kidneys from living donors. This group has reported that in the majority of patients tacrolimus could be partially weaned by decreasing the dose and/or increasing the interval between doses. Importantly, neither of these groups has withdrawn all maintenance immunosuppression successfully from patients treated with alemtuzumab. Thus, although alemtuzumab appears to be an excellent agent for facilitating the minimization of maintenance immunosuppression, it remains to be determined whether this approach will lead to true, immunosuppression-free transplantation tolerance.

The only approach thus far shown to lead to transplantation tolerance in human beings is combined nonmyeloablative bone marrow and kidney transplantation.[31] Six patients with renal failure associated with multiple myeloma underwent simultaneous kidney and bone marrow transplantations from human leukocyte antigen–identical siblings after nonmyeloablative

Table 1. Barriers to Tolerance

High frequency of alloreactive T cells
Homeostatic proliferation of lymphocytes after depletion
Alloreactive memory lymphocytes
Heterologous or cross-reactive memory T cells
Inflammation mediated by the innate immune system

conditioning. Each patient received a short course of maintenance immunosuppression with cyclosporine. All 6 patients have accepted the transplanted kidney with follow-up periods ranging from 1.3 to 7 years. In contrast to rodent models, long-term acceptance was associated with the persistence of chimerism in only 2 of the 6 patients, both of whom required maintenance therapy with cyclosporine for treatment of graft-versus-host disease. A modification of this regimen has been used more recently in the setting of haploidentical living-donor renal transplantation. Although the majority of patients have been weaned successfully from immunosuppression, episodes of humoral rejection appear common.[32] In sum, these studies show that although tolerance is making its way into clinical practice, a number of as yet poorly understood barriers exist that will need to be overcome before the broader application of these regimens.

BARRIERS TO TOLERANCE

Animal studies have identified a number of barriers to achieving tolerance after organ transplantation (Table 1). Unlike the relatively low precursor frequency of T cells capable of responding to a given nominal antigen, which is estimated to be approximately 1 in 100,000, experimental studies have shown that approximately 7% of the recipient T-cell pool responds to fully allogeneic grafts as indicated by proliferation.[33] This observation may provide some insight into the difficulty of achieving tolerance via strategies aimed at depleting recipient lymphocytes. Even after treatment with highly effective depleting agents such as alemtuzumab, the small percentage of T cells remaining may represent a relatively large absolute number of T cells that exceeds the threshold for the number of cells necessary to trigger a rejection episode. The effectiveness of depletional strategies is limited further by homeostatic proliferation of the residual lymphocytes. Homeostatic proliferation refers to the property of T cells residing in a lymphopenic environment that causes them to spontaneously divide and repopulate the host with numbers of T cells that more closely approximate lymphocyte numbers in normal individuals. Homeostatic proliferation of T cells has been shown to present a significant barrier to allograft survival at least in part owing to the memory-like phenotype acquired by homeostatically expanded T cells and their relative resistance to certain immunosuppressive agents such as agents that block T-cell costimulation.[34]

Similar to memory-like T cells that arise as a result of homeostatic proliferation, conventional memory T cells generated during an immune response also pose a barrier to the development of tolerance.[35] Several properties of memory T cells likely contribute to their ability to prevent the development of tolerance including their lower activation threshold, their ability to be activated outside of secondary lymphoid organs and independent of professional antigen-presenting cells, and their relative lack of dependence on conventional costimulatory signals. Although memory cells arising from previous exposure to alloantigens clearly inhibit the development of tolerance, memory T cells arising from exposure to non-HLA antigens such as viral antigens or environmental antigens also have been shown to prevent the development of tolerance.[36] This phenomenon, termed *heterologous immunity*, may help explain the resistance of supposedly naive (at least with respect to previous exposure to alloantigens) recipients to tolerance induction as well as the relative resistance of NHPs and human beings to tolerance induction compared with rodents housed in specific pathogen-free barrier facilities.

Although components of the adaptive immune system have long been considered to be the major factor responsible for the prevention of transplantation tolerance, the barrier to tolerance posed by innate immunity only recently

has been appreciated. Initially the innate immune system was thought to link early nonspecific allograft injury mediated by processes such as ischemia-reperfusion injury to the adaptive immune response, thereby amplifying antidonor alloimmunity. More recently it was shown that disruption of toll-like receptor (TLR) signaling facilitates the development of tolerance and that this effect is at least partially caused by impaired dendritic cell responses that tip the balance of the immune response after transplantation in favor of $CD4^+CD25^+$ regulatory cells.[37] It has been proposed that the resistance of some organs and tissues such as intestine, lung, and skin to tolerance induction after the blockade of costimulatory pathways is a consequence of enhanced TLR signaling in response to the environmental microorganisms to which these organs constantly are exposed. Chen et al[38] recently showed that augmenting TLR signaling prevented tolerance after heart transplantation in mice treated with anti-CD40L (a model in which tolerance routinely is achieved), whereas disruption of TLR signaling caused skin allografts (which normally are resistant to tolerance induction by anti-CD40L) to be accepted indefinitely. These reports suggest that the successful induction of tolerance may require targeting key components of the innate immune system as well as components of the adaptive immune system.

MECHANISMS OF TOLERANCE

The development of tolerance-inducing regimens in transplantation as well as the identification of biomarkers or assays predictive of tolerance would be facilitated greatly by an understanding of the mechanisms responsible for the induction and maintenance of tolerance. Although the specific mechanisms responsible for the rare occurrence of tolerance in human beings have not been elucidated, 4 mechanisms appear to account for nearly all cases of tolerance observed in experimental transplant models (Table 2). Ignorance refers to the observation that T cells failing to encounter donor antigen in the correct environment are not activated appropriately and hence do not contribute to the antidonor immune response. The best example of ignorance as a mechanism promoting tolerance is the long-term survival of heart allografts in splenectomized mice that lack all lymph nodes as a result of a genetic mutation.[39] In the complete absence of secondary lymphoid organs, recipient T cells fail to undergo initial activation when they encounter donor alloantigens. Although likely important, ignorance as a mechanism of tolerance is unlikely to be the main mechanism promoting tolerance because memory T cells can be activated fully in the absence of recipient secondary lymphoid organs, and inflammation unrelated to alloantigen (ie, viral infections) may alter T-cell migration such that ignorant T cells encounter previously sequestered donor antigens. *Anergy* refers to the state of T cells that are not actively in the process of dying but remain unresponsive to antigenic stimulation. Although best described as a mechanism for maintaining tolerance to self-antigens, anergy likely contributes to transplantation tolerance.

Table 2. Mechanisms of Tolerance

Ignorance
Anergy
Regulation or suppression
Deletion of alloantigen-specific lymphocytes

The 2 mechanisms most widely held to be responsible for tolerance after transplantation are regulation and deletion. *Regulation* refers to an active process in which components of the recipient immune system respond to donor antigen in a manner that inhibits or suppresses injurious effector mechanisms. Numerous cell types may have regulatory properties including naturally occurring $CD4^+CD25^+$ regulatory cells, natural killer cell 1.1 (NK1.1), $C8^+CD28^-$, and $CD3^+CD4^-CD8^-$ cells. Several cell surface molecules including CD45RB, glucocorticoid-induced TNFR-related protein (GITR), CTLA4, and CD103 all have been associated with various populations of cells that display a regulatory function, but the transcription factor forkhead box protein 3 (FOXP3) is considered to be a critical controller and major marker of at least naturally occurring regulatory T cells. The regulatory effects of T cells may be mediated by

the production of suppressive cytokines such as TGF-β or interleukin-10, or in the case of naturally occurring regulatory cells caused by as yet incompletely understood cell contact–dependent mechanisms. The observation that regulatory T cells can be detected in a significant number of renal transplant recipients as early as 3 months after transplantation and that they may persist for many years[40] suggests that tolerance, if it is maintained by regulatory cells, depends on the balance between recipient cells with regulatory properties and those with effector properties. The contribution of regulatory mechanisms to tolerance after transplantation is supported by the observation that 3 tolerant transplant recipients all displayed regulation that was dependent on the production of TGF-β and/or interleukin-10, as assessed using the trans-vivo delayed type hypersensitivity (DTH) assay.[41]

Deletion is also an active process in which T cells responding to donor antigens are selectively purged from the recipient T-cell repertoire. Deletional mechanisms are known to be critical for establishing and maintaining self-tolerance and are the dominant mechanisms by which tolerance to transplanted organs is maintained in many experimental models of combined organ and bone marrow transplantation. Several different processes can cause alloreactive T cells to undergo apoptosis or programmed cell death and thus be deleted, including clonal exhaustion as a result of chronic antigenic stimulation, activation in suboptimal conditions such as the lack of critical costimulatory signals, cytokine withdrawal, or signaling through death receptors belonging to the tumor necrosis factor–receptor superfamily.

Most reviews of tolerance mechanisms discuss each potential mechanism as if it were a discreet process. However, it is likely that multiple mechanisms contribute to the development and maintenance of tolerance for any given individual. It seems equally likely that these mechanisms change over time, vary between seemingly similar individuals, and differ depending on the organ transplanted and the previous immunologic history of the individual. In our experience it is clear that tolerance is not a permanent end point but rather a state of varying duration that unfortunately may wane with time, resulting in immunologic allograft damage or loss. In recognition of this phenomenon some investigators have preferred the term *metastable tolerance*.

POTENTIAL TOLERANCE ASSAYS

Current approaches to managing immunosuppression are largely empiric and reactive rather than proactive. This is the result of our inability to predict accurately how the recipient immune system will respond to a given organ allograft. Usually immunosuppression is selected based on broad clinical criteria. Deviations from standard protocols occur only in response to significant changes in the recipient's clinical course. A more ideal situation would be to develop assays to adequately characterize the status of the recipient immune system. By using these assays to monitor the recipient immune system routinely, changes in immunosuppression could be made before the development of immunologic graft injury or the occurrence of the unwanted consequences of overimmunosuppression. The development of immune monitoring assays would allow the safe individualization of immunosuppression and greatly facilitate tolerance studies.

The large number and complexity of assays that have been proposed for monitoring the immune response after transplantation (Table 3) precludes their comprehensive discussion in this article. Interested readers are referred to recent reviews of potential tolerance assays.[42,43] Briefly, assays can be categorized as either antigen-specific or non–antigen-specific and as being based on either cellular or molecular methods.

Antigen-specific assays have the advantage that they measure the response of the immune system to antigens expressed uniquely by the transplanted organ. As discussed, transplantation tolerance implies a state of donor-specific hyporesponsivness or unresponsiveness rather than a state of global immune suppression. Thus, donor-specific assays offer significant conceptual advantages. However, practical limitations such as the requirements for large numbers of viable recipient lymphocytes as well as

Table 3. Potential Tolerance Assays

Antigen specific
ELISPOT
Trans-vivo delayed-type hypersensitivity assay
Limiting dilution assay
CFSE mixed lymphocyte reaction–proliferation assay
Intracellular cytokine staining after donor antigen stimulation in vitro
Determination of antidonor antibodies
Antigen nonspecific
Characterization of T-cell receptor repertoire (TcLandscape, Nantes, France)
Phenotypic characterization of peripheral blood mononuclear cells by flow cytometry
Gene polymorphisms
Gene transcription/transcriptional profiling
Microarrays
Real-time reverse-transcriptase polymerase chain reaction
Urine proteomics
Cylex (Columbia, MD) immune cell function assays–polyclonal stimulation with PHA in vitro

Abbreviations: CFSE, 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester; PHA, phytohemagglutinin.
Reviewed in Newell and Larsen[42] and Najafian et al.[43]

large numbers of donor cells to use as stimulators complicate the routine use of these assays.

Antigen-nonspecific assays avoid some of these potential difficulties but may be reflective of changes in the immune system not specifically arising from exposure to the transplanted organ. The rapid evolution of molecular techniques such as determination of gene expression using microarrays (genomics), determination of protein expression (proteomics), metabolomics, or analysis of gene polymorphisms has created unique opportunities and problems. Instead of limiting investigators to testing hypotheses based on known paradigms, these methodologies allow investigations of many pathways of unknown importance simultaneously.

These hypothesis-generating approaches may discover new unrecognized pathways that will help to determine the fate of transplanted organs. However, the vast amounts of data these methods generate have challenged investigators to develop new approaches for data management and analysis. Given the likelihood that a number of immunologic mechanisms contribute to the acquisition and maintenance of tolerance and that these mechanisms vary between individuals and change over time, it seems likely that a battery of sequentially performed assays will be necessary to create a reliable picture of the recipient immune system after transplantation and to detect the emergence of tolerance and its impending loss. The importance of developing monitoring assays in transplantation in general and tolerance assays in particular is shown by the large number of multicenter, cooperative studies that aim to develop monitoring assays including the Cooperative Trials in Organ Transplantation (available at: https://www.ctotstudies.org/index.htm), the Registry of Tolerant Kidney Transplant Recipients (available at: http://www.immunetolerance.org/registry/), and the Indices of Tolerance Study (available at: http://www.transplant-tolerance.org.uk/introduction.aspx).

CONCLUSIONS

The failure of long-term outcomes to improve at the same pace as improvements in short-term outcomes after organ transplantation together with the costs and toxicities of chronic, nonspecific immunosuppression provide the impetus to work toward the development of transplantation tolerance in at least selected subsets of transplant recipients. In this regard it may be important to recognize that tolerance is not a final, immutable end point but rather a continuum ranging from transient hyporesponsiveness to sustained unresponsiveness. As our understanding of the mechanisms of transplantation tolerance and the barriers to tolerance improves, tolerance studies should become increasingly feasible and successful. Preclinical studies in NHPs will play a major role in selecting the safest and most promising regimens to induce tolerance in human beings and in identifying those factors limiting success. Integral to the safe and successful development of clinical transplantation tolerance will be the

development of immune monitoring or tolerance assays that can be feasibly introduced for the routine management of immunosuppression after transplantation.

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