REVIEW-THEMED ISSUE

New perspectives on mTOR inhibitors (rapamycin, rapalogs and TORKinibs) in transplantation

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The macrolide rapamycin and its analogues (rapalogs) constitute the first generation of mammalian target of rapamycin (mTOR) inhibitors. Since the introduction of rapamycin as an immunosuppressant, there has been extensive progress in understanding its complex mechanisms of action. New insights into the function of mTOR in different immune cell types, vascular endothelial cells and neoplastic cells have opened new opportunities and challenges regarding mTOR as a pharmacological target. Currently, the two known mTOR complexes, mTOR complex (mTORC) 1 and mTORC2, are the subject of intense investigation, and the introduction of second-generation dual mTORC kinase inhibitors (TORKinibs) and gene knockout mice is helping to uncover the distinct roles of these complexes in different cell types. While the pharmacological profiling of rapalogs is advanced, much less is known about the properties of TORKinibs. A potential benefit of mTOR inhibition in transplantation is improved protection against transplant-associated viral infections compared with standard calcineurin inhibitor-based immunosuppression. Preclinical and clinical data also underscore the potentially favourable antitumour effects of mTOR inhibitors in regard to transplant-associated malignancies and as a novel treatment option for various other cancers. Many aspects of the mechanisms of action of mTOR inhibitors and their clinical implications remain unknown. In this brief review we discuss new findings and perspectives of mTOR inhibitors in transplantation.

Introduction

Rapamycin was isolated in 1975 as an antibiotic product of the actinomycete *Streptomyces hygroscopicus*, obtained from a soil probe collected on Easter Island (Rapa Nui), and was investigated initially for its antifungal properties [1]. Since the first description of its immunosuppressive activity in 1977 [2], much has been learned about the complex mechanisms of action of this macrolide and its site of action, the mammalian target of rapamycin (mTOR) [3]. mTOR is an evolutionarily conserved intracellular serine–threonine kinase that plays a central role in the regulation of cell growth, metabolism and proliferation

[4–6]. The catalytic activity of mTOR occurs via at least two distinct complexes – mTOR complex (mTORC) 1 and mTORC2 [7]. Compared with mTORC1, comparatively little is known about the function of mTORC2. To exert its function, rapamycin forms a complex with the intracellular immunophilin FK506 binding protein 1 A 12 kDa (FKBP12) [8]. This complex inhibits the kinase activity of mTOR by directly blocking substrate recruitment and restricting active site access [9]. While rapamycin and its analogues, or 'rapalogs', almost completely inhibit mTORC1, mTORC2 is affected only after long exposure [10]. Specific deletion of genes encoding mTORC1 or mTORC2, and the use of new-

generation dual mTOR kinase inhibitors, known as 'TORKinibs' [11, 12], have opened up new possibilities to investigate the discrete functions of each mTOR subunit in immune cells, with implications for their roles in transplantation. Comprehensive review of the role of mTOR in the regulation of immune responses [13, 14], pharmacokinetic and dynamic aspects of rapamycin in transplantation [15], and the advantages and disadvantages of mTOR inhibitors in renal transplantation [16] have been published. In the present brief review, we highlight recent insights that have been gained into the immunobiology and pharmacology of mTOR and its role in transplantation.

Molecular biology of mTORCs

The molecular components of mTORC1 and mTORC2, and the factors and pathways that influence their function, are depicted in Figure 1.

(i) mTORC1

mTORC1 is formed by mTOR, mammalian lethal with SEC13 protein 8 (mLST8), proline-rich Akt substrate of 40 kDa

(PRAS40), Dep domain-containing mTOR-interacting protein (Deptor) and the regulatory associated protein of mTOR (Raptor). The activity of mTORC1 is controlled by tuberous sclerosis complex (TSC) 1 and TSC2, which act as its main upstream inhibitors. TSC1/2 control the activity of the guanosine triphosphate GTP)-ase Ras homologue enriched in the brain (RHEB), a protein that interacts directly with mTORC1. Cytokines, growth factors, nutrients [17], costimulatory molecules, as well as cellular energy level and stress influence the activity of TSC1/2. There is evidence that amino acids can directly regulate mTORC1 via Ragulator–Rag GTPases by binding to Raptor and leading it to the surface of lysosomes [18]. Recent studies have identified a member of the solute carrier family 38 (SLC38A9) as a key transmembrane protein in this process [19]. In dendritic cells (DCs), the late endosomal lysosomal adaptor and mitogen-activated protein kinase and mTOR 2 (LAMTOR2) complex has been identified as an essential regulator of Langerhans cell homeostasis *in vivo* [20], suggesting that mTORC1 is important in the immunological regulation of these important antigen (Ag)-presenting cells (APCs). New studies investigating the role of Rac1 have shown that, by binding directly to mTOR, this member of the Rho family of GTPases is able to activate both mTORC1 and mTORC2, facilitating localization to cellular membranes [21].
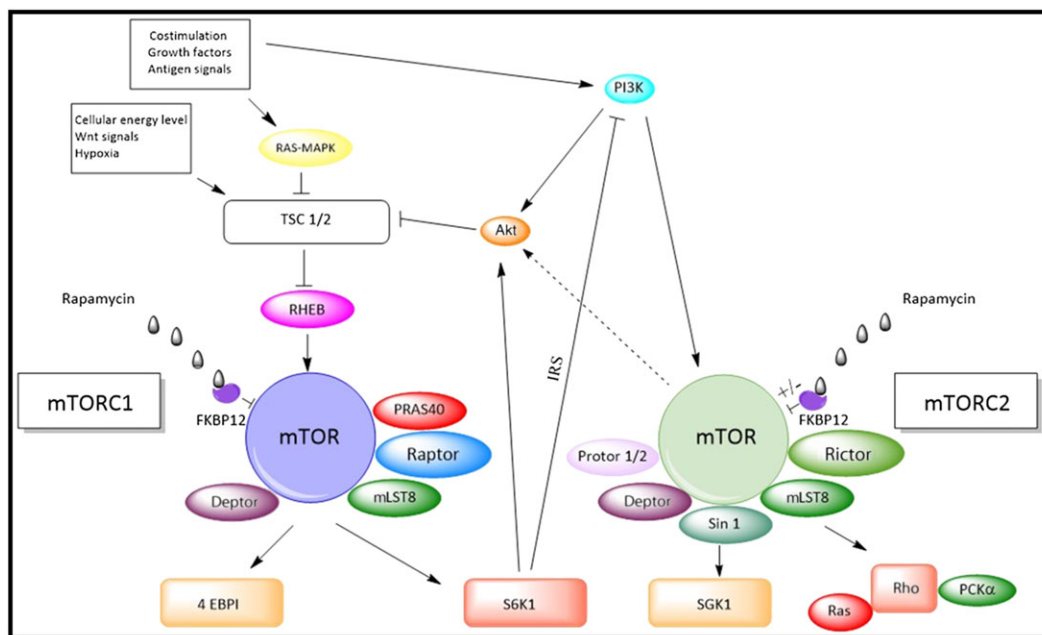

Figure 1

The mammalian target of rapamycin (mTOR) complexes. mTOR complex (mTORC) 1 is formed by mTOR, mammalian lethal with SEC13 protein 8 (mLST8), proline-rich Akt substrate of 40 kDa (PRAS40), Dep domain-containing mTOR-interacting protein (Deptor) and the regulatory associated protein of mTOR (Raptor). Cytokines, growth factors, various nutritional cues and Akt influence the activity of tuberous sclerosis complex (TSC) 1 and TSC2, which control the activity of the GTPase RAS homologue enriched in the brain (RHEB). The interaction with RHEB is followed by phosphorylation of mTOR and leads to mRNA translation by stimulating s6 kinase 1 (S6K1) and phosphorylating eukaryotic translation initiation factor-binding protein 1 (4E-BP1), dissociating the inhibitory effect of 4E-BP1 on eIF4E, a cap-dependent mRNA translation, in mTORC1 signalling. The activation of S6k1 leads to a negative feedback loop over the phosphatidylinositol 3-kinase (PI3K)–Akt axis via insulin receptor substrate. mTORC2 is formed by the additional components protein observed with Rictor (Protor) 1/2 and stress-activated protein kinase-interacting protein 1 (Sin 1). It is activated by PI3K and is only partially inhibited by rapamycin. Activation of mTORC2 regulates cytoskeletal changes via small GTPase Ras homologue (Rho) and protein kinase Cα (PKCα). Activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1) by mTORC2 regulates the epithelial Na$^+$ channel in kidneys. mTORC2 phosphorylates Akt and can influence the activity of TSC1/2. MAPK, mitogen-activated protein kinase

(ii) mTORC2

In mTORC2, mTOR forms a complex with rapamycin-insensitive companion of mTOR (Rictor), mLST8, stress-activated protein kinase-interacting protein 1 (SIN1) and protein observed with Rictor (Protor) 1 and 2 [22]. Whereas mTORC1 phosphorylates S6 kinase, mTORC2 phosphorylates Akt, protein kinase Cα (PKCα) and serum- and glucocorticoid-induced protein kinase 1 (SGK-1), leading to Raptor-independent rearrangement of the actin cytoskeleton [7] and to the regulation of cell metabolism and survival. While rapamycin is a potent inhibitor of mTORC1, mTORC2 is only partially affected after long-term exposure [10]. A recent study has shown that relative expression of FKBP12 and FKBP51 determines the sensitivity of a cell or tissue to mTORC2 inhibition by rapamycin [23].

Influence of mTOR inhibition on different immune cell populations

Table 1 summarizes the known effects of mTORC1 and mTORC2 inhibition in different immune cell populations.

(i) (DCs)

DCs are important 'professional' APCs that play critical roles in the induction and regulation of immunity. They comprise both conventional myeloid DCs (cDCs) and 'non-conventional' plasmacytoid DCs (pDCs), the latter being important sources of type-1 interferons (IFNs). Multiple studies have explored the influence of rapamycin on DC differentiation and function, and analysed the impact of mTOR inhibition on DC function during transplantation. The stage of DC differentiation, their state of activation, and the duration and timing of their exposure to rapamycin determines the nature of the response. Use of new ATP-competitive dual mTORC1 and mTORC2 inhibitors is currently enabling new insights into mTOR signalling in DCs and allowing investigators to pose further questions about the roles of these complexes in DCs. In conventional DCs, rapamycin-induced mTOR inhibition suppresses cell maturation [24], Ag uptake [25], and macro- and endocytosis [26] and induces their apoptosis [27]. These effects lead to reduced activation and proliferation of alloreactive T cells by DCs [28] via impaired

Table 1

Effects of mammalian target of rapamycin complex (mTORC) 1 and mTORC2 inhibition on different immune cell types

Cell type	mTORC 1 inhibition [References]	mTORC 2 inhibition [References]
Dendritic cells (DCs)		
- Conventional DCs	Suppresses maturation, antigen uptake and micropinocytosis, and induces apoptosis [24–26]; paradoxical augmentation of proinflammatory cytokine production [124]	Augments ability to polarize Th1 and Th17; mTORC2 restrains proinflammatory function of activated DCs [33]
- Plasmacytoid DCs	Inhibits activation, modifies cytokine production, enhances Tmem and Treg proliferation [38]	Unknown
T cells	Controls Th1 and Th 17 differentiation [38]	Controls Th2 differentiation [125]
- Effector T cells		
– CD8+ memory cells	Augments CD8+ Tmem responses in infection [126]	Regulates development of CD8+ cells, altering the quantity and quality of receptors important for cell differentiation [45]
– Tregs	Promotes Treg expansion, differentiation and function [50, 78]	Maintains Treg cell stability and coordinates Treg-mediated control of effector responses [127]
NKT cells	Decreases terminal differentiation, reduces peripheral invariant NKT cells, impairs cytokine production [54]	Reduces NKT-17 cell differentiation, reduces thymic and peripheral NKT cells [55]
B cells	Reduces marginal zone formation, decreases antibody (Ab) class switching, alters Ab repertoire [128]	Affects development, survival and function of mature B lineage cells, impairs Ab production [58]
MDSCs	Induces T cell suppression by MDSCs, higher expression of iNOS, upregulation of Tregs [42]	Unknown
Endothelial cells	Lessens proliferation and cytokine secretion by allogeneic CD4+, upregulates Tregs and reduces infiltration of allogeneic effector T cells into the arterial intima [62]	Antagonizes TNF induction of VCAM-1 [63]

iNOS, inducible nitric oxide synthase; MDSC, myeloid-derived suppressor cells; NKT, natural killer T cells; Th, T helper cell; Tmem, T memory cell; TNF, tumour necrosis factor; Tregs, antigen-specific regulatory T cells; VCAM-1, vascular cell adhesion molecule-1.

cytokine-driven activation and differentiation of T helper (Th) 1, Th2 and Th17 cells [29]. In contrast to these effects, the stimulation of rapamycin-preconditioned murine bone marrow (BM)-derived DCs with the Toll-like receptor 4 ligand lipopolysaccharide (LPS) results in increased secretion of the proinflammatory cytokine interleukin (IL) 12/p70, although the stimulatory effects of these rapamycin-conditioned DCs on $CD4^+$T cell activation remain low [30]. In a recent study [31] that investigated the extended lifespan of mouse BM-derived DCs after mTOR inhibition, it was found that mTOR inhibition suppressed the induction of LPS-mediated inducible nitric oxide synthase (iNOS) in DCs. The reduced transcription of iNOS allowed the cells to continue to use their mitochondria to generate ATP and to use fatty acids or glucose as nutrients to promote metabolism. The same group [32] demonstrated improved outcome after autologous DC vaccination in a murine model using mTOR inhibition, owing to the extended lifespan and prolonged period during which the DCs exhibited an activated phenotype.

In a recent report investigating the role of mTORC2 in DC function, Rictor$^{-/-}$ murine BM-derived DCs were stimulated with different activating agents. Compared with wild-type DCs, Rictor$^{-/-}$ DCs displayed an augmented ability to polarize alloreactive Th1 and Th17 cells, both *in vitro* and *in vivo* [33], with the implication that mTORC2 activity restrains the proinflammatory function of activated DCs. The Fms-like tyrosine kinase 3 (Flt3) receptor and its ligand (Flt3L) play an important role in DC development and differentiation [34]. Administration of Flt3L, induces marked expansion of all DC subsets, including pDCs and both $CD8^+$ and $CD8^-$ cDCs in mouse spleen and bone marrow [35, 36]. Flt3L signalling in DCs is only partially understood but is believed to function via the phosphatidylinositol 3-kinase (PI3K)–Akt–mTOR pathway [37]. $CD8^+$ cDCs were particularly responsive to Flt3 signalling, a mechanism that is explained by higher levels of mTOR activity in this DC subset. Recent work has demonstrated a synergistic effect of mTOR inhibition with rapamycin and Flt3L administration on the induction of Ag-specific regulatory T cells (Tregs) in mice [38]. This effect appears to be mediated via the selective expansion of 'tolerogenic' pDCs [38]. Moreover, a combination of rapamycin and Flt3L promotes organ allograft survival in mice by inducing regulatory DC and allograft autophagy [39].

(ii) Myeloid-derived suppressor cells (MDSCs)

MDSCs differentiate from monocyte and granulocyte precursors under the influence of multiple environmental cues. There is emerging evidence that they play a key role in the regulation of alloimmunity and the induction of experimental organ transplant tolerance [40, 41]. Exposure to rapamycin induces T cell suppression by MDSCs and higher expression of iNOS. Moreover, the transcoronary transfer of rapamycin-conditioned MDSCs can prolong heart allograft survival and upregulate Tregs in mice [42].

(iii) Th cells

mTOR inhibitors exert multiple influences on T cell development, homeostasis, activation, differentiation, function and migration. Detailed information about the role of mTOR in T cells can be found in recent reviews [13, 14, 43]. The role of mTOR in T cells is evident during the early stages of their maturation

in the thymus in response to various environmental cues. The impact of mTOR inhibition is reflected in thymic atrophy in rodents after rapamycin administration [44]. mTORC2 appears to be involved in the co/post-translational processing of membrane-expressed αβ T cell receptors (TCRs) during thymocyte development. Thus, mTORC2 inhibition affects T cell development via regulation of the quantity and quality of receptors that are important for their functional differentiation [45]. In knockout (KO) mice, specific deletion of mTORC1 leads to failure of Th1 and IL-17-producing Th (Th17) effector cell differentiation, while mTORC2-deleted T cells fail to differentiate into Th2 cells [46]. Another recent report [47] has revealed a critical role for Akt isoforms and both mTOR complexes in the control of Th17 subset development.

New studies on the role of mTORC1 in Th1 and follicular B helper T (Tfh) cells have demonstrated an important role of the IL-2-mTORC1 axis in the signalling, differentiation and metabolism of these cells. Tfh cells display reduced metabolic activity, mitochondrial function and mTOR kinase activity compared with Th1 cells. IL-2 activation of Akt and mTOR signalling is critical in orchestrating the reciprocal differentiation of Th1 and Tfh cells, and thus IL-2 conducts two signalling arms that are important for T cell differentiation via signal transducer and activator of transcription 5 (STAT5) and PI3K [48].

(iv) Effector memory T cells

Recent studies in mice have defined specific roles for mTORC1 and mTORC2 that link metabolism and $CD8^+$ T effector and memory cell generation, suggesting that these functions could be targeted to promote vaccine efficiency and antitumour immunity [49].

(v) Tregs

Naturally occurring Tregs are $CD4^+$ cells that develop in the thymus. Under normal activating conditions, T cells that lack mTOR differentiate into forkhead box protein (Fox) p3$^+$ Tregs [50], regulate immune responses *in vitro* and *in vivo*, and are now the subject of clinical trials for the treatment of graft-versus-host disease (GVHD) after BM transplantation and organ allograft rejection. Although multiple studies have reported augmentation of Tregs in response to mTOR inhibition, seemingly paradoxical effects were observed after deletion of Raptor in murine Tregs, resulting in a profound loss of their suppressive activity *in vivo* and the development of a fatal, early-onset inflammatory disorder [51]. In a nonhuman primate (NHP) model, repeated infusion of Tregs after their *ex vivo* expansion resulted in longer survival of allogeneic renal transplants when combined with low-dose rapamycin and antithymocyte globulin [52]. Pulsing Tregs from NHPs with rapamycin can enhance their ability to inhibit the proliferation of multiple T cell subpopulations, including $CD4^+$ and $CD8^+$ T cells, as well as Ag-experienced CD28$^+$CD95$^+$ memory and CD28$^-$CD95$^+$ effector cell subpopulations [53].

(vi) Natural killer T (NKT) cells

NKT cells play a central role in viral and bacterial immune responses, depending on secreted cytokines to induce

inflammation or promote immune tolerance. The differentiation and effector function of invariant NKT cells (iNKT), a group of T cells with unique α and β TCR chains, has been shown to be dependent on mTORC1 signalling [54]. New studies in murine KO models have identified a crucial role for mTORC2 in NKT cell development, indicating that deficiency in Rictor (and thus the mTORC2 pathway) decreases thymic and peripheral NKT cells and abolishes NKT17 (a NKT effector lineage producing IL-17) [55]. However, deletion of phosphatase and tensin homologue (Pten), which upregulates mTORC2 activity, enhanced NKT17 generation. By contrast, mTORC1 was dispensable for NKT17 generation. Another recent study [56] has investigated the influence of IL-10 and transforming growth factor β on different rapamycin-treated iNKT lines and found that the suppressive function of iNKT depended on the nuclear localization of Foxp3. While the expression of Foxp3 was mainly dependent on IL-10 stimulation, rapamycin was required to promote the nuclear localization of Foxp3.

(vii) B cells

mTOR inhibition affects the development and function of B cells. Deletion of TSC-1 results in a significant reduction in the number of marginal zone (MZ) B cells, an effect that is corrected by administration of rapamycin [57]. New studies on the function of mTOR inhibitors in B cells have revealed an important role of mTORC2 in B cell homeostasis. In a KO mouse model, Rictor deletion early in B lymphoid ontogeny had, at most, a modest effect on pro- and pre-B cell progression in the BM. By contrast, striking effects were observed in the development, survival and function of mature B lineage cells, with antibody (Ab) production severely impaired when mature B cells lacked Rictor expression after complete development [58]. The blocking of mTORC1 and mTORC2, using the TORKinib AZ8055, resulted in a higher fraction of class-switching B cells in a dose-dependent manner [59]. Interestingly, vaccine studies have shown that the treatment of mice infected with influenza virus subtype H3N2 (a relatively avirulent subtype of the influenza A virus) with rapamycin results in enhanced protection against lethal infection with the H5N1 virus. This effect was promoted by reduced germinal centre formation and decreased Ab class switching, leading to more cross-reactive responses owing to an altered Ab repertoire [60].

Influence of mTOR inhibition on endothelial cells (ECs)

Vascular ECs express major histocompatibility complex (MHC) I and II molecules and produce multiple immunostimulatory and inhibitory signals that activate memory CD4$^+$ cells, inducing graft rejection [61]. Recent studies of the influence of rapamycin on ECs have shown that, in vitro, rapamycin-pretreated ECs stimulate less proliferation and cytokine secretion by allogeneic CD4$^+$ cells, owing to the upregulation of programmed death ligand (PD-L) 1 and PD-L2. Rapamycin preconditioning of ECs also results in their preferential activation of allogeneic CD4$^+$ CD25^{hi} CD127^{lo} Foxp3$^+$ (Treg) cells and reduced infiltration of allogeneic effector T cells into the arterial intima in vivo [62]. Rapamycin-pretreated ECs also show a reduced ability to capture T cells during venular flow by inhibiting tumour necrosis factor-induced expression of vascular cell adhesion molecule-1

(VCAM-1) on ECs. This effect was shown to be dependent on the inhibition of Rictor, suggesting mTORC2 inhibition as a new therapeutic option to reduce vascular rejection [63]. Inhibition of mTORC2 leads to the upregulation of extracellular signal-regulated kinase 1/2, reducing VCAM-1 expression by repressing the induction of the transcription factor interferon regulatory factor 1 (IRF-1), an effect that could be shown in vitro and in vivo after exposure to rapamycin in mice. In an analysis of renal transplant recipients with antiphospholipid syndrome, an autoimmune disease leading to vascular thrombosis and obstetric complications, biopsies from patients treated with rapamycin were compared with those from patients undergoing other immunosuppressive therapy [64]. In this study, the formation of intimal hyperplasia by immunoglobulin G Abs was associated with the activation of mTORC1 and mTORC2 in ECs. Patients with antiphospholipid syndrome nephropathy who required transplantation and were treated with rapamycin (sirolimus) had no recurrence of vascular lesions and showed decreased vascular proliferation on biopsy, compared with patients with antiphospholipid Abs who were not receiving rapamycin [64].

Pharmacological aspects of mTOR inhibition

The most commonly used mTOR inhibitors are sirolimus and everolimus. Everolimus is a sister drug of sirolimus that, instead of a hydrogen atom at position 40, has a 2-hydroxethyl chain substitution, which improves its solubility and bioavailability [65]. The main difference between the two agents lies in their pharmacokinetic characteristics and interindividual differences in pharmacokinetics.

(i) Sirolimus

After oral administration of 2.5 mg sirolimus, the drug is absorbed rapidly, reaching a maximal whole blood concentration (C_{max}) of 40.5 ± 22.2 µg l^{-1} (standard deviation) after an average period of 2.7 ± 2.1 h (T_{max}), dependent on dose. The exact bioavailability of sirolimus is unknown but has been estimated to be 15 ± 9%, with inter- and intraindividual variations depending on intestinal cytochrome P450 (CYP) 3A content [66]. The metabolism of sirolimus occurs mainly via CYP3A4, CYP3A5 and CYP2C8 [67], with associated interindividual variability due to different expression of these enzymes. An important difference in clearance is found between the Afro-Caribbean and non-Afro-Caribbean populations, with significantly higher metabolism in the former group [68].

(ii) Everolimus

Oral administration of 2.5 mg everolimus reaches whole blood C_{max} levels of 45 (SD ±21) µg l^{-1} after an average time of 1.3 (±0.4 h) [69]. The total bioavailability is estimated to be about 16%, with interindividual and intraindividual variation [70]. Everolimus is metabolized by the same enzymes as sirolimus [71]. In contrast to sirolimus, recent findings describe slower metabolism of everolimus in Afro-Caribbean patients [72].

(iii) TORKinibs

Early pharmacokinetic studies have been performed on these dual mTORC1 and mTORC2 inhibitors in cancer

patients [73, 74] and in rodents [75, 76]. Pharmacokinetic data for the TORKinib AZD2014 show rapid absorption after oral intake, with a median time to peak of 0.5 h and 1 h following a single dose between 50 mg and 100 mg (C_{max}: 1664 ng ml^{-1}). Although the elimination half-life was approximately 3 h, large interpatient variability was seen [77].

Drug combination/conversion strategies in clinical organ transplantation

New immunosuppressive protocols in organ transplantation involve a switch from calcineurin inhibitors (CNIs) to mTOR inhibitors. Recent studies examining changes in T cell subsets in kidney transplant recipients after conversion to rapamycin have shown upregulation of Tregs, accompanied by a reduction in Th17 cells. An increased proportion of CD8$^+$CCR7$^+$ T cells (CD8$^+$ Tregs) that could suppress CD4$^+$ T cell activation *in vitro* has been found [78]. Newer immunosuppressive protocols in renal transplantation have investigated the combination of mTOR inhibition and costimulation (B7-CD28) blockade (with belatacept) [79]. Induction therapy with rabbit antithymocyte globulin in combination with these two agents results in a favourable ratio of Foxp3$^+$ Tregs over the memory T cell compartment 1 year post-transplant. Despite the presence of alloreactive CD4$^+$ cells in pretransplant donor-specific memory/effector T cell immune monitoring, no patient showed signs of clinical rejection during the first year. The combination of rapamycin (sirolimus) and belatacept successfully suppressed both donor-specific and naïve effector T cell responses.

New perspectives of mTOR inhibition in experimental organ transplantation

A new-generation TORKinib, which blocks both mTORC1 and mTORC2, has recently been shown to prolong allograft survival in a murine organ transplant model [75]. In this latter study, simultaneous blocking of both mTOR complexes led to similar immunomodulatory effects as described for rapamycin (upregulation of Foxp3$^+$ Tregs and inhibition of IFNγ production), while the production of cytokines by Th1 and Th17 cells was increased. Recent findings in a rat model showed that chronic rejection could be eliminated through the delivery of a MHC class I molecule into ACI strain recipients of Wistar–Furth hearts at the time of transplantation, together with subtherapeutic cyclosporine. Deregulation of two parallel (RhoA and Rac1) actin pathways played a crucial role in the inhibition of chronic rejection. This implies that both mTORC1 and mTORC2 control cell motility through changes in the organization of the cellular cytoskeleton/RhoA/Rac1 pathway components [80].

New perspectives of mTOR inhibition in clinical organ transplantation

(i) Kidney transplantation

In renal transplantation, special interest in the use of mTOR inhibitors has arisen from their low nephrotoxicity when compared with CNIs [81], and much has been learned since their introduction [82] into clinical transplantation [68]. Recent studies report that an early switch to sirolimus in combination with a low-dose CNI [83] or mTOR monotherapy [84] significantly improves renal graft function and gives rise to a similar risk of acute rejection compared with standard CNI protocols. The use of mTOR inhibitors as monotherapy is still a point of discussion; in a clinical trial comparing CNI-based immunosuppression with 'early conversion' to everolimus-based CNI-free therapy, the occurrence of donor-specific human leucocyte antigen Abs and Ab-mediated rejection after low-risk kidney transplantation was increased in the everolimus group [85]. A 5-year follow-up clinical trial after conversion from a CNI to everolimus at 4.5 months showed significantly improved graft function, along with similar risks of graft loss, mortality, serious adverse events and neoplasms postrandomization [86].

(ii) Heart and lung transplantation

In lung transplantation, a large multicentre, randomized, open-label controlled trial comparing sirolimus with azathioprine in a tacrolimus-based immunosuppressive regimen including 181 patients showed no significant difference in the overall rate of acute rejection after 1 year [87]. In a subgroup analysis by the same investigators, the incidence of cytomegalovirus (CMV) events in transplanted patients given rapamycin (sirolimus) remained significantly lower than in the azathioprine arm, even after adjustment for confounding factors [88]. In heart transplantation, a new randomized trial comparing patients undergoing complete CNI withdrawal after 7–11 weeks with standard cyclosporine treatment showed a significant reduction in cardiac allograft vasculopathy after 1 year [89]. A long-term follow-up comparing the combination of sirolimus and tacrolimus with tacrolimus and mycophenolate mofetil did not show any differences in cardiac allograft vasculopathy progression after 8 years, indicating the need for large randomized clinical trials to compare different drug combinations [90]. In addition, in heart transplantation, as in lung and kidney transplantation, treatment with everolimus was found to be associated with a lower incidence of CMV infection compared with azathioprine and mycophenolate mofetil [91].

(iii) Infection

Next to improvement in graft function, clinical trials have described a reduced incidence of CMV infection in renal transplant patients under mTOR inhibition [92]. In a recent trial conducted in low/moderate immunological risk kidney transplant recipients receiving no CMV prophylaxis, the incidence of CMV infection/disease was significantly lower in those receiving everolimus and reduced-dose tacrolimus compared with mycophenolate mofetil and standard-dose tacrolimus [93]. One possible explanation for these findings is the selective effect of mTOR inhibitors on T cell differentiation, especially CD8$^+$ T cells [94]. In addition, Poglitsch *et al.* [95] investigated the effect of mTOR inhibition in CMV-infected macrophages and demonstrated that mTOR is essential for virus replication during the late phases of the viral cycle in myeloid cells. This complex interaction has recently been clarified by *in vitro* studies in which it could be shown that CMV immediate early proteins can activate PI3K–Akt, resulting in activation of mTORC1 and mTORC2

[96]. In this study, it was reported that the inhibitory effects of rapamycin on viral growth are due primarily to the presence of Rictor, not Raptor, and that Rictor- and Raptor-containing complexes are modified such that their substrate specificities and rapamycin sensitivities are altered. In another study, where mTOR activity was completely blocked using the dual mTORC1 and mTORC2 inhibitor Torin 1, replication of representative members of the α-, β- and γ-herpesvirus families was inhibited [97]. Recent findings in a murine γ-herpesvirus infection model, in which treatment with belatacept resulted in increased viral burden, showed that the addition of rapamycin could maintain the number and function of virus-specific CD8^{+} T cells [98]. The molecular mechanisms underlying these findings remain unclear. Possible explanations are that suppression of Akt and mTOR and augmentation of PD-1 expression via increased FoxO1 are both a normal and necessary part of the progression of cytotoxic T cell exhaustion that serves not only to prevent excessive immunopathology, but also to sustain virus-specific cytotoxic T cells during persistent Ag stimulation.

mTOR inhibition and malignant disease

(i) Non-transplant-associated malignancies

The mTOR pathway is currently the subject of intense investigation in cancer research, owing to its central role in cell metabolism and proliferation [99]. Owing to the complexity of this topic, only a brief overview is presented here. mTOR inhibitors have shown clinical efficacy against a number of malignancies, especially renal cell carcinoma [100], breast cancer [101] and hepatocellular carcinoma (HCC) [102]. In a large phase III clinical trial of patients with advanced clear cell renal cell carcinoma (ccRCC), treatment with everolimus prolonged progression-free survival, with mild or moderate adverse events [103]. The largest prospective randomized clinical trial to determine if sirolimus can improve HCC recurrence-free patient survival (RFS) in liver transplant recipients [Sirolimus in Liver Transplant Recipients with HCC study (SiLVER); 525 patients] has revealed that sirolimus does not improve long-term RFS beyond 5 years [104]. However, a RFS and overall survival benefit was evident in the first 3–5 years, especially in low-risk patients. Preclinical data support the use of dual mTOR inhibitors for ccRCC, with evidence of increased cell apoptosis *in vivo* and *in vitro* [105]. A possible explanation for this finding is that negative feedback loops, which regulate PI3K–Akt signalling, are inhibited by rapalogs and may counteract their anticancer efficacy [105]. Additionally, the toxicity profiles of new-generation ATP-competitive TORKinibs are more attractive compared with those of rapalogs. New phase II trials investigating the use of TORKinibs for advanced vascular endothelium growth factor-refractory ccRCC have, however, shown AZD2014 to be inferior compared with everolimus regarding progression-free survival and overall survival [106]. The discrepancy between preclinical data and clinical findings is still unclear and raises new questions regarding the role of mTORC1 and mTORC2 in tumour biology.

(ii) Transplant- associated malignancies

Recent studies investigating the use of rapamycin in liver transplantation in selected patients with HCC report lower tumour recurrence in comparison with conventional immunosuppression [107, 108]. Retrospective analysis of the prognosis of patients who developed *de novo* solid organ tumours after liver transplantation for alcoholic liver disease has shown a significant improvement under everolimus-based immunosuppression compared with other immunosuppressive protocols [109]. In HCC, mTORC1 and mTORC2 pathways, including phosphorylated ribosomal protein S6, p-Akt, insulin-like growth factor-1R and Rictor, are upregulated in 40–50% of the tumours [110]. While use of sirolimus did not improve the survival of patients with advanced HCC after the failure of the multikinase inhibitor sorafenib [111], studies using novel dual mTORC1 and mTORC2 inhibitors have shown promising *in vitro* results. Systematic reviews and meta-analyses have revealed a reduced risk of malignancies in kidney transplant patients given sirolimus immunosuppression [112, 113]. Although overall mortality in one study was increased compared with controls, there may be a subset of patients that benefit from mTOR inhibition. Next to nonskin cancer malignancies, there is also evidence that secondary skin cancer can be prevented by sirolimus treatment in kidney transplant patients [114].

Side effects of mTOR inhibition

Since the introduction of rapamycin and rapalogs for transplantation and other indications such as TSC, various adverse events have been described. Hypertriglyceridaemia is one of the most common metabolic side effects of rapalogs, making dietary and pharmacological treatment (e.g. statins) necessary in many patients [115]. Haematological complications described in association with mTOR inhibition include thrombocytopenia, leukopenia, neutropenia, lymphopenia and anaemia [116]. These findings were consistent in a systematic review, showing a significant increase in hypercholesterolaemia and anaemia in renal transplant patients after conversion to mTOR inhibitor-containing immunosuppressive therapy [117]. Women treated with mTOR inhibitors for polycystic kidney disease reported menstrual cycle disturbances 3–5 times more commonly than untreated controls; some patients reported amenorrhoea, leading to drug withdrawal in a renal transplant setting [118, 119]. Increased risks for wound complications and postoperative lymphocoeles after renal and heart transplantation have been described in recent reviews of the literature. These studies underline the need for meticulous surgical techniques, especially in obese patients with mTOR inhibitory treatment regimens [120, 121]. One of the most dreaded complications associated with the use of mTOR inhibitors is noninfectious pneumonitis (NIP), which can result in life-threatening respiratory distress. In a randomized trial comparing treatment with everolimus *vs*. placebo in patients with renal cell carcinoma, 16% developed grade 2 (not interfering with daily living) and 3.6% grade 3 (interfering with daily living or oxygen indicated) NIP in the everolimus group [122]. Therapy for NIP consists of dose reduction, discontinuation or corticosteroid treatment. Less is known about the side effects of TORKinibs as these new agents have only been used for short periods of time in oncology patients. In a phase I study of patients with advanced solid tumours, those receiving the TORKinib AZD2014 commonly developed nausea, mucositis, diarrhoea

and anaemia [77]. In a randomized phase II study of everolimus versus AZD2014 in vascular endothelium growth factor-refractory metastatic ccRCC, grade 3–4 adverse events (most commonly, anaemia, fatigue and nausea) occurred in 35% and 48% of AZD2014 and everolimus patients, respectively [106]. It remains unclear whether the perioperative use of TORKinibs would be well tolerated and safe. More detailed elaboration on the safety considerations of mTOR inhibitors can be found in a recent review [123].

Conclusions

In recent years, our knowledge of the complex implications of mTOR inhibition has grown through both basic research and clinical experience. Selective gene KO models and ATP-competitive mTOR inhibitors have provided new insights into the distinct roles of mTORC1 and mTORC2 in cell growth, metabolism and function. As exclusive mTORC2 inhibitors are not currently available, our understanding of the influence of selective mTORC2 inhibition remains limited to genetic deletion models and RNA silencing. The precise roles of mTORC1 and mTORC2 in many immune cells remain unclear, and interactions between these complexes remain to be studied.

The ability of mTORC1 inhibition to modify the behaviour of APCs, suppress alloreactive effector T cell responses and promote Tregs has bestowed 'tolerogenic' properties on rapamycin. However, paradoxical immunostimulatory effects of mTOR inhibition on APCs and T cells, paired with limited immunosuppressive potency, makes further research and refinement of mTOR inhibition strategies essential. Low nephrotoxicity, lower incidences of viral infection and beneficial effects of mTOR inhibition on EC proliferation need to be balanced against known side effects and optimal strategies devised to maximize the therapeutic potential of these important agents.

Competing Interests

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