Immunosuppressive potency of mechanistic target of rapamycin inhibitors in solid-organ transplantation

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Abstract

Mammalian target of rapamycin, also known as mechanistic target of rapamycin (mTOR) is a protein kinase that belongs to the PI3K/AKT/mTOR signaling pathway, which is involved in several fundamental cellular functions such as cell growth, proliferation, and survival. This protein and its associated pathway have been implicated in cancer development and the regulation of immune responses, including the rejection response generated following allograft transplantation. Inhibitors of mTOR (mTORi) such as rapamycin and its derivative everolimus are potent immunosuppressive drugs that both maintain similar rates of efficacy and could optimize the renal function and diminish the side effects compared with calcineurin inhibitors. These drugs are used in solid-organ transplantationtoinduceimmunosuppression while also promoting the expansion of CD4+CD25+FOXP3+ regulatory T-cells that could favor a scenery of immunological tolerance. In this review, we describe the mechanisms by which inhibitors of mTOR induce suppression by regulation of these pathways at different levels of the immune response. In addition, we particularly emphasize about the main methods that are used to assess the potency of immunosuppressive drugs, highlighting the studies carried out about immunosuppressive potency of inhibitors of mTOR.

Key words: Everolimus; Immunosuppression; Mechanistic target of rapamycin inhibitor; Rapamycin; Tolerance

Core tip: Inhibitors of mechanistic target of rapamycin (mTOR), rapamycin and its derivative everolimus, have been used as immunosuppressive drugs during the last decade. Several reviews have been written on the use of these drugs compared to classical calcineurin inhibitors, however few has been reviewed about

immunosuppressive potency of such compounds. Our aim is to summarize the principal studies about potency of the immunosuppressants, highlighting the studies carried out with inhibitors of mTOR.

INTRODUCTION

The elucidation, at the molecular level, of T-cell-mediated rejection, explained by the three-signal model of lymphocyte activation, has facilitated the development of novel immunosuppressive drugs (Figure 1). Advances in immunosuppressive therapy have had a great impact on the evolution and success of solid-organ transplantation. Rejection responses after transplantation can be minimized by optimally matching major histocompatibility complex (MHC) antigens, by administration of drugs that generally suppress the immune system, or by inducing a state of tolerance[1]. With the introduction of newer immunosuppressive pharmacological agents, the incidence of acute cellular allograft rejection has decreased to low levels, and one and five-year patient survival rates are approaching 85% and 68%, respectively, with a 10-year survival closer to 50%[2].

Immunosuppressive drugs can be classified into two categories: Biologic agents, such as polyclonal and monoclonal anti-lymphocyte antibodies; and pharmacological or small-molecule drugs, such as corticosteroids and inhibitors of nucleotide synthesis, calcineurin inhibitors or mammalian target of rapamycin inhibitors (mTORi) (Table 1 and Figure 1)[1,3]. These drugs are used in combinations that are intended to maximize immunosuppression while reducing the adverse effects of each individual drug[4].

Calcineurin inhibitors (CNI), such as tacrolimus and cyclosporine, have become the cornerstone of immunosuppressive therapy in solid organ transplantation[5]. Their use resulted in lower rejection rates and improved short-term patient and allograft survival rates. However, long-term improvements in graft survival have been more difficult to achieve with these drugs. The main reason for this observation is that prolonged CNI exposure is associated with nephrotoxicity[6], neurotoxicity[7], increased risk for cancer[8], metabolic complications[9], and hypertension[10], which are an important cause of long-term morbidity and mortality. Nevertheless, the limitation in the long-term survival of patients with transplantation depends on other factors not directly related to the immunosupression, such as recurrence of basal disease and death with a functioning graft for reasons beyond to the own transplantation. Reducing CNI exposure is the main strategy to lower these adverse events, for example combining immunosuppressants with different mechanism of action to minimize the adverse events while maintaining immunosuppressive efficacy.

The mTORi, such as rapamycin and its derivate everolimus, are powerful nonnephrotoxic agents with a different toxicity profile respect to CNI, specially affecting to a gastrointestinal, respiratory and hematological level, in addition to a different mechanism of action than CNI. Meanwhile CNI block the production of proinflammatory cytokines such as IL-2 and, subsequently, inhibition of T-cell activation, mTORi reduce T-cell activation later in the cell cycle by blocking growth-factor-mediated cell proliferation in the cellular response to alloantigen[11,12] (Figure 1). The distinct mechanism of action and favorable nephrotoxicity profile has led to mTORi-containing regimens being developed with the aim of minimizing, eliminating, or avoiding exposure to CNI, although many trials failed because of the high incidence of antibody-mediated rejection[13].

Rapamycin is an immunosuppressive drug that was approved by the United States Food and Drug Administration (FDA) in 1999 and by the European Medicines Agency (EMA) in 2000 as an immunosuppressive agent for renal transplantation patients once its T-cell suppression characteristics were recognized[14]. Later, everolimus was approved in 2003 for the prophylaxis of organ rejection in kidney and heart transplant recipients in many European countries, followed by FDA approval for kidney transplantation in 2010[15]. Everolimus was developed to improve the pharmacokinetic profile of rapamycin. At position 40 of the rapamycin molecule, everolimus has a covalently bound 2-hydroxyethyl group that provides a pharmacokinetic advantage, conferring faster absorption and a shorter half-life in comparison to rapamycin[16,17]. These properties allow everolimus to be formulated as an oral agent, while maintaining immunosuppressive and anti-neoplastic activities similar to rapamycin[18,19]. In addition, unlike rapamycin, no loading dose is required for everolimus, and the twice-daily dosing schedule enables accurate dose adjustments[20].

In this review, we summarize some of the main methods that are used to assess the potency of immunosuppressive drugs, highlighting the studies about immunosuppressive potency of mTORi.

ROLE OF mTOR IN THE IMMUNE RESPONSE AND EFFECTS OF mTORi IN THE IMMUNE SYSTEM

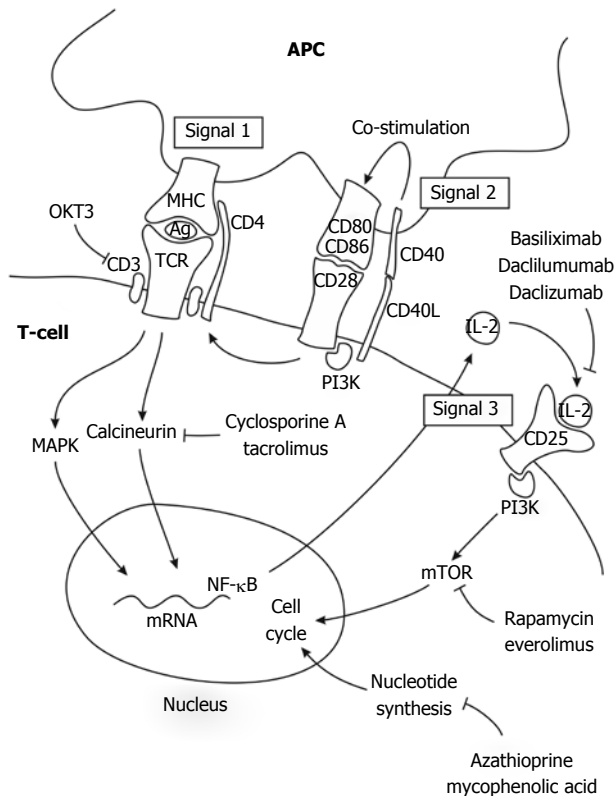mTOR is a protein kinase involved in the signal 3

Figure 1 Three-signal pathway of lymphocyte activation and targets of inhibitory agents. The elucidation of lymphocyte activation pathways has facilitated the development of novel immunosuppressive drugs. At the molecular level, T-cell-mediated rejection is explained by the three-signal model of lymphocyte activation. Signal 1 occurs when alloantigen-bearing APCs engage alloantigen-reactive naïve and memory T-cells and trigger their activation; alloantigen recognition is transduced through the TCR-CD3 complex. Signal 2 occurs when CD80 and CD86 on the surface of APCs engage CD28 on T-lymphocytes, providing T-lymphocyte co-stimulation. Together, signals 1 and 2 activate several signal transduction pathways, including the calcium-calcineurin pathway, the MAPK pathway, and the NF-κB pathway, which in turn, trigger the expression of many cytokines. Several of these cytokines (IL-2, IL-4, IL-7, IL-15, and IL-21) induce proliferation (signal 3) through PI3K and mTOR pathways. Ag: Antigen; APC: Antigen-presenting cell; MAPK: Mitogen-activated protein kinase; MHC: Major histocompatibility complex; mTOR: Mechanistic target of rapamycin; NF-κB: Nuclear factor kappa B; PI3K: Phosphatidylinositol 3-kinase; TCR: T-cell receptor.

pathway of lymphocyte activation[3] (Figure 1). More specifically, mTOR belongs to the PI3K pathway, which is involved in several fundamental cellular functions such as cell growth, proliferation, and survival. The mTOR protein interacts with several proteins to form two distinct complexes: mTOR complex 1 (mTORC1) and 2 (mTORC2)[21]. Both complexes share the catalytic mTOR subunit, mammalian lethal with Sec13 protein 8 (mLST8), DEP domain-containing mTOR-interacting protein (DEPTOR), and the Tti1/tel2 complex. Furthermore, mTORC1 is composed uniquely of regulatory-associated protein of mTOR (RAPTOR) and the proline-rich AKT substrate 40 kDa (PRAS40). By contrast, mTORC2 uniquely contains the scaffolding protein rapamycin-insensitive companion

of mTOR (RICTOR), mammalian stress-activated map kinase-interacting protein 1 (mSIN1), and the protein observed with RICTOR 1 and 2 (PROTOR1/2)[21]. Located adjacent to the kinase domain of mTOR is the FKBP12-rapamycin-binding (FRB) domain[22].

mTORC1 participates in the translocation and synthesis of cell-cycle regulating and ribosomal proteins, as well as the synthesis of lipids that are required for proliferating cells to generate membranes[23-25]. However, mTORC2 activates protein kinase B (AKT), which is the central mediator of the PI3K pathway and promotes cell growth and survival *via* several mechanisms[26] (Figure 2).

In addition, mTOR has an important role as a central regulator of the immune response, functioning as a central node in a signaling cascade that directs the integration of diverse environmental inputs in the immune microenvironment. mTOR regulatesthe function of diverse immune cell types, including dendritic cells, B cells or regulatory and effector T-cells[27-30].

mTORi (rapamycin and everolimus) are immunosuppressive drugs that interact with and inhibits mTOR, but only when it is part of mTORC1 and not mTORC2[21]. These drugs bind to the cytosolic protein FKBP12. This complex binds to the FRB domain of mTOR, which blocks the ability of RAPTOR to bind to mTOR, thereby inhibiting formation of mTORC1[31]. However, prolonged treatment with rapamycin has also revealed the inhibition of mTORC2 signaling[32]. Rapamycin mediates immunosuppressive effects through multiple immune cell types and processes. Inhibition of mTOR by rapamycin suppresses the immune response by preventing cell cycle progression from G1 to S phase, thereby blocking proliferation[33]. In addition, rapamycin can promote T-cell anergy independently of the inhibition of proliferation even in the presence of TCR activation and co-stimulation by CD28 and IL-2[34,35].

Rapamycin inhibits the ability of dendritic cells to mature into APCs that can strongly stimulate T-cells. Immature dendritic cells promote the expansion of regulatory T-cells while concomitantly suppressing conventional T-cell responses by inducing T-cell anergy and apoptosis, thus promoting tolerance to the graft[36]. Furthermore, rapamycin has beneficial effects on the survival and proliferation of regulatory T-cells[37]. Many studies have confirmed the beneficial effects of rapamycin or everolimus on regulatory T-cell biology[38-40]. By contrast, CNI impair the number, function and phenotype of regulatory T-cells, potentially acting as a barrier to the achievement of host tolerance to an allograft[38,39,41]. However, this issue is controversial, because some studies have shown how CNI does not affect or improve the expansion of Treg[42,43]. Likewise, everolimus can inhibit humoral responses both directly, by suppressing B cell proliferation and differentiation, and indirectly, by suppressing T-cell help[44,45].

Table 1 Classification of biological and pharmacological immunosuppressive agents[1,3]

Biologic immunosuppressive agents	Function
Lymphocyte-depleting agents	
Monoclonal anti-CD20 (rituximab)	Depletion of B-cells
Monoclonal anti-CD52 (alemtuzumab)	Depletion of T-cells, monocytes, macrophages and natural killer cells
Monoclonal anti-CD3 (OKT3)	Interference with signal 1 in T-cells
Anti-thymocyte globulin	Interference with signals 1, 2 and 3 in T-cells
Non-lymphocyte-depleting agents	
Anti-IL-2 receptor (basiliximab, daclilumumab)	Inhibition of T-cell proliferation and signal 3
Belatacept	Inhibition of signal 2 in T-cells (competition with CD28 for CD80/CD86 binding) inhibiting T-cell co-stimulation
Daclizumab	Inhibition of signal 2 in T-cells (binds to CD25, the alpha subunit of the IL-2 receptor) preventing IL-2-induced T-cell activation
Pharmacological drugs	**Function**
Corticosteroids	Inhibition of cytokine transcription by APCs
Azathioprine	Inhibition of nucleotide synthesis, blocking lymphocyte proliferation
Mycophenolic acid	Inhibition of nucleotide synthesis, blocking lymphocyte proliferation
Calcineurin inhibitors	Inhibition of signal 2 transduction in T-cells [inhibits calcineurin *via* cyclophilin (cyclosporine A)
(cyclosporine A, tacrolimus)	or *via* FKBP12 (tacrolimus)], blocking IL-2 transcription
FK778 (manitimus)	Inhibits dihydro-orotate dehydrogenase, interrupting *de novo* pyrimidine synthesis, thereby acting on both B-cells and T-cells beyond the early S phase of the cell cycle, differentially from calcineurin inhibitors
mTOR inhibitors (rapamycin, everolimus)	Inhibition of signal 3 transduction in T-cells (inhibits mTOR), preventing IL-2-induced T-cell proliferation

APC: Antigen-presenting cell; IL-2: Interleukin-2; mTOR: Mammalian target of rapamycin.

METHODS TO MEASURE IMMUNOSUPPRESSIVE POTENCY. SCIENTIFIC EVIDENCE FOR THE IMMUNOSUPPRESSIVE AND IMMUNOREGULATORY POTENCY OF mTORi IN TRANSPLANTATION

No standardized methods are available to measure the immunosuppressive potency of drugs that are used to improve transplantation outcomes. To date, routine clinical use of immunosuppressive drugs has relied on blood concentration measurements (pharmacokinetics) rather than on biologically relevant analysis of drug effects on immune-cell function (pharmacodynamics)[46,47]. However, several methods are used to evaluate and monitor the pharmacodynamics of immunosuppression in transplantation in the context of research studies[48]. Some of these methods include changes in lymphocyte markers, measure of cytokine levels, soluble CD30 or intracellular ATP.

The immunosuppressive potency of mTORi, such as rapamycin and everolimus, has been evaluated in several studies using various methods. The studies can be categorized into three groups: Studies that examined inhibition of T-lymphocyte proliferation, studies that analyzed inhibition of B-lymphocyte proliferation, and studies that evaluated immunoprotective capabilities.

Measurement of changes in T-cell subsets: Inhibition of T-lymphocyte proliferation
Fluorescent-activated cell sorting (FACS) analysis can be used for the quantification of T-lymphocyte subsets. This simple and sensitive method involves sorting and quantification of lymphocyte subsets by fluorescent labelling of cell surface markers. Using this approach, reductions in the number of regulatory T-cells have been reported in kidney transplant recipients in which recipients were treated with CNI compared with those patients treated with rapamycin[49]. One study that investigated inhibition of T-lymphocyte proliferation evaluated the pharmacodynamics of everolimus at varying doses (0.75-10 mg) when combined with cyclosporine A and prednisolone in human renal transplant recipients[50]. T-lymphocytes isolated from peripheral blood one day before everolimus treatment (baseline), 1 d after and 21 d later, were stimulated *in vitro* using monoclonal anti-CD3 antibodies. Lymphocyte proliferation was measured by cell viability through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In contrast to placebo, T-cell proliferation was significantly reduced by a single dose of everolimus by 2-6 h, but had returned to baseline values by 10 h. In addition, lymphocyte proliferation of everolimus-treated patients decreased significantly on day 1 after everolimus intake by 25.4% ($P < 0.05$), and on day 21 by 53.3% ($P < 0.01$) compared to placebo. Patients receiving a placebo showed no meaningful changes in lymphocyte proliferation rates over the whole study period. By day 42, 21 d after the last everolimus intake, decreased lymphocyte proliferation returned to baseline values. Moreover, everolimus reduced the production of IL-10 from supernatants of peripheral blood mononuclear cells, as measured by enzyme-linked immunosorbent
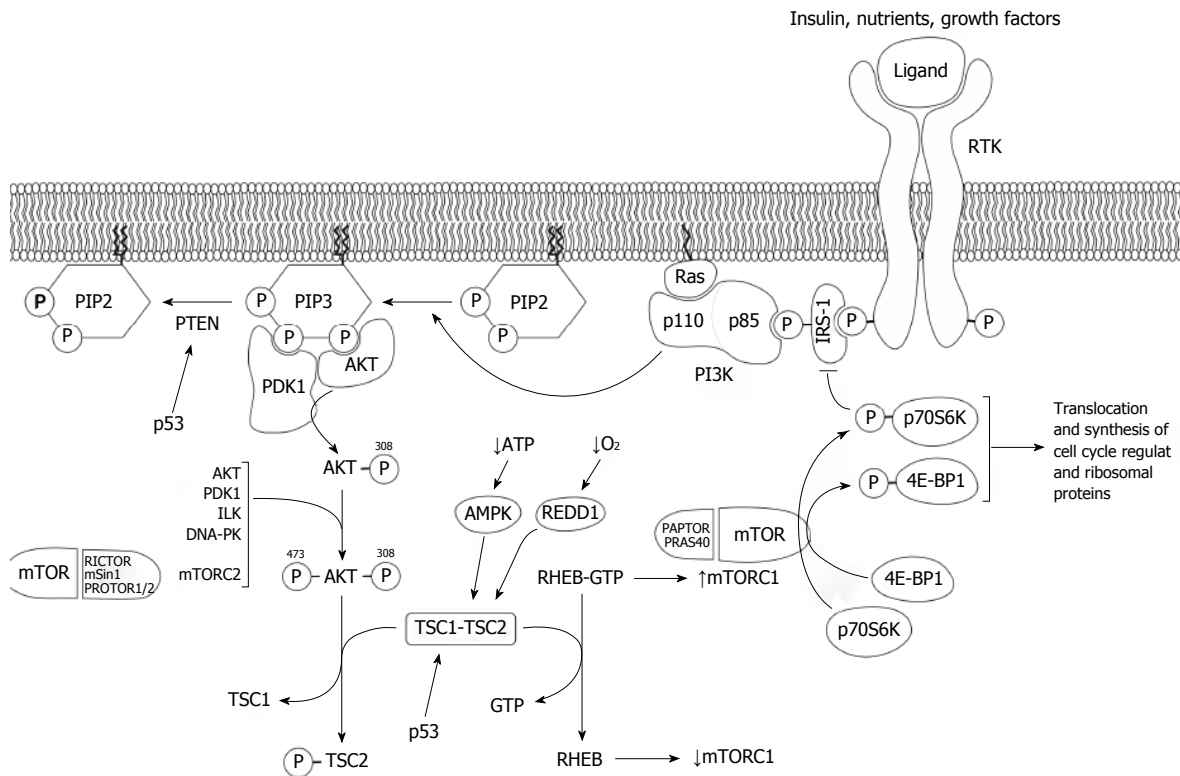
Figure 2 PI3K/AKT/mTOR signaling pathways. PI3K is activated by growth factor stimulation through RTK. The regulatory subunit of PI3K, p85, binds directly to phosphotyrosine residues on RTK and/or adaptors, such as the IRS-1. This binding relieves the intermolecular inhibition of the p110 catalytic subunit of PI3K by p85 and allows it to move toward PI3K to the plasma membrane where its substrate, PIP2, resides. The catalytic subunit can also be activated by activated RAS, which binds directly to p110, and by G protein-coupled receptors. PI3K phosphorylates PIP2 to produce PIP3. In addition, the tumor suppressor PTEN dephosphorylates PIP3 to PIP2, thereby regulating PI3K-dependent signaling in a negative manner. Following PIP3 formation, PDK1 and AKT bind to PIP3 through its pleckstrin homology domains into close proximity at the cell plasma membrane. PDK1 activates AKT by phosphorylating AKT at threonine 308. After phosphorylation, AKT is fully activated by the subsequent phosphorylation at serine 473 by several protein kinases such as PDK1, the complex mTORC2, or AKT itself. AKT phosphorylates TSC2, thereby inhibiting the GTPase activity of the TSC1-TSC2 dimer, and the GTP-binding protein RHEB remains in its active GTP-bound state, causing a rise in mTORC1. In the mTORC1 complex, mTOR phosphorylates p70S6K and 4E-BP1, leading to an increase in the translation and synthesis of cell cycle-regulating and ribosomal proteins. Activated p70S6K also participates in a negative feedback loop, reducing the activation of the PI3K pathway through the phosphorylation and subsequent inhibition of ISR-1. 4E-BP1: eIF4E-binding protein; AMPK: AMP-activated kinase; AKT: Protein kinase B; IRS-1: Insulin-like growth factor-1; p70S6K: 70 kDa ribosomal protein S6 kinase; PDK1: Phosphoinositide-dependent kinase 1; PI3K: Phosphatidylinositol 3-kinase; PIP2: Phosphatidylinositol 4,5-bisphosphate; PIP3: Phosphatidylinositol 3,4,5-trisphosphate; PTEN: Phosphatase and tensin homolog; mTOR: Mechanistic target of rapamycin; RAPTOR: Regulatory-associated protein of mTOR; REDD1: Factor protein regulated in the development of DBA damage response 1; RHEB: RAS homolog enriched in brain; RICTOR: Rapamycin-insensitive companion of mTOR; RTK: Receptor tyrosine kinase; TSC1-TSC2: Tuberous sclerosis protein 1 and 2.

assay (ELISA), by 23.7% on day 1 ($P < 0.05$) and 62.2% on day 21 ($P < 0.01$) in renal-allograft recipients compared to baseline. It is believed that IL-2 induces expression of IL-10[51]. Thus, mTORi interfere with IL-2-dependent signal transduction and inhibit IL-10 expression.

Another study investigated the *in vitro* effects of several doses of everolimus and intravenous immunoglobulin, widely used for treatment of autoimmune and systemic inflammatory disorders[52], on induction of lymphocyte proliferation [by two-way mixed lymphocyte reaction (MLR)] and apoptosis (by terminal deoxynucleotidyltransferase dUTP nick-end labeling and annexin V assays)[53]. Everolimus and intravenous immunoglobulin alone each inhibited cell proliferation in a dose-dependent manner: Everolimus decreased it from 16% to 67%, and intravenous immunoglobulin from 12% to 66%. In addition, intravenous immunoglobulin induced apoptosis in B and T-cells, but everolimus did not. The study concluded that everolimus is a potent inhibitor of immune cell proliferation but does not act additively or synergistically with intravenous immunoglobulin under the *in vitro* conditions used in the study.

A prospective study determined whether systemic signatures of immunoregulation are promoted by switching liver transplant patients from treatment with the CNI tacrolimus to rapamycin[41]. The investigators argued that immunosuppression withdrawal from CNI is possible in only approximately 20% of all liver transplant recipients. However, mTORi such as rapamycin appear to be more immunoregulatory than CNI and might promote a tolerant state to enable withdrawal. Several assays were conducted before and after converting to rapamycin treatment. Flow cytometry revealed a significant increase in the number of regulatory T-cells in peripheral blood mononucleated cells (PBMC) and in bone marrow, and

in the number of regulatory dendritic cells in PBMC after conversion. Immunohistochemical analysis of liver biopsy showed that the ratios of FOXP3:CD3 and CD4:CD8 were higher following conversion to rapamycin treatment, with an increase the proliferation of new or existing FOXP3+ cells. Both tacrolimus and rapamycin treatment were associated with inhibition of lymphocyte proliferation as measured by an MLR, although only tacrolimus suppressed regulatory T-cells generation. Finally, 289 novel genes and 22 proteins, some of which have been implicated in immunoregulatory pathways, were expressed after conversion to rapamycin treatment. The study concluded that conversion from tacrolimus to rapamycin treatment increases the number of systemic regulatory T-cells and regulatory dendritic cells, and induces an immunoregulatory proteogenomic signature in liver transplant recipients.

Another study evaluated the capacity of FK778 administered either alone or in combination with tacrolimus, rapamycin or everolimus, to inhibit the clonal expansion of T-lymphocytes and the expression of lymphocyte-activation antigens[54]. FK778 is a malononitrilamide which has been found to prevent acute allograft rejection in multiple experimental transplantation models[55]. Cell proliferation was assessed by ^{3}H-thymidine incorporation in whole blood cultures stimulated with concanavalin A, whereas the effect on the alloresponse in a MLR, and the expression of lymphocyte surface antigens by flow cytometry. All four of the drugs showed a high capacity to inhibit lymphocyte proliferation in a dose-dependent manner, and FK778 had an additive effect when combined with the other three immunosuppressive drugs that is similar to that found in mycophenolic acid combinations. Furthermore, FK778 inhibited the expression of lymphocyte surface antigens that have been implicated in activation, co-stimulation and apoptosis of T-cells. The authors suggested that these combinations appear promising, especially the combination of FK778 and mTORi for transplant patients with renal failure, because they are non-nephrotoxic.

In another study, the potency and efficacy of different concentrations of cyclosporine A and tacrolimus, rapamycin and mycophenolate mofetil, administered alone or in combination, were analyzed to develop a human whole blood assay for flow cytometric assessment of T-cell function, proliferation and the expression of surface antigens[56]. Whole cell cultures were stimulated with concanavalin A and then analyzed by flow cytometry to detect lymphocyte proliferation and activation by bivariate expression of proliferating cell nuclear antigen (PCNA)/DNA content and T-cell-surface activation markers such as CD25, CD95 and CD154. Rapamycin alone had the most potent effect on proliferation of the drugs used in the study, followed by tacrolimus, cyclosporine A

and mycophenolate mofetil, as rapamycin required a lower dose than the other drugs to achieve the same inhibition. In particular, rapamycin showed a synergistic effect on proliferation and activation marker expression when added to cyclosporine A at various concentrations. Rapamycin also synergistically inhibited proliferation and activation marker expression when combined with low concentrations of tacrolimus. However, when combined with high concentrations of tacrolimus, rapamycin acted antagonistically. Rapamycin combined with mycophenolate mofetil further increased the inhibition of lymphocyte function compared to treatment with either drug alone.

Inhibition of B-lymphocyte proliferation
As antibody-secreting plasma cells can develop from B-cells with or without the help of T-cells in response to donor antigens[57], it is imperative to understand the mode of drug action during B-lymphocyte differentiation (*i.e.,* independent of drug effects on T-cells). Therefore, B-lymphocytes are therapeutic targets for immunosuppressive drugs. However, although T-cell assays such as the MLR (to measure proliferation) and ELISPOT (to measure cytokine production) have been well established, the B-cell responses have been more difficult to measure.

A study analyzing the effect of sotrastaurin (a protein kinase C inhibitor for the prevention of transplant rejection and treatment of psoriasis), mycophenolic acid or everolimus assessed proliferation, apoptosis, CD80/CD86 expression, and immunoglobulin and IL-10 production in primary stimulated B-cells *in vitro*. Additionally, B-cells were co-cultivated with pre-activated T-cells with anti-CD28 monoclonal antibody to evaluate the effects of these immunosuppressive drugs on T-cell-dependent immunoglobulin production[44]. Everolimus and mycophenolic acid but not sotrastaurin strongly inhibits B-cell functions in a dose-dependent manner, but all three agents decreased T-cell-dependent immunoglobulin production. The study concluded that although sotrastaurin can affect B-cell function only indirectly by suppressing T-cell help, everolimus and mycophenolic acid can inhibit humoral responses both directly and indirectly.

The effects of everolimus, mycophenolic acid, or prednisolone were analyzed in a three-step *in vitro* culture system developed to promote the proliferation and differentiation of peripheral CD19+ B-cells into plasma cells that produce IgG antibodies[45]. The inhibitory effect of everolimus, mycophenolic acid, and prednisolone on cell proliferation was examined in each step of a three-step culture model. This culture model consisted of: B-cell activation (step 1, days 0-4), plasmablasts generation (step 2, days 4-7), and plasma cell generation (step 3, days 7-10). On day 10, IL-10 production was analyzed by ELISA and cell proliferation by flow cytometry analysis. Although both everolimus and mycophenolic acid efficiently

suppressed cell proliferation and differentiation in step 1, everolimus suppressed B-cell differentiation in step 2. IgG production on day 10 was significantly suppressed by everolimus, mycophenolic acid, and prednisolone, but not cyclosporine. These results suggest that suppression of IgG production by plasma cells could avoid antibody-mediated rejection facilitated by donor-specific antibodies, thus precluding one of the main causes of acute or chronic allograft dysfunction that leads to graft loss. However, these results were obtained from *in vitro* assays and so this hypothesis must be validated in clinical settings.

Immunoprotection

We have described the evidence that mTORi inhibit lymphocyte proliferation and cytokine and antibody production, but mTORi also induce other important immunomodulatory effects. As discussed above, mTORi selectively promote the expansion of regulatory T-cells, which may contribute to the immunoprotective effects of mTORi[37,58-60]. In this section, we review studies indicating that mTORi protect transplant recipients against cytomegalovirus infection and disease, which is a major complication in transplant recipients, and how they aid in DNA repair, thereby lowering cancer risk.

A review explained how mTORi may increase immunity against cytomegalovirus infection[61]. Specifically, activation of mTOR in host cells is essential for cytomegalovirus to propagate viral proteins successfully, even under conditions that normally block mTOR activity[62]. A recent study investigated why patients treated with an mTORi are protected against cytomegalovirus disease, even while graft rejection is prevented[63]. The study was conducted among renal transplant recipients who were treated with prednisolone, cyclosporine A, and mycophenolate sodium for the first 6 mo after transplantation, followed by double therapy with prednisolone and everolimus, prednisolone and mycophenolate sodium, or prednisolone and cyclosporine A. All patients tested cytomegalovirus-seropositive before transplantation. The study observed a significant increase in cytomegalovirus-specific effector-type CD27-CD8+ and CD28-CD27-CD4+ T-cell counts in patients treated with everolimus, but not among those treated with the other drugs. Furthermore, everolimus strongly inhibited allo-responses *in vitro*, whereas it did not affect cytomegalovirus-specific responses. Cyclosporine A and mycophenolate sodium dose-dependently reduced virus-specific proliferation, although less effectively as the allo-responses. Another study investigating cardiac transplant recipients treated with everolimus and cyclosporine, or mycophenolate mofetil and cyclosporine, achieved similar results related to cytomegalovirus infection[64]. Patients in this study treated with the everolimus regimen had a significantly lower incidence of any cytomegalovirus event, infection or cytomegalovirus syndrome, than patients treated with the other regimen.

Other study compared the effect of rapamycin on CD8+ T-cells responding to a graft *vs* a pathogen using a transgenic mice system in which the same monoclonal TCR transgenic T-cells responded to a bacterial pathogen infection or a skin graft[65]. Whereas treatment with rapamycin increased the antigen-specific CD8+ T-cell response to the pathogen, the same T-cell population did not show an enhanced response in the context of a graft.

The results of another study in mice treated with rapamycin have suggested that antigen-specific T-cells responding to a pathogen express CD62L, which is associated with the development of a memory phenotype, whereas antigen-specific T-cells responding to a graft do not express this marker[66]. These results suggest that the conditions under which T-cells are stimulated can profoundly modify the impact of rapamycin on antigen-specific T-cell responses. The mechanism underlying this effect might be linked to the ability of rapamycin to enhance fatty acid oxidation in responding T-cells, and to reduce glucose utilization, a change that has been shown to be crucial for an effector-to-memory transition in CD8+ T-cells[67]. Thus, minimizing the generation of memory cells by treatment with an mTORi could decrease graft rejection responses, and indirectly promote an environment where tolerance could be established.

CONCLUSION

In this review, we have discussed how the mTORi rapamycin and everolimus mediate a potent immunosuppression while concomitantly promoting the expansion and survival of CD4+CD25+FOXP3+ regulatory T-cells after transplantation, which could help to induce tolerance to the graft. However, although the tolerogenic properties of mTORi have been well demonstrated in rodent transplant models, they have not been shown to induce regulatory T-cell-mediated tolerance in humans. The pathogen-activated pro-inflammatory response in humans, which is enhanced by mTOR inhibition, may counterbalance the tolerogenic potential of regulatory T-cell expansion. Future immunomodulatory protocols based on mTORi should combine other immunomodulatory molecules to limit the capacity of mTORi to promote anti-pathogen responses while further supporting regulatory T-cell expansion and stability.

Our review of methods used to quantify the potency of immunosuppressive agents indicates that the available options are not yet sufficiently sensitive for that, or their utility is supported by only a few studies. Until better approaches are developed, a combination of methods may be the most effective way to accurately quantify the potency of immu-

nosuppressive agents. However, from the studies on immunosuppressive potency it can be deduced that mTORi are immunosuppressive drugs with significant power similar to that of CNI.

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REFERENCES

1   **Geissler EK**, Schlitt HJ. Immunosuppression for liver transplantation. *Gut* 2009; **58**: 452-463 [PMID: 19052024 DOI: 10.1136/gut.2008.163527]

2   **United Network for Organ Sharing**. Available from: URL: http://www.unos.org

3   **Wiesner RH**, Fung JJ. Present state of immunosuppressive therapy in liver transplant recipients. *Liver Transpl* 2011; **17** Suppl 3: S1-S9 [PMID: 21850697 DOI: 10.1002/lt.22410]

4   **Nevins TE**. Overview of new immunosuppressive therapies. *Curr Opin Pediatr* 2000; **12**: 146-150 [PMID: 10763765]

5   **Knops N**, Levtchenko E, van den Heuvel B, Kuypers D. From gut to kidney: transporting and metabolizing calcineurin-inhibitors in solid organ transplantation. *Int J Pharm* 2013; **452**: 14-35 [PMID: 23711732 DOI: 10.1016/j.ijpharm.2013.05.033]

6   **Ojo AO**, Held PJ, Port FK, Wolfe RA, Leichtman AB, Young EW, Arndorfer J, Christensen L, Merion RM. Chronic renal failure after transplantation of a nonrenal organ. *N Engl J Med* 2003; **349**: 931-940 [PMID: 12954741 DOI: 10.1056/NEJMoa021744]

7   **Gijtenbeek JM**, van den Bent MJ, Vecht CJ. Cyclosporine neurotoxicity: a review. *J Neurol* 1999; **246**: 339-346 [PMID: 10399863]

8   **Tjon AS**, Sint Nicolaas J, Kwekkeboom J, de Man RA, Kazemier G, Tilanus HW, Hansen BE, van der Laan LJ, Tha-In T, Metselaar HJ. Increased incidence of early de novo cancer in liver graft recipients treated with cyclosporine: an association with C2 monitoring and recipient age. *Liver Transpl* 2010; **16**: 837-846 [PMID: 20583092 DOI: 10.1002/lt.22064]

9   **Subramanian S**, Trence DL. Immunosuppressive agents: effects on glucose and lipid metabolism. *Endocrinol Metab Clin North Am* 2007; **36**: 891-905; vii [PMID: 17983927 DOI: 10.1016/j.ecl.2007.07.003]

10 **Bianchi G**, Marchesini G, Marzocchi R, Pinna AD, Zoli M. Metabolic syndrome in liver transplantation: relation to etiology and immunosuppression. *Liver Transpl* 2008; **14**: 1648-1654 [PMID: 18975273 DOI: 10.1002/lt.21588]

11 **Johnson RW**, Kreis H, Oberbauer R, Brattström C, Claesson K, Eris J. Sirolimus allows early cyclosporine withdrawal in renal transplantation resulting in improved renal function and lower blood pressure. *Transplantation* 2001; **72**: 777-786 [PMID: 11571437]

12 **Nashan B**, Curtis J, Ponticelli C, Mourad G, Jaffe J, Haas T. Everolimus and reduced-exposure cyclosporine in de novo renal-transplant recipients: a three-year phase II, randomized, multicenter, open-label study. *Transplantation* 2004; **78**: 1332-1340 [PMID: 15548972]

13 **Salvadori M**, Bertoni E. Is it time to give up with calcineurin inhibitors in kidney transplantation? *World J Transplant* 2013; **3**: 7-25 [PMID: 24175203 DOI: 10.5500/wjt.v3.i2.7]

14 **Chueh SC**, Kahan BD. Clinical application of sirolimus in renal transplantation: an update. *Transpl Int* 2005; **18**: 261-277 [PMID: 15730485 DOI: 10.1111/j.1432-2277.2004.00039.x]

15 **Ganschow R**, Pollok JM, Jankofsky M, Junge G. The role of everolimus in liver transplantation. *Clin Exp Gastroenterol* 2014; **7**: 329-343 [PMID: 25214801 DOI: 10.2147/CEG.S41780]

16 **Schuler W**, Sedrani R, Cottens S, Häberlin B, Schulz M, Schuurman HJ, Zenke G, Zerwes HG, Schreier MH. SDZ RAD, a new rapamycin derivative: pharmacological properties in vitro and in vivo. *Transplantation* 1997; **64**: 36-42 [PMID: 9233698]

17 **Kirchner GI**, Meier-Wiedenbach I, Manns MP. Clinical pharmacokinetics of everolimus. *Clin Pharmacokinet* 2004; **43**: 83-95 [PMID: 14748618 DOI: 10.2165/00003088-200443020-00002]

18 **Eisen HJ**, Tuzcu EM, Dorent R, Kobashigawa J, Mancini D, Valantine-von Kaeppler HA, Starling RC, Sørensen K, Hummel M, Lind JM, Abeywickrama KH, Bernhardt P. Everolimus for the prevention of allograft rejection and vasculopathy in cardiac-transplant recipients. *N Engl J Med* 2003; **349**: 847-858 [PMID: 12944570 DOI: 10.1056/NEJMoa022171]

19 **Boulay A**, Zumstein-Mecker S, Stephan C, Beuvink I, Zilbermann F, Haller R, Tobler S, Heusser C, O'Reilly T, Stolz B, Marti A, Thomas G, Lane HA. Antitumor efficacy of intermittent treatment schedules with the rapamycin derivative RAD001 correlates with prolonged inactivation of ribosomal protein S6 kinase 1 in peripheral blood mononuclear cells. *Cancer Res* 2004; **64**: 252-261 [PMID: 14729632]

20 **Salvadori M**, Bertoni E. Long-term outcome of everolimus treatment in transplant patients. *Transpl Res Risk Manag* 2011; **3**: 77-90

21 **Laplante M**, Sabatini DM. mTOR signaling in growth control and disease. *Cell* 2012; **149**: 274-293 [PMID: 22500797 DOI: 10.1016/j.cell.2012.03.017]

22 **Yang H**, Rudge DG, Koos JD, Vaidialingam B, Yang HJ, Pavletich NP. mTOR kinase structure, mechanism and regulation. *Nature* 2013; **497**: 217-223 [PMID: 23636326 DOI: 10.1038/nature12122]

23 **Beretta L**, Gingras AC, Svitkin YV, Hall MN, Sonenberg N. Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. *EMBO J* 1996; **15**: 658-664 [PMID: 8599949]

24 **Chung J**, Kuo CJ, Crabtree GR, Blenis J. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell* 1992; **69**: 1227-1236 [PMID: 1377606]

25 **Laplante M**, Sabatini DM. An emerging role of mTOR in lipid biosynthesis. *Curr Biol* 2009; **19**: R1046-R1052 [PMID: 19948145 DOI: 10.1016/j.cub.2009.09.058]

26 **Sarbassov DD**, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 2005; **307**: 1098-1101 [PMID: 15718470 DOI: 10.1126/science.1106148]

27 **Delgoffe GM**, Powell JD. mTOR: taking cues from the immune microenvironment. *Immunology* 2009; **127**: 459-465 [PMID: 19604300 DOI: 10.1111/j.1365-2567.2009.03125.x]

28 **Mills RE**, Jameson JM. T cell dependence on mTOR signaling. *Cell Cycle* 2009; **8**: 545-548 [PMID: 19182526]

29 **Weichhart T**, Säemann MD. The multiple facets of mTOR in immunity. *Trends Immunol* 2009; **30**: 218-226 [PMID: 19362054 DOI: 10.1016/j.it.2009.02.002]

30 **Thomson AW**, Turnquist HR, Raimondi G. Immunoregulatory functions of mTOR inhibition. *Nat Rev Immunol* 2009; **9**: 324-337 [PMID: 19390566 DOI: 10.1038/nri2546]

31 **Abraham RT**, Wiederrecht GJ. Immunopharmacology of rapamycin. *Annu Rev Immunol* 1996; **14**: 483-510 [PMID: 8717522 DOI: 10.1146/annurev.immunol.14.1.483]

32 **Sarbassov DD**, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, Sabatini DM. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell* 2006; **22**: 159-168 [PMID: 16603397 DOI: 10.1016/j.molcel.2006.03.029]

33 **Mondino A**, Mueller DL. mTOR at the crossroads of T cell proliferation and tolerance. *Semin Immunol* 2007; **19**: 162-172 [PMID: 17383196 DOI: 10.1016/j.smim.2007.02.008]

34 **Powell JD**, Lerner CG, Schwartz RH. Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation. *J Immunol* 1999; **162**: 2775-2784 [PMID: 10072524]

35 **Allen A**, Zheng Y, Gardner L, Safford M, Horton MR, Powell JD. The novel cyclophilin binding compound, sanglifehrin A,

disassociates G1 cell cycle arrest from tolerance induction. *J Immunol* 2004; **172**: 4797-4803 [PMID: 15067056]

36   **Steinman RM**, Nussenzweig MC. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc Natl Acad Sci USA* 2002; **99**: 351-358 [PMID: 11773639 DOI: 10.1073/pnas.231606698]

37   **Strauss L**, Czystowska M, Szajnik M, Mandapathil M, Whiteside TL. Differential responses of human regulatory T cells (Treg) and effector T cells to rapamycin. *PLoS One* 2009; **4**: e5994 [PMID: 19543393 DOI: 10.1371/journal.pone.0005994]

38   **Akimova T**, Kamath BM, Goebel JW, Meyers KE, Rand EB, Hawkins A, Levine MH, Bucuvalas JC, Hancock WW. Differing effects of rapamycin or calcineurin inhibitor on T-regulatory cells in pediatric liver and kidney transplant recipients. *Am J Transplant* 2012; **12**: 3449-3461 [PMID: 22994804 DOI: 10.1111/j.1600-6143.2012.04269.x]

39   **Roat E**, De Biasi S, Bertoncelli L, Rompianesi G, Nasi M, Gibellini L, Pinti M, Del Giovane C, Zanella A, Di Benedetto F, Gerunda GE, Cossarizza A. Immunological advantages of everolimus versus cyclosporin A in liver-transplanted recipients, as revealed by polychromatic flow cytometry. *Cytometry A* 2012; **81**: 303-311 [PMID: 22311717 DOI: 10.1002/cyto.a.22019]

40   **Wang GY**, Yang Y, Li H, Zhang J, Li MR, Zhang Q, Chen GH. Rapamycin combined with donor immature dendritic cells promotes liver allograft survival in association with CD4(+) CD25(+) Foxp3(+) regulatory T cell expansion. *Hepatol Res* 2012; **42**: 192-202 [PMID: 22103959 DOI: 10.1111/j.1872-034X.2011.00909.x]

41   **Levitsky J**, Mathew JM, Abecassis M, Tambur A, Leventhal J, Chandrasekaran D, Herrera N, Al-Saden P, Gallon L, Abdul-Nabi A, Yang GY, Kurian SM, Salomon DR, Miller J. Systemic immunoregulatory and proteogenomic effects of tacrolimus to sirolimus conversion in liver transplant recipients. *Hepatology* 2013; **57**: 239-248 [PMID: 22234876 DOI: 10.1002/hep.25579]

42   **Meloni F**, Morosini M, Solari N, Bini F, Vitulo P, Arbustini E, Pellegrini C, Fietta AM. Peripheral CD4+ CD25+ Treg cell expansion in lung transplant recipients is not affected by calcineurin inhibitors. *Int Immunopharmacol* 2006; **6**: 2002-2010 [PMID: 17161354 DOI: 10.1016/j.intimp.2006.07.019]

43   **Ruppert SM**, Falk BA, Long SA, Bollyky PL. Regulatory T Cells Resist Cyclosporine-Induced Cell Death via CD44-Mediated Signaling Pathways. *Int J Cell Biol* 2015; **2015**: 614297 [PMID: 26448755 DOI: 10.1155/2015/614297]

44   **Matz M**, Lehnert M, Lorkowski C, Fabritius K, Unterwalder N, Doueiri S, Weber UA, Mashreghi MF, Neumayer HH, Budde K. Effects of sotrastaurin, mycophenolic acid and everolimus on human B-lymphocyte function and activation. *Transpl Int* 2012; **25**: 1106-1116 [PMID: 22816666 DOI: 10.1111/j.1432-2277.2012.01537.x]

45   **Haneda M**, Owaki M, Kuzuya T, Iwasaki K, Miwa Y, Kobayashi T. Comparative analysis of drug action on B-cell proliferation and differentiation for mycophenolic acid, everolimus, and prednisolone. *Transplantation* 2014; **97**: 405-412 [PMID: 24445924 DOI: 10.1097/01.TP.0000441826.70687.f6]

46   **Mohammadpour N**, Elyasi S, Vahdati N, Mohammadpour AH, Shamsara J. A review on therapeutic drug monitoring of immunosuppressant drugs. *Iran J Basic Med Sci* 2011; **14**: 485-498 [PMID: 23493821]

47   **Kahan BD**, Keown P, Levy GA, Johnston A. Therapeutic drug monitoring of immunosuppressant drugs in clinical practice. *Clin Ther* 2002; **24**: 330-350; discussion 329 [PMID: 11952020]

48   **Barraclough KA**, Staatz CE, Isbel NM, McTaggart SJ. Review: Pharmacodynamic monitoring of immunosuppression in kidney transplantation. *Nephrology* (Carlton) 2010; **15**: 522-532 [PMID: 20649871 DOI: 10.1111/j.1440-1797.2010.01349.x]

49   **Segundo DS**, Ruiz JC, Izquierdo M, Fernández-Fresnedo G, Gómez-Alamillo C, Merino R, Benito MJ, Cacho E, Rodrigo E, Palomar R, López-Hoyos M, Arias M. Calcineurin inhibitors, but not rapamycin, reduce percentages of CD4+CD25+FOXP3+ regulatory T cells in renal transplant recipients. *Transplantation* 2006; **82**: 550-557 [PMID: 16926600 DOI: 10.1097/01.tp.0000229473.95202.50]

50   **Böhler T**, Waiser J, Lichter S, Schumann B, Neumayer HH, Kamar

N, Budde K. Pharmacodynamic effects of everolimus on anti-CD3 antibody-stimulated T-lymphocyte proliferation and interleukin-10 synthesis in stable kidney-transplant patients. *Cytokine* 2008; **42**: 306-311 [PMID: 18440821 DOI: 10.1016/j.cyto.2008.02.015]

51   **Cohen SB**, Parry SL, Feldmann M, Foxwell B. Autocrine and paracrine regulation of human T cell IL-10 production. *J Immunol* 1997; **158**: 5596-5602 [PMID: 9190906]

52   **Kazatchkine MD**, Kaveri SV. Immunomodulation of autoimmune and inflammatory diseases with intravenous immune globulin. *N Engl J Med* 2001; **345**: 747-755 [PMID: 11547745 DOI: 10.1056/NEJMra993360]

53   **Amet N**, Gacad M, Petrosyan A, Pao A, Jordan SC, Toyoda M. In vitro effects of everolimus and intravenous immunoglobulin on cell proliferation and apoptosis induction in the mixed lymphocyte reaction. *Transpl Immunol* 2010; **23**: 170-173 [PMID: 20609387 DOI: 10.1016/j.trim.2010.06.012]

54   **Millán O**, Jiménez O, Fortuna V, Barceló JJ, Brunet M. Role of FK778 alone or in combination with tacrolimus or mTOR inhibitors as an immunomodulator of immunofunctions: in vitro evaluation of T cell proliferation and the expression of lymphocyte surface antigens. *Int J Immunopathol Pharmacol* 2006; **19**: 317-330 [PMID: 16831299]

55   **Kaplan MJ**. FK-778 Astellas. *Curr Opin Investig Drugs* 2005; **6**: 526-536 [PMID: 15912968]

56   **Barten MJ**, Dhein S, Chang H, Bittner HB, Tarnok A, Rahmel A, Mohr FW, Gummert JF. Assessment of immunosuppressive drug interactions: inhibition of lymphocyte function in peripheral human blood. *J Immunol Methods* 2003; **283**: 99-114 [PMID: 14659903]

57   **Shapiro-Shelef M**, Calame K. Regulation of plasma-cell development. *Nat Rev Immunol* 2005; **5**: 230-242 [PMID: 15738953 DOI: 10.1038/nri1572]

58   **Bensinger SJ**, Walsh PT, Zhang J, Carroll M, Parsons R, Rathmell JC, Thompson CB, Burchill MA, Farrar MA, Turka LA. Distinct IL-2 receptor signaling pattern in CD4+CD25+ regulatory T cells. *J Immunol* 2004; **172**: 5287-5296 [PMID: 15100267]

59   **Battaglia M**, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 2005; **105**: 4743-4748 [PMID: 15746082 DOI: 10.1182/blood-2004-10-3932]

60   **Kang J**, Huddleston SJ, Fraser JM, Khoruts A. De novo induction of antigen-specific CD4+CD25+Foxp3+ regulatory T cells in vivo following systemic antigen administration accompanied by blockade of mTOR. *J Leukoc Biol* 2008; **83**: 1230-1239 [PMID: 18270248 DOI: 10.1189/jlb.1207851]

61   **Nashan B**, Gaston R, Emery V, Säemann MD, Mueller NJ, Couzi L, Dantal J, Shihab F, Mulgaonkar S, Seun Kim Y, Brennan DC. Review of cytomegalovirus infection findings with mammalian target of rapamycin inhibitor-based immunosuppressive therapy in de novo renal transplant recipients. *Transplantation* 2012; **93**: 1075-1085 [PMID: 22683823 DOI: 10.1097/TP.0b013e31824810e6]

62   **Kudchodkar SB**, Yu Y, Maguire TG, Alwine JC. Human cytomegalovirus infection induces rapamycin-insensitive phosphorylation of downstream effectors of mTOR kinase. *J Virol* 2004; **78**: 11030-11039 [PMID: 15452223 DOI: 10.1128/JVI.78.20.11030-11039.2004]

63   **Havenith SH**, Yong SL, van Donselaar-van der Pant KA, van Lier RA, ten Berge IJ, Bemelman FJ. Everolimus-treated renal transplant recipients have a more robust CMV-specific CD8+ T-cell response compared with cyclosporine- or mycophenolate-treated patients. *Transplantation* 2013; **95**: 184-191 [PMID: 23222818 DOI: 10.1097/TP.0b013e318276a1ef]

64   **Viganò M**, Dengler T, Mattei MF, Poncelet A, Vanhaecke J, Vermes E, Kleinloog R, Li Y, Gezahegen Y, Delgado JF. Lower incidence of cytomegalovirus infection with everolimus versus mycophenolate mofetil in de novo cardiac transplant recipients: a randomized, multicenter study. *Transpl Infect Dis* 2010; **12**: 23-30 [PMID: 19744284 DOI: 10.1111/j.1399-3062.2009.00448.x]

65   **Ferrer IR**, Wagener ME, Robertson JM, Turner AP, Araki K, Ahmed R, Kirk AD, Larsen CP, Ford ML. Cutting edge: Rapamycin augments pathogen-specific but not graft-reactive CD8+ T cell

responses. *J Immunol* 2010; **185**: 2004-2008 [PMID: 20631309 DOI: 10.4049/jimmunol.1001176]

66   **Araki K**, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, Larsen CP, Ahmed R. mTOR regulates memory CD8 T-cell differentiation. *Nature* 2009; **460**: 108-112 [PMID: 19543266

DOI: 10.1038/nature08155]

67   **Pearce EL**, Walsh MC, Cejas PJ, Harms GM, Shen H, Wang LS, Jones RG, Choi Y. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature* 2009; **460**: 103-107 [PMID: 19494812 DOI: 10.1038/nature08097]

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