The Evolving Role of mTOR Inhibition in Transplantation Tolerance

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The ultimate goal in transplantation is long-term graft acceptance by achieving graft tolerance in the recipient with minimal or no immunosuppression. The mammalian target of rapamycin (mTOR) pathway plays a central role in the activation and proliferation of T cells. The mTOR inhibitor, rapamycin, has been used clinically to prevent graft rejection since being approved for use in transplantation in 1999.1 Increasing experimental data also suggest that blocking the mTOR pathway promotes immunological tolerance. This review summarizes the molecular mechanisms by which mTOR inhibitors promote tolerance. We discuss the clinical relevance of these mechanisms and suggest how they might be used in the design of future protocols to induce tolerance.

THE mTOR PATHWAY

Rapamycin, also known as sirolimus, is a macrocyclic antibiotic that was first discovered in the soil of Easter Island (Rapa Nui), from which it derives its name. It is produced by the bacterium Streptomyces hygroscopicus, and, although it was initially proposed as an anti-fungal agent,2 it was subsequently developed as an immunosuppressive drug.3,4 Recognition of antigen by the T cell receptor (TCR) in the presence of CD28 co-stimulation leads to downstream activation of mTOR, which is a 289-kD serine/threonine protein kinase.5 This ultimately leads to increased translation of multiple proteins important for cell division. mTOR inhibitors prevent progression of the cell cycle from the G1 to the S phase and thus block proliferation of T cells.5 Rapamycin binds and forms a complex with the FK506 binding protein, which inhibits mTOR.6 In response to growth factors, phosphatidylinositol 3-kinase (PI3K) phosphorylates Akt, which in turn inactivates tuberous sclerosis complex 1, an inhibitor of mTOR7 (Figure 1). mTOR forms two distinct complexes: mTORC1 and mTORC2.8,9 mTORC1 is sensitive to rapamycin and is involved in the regulation of cell growth by controlling translation, transcription, and nutrient transport.10 mTORC2 is classically thought to be rapamycin insensitive, but has recently been shown to be disrupted after long-term treatment.11 mTORC2 regulates the spatial aspects of cell growth through its effects on the actin cytoskeleton and also phosphorylates and activates Akt.12

RAPAMYCIN AND T CELL ANERGY

Naïve T cells encountering antigen require co-stimulation for full activation. When the TCR is stimulated in the absence of co-stimulation, T cells do not proliferate or produce IL-2, and they remain unresponsive on re-challenge with the same antigen.13 This state is called T cell clonal anergy, and it plays an important role in tolerance to self-antigens and hence the prevention of autoimmunity. An immunosuppressive protocol that induces anergy in donor-reactive T cells

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ABSTRACT

The mammalian target of rapamycin (mTOR) plays a key role in the immune response. mTOR inhibitors suppress T cell activation and proliferation and are effective immunosuppressants. Today there is growing interest in their potential role in inducing tolerance after transplantation. mTOR inhibitors induce anergy in naïve T cells, promote the expansion of regulatory T cells, and inhibit the maturation of dendritic cells, thus promoting immunologic tolerance. Here we review the mechanisms by which mTOR inhibitors promote tolerance. We discuss the clinical relevance of these mechanisms and suggest how they might be used in the design of future protocols to induce tolerance.

could allow for long-term graft acceptance without maintenance immunosuppression. Inhibition of mTOR with rapamycin induces anergy in naïve T cells. T cells treated with rapamycin in vitro, even in the presence of normal TCR/CD28/IL-2R co-stimulation, become anergic and fail to produce IL-2 on repeated stimulation. The key role played by mTOR activation, as measured by the phosphorylation of S6 kinase, is significantly reduced in anergic T cells, whereas T cells that express a mutant rapamycin-resistant mTOR do not become anergic in the presence of rapamycin. Anergic state is reversed if the cells are subsequently treated with exogenous IL-2. IL-2-mediated reversal of anergy is also mTOR dependent. Anergic T cells stimulated in vitro after treatment with IL-2 respond similarly to control cells. However, when these cells are treated with IL-2 in the presence of rapamycin, they fail to respond to stimulation and remain anergic. In contrast, calcineurin inhibitors such as cyclosporine inhibit the induction of clonal anergy in vitro, even when T cells are simultaneously treated with rapamycin.

Similar effects have been noted in vivo. Rapamycin treatment during antigen stimulation induces clonal anergy in mouse T cells. This is seen even in the presence of CTLA-4 blockade, which prevents anergy induction. In a mouse model, rapamycin, combined with co-stimulation blockade in the form of CTLA-4Ig plus anti-CD40L, leads to long-term survival of skin allografts. These mice subsequently accept second skin allografts from the same donor, whereas third party grafts are rejected, suggesting this combination induces tolerance. Treatment with rapamycin, co-stimulation blockade, or cyclosporine alone leads to rapid rejection of the grafts.

**RAPAMYCIN AND REGULATORY T CELLS**

Rapamycin may also be useful in promoting tolerance through its effects on regulatory T cells (Tregs). Tregs were first identified in the 1990s when it was discovered that depletion of a subset of CD4+ T cells leads to autoimmune disease in mice. These cells highly express the IL-2 receptor α-chain (CD25). Nude mice inoculated with CD4+ T cells depleted of CD25 develop autoimmune disease, which is prevented by the inoculation of CD4+, CD25+ T cells. Tregs are anergic and do not produce proinflammatory cytokines when stimulated in vitro. Tregs play a role in the prevention of autoimmunity by suppressing T cell responses to self-antigens and by limiting the response to foreign antigens.

Tregs are also characterized by the expression of the forkhead family transcription regulator, Foxp3. Foxp3 is vital for the development and function of Tregs, and the lack of Foxp3 leads to absence of Tregs in mice. Defects in the Foxp3 gene associate with autoimmune disease in mice, and humans with mutations in Foxp3 develop a similar autoimmune disease called IPEX. The role of Foxp3 in Treg development was shown when naïve CD4+, CD25+ T cells developed regulatory activity after retroviral gene transfer of Foxp3. Foxp3 acts in T cells in part by blocking NFAT-mediated transcription of IL-2. IL-2 is necessary for the survival and function of Tregs. Mice lacking the IL-2 receptor develop autoimmune disease, and the number of mature, peripheral Tregs is reduced in IL-2−/− deficient mice. IL-2 also induces proliferation of Tregs when accompanied by TCR stimulation. This has implications for the choice of immunosuppressive therapy, because calcineurin inhibitors reduce the production of IL-2 by activated T cells, whereas rapamycin, in contrast, inhibits cellular responses to IL-2. Distinguishing Tregs from conventional T cells (Tconv) is complicated by the fact that the markers for Tregs have limited specificity. For example, stimulated CD4+, CD25+ Tconv transiently express Foxp3. Also, Tregs that are expanded in vitro lose Foxp3 expression and hence their regulatory phenotype on repeated stimulation.

Rapamycin has beneficial effects on...
the survival and proliferation of Tregs *in vivo* and *in vitro*. Mice treated with a combination of an IL-2 agonist, an IL-15 antagonist, and rapamycin increase the apoptosis of Tconv and increase the percentage of CD4<sup>-</sup>, CD25<sup>+</sup> cells. This regimen promotes long-term engraftment of skin, heart, and islet cell allografts. These mice are tolerant to subsequent skin transplants from the same donors even after cessation of immunosuppression, whereas grafts from new donors are promptly rejected. Subsequent studies showed that rapamycin expands Tregs *in vitro* and *in vivo*, that these Tregs are anergic on TCR stimulation, and that they retain their suppressive ability. Although rapamycin preserves these T cell subsets, when mice are treated with cyclosporine, the number of peripheral Tregs and the maturation of thymic Foxp3<sup>+</sup> cells are significantly reduced; cyclosporine treatment in the first week of life also leads to autoimmune disease similar to that seen in IL-2-deficient mice. Rapamycin in addition promotes the differentiation of naïve T cells into Tregs in the periphery. When Foxp3<sup>-</sup> T cells are activated *in vitro*, 2 to 3% expressed Foxp3 and developed a regulatory phenotype. However, when these cells are treated with IL-2 and rapamycin at the time of activation, the percentage of cells expressing Foxp3 increases to 7%. Similarly, Foxp3 expression induces *in vitro* when Foxp3<sup>-</sup> T cells activate in the presence of TGFβ or rapamycin, whereas cyclosporine fails to induce Foxp3. In the same study, when naïve T cells are adoptively transferred into mice, 2% became Foxp3<sup>+</sup>. After treatment with rapamycin, 6% expressed Foxp3, and the combination of rapamycin and anti-CD154 (CD40L) induced Foxp3 expression in 25% of T cells. In contrast, when the mice were treated with cyclosporine, none of the T cells became Foxp3<sup>+</sup>. Rapamycin and anti-CD154 treatment for 14 days promotes tolerance of skin allografts (survival >100 days), whereas cyclosporine-treated mice eventually reject their grafts after cessation of immunosuppression, with a mean survival of 45 days.

Rapamycin also expands human Tregs. Tregs from normal humans proliferate in response to rapamycin, whereas the proliferation of Tconv does not. These expanded Tregs express high levels of CD25 and Foxp3, are resistant to apoptosis, and have suppressive activity *in vitro*. Rapamycin also expands a highly suppressive subset of human CD4<sup>+</sup>, CD25<sup>+</sup> T cells that are CD27<sup>+</sup>. These cells do not expand in the presence of cyclosporine. Interestingly, human Tregs cultured in the presence of cyclosporine lose their suppressive capabilities within 48 hours, whereas rapamycin-treated Tregs retain this functionality. Tregs expanded *in vitro* may have a role in the future treatment of autoimmune disease, because the suppressive activity of Tregs expanded *in vitro* from patients with autoimmune disease remains. In one study of patients with multiple sclerosis, rapamycin expanded Tregs from both normal donors and those with the disease, and the suppressive phenotype was retained in each. Similarly, Tconv isolated from patients with type I diabetes are highly sensitive to the anti-proliferative effects of rapamycin, whereas Tregs expand to be suppressive. In contrast to other studies, rapamycin treatment did not promote anergy.

Unlike cyclosporine, corticosteroids do not inhibit Tregs and may promote their expansion. The combination of dexamethasone and IL-2 treatment expands Tregs in mice. Similarly, an increased percentage of Tregs is seen in the thymus and spleen of mice receiving dexamethasone relative to controls. However, this may be the result of increased resistance to dexamethasone-induced apoptosis in Tregs relative to Tconv, because their absolute numbers were reduced. Addition of dexamethasone and IL-7 to human CD4<sup>+</sup>, CD25<sup>+</sup> cells results in an increased percentage of CD4<sup>+</sup>, CD25<sup>+</sup> cells in the culture, whereas increased Foxp3 expression is noted in CD4<sup>+</sup> T cells of asthmatic patients after systemic treatment with glucocorticoids. The few studies assessing the effect of mycophenolate mofetil on Tregs show little or no effect on their function. One study showed reduced apoptosis of Tregs in the presence of mycophenolate mofetil compared with cyclosporine. Mycophenolate mofetil also preserves Treg function in a mouse model of allogeneic bone marrow transplantation, particularly compared with cyclosporine. After conversion from cyclosporine to mycophenolate mofetil, liver transplant recipients were found to have higher numbers of circulating Tregs.

**INTRACELLULAR MECHANISMS UNDERLYING THE EFFECTS OF RAPAMYCIN ON TREGS**

Several mechanisms may explain rapamycin’s preferential expansion of Tregs compared with Tconv. One is the differential effect of IL-2 receptor signaling. A number of pathways are activated in response to IL-2R signaling, including the JAK/STAT, MAPK, and the PI3K/Akt/mTOR pathways. In Tregs, JAK/STAT signaling is induced preferentially, whereas PI3K/Akt/mTOR signaling is reduced relative to Tconv (Figure 2). STAT5 binds to the Foxp3 promoter in Tregs, thus regulating Foxp3 expression. Phosphatase and tensin homolog (PTEN) is an endogenous inhibitor of PI3K. Tregs constitutively express PTEN and, therefore, when the IL-2 receptor is activated, there is minimal downstream activation of mTOR. In contrast, PTEN activity is low in Tconv, so the PI3K/Akt/mTOR pathway activates in response to IL-2 receptor signaling. Thus, rapamycin treatment inhibits the PI3K/Akt/mTOR pathway in Tconv, whereas Tregs, because this pathway is not activated, are relatively insensitive to the anti-proliferative effects of rapamycin. There is also evidence that activation of Akt may itself be detrimental to Tregs. When naïve T cells are treated *in vitro* with IL-2 and TGFβ, Foxp3 is induced. However, when Akt is overexpressed in these cells, Foxp3 expression is inhibited. This effect is partially reversed by treatment with rapamycin. This latter finding suggests the decreased activation of the PI3K/Akt/mTOR pathway in Tregs helps ensure expression of sufficient...
levels of Foxp3 to allow their proliferation and survival.

Rapamycin also influences the expression of pro- and anti-apoptotic proteins on T cells. Tregs cultured in the presence of rapamycin express high levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL. In contrast, when Tconv are cultured with rapamycin, they down-regulate the expression of the anti-apoptotic proteins and upregulate the expression of pro-apoptotic Bax. As a result, after treatment with rapamycin, Tregs are relatively resistant to apoptosis compared with Tconv.53

A third mechanism for the relative resistance of Tregs to rapamycin is through the Pim-2 pathway. Pim-2 is a serine-threonine kinase that shares downstream targets of mTOR and Akt, including Bad and 4-EBP1.55 In Tconv, Pim-2 is activated in response to cytokine stimulation through the JAK/STAT pathway and expression is quickly downregulated in the absence of these cytokines. Absence of Pim-2 causes Tconv to be highly sensitive to rapamycin.56 In Tregs, however, Pim-2 expression is controlled by Foxp3, and it is constitutively expressed.57 Thus, in Tregs, Pim-2 confers resistance to the anti-proliferative effects of rapamycin by offering an alternative pathway independent of PI3K/Akt/mTOR.

**REGULATORY T CELLS AND TRANSPLANTATION**

Higher levels of circulating Tregs associate with better outcomes after solid-organ transplantation. Decreased numbers of Tregs are found in the blood of lung transplant recipients with bronchiolitis obliterans compared with those with normal grafts 3 years after transplant. None of the recipients were treated with rapamycin.58,59 Similarly, decreased numbers of Tregs are seen in liver transplant recipients who have an episode of acute rejection in the first year after transplant.60 Another study of liver transplant recipients reported the numbers of Tregs before and after withdrawal of cyclosporine. In those patients who tolerated withdrawal of immunosuppression, there was a threefold increase in Tregs compared with when they were on immunosuppression. In contrast, there was no increase in the number of Tregs in those who had an episode of acute rejection after withdrawal, suggesting that increased Tregs associate with tolerance to the graft.61

In a study of 37 patients with subclinical rejection diagnosed on protocol renal biopsies 3 years after transplant, a higher percentage of Tregs in the T cell infiltrates correlated with better renal function. Most of the patients with increased Tregs were on rapamycin, although even in the group taking calcineurin inhibitors, Treg numbers correlated with renal function.62 Increased expression of Foxp3 was noted in T cells of renal allograft recipients with long-term (8 years) stable renal function relative to those who had chronic rejection.63 Increased Foxp3 expression was also noted in renal transplant recipients with donor-specific hyporesponsiveness, and all of the hyporesponsive patients were taking rapamycin.64 When T cells are depleted with the anti-CD52 mAb, Campath-1H, T cell numbers slowly recover over the first year. The use of a rapamycin-containing regimen associates with higher numbers of circulating Tregs relative to those patients treated with cyclosporine. These Tregs are capable of suppressing T cell alloreactivity to donor antigens in vitro, and this hyporesponsiveness is reversed when CD4+ and CD25+ T cells are depleted.65 However, the increased numbers of Tregs is not associated with better outcomes in a small number of patients 3 years after transplant. In fact, higher tubular CD4 staining and increased clinical proteinuria were noted in the patients treated with rapamycin.66

Rapamycin is effective both in treating established graft versus host disease (GVHD)67 and for the prophylaxis of GVHD68,69 in allogeneic bone marrow transplantation in humans. The benefits may be partially mediated through the effect of rapamycin on Tregs. For example, interstitial Tregs are increased in the skin of mice treated with rapamycin after bone marrow transplantation.70 Human studies showed in patients with acute GVHD that there are reduced numbers of Tregs in the periphery and in the af-

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**Figure 2.** In Tconv, stimulation of the IL-2 receptor leads preferentially to activation of the PI3K/Akt/mTOR pathway. Because of their dependence on this pathway for proliferation, Tconv are sensitive to the effects of rapamycin. In contrast, because of their high baseline expression of PTEN, in Tregs, IL-2R stimulation leads to activation of JAK/STAT signaling, which regulates Foxp3. The Pim-2 pathway is not sensitive to rapamycin and is constitutively expressed in the presence of Foxp3. This allows expansion of Tregs in the presence of rapamycin while suppressing the proliferation of Tconv.
RAPAMYCIN AND DENDRITIC CELLS

Dendritic cells (DCs) are bone marrow–derived antigen-presenting cells that play an important role in the immune response by regulating T cells. The nature of this response is determined by the cytokines produced by DCs along with the specific MHC and co-stimulatory molecules expressed on their surface. This depends on the degree of maturation of the DCs. When immature DCs stimulate T cells, they suppress T cell responses by inducing anergy and apoptosis, thus promoting tolerance. Immature, semi-mature, and mature DCs also induce expansion of Tregs. These DC-expanded Tregs are immunosuppressive in vivo and can prolong skin allograft survival and prevent GVHD in animal models. The mTOR pathway plays an important role in DC maturation and function. Rapamycin treatment inhibits the ability of immature DCs to endocytose antigens. Rapamycin also inhibits the expression of MHC class II and co-stimulatory molecules on immature DCs, thus preventing maturation. In contrast, rapamycin treatment has no effect on the maturation of DCs, and rapamycin does not affect the production of cytokines by DCs. Immature DCs cultured with rapamycin produce reduced amounts of IL-10 and IL-12. Mature DCs, in contrast, secrete little IL-10 but increased IL-12. However, rapamycin blocks the production of the pro-inflammatory cytokine IL-18 by mature DCs in response to lipopolysaccharide. Cyclosporine treatment has no effect on IL-18 production. Rapa-DCs also prolong graft survival in mice. Multiple infusions of alloantigen-pulsed, rapa-DCs lead to long-term cardiac allograft survival. In the same model, a single infusion of rapa-DCs in combination with a short postoperative course of rapamycin promotes indefinite graft survival. This associates with a marked infiltration of Tregs into grafts with minimal transplant vasculopathy. Thus, rapa-DCs may have a role to play in the prevention of transplant rejection.

CONCLUSION

The mTOR pathway is vital for the full activation of Tconv. Rapamycin treatment inhibits the proliferation of T cells and induces anergy in activated Tconv. In contrast, Tregs are not dependent on the mTOR pathway for activation and proliferation in response to rapamycin treatment. The ability of mTOR inhibitors to induce anergy and promote the selective expansion of Tregs suggests an important role for this class of drugs in tolerance-inducing protocols in transplantation. However, in clinical practice, the use of mTOR inhibitors has not been associated with substantially improved long-term graft survival. This may be in part because they are primarily used in combination with drugs that inhibit their tolerogenic properties. Cyclosporine, for example, prevents T cell anergy induced by rapamycin and also inhibits the expansion of Tregs. This underscores the importance of considering the effects of these interactions when designing immunosuppressant protocols that include mTOR inhibitors, with the final aim of achieving transplant tolerance.

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M.R.W. has served as a scientific advisor to Wyeth.
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