

## Is Tolerance in Renal Transplantation Possible?

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### Abstract

***Kidney transplantation remains the optimal treatment for end-stage renal failure by improving patient survival and quality of life. Although modern immunosuppressives have largely overcome the problem of acute rejection, long-term allograft survival rates have not improved since the 1990s as the immunosuppressive drugs that we use to protect the graft ultimately lead to its destruction by causing chronic allograft nephropathy. It is for these reasons that the induction of tolerance remains the ultimate goal in transplantation. While we have made major advances towards achieving tolerance in renal transplantation using animal models, there are still several major hurdles that must be overcome to allow the translation of data from experimental models into clinical trials. This review discusses the four strategies used to achieve tolerance, namely mixed chimerism, co-stimulation blockade, lymphocyte depletion and regulatory T-cell immunotherapy. We examine each technique's strengths and pitfalls and argue that immunologic tolerance may be possible by using a combination of strategies.*** (Trends in Transplant. 2008;2:117-28)

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### Key words

***Transplantation. Tolerance. Mixed chimerism. Co-stimulation blockade. Lymphocyte depletion. Regulatory T-cell.***

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## Introduction

One of the goals in transplantation is to achieve tolerance to the transplanted organ. Ray Owen<sup>1,2</sup> together with Billingham, Brent and Medawar<sup>3</sup> demonstrated this concept of transplantation immunologic tolerance. In this now classic citation, Billingham, et al. demonstrated that tolerance could be induced deliberately by the introduction of donor-specific antigen into neonatal mice and this allowed them as adults to accept skin allografts from the same donor<sup>3</sup>. While this was a very promising start, the translation of tolerance from animal models to the clinical setting has remained very challenging and elusive.

## Definitions of transplant tolerance

The definition of transplant tolerance is much debated. The definition of “true” tolerance is the long-term acceptance of a transplant in the absence of any immunosuppressive drugs in an immunocompetent host, with the transplant displaying normal histologic characteristics and function<sup>4</sup>. In experimental models, this definition of tolerance also requires that the recipient be able to accept a second graft from the same donor, while being able to reject a third-party graft. This “functional” definition does not attempt to assign a strategy for the induction, nor a mechanism that is responsible for the tolerant state, and therefore can most probably be accepted by the majority of clinicians and scientists working in this very active field.

Achieving tolerance in clinical transplantation, however, is clearly a formidable task and may only be possible in specific groups of transplant recipients. Nevertheless, striving for this goal in clinical transplantation is important as, in the process of defining strategies that will result in transplant tolerance and the mechanisms that are brought into play,

this will lead to innovations in therapy and clinical care that will benefit transplant recipients in the future. For example, the concept that lifelong, high-dose immunosuppression is essential in maintaining graft function in everyone who receives a transplant is being challenged as there are clearly subgroups of transplant recipients, notably liver recipients, who can slowly be weaned off immunosuppression<sup>5</sup>. This weaning off immunosuppression produces a clinical state termed “operational tolerance”, which is defined as the long-term survival of a graft with stable function in the absence of maintenance immunosuppressive drugs<sup>6</sup>. This may be a more realistic goal to strive for as opposed to “true” tolerance. It may not, however, be possible in all transplant recipients, and reducing or minimizing immunosuppression in the long term may be achieved in these recipients instead.

The Souillou group have described a subset of renal transplant recipients that exhibit spontaneous “operational tolerance”<sup>6</sup>. They have studied these patients and identified clinical factors as well as a gene signature that is associated with this state<sup>7</sup>. These could prove very useful tools in identifying the subgroup of patients that could successfully be weaned off immunosuppression.

## The importance of achieving tolerance

The introduction of cyclosporine, a calcineurin inhibitor (CNI), in 1970, revolutionized transplantation by overcoming the problem of acute rejection and allowing long-term graft acceptance. Since then, a variety of immunosuppressive drugs, such as tacrolimus, mycophenolate mofetil, and sirolimus, just to name a few, have been developed. Why is it then that the quest for tolerance is still so sought after, given that we have an array of immunosuppressive drugs? This is because although the CNI (cyclosporine and tacrolimus) have

dramatically improved patient and graft survival after transplantation, they are often used as part of a cocktail of immunosuppressants that must be continued indefinitely posttransplantation, each component bearing a significant side-effect profile. Among the most severe side effects are infections, malignancy, nephrotoxicity, hypertension, and diabetes mellitus<sup>8</sup>. Furthermore, these very drugs that we rely on to protect the graft actually cause damage in the long term by leading to chronic allograft nephropathy in renal transplant recipients. Moreover, it has been shown that some immunosuppressive drugs, notably steroids and CNI, may have a negative impact on the natural mechanisms the immune system uses to promote the generation of tolerance<sup>9</sup>, while others, such as sirolimus, may promote tolerance via the development of regulatory T-cells ( $T_{reg}$ )<sup>10</sup>. Therefore, one of the major goals of new immunosuppressive regimens being developed is to reduce the dose of immunosuppressants and avoid the long-term use of steroids and CNI. Instead, they aim to maintain graft function in the long term by encouraging the development of specific unresponsiveness or tolerance to the transplant.

### **Why has achieving tolerance in the clinic proved such a formidable task?**

The human immune system is a complex interplay of multiple cell types and pathways. The more we understand about this system, the clearer it becomes that our immune system has evolved to include a redundancy within it. Therefore, to achieve tolerance in humans, multiple pathways need to be targeted.

The vast experience of the human immune system, acquired as a result of a constant exposure to environmental antigens, could be one of the critical differences between naive animal models and humans. This

may explain why tolerance induction protocols that are effective in animal models have proved less successful in the clinic. Most tolerance studies have been performed in pathogen-free rodents and nonhuman primates, which lack a large pool of memory T-cells that are present in human transplant recipients. If memory T-cells hinder the induction of transplant tolerance, then we would expect children (who have a relatively small memory T-cell pool) to have better outcomes following transplantation. Support for this theory comes from two large cohort studies following liver transplant recipients, in which pediatric age at transplantation was associated with ability to successfully withdraw immunosuppression<sup>11,12</sup>.

While immunologic memory is a critical component in the fight against infection, in clinical transplantation it acts as a barrier to tolerance induction due to the presence of alloreactive memory T-cells. These cells are generated pretransplant in sensitised individuals due to previous transplants, blood transfusions or pregnancies, and in non-sensitised individuals as a result of cross-reactivity with viral antigens (heterologous immunity)<sup>13,14</sup> or via homeostatic proliferation<sup>15</sup>. Memory T-cells also contribute to late graft loss as memory T-cells that develop after a rejection episode are refractory to current drug therapy<sup>16</sup>. Furthermore, memory T-cells have different costimulatory requirements than naive T-cells<sup>17</sup>, and therefore strategies that target the classical CD28-B7 or CD40-CD154 co-stimulatory interactions are likely to be ineffectual in controlling the memory cell pool.

### **Strategies to achieve transplant tolerance**

Tolerance can be categorized as either central or peripheral tolerance. Central tolerance refers to the intra-thymic deletion of alloreactive T-cells. In experimental studies, mixed chimerism has been shown to be an

example of central tolerance. Peripheral tolerance is mediated by a combination of mechanisms, including deletion, anergy, ignorance, or regulation<sup>18</sup>. Strategies such as T-cell depletion, co-stimulatory blockade and T<sub>reg</sub> immunotherapy attempt to achieve tolerance via peripheral tolerance mechanisms.

## Mixed chimerism

Chimerism occurs when foreign (donor) hematopoietic cells are present in an individual. Microchimerism occurs when representation of donor hematopoietic cells is < 1%, whereas macrochimerism occurs when donor cells constitute > 1% of host hematopoietic cells. Within macrochimerism, complete chimerism occurs when all hematopoietic cells are of donor origin (for example following myeloablation and transplantation of donor hematopoietic cells), whereas in mixed chimerism, donor cells constitute > 1% but < 100% of the total, which can occur following non-myeloablative host conditioning.

Microchimerism occurs in some organ transplant recipients when donor hematopoietic cells from the transplanted organ persist, typically at levels detectable only by polymerase chain reaction<sup>19</sup>. Theoretically, microchimerism can result in modulation of the immune response to donor antigens. However, the significance of microchimerism *in vivo* remains unclear, as there does not appear to be any clear correlation with acceptance or rejection. In one study, only one-third of patients with long-term graft survival demonstrated microchimerism<sup>20</sup>, whereas in others, microchimerism could still be detected in patients experiencing allograft rejection<sup>21,22</sup>, with the level of chimerism fluctuating with time<sup>22</sup>.

Individuals who have complete chimerism after myeloablative therapy and bone marrow transplantation subsequently accept solid organ allografts from the same donor<sup>23</sup>.

However, the morbidity and mortality associated with complete myeloablation has precluded the clinical translation of protocols that lead to full chimerism. Mixed chimerism, however, is a more promising alternative as it is associated with a reduced susceptibility to graft-versus-host disease (GVHD)<sup>24</sup>, whilst maintaining improved immunocompetence<sup>25</sup>. Consequently, there has been an intense research focus on strategies to augment mixed chimerism following transplantation.

To achieve tolerance, both the preexisting mature donor-reactive T-cells and the developing donor-reactive T-cells need to be eliminated or inactivated in a sustained manner. To achieve the former, experimental models of mixed chimerism utilize total body irradiation (TBI)<sup>26</sup>, cytotoxic drugs, T-cell-depleting antibodies<sup>27,28</sup>, or the induction of peripheral clonal deletion using co-stimulatory blockade<sup>29</sup>. Once peripheral donor-reactive T-cells are rendered ineffective, central tolerance in mixed-chimerism models is achieved by the following mechanism: donor stem cells engraft in the recipient's bone marrow, giving rise to cells of multiple hematopoietic lineages including hematopoietic progenitor cells that seed the thymus. In the thymus, these cells can develop into specialized thymic dendritic cells<sup>30</sup> that have the capacity to induce deletion of antigen-specific T-cells (clonal deletion). Developing thymic T-cells reactive to antigens expressed on hematopoietic cells undergo negative selection. Consequently, in mixed chimerism, both host and donor hematopoietic cells mediate intra-thymic deletion of host and donor reactive T-cells<sup>31</sup>. This renders the host tolerant to host and donor antigens, as long as the donor hematopoietic stem cells persist in the bone marrow.

Early work demonstrated that mice reconstituted with a mixture of recipient and donor bone marrow after TBI, develop mixed chimerism and robust tolerance to donor skin grafts<sup>26</sup>. Subsequent studies aimed at reducing

the toxicity of the host conditioning demonstrated similar results, using either depleting anti-CD4 and anti-CD8 monoclonal antibodies (MAb)<sup>27</sup> or co-stimulatory blockade<sup>32</sup> prior to a non-myeloablative dose of TBI. Immunocompetence is demonstrated by the fact that these mice are able to reject third-party grafts<sup>27</sup>, whereas protocols that induce full chimerism also induce a degree of immunoincompetence<sup>33</sup>. Interestingly, while mixed chimerism is often sustained indefinitely in mice using these protocols, in nonhuman primates (NHP), mixed chimerism is maintained for only a few weeks<sup>34</sup> and yet long-term graft survival is maintained.

These promising animal results posed an interesting ethical dilemma that delayed its translation into human studies: namely, does the benefit of long-term immunosuppression-free graft survival outweigh the risks of bone marrow ablative therapy in patients with end-stage renal disease (ESRD) and normal bone marrow? This question remained unanswered until 1998, when a trial began in patients with renal failure due to myeloma, who therefore required both bone marrow and renal transplants. Using a protocol developed in NHP, patients underwent thymic irradiation together with antithymocyte globulin (ATG), but with cyclophosphamide replacing TBI prior to simultaneous human leukocyte antigen (HLA)-matched bone marrow and renal transplantation. So far, six such transplants have been performed, with all patients accepting their grafts long term<sup>35</sup>. Interestingly, three patients lost detectable chimerism but maintained graft function without immunosuppression or rejection episodes for up to seven years<sup>35</sup>. This approach has since been extended to five patients with ESRD but without bone marrow disease. The protocol employed was similar to the previous study, except ATG was replaced with anti-CD2 MAb, and bone marrow was from HLA single-haplotype mismatched living-related donors<sup>36</sup>. All five patients developed transient microchimerism, with four recipients demonstrating excellent renal function

for up to five years posttransplantation following withdrawal of all immunosuppressive therapy<sup>36</sup>. The mechanism responsible for such excellent results using this protocol remains unclear. *In vitro* testing of T-cells from the tolerant recipients showed donor-specific unresponsiveness, which is consistent with a central deletion mechanism (as outlined earlier), whereas allograft biopsies performed after withdrawal of immunosuppression had high levels of forkhead box protein 3 (Foxp3) messenger RNA, indicating a role of T<sub>reg</sub> cells in peripheral tolerance. Another important clinical aspect of this study is that it successfully employed HLA-mismatched bone marrow donors, without any evidence of GVHD. Unfortunately, one patient experienced acute humoral rejection on day 10 and subsequently underwent retransplantation using a conventional regimen.

A similar ongoing trial reported persistent mixed chimerism in one patient who received combined renal transplantation and HLA-matched donor hematopoietic cells, following a conditioning regime of total lymphoid irradiation, ATG, and mycophenolate mofetil for one month posttransplantation<sup>37</sup>. Allograft function has been normal for more than two years since discontinuation of immunosuppression, with no episodes of rejection. Analysis of this patient's T-cells after transplantation demonstrated that naive CD8<sup>+</sup> T-cells repopulated the periphery faster than naive CD4<sup>+</sup> T-cells. This was thought to be due to peripheral expansion rather than thymic generation of new T-cells. Additional analysis indicated that donor lymphocytes present in the recipient were of thymic origin, suggesting a central deletion mechanism<sup>37</sup>. This study supports earlier work demonstrating that pretransplant total lymphoid irradiation can induce mixed chimerism and immune tolerance to cadaveric renal allografts<sup>38</sup>, whereas posttransplant total lymphoid irradiation produces transient microchimerism and acute rejection following withdrawal of immunosuppression<sup>39</sup>.

Taken together, these studies show that protocols which induce mixed chimerism can lead to long-term donor-specific tolerance following renal transplantation. Although the mechanism remains incompletely understood, the potential therapeutic application of these approaches is enormous.

## Co-stimulatory blockade

After binding peptide presented by the major histocompatibility complex (MHC) of antigen presenting cells (APC), an effective T-cell response can only be generated if the T-cell receptor signal is accompanied by a second, co-stimulatory signal. Without this co-stimulatory signal the T-cell becomes anergic. Consequently, there has been an intense research effort to harness the therapeutic potential of co-stimulatory blockade.

Naive T-cells constitutively express the co-stimulatory receptor CD28, which engages CD80 and CD86 proteins on APC, whereas activated T-cells express CD154 (CD40 ligand), which interacts with CD40 on APC. Whereas CD28 augments T-cell function when bound simultaneously with the T-cell receptor, CTLA4 (cytotoxic T lymphocyte antigen 4, a CD28 family receptor) inhibits T-cell functioning. Although other co-stimulatory molecules have been identified, CD154 and CD28 MAb have been the most extensively studied in humans.

Early work demonstrated that simultaneous blockade of the B7-CD28 and CD40-CD154 pathways produced long-term graft survival in rodents<sup>40</sup>, and CD154 blockade alone was shown to prevent renal allograft loss in rhesus monkeys<sup>41</sup>. However, subsequent follow-up studies found that only 50% of recipients in the former study experienced permanent engraftment<sup>42</sup> and in the later study repeated anti-CD154 was required to prevent acute rejection<sup>43</sup>. Furthermore, a repeat of Kirk's rhesus monkey study using older

animals failed to produce permanent engraftment, which was attributed in part to the presence of memory cells in the older host, as secondary immune responses are less amenable to co-stimulatory blockade<sup>44</sup>. In addition to age-related memory cell disparity, environmental exposure and intrinsic immune system differences contribute to the divergent outcomes following co-stimulatory blockade in rodent and NHP models. For example, viral infection at the time of transplantation can activate toll-like receptors on T-cells, which renders alloreactive CD8<sup>+</sup> T-cells resistant to co-stimulatory blockade-induced apoptosis<sup>45</sup>.

Anti-CD154 alone is clearly not sufficient to induce tolerance. However, administration of T-cell-replete bone marrow at the time of anti-CD154 co-stimulatory blockade leads to robust donor-specific tolerance in mice<sup>46</sup>, and to a lesser extent in NHP, as although all eight NHP recipients demonstrated graft survival of up to five years following discontinuation of immunosuppression, three had late episodes of chronic rejection<sup>47</sup>.

Human trials utilizing CD154 blockade have been less encouraging. Acute rejection rates were unexpectedly high, and thrombotic complications limited further use<sup>48</sup>. Thorough pathologic analysis of seven NHP treated with anti-CD154 has revealed that two had thrombotic complications, suggesting that such complications may be intrinsically related to anti-CD154 treatment rather than being a human-specific effect<sup>49</sup>. The CD154 is expressed on platelets and activated endothelium, and has recently been found to play an essential role in stabilization of arterial thrombi<sup>50</sup>. Unless this intrinsic complication can be overcome, it is unlikely that anti-CD154 therapy will make the transition from bench to bedside.

The use of CTLA4-Ig on its own has failed to produce tolerance in murine and NHP models. This may be related to its lower affinity for CD86 than CD80. Consequently, by modifying

the CTLA4-binding domain with amino acid substitutions, affinity for CD80 and CD86 is increased twofold and fourfold, respectively<sup>40</sup>. This modified CTLA4-Ig (belatacept) produced significant prolongation of renal allograft survival in a NHP model<sup>40</sup>. However, in this model, belatacept cannot be viewed as tolerogenic, as maintenance therapy with mycophenolate and steroids was required.

Similar to anti-CD154 treatment, CTLA4-Ig alone does not appear to induce tolerance, but its low incidence of side effects makes it an attractive alternative to traditional maintenance immunosuppression. Belatacept produces long-term survival of pancreatic islet cells when combined with sirolimus in a NHP model by preventing the priming of anti-donor T- and B-cell responses<sup>51</sup>. In contrast to the anti-CD154 experience, similar results have been found in humans: in a randomized trial powered to show lack of inferiority of belatacept versus cyclosporine, chronic administration of belatacept improved glomerular filtration rates and decreased chronic allograft nephropathy after 12 months, with similar incidences of acute rejection when compared to cyclosporine-based maintenance therapy<sup>52</sup>.

Concerns have been raised regarding the safety of chronic immunoglobulin administration. However, to date there appears to be no excess incidence of posttransplant lymphoproliferative disease<sup>52</sup>, and two large-scale phase III trials are underway to definitively establish the efficacy and safety of belatacept. Thus, belatacept currently appears to provide a promising alternative to traditional maintenance immunosuppressive therapy. It remains undetermined whether or not belatacept can ultimately be used to induce tolerance. A proof of concept trial by the Immune Tolerance Network aims to address this question by withdrawal of sirolimus after one year and belatacept after two years in living-related renal transplant recipients who have had no episodes of rejection and no evidence of antidonor alloreactivity<sup>52</sup>.

In summary, whether or not co-stimulatory blockade can induce tolerance remains unknown. To date, none of the studies have shown robustly that it does ultimately produce tolerance. It may, however, be useful as an alternative to conventional immunosuppressive drugs, if their long-term side-effect profile is shown to be favorable.

## **Lymphocyte depletion**

As many as one in 10 naive T-cells are able to recognize allogeneic MHC antigens. This results in a large alloreactive T-cell pool that is able to reject an organ on transplantation<sup>53</sup>. Theoretically, depletion of this pool of T-cells at the time of transplantation prevents rejection by preventing immune engagement at a time when “danger signals” are maximal. Instead, the encounter between the allograft and the alloreactive pool is delayed and occurs within a more quiescent milieu. The theoretical basis for lymphocyte depletion is that this later encounter may shift the immune response towards unresponsiveness rather than rejection<sup>18</sup>.

Depletion strategies used clinically today take the form of polyclonal preparations such as ATG, or MAb such as alemtuzumab (Campath-1H). Despite the profound depletion effects of both these preparations, they unfortunately do not lead to long-term tolerance. Instead, they are used as induction agents at the time of transplantation to allow for lower doses of maintenance immunosuppression.

Campath-1H is a powerful, depleting, humanized MAb that targets CD52, one of the most abundant proteins on the lymphocyte surface<sup>54</sup>. Depletion of lymphocytes by Campath-1H at the time of transplantation has been shown to induce a state in which patients are able to maintain stable transplant function with minimal immunosuppression<sup>55,56</sup>. Importantly,

some recipients could be weaned off immunosuppression and a state of donor-specific unresponsiveness demonstrated<sup>57</sup>. The use of Campath-1H alone, however, at the time of kidney transplant has unfortunately been unsuccessful and uniformly leads to acute rejection of the graft<sup>58</sup>. This phenomenon has been attributed to memory T-cells that are relatively resistant to depletion and prevalent at the time of rejection of the allograft<sup>59,60</sup>.

Polyclonal ATG is produced from purifying the IgG fraction of the serum of rabbits or horses that have been immunized with thymocytes or T-cell lines. Rabbit ATG (thymoglobulin) and horse ATG (lymphoglobulin) are the most widely used preparations<sup>61</sup>. As with Campath-1H, rabbit ATG use in humans results in a profound depletion that facilitates lower doses of maintenance immunosuppression to be used posttransplantation. Of the two agents, Campath-1H was found to be a more effective agent for induction<sup>56</sup>.

The effects of Campath-1H on  $T_{reg}$  cells appears to be inconclusive, with some studies showing an increased ratio of  $T_{reg}$  to T-effector cells<sup>62</sup>, and others showing a depletion of  $T_{reg}$  cells<sup>59</sup>. Pearl, et al. also showed that rabbit ATG efficiently depleted  $T_{reg}$  cells<sup>59</sup>.

Therefore, in summary, lymphocyte depletion has allowed a reduction in maintenance immunosuppression in renal transplant recipients, but has failed to lead to tolerance to the allograft. However, this strategy may be useful in combination with other strategies, such as  $T_{reg}$  immunotherapy, to render a patient tolerant to his allograft.

## Regulatory T-cell immunotherapy

The concept of the suppressor, or regulatory T-cell as it is now more commonly known, was initially conceived in the early 1970s when Gershon and Kondo found that a subset of

T-cells were able to suppress the immune response and were distinct from T-cells that augmented and propagated an immune response<sup>63</sup>. However, it has taken us more than 30 years to establish their presence beyond reasonable doubt and to begin to attempt to translate their use into clinical practice. Today, it is well accepted that they play a crucial role in active regulation of the immune system to self antigens, thereby preventing autoimmune diseases<sup>64</sup>. This physiologic role of the  $T_{reg}$  cells has now been exploited and manipulated in various experimental models to allow the host to be tolerant to alloantigens in the form of a transplanted organ.

## Subsets of regulatory T-cells

Two main types of  $T_{reg}$  subsets have been delineated – the thymus-derived or natural  $T_{reg}$  cells and the adaptive or induced  $T_{reg}$  cells. Natural  $T_{reg}$  cells are thymus-derived cells that are  $CD4^+CD25^+$  and  $Foxp3^+$  also<sup>65</sup>. The forkhead transcription factor,  $Foxp3$ , was identified in 2003 as critical for  $T_{reg}$  development and function<sup>66,67</sup>.  $Foxp3$  mutations occur spontaneously in Scurfy mice and humans suffering from IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome. Both these disorders exhibit a lack of  $T_{reg}$  cells and severe lymphoproliferation and autoimmune disorders<sup>68,69</sup>. This has proved a very useful marker in identifying  $T_{reg}$  cells in mice. It has, unfortunately, proven less reliable in humans as it has recently been shown that human T-cells turn on  $Foxp3$  upon activation<sup>65</sup>. This lack of exclusivity does not diminish the importance of  $Foxp3$  in  $T_{reg}$  biology, but it does mean that this marker cannot be used reliably for identifying  $T_{reg}$  cells in humans.

Adaptive  $T_{reg}$  cells are generated in the periphery by conversion of  $CD4^+CD25^-FoxP3^-$  T-cells into  $CD4^+CD25^+Foxp3^+$   $T_{reg}$  cells that also possess suppressive capabilities. The



generation of these cells can occur during an immune response to control that response and requires the correct conditions such as antigenic exposure in the presence of transforming growth factor- $\beta$ <sup>70</sup> or interleukin-10<sup>71</sup>. There is therefore a dichotomy of roles, with naturally occurring T<sub>reg</sub> cells potentially playing a more significant role in immune homeostasis and controlling autoimmunity, while adaptive T<sub>reg</sub> cells control and limit an ongoing immune response.

### ***Experimental models of tolerance with regulatory T-cells***

Numerous groups have shown that T<sub>reg</sub> cells are able to prolong graft survival in murine models of transplant tolerance. Our group has shown that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells generated by pretreating mice with a non-depleting anti-CD4 MAb plus a donor-specific transfusion are able to suppress cardiac and skin-graft rejection in an adoptive transfer model<sup>72,73</sup>. Several groups in 2002 showed that adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells were able to prevent GVHD in mouse bone marrow transplantation models<sup>74-76</sup>. Battaglia, et al. and Gregori, et al. have also shown that T<sub>reg</sub> cells are able to prevent allogeneic islet-cell transplant rejection in mice<sup>77,78</sup>.

It has recently been appreciated that although T<sub>reg</sub> cells are able to prevent acute rejection and prolong graft survival long-term (to > 100 days), they may not be able to suppress chronic rejection. An interesting study by Joffre, et al. utilizing a combination protocol of hematopoietic chimerism as well as T<sub>reg</sub> cells in a murine model has been able to show that T<sub>reg</sub> cells that are able to recognize donor antigen via the direct pathway of allorecognition are able to effectively prevent acute rejection, but these grafts showed histologic damage that correlates with chronic rejection. On the other hand, T<sub>reg</sub> cells that can recognize donor antigen via both the direct and indirect

pathways are able to prevent both acute and chronic rejection of skin and heart transplants in mice. The grafts showed little or no histologic damage after 100 days<sup>79</sup>.

Therefore, in this study, the induction of mixed chimerism allowed T<sub>reg</sub> cells specific for donor-type antigen to graft well in the recipients, and allowed not only the acceptance of skin and heart transplants in immunocompetent mice, but also prevented chronic rejection, which has been a major hurdle in transplantation. This is the closest we have come to a tolerant state while not generally immunosuppressing the mice. This regime also allows a realistic possibility of being translated to NHP in the first instance, and then into clinical trials.

### ***Regulatory T-cells in clinical practice***

To date, there is no published data on T<sub>reg</sub> cells and transplantation in humans. The first such clinical studies are currently in progress for the prevention of GVHD in bone marrow transplantation and rejection in solid organ transplantation.

In order to harness the useful properties of T<sub>reg</sub> cells, however, a number of controversial issues still need addressing. We have not yet identified a surface marker that identifies T<sub>reg</sub> cells in humans exclusively. The problem with Foxp3 is that it is an intracellular marker and is also expressed on activated T-cells in humans. It has been proposed that low levels of expression of CD127 may be a useful additional marker, and this is proving a promising tool to identify T<sub>reg</sub> cells in addition to CD4 and CD25<sup>65</sup>.

There is still controversy in the area of antigen-specificity of T<sub>reg</sub> cells as both antigen-specific and polyclonal T<sub>reg</sub> cells have been shown to be capable of promoting

tolerance in experimental models. However, when translated into clinical practice, the use of  $T_{reg}$  cells that are not antigen-specific could mean that should a patient develop a concurrent infection, the  $T_{reg}$  cells will also suppress the immune response that is mounted against the infection. Will this result in unchecked infectious and malignant complications in these patients? These questions are unanswered at present and careful analysis of patients treated with  $T_{reg}$  cell therapy is required.

A further related issue is the applicability of data from mouse studies on  $T_{reg}$  cells to the translation of this approach to human clinical practice. There already exist significant differences between *in vivo* and *in vitro* mouse  $T_{reg}$  studies. So how do we translate this to the human setting? Perhaps, a more realistic step would be to carry out these experiments in humanized mouse models and NHP prior to translation into clinical practice.

Further issues that require clarification are whether  $T_{reg}$  cells should be expanded *in vivo* or *ex vivo*? What number of cells should be used? How and when should they be administered? How do we monitor the effect of the  $T_{reg}$  cells *in vivo*? Do we need to include suicide genes into the  $T_{reg}$  cells to prevent deleterious side effects?

In conclusion,  $T_{reg}$  cells hold much promise in being able to mediate operational tolerance to transplants. They may, however, be insufficient to control the large numbers of effector T-cells that are able to recognize allogeneic MHC. Perhaps a combination of strategies each using a different mechanism would be more feasible in achieving tolerance. An effective strategy that could control this overwhelming allogeneic response is a combination of deletion and regulation. Depletion of effector T-cells at the time of transplantation with Campath or ATG along with the use of conventional immunosuppression may allow a window of opportunity in which  $T_{reg}$  cells from

the recipient can be expanded either *in vivo* or *ex vivo* and be used to control the subsequent immune response when the post-depletional T-cells begin to recover.

## Conclusion

It is clear that we have made significant advances in our quest to achieve tolerance to renal transplants in the last few years. More translational research in the form of clinical trials is currently underway, and these studies will further extend our knowledge in the near future.

The most promising tolerance-inducing strategies are mixed chimerism and  $T_{reg}$  therapy. Co-stimulatory blockade and lymphocyte depletion, while not tolerogenic on their own, could also prove very useful as adjunctive treatments. The complexity and innate redundancy of the human immune system means that multiple pathways need to be targeted. For this reason, an approach that involves a combination of strategies is more likely to be successful than one that involves a single strategy. Combining mixed chimerism and  $T_{reg}$  therapy would allow both central and peripheral tolerance, and may allow mixed chimerism to be achieved with less intensive preconditioning. An alternative strategy would be to use lymphocyte depletion prior to  $T_{reg}$  therapy. The lymphocyte depletion can be used to shift the effector to regulatory T-cell ratio to one that is more favorable to tolerance, and this could be followed by a  $T_{reg}$  infusion that could maintain tolerance by controlling the effector T-cells that gradually repopulate the immune system.

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