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Immunoregulatory Functions of mTOR Inhibition

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Abstract

The potent immunosuppressive action of rapamycin is commonly ascribed to inhibition of growth-factor-induced T-cell proliferation. However, it is now evident that the serine/threonine protein kinase mammalian target of rapamycin (mTOR) has an important role in the modulation of both innate and adaptive immunity. mTOR regulates diverse functions of professional antigen-presenting cells, such as dendritic cells (DCs), and has important roles in the activation of conventional T cells and the function and proliferation of regulatory T cells. Here, we review our current understanding of the mTOR pathway and the consequences of mTOR inhibition, both in DCs and T cells, including new data on the regulation of FOXP3 expression.

Rapamycin was isolated in the early 1970s from a soil sample obtained on Easter Island (Rapa Nui) and identified as a potent anti-fungal metabolite [1]. This macrolide, which is produced by *Streptomyces hygroscopicus*, was found to inhibit cell proliferation and to have potent immunosuppressive activity. It is used for prevention of kidney transplant rejection[2]. Rapamycin and its derivatives are undergoing clinical testing for prophylaxis of graft rejection[3] and graft-versus-host disease (GVHD) **[G]**[4], chemotherapy of some cancers[5] and the prevention of restenosis following angioplasty[6].

Our understanding of the mechanisms that underlie the unique immunosuppressive profile of rapamycin continues to evolve. In line with this, the central and pervasive role of the serine/threonine kinase 'mammalian target of rapamycin' (mTOR) in innate and adaptive immunity is becoming apparent. Blockade of mTOR by rapamycin impairs dendritic cell (DC) maturation and function, and inhibits T-cell proliferation, a mechanism that underpins its immunosuppressive effect. There is now strong evidence that mTOR is crucial for the regulation of antigen responsiveness in $CD4^+$ T cells. This effect seems to be mediated by an influence of mTOR inhibition on naturally-occurring regulatory T (T_{Reg}) cells, which have a key role in immunological tolerance. Exciting information has emerged regarding the role of the phosphatidylinositol-3-kinase (PI3K)–AKT–mTOR pathway in regulating DC and T-cell function, particularly in relation to the expression of forkhead box P3 (FOXP3) and the differentiation of T_{Reg} cells. Here, we review the remarkable recent progress in elucidation of the mechanisms by which mTOR inhibition affects intracellular signalling pathways in immune cells, particularly DCs and T cells, and how this influences immunity.

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The mTOR signalling pathway

Discovery of mTOR

Genetic screening of *Saccharomyces cerevisiae* identified two targets of rapamycin (TOR) genes (*TOR1* and *TOR2*) and gave an early indication of the importance of TOR in control of cell proliferation[7]. Rapamycin forms a complex with the immunophilin **[G]** FK506-binding protein 1A, 12 kDa (FKBP12) to inhibit TOR function[8] **(**BOX 1**)**. A single mTOR gene was subsequently identified in rat, mouse and human cells, with a high degree of homology to yeast TORs[9-11]. Subsequent work has identified mTOR as an atypical serine/threonine protein kinase (BOX 1**)** with a central role in mammalian development[12, 13]. It is a crucial regulator of cell growth and proliferation, and of physiological events such as transcription, mRNA turnover, translation, ribosomal biogenesis, vesicular trafficking, autophagy, cytoskeletal organization and cell size[14].

mTOR exists in at least two complexes, mTOR complex 1 (mTORC1) and mTORC2 (BOX 1), that have distinct relationships both to upstream and downstream effectors and to each other **(**FIG. 1**)**. mTOR in mTORC1 is exquisitely sensitive to inhibition by rapamycin, whereas mTOR in mTORC2 is resistant to rapamycin, for an unknown reason. Notably, there are also rapamycin-insensitive functions of mTORC1 in mouse embryonic fibroblasts that have been identified recently using a new inhibitor (Torin 1) of both mTORC1 and mTORC2[15]. Also prolonged exposure to rapamycin can impede the assembly of mTORC2 in some cells[5, 16, 17].

Signalling through mTORC1

In immune cells, mTORC1 is a regulator of cell growth and other processes downstream of PI3K–AKT, Wingless and Integrase-1 (WNT)–glycogen synthase kinase 3 (GSK3) and AMP-activated protein kinase (AMPK) signalling (FIG. 1). Details of mTORC1 signalling can be found in recent comprehensive reviews[14, 18, 19]. Tuberous sclerosis complex 1 (TSC1) and TSC2 together form a functional complex that acts as the upstream inhibitor of mTORC1. Growth factors, cytokines, co-stimulatory molecules and antigen receptors activate PI3K, which subsequently activates AKT. Fully activated AKT inhibits TSC2 by phosphorylation[20], thereby negating its inhibitory effect on mTORC1. Activation of the RAS–mitogen-activated protein kinase (MAPK) pathway also leads to inhibition of the TSC1–TSC2 complex[14]. Alternatively, cellular stresses and DNA damage can inhibit mTORC1 by promoting the regulatory capacity of TSC1–TSC2[14, 19]. The inhibitory activity of the TSC1–TSC2 complex is mediated by targeting RHEB (RAS homologue enriched in brain), a RAS-like guanosine triphosphatase (GTPase) and a positive regulator of mTORC1[18, 19].

Removal of mTORC1 inhibition results in its phosphorylation of S6 kinase 1 (S6K1) and the eukaryotic initiation factor 4EBP1 (eIF4E binding protein 1). Phosphorylated S6K1 promotes mRNA translation and cell growth by enhancing the biosynthesis of the translational apparatus in the cell[14, 19]. The phosphorylation of 4EBP1 prevents its inhibition of eIF4E, which also stimulates translation[14]. WNT proteins bind to the Frizzled family of receptors, which are involved in regulation of effector T-cell development, T_{Reg}-cell activation and DC maturation[21]. The WNT pathway influences the mTORC1 pathway by inhibiting GSK3, which in the absence of WNT signaling, is an additional negative regulator of mTORC1[22].

Signalling through mTORC2

Knowledge of the more recently defined mTORC2 is limited compared with mTORC1, given the lack of a mTORC2-specific inhibitor. Studies targeting Rictor (rapamycin-insensitive companion of mTOR; a defining component of mTORC2; BOX 1) through small interfering RNA (siRNA) **[G]**[23, 24] and studies of Rictor knock-out mice[13] have shown the importance

of mTORC2 in mammalian development and several cellular processes. mTORC2-mediated phosphorylation of AKT is stimulated by insulin and can be blocked by PI3K inhibitors[25]. However, knockdown of Rictor does not decrease S6K1 activation[24], indicating that mTORC2 does not activate mTORC1[24].

Targeting *Rictor* has also established that mTORC2 regulates the actin cytoskeleton through the small GTPase RHO and protein kinase C (PKC)[14]. TSC1–TSC2 has been shown to regulate cell adhesion and migration[26], but it is not clear whether TSC1–TSC2 signals to and regulates mTORC2 directly. Regulation of cell movement and adhesion is an important feature of effective immune responses, so it is possible that mTORC2 might also be shown to modulate immune reactivity when specific inhibitors become available.

S6K1-mediated regulation of mTOR signalling

mTOR is a crucial coordinator of signalling pathways, so it is not unexpected that feedback inhibition is an important component of the pathway. Activated S6K1, the main effector of uninhibited mTORC1, negatively regulates input from PI3K–AKT to mTORC1[19, 27-29] by phosphorylating and initiating the degradation of insulin receptor substrate 1 (IRS1), which is the molecular intermediate between the insulin receptor and PI3K[19, 27-31]. Whether S6K1 can also negatively regulate input from other receptor systems that activate mTOR through PI3K has not been defined. Activated S6K1 can also positively regulate mTOR activation. GSK3, a unique kinase that is constitutively activated in the absence of growth factors, negatively regulates the mTOR pathway by stimulating the TSC1–TSC2 complex. Under certain conditions, activation of S6K1 can negatively regulate GSK3[32, 33], thus facilitating cell proliferation.

Overall, the mTOR kinase, functioning in mTORC1 and mTORC2, acts as a coordinator of signalling pathways that shape the response of cells to various stimuli. Immune cells, using receptors that signal through mTOR directly or indirectly, modulate host responses based on their “perception” of environmental danger. We focus here on the role of mTOR in DCs, macrophages and T cells, but it is worth noting that the inhibition of mTOR by rapamycin has effects on other immune cells (BOX 2) that are outside the scope of this Review.

mTOR in DCs and macrophages

DCs and macrophages are phagocytic cells, resident in almost all tissues. Both cell types degrade pathogens and function as antigen-presenting cells. Whereas macrophages have a scavenging role and activate and recruit other immune cells, DCs are uniquely well-equipped to present antigen to T cells and initiate adaptive immune responses.

DC differentiation and maturation

The suppressive effects of mTOR inhibition on DC differentiation and maturation are well-documented *in vitro*. In addition, rapamycin inhibits the differentiation and mobilization of mouse DCs *in vivo* in response to administration of Fms-like tyrosine kinase 3 ligand (FLT3L) **[G]**[34]. Whereas an early study suggested that rapamycin did not affect the phenotypic differentiation or CD40L-induced maturation of human monocyte-derived DCs *in vitro*, but rather promoted the apoptosis of these DCs and $CD34^+$-derived DCs[35], studies of mouse bone marrow-derived DCs[34] showed that mTOR inhibition suppressed IL-4-dependent maturation through the post-transcriptional downregulation of the IL-4R complex. *In vitro* and *in vivo*, rapamycin inhibited costimulatory molecule expression by DCs, and suppressed IL-4-induced IL-12 and TNF production and T-cell stimulatory function[34]. Further work has shown that mTOR inhibition during DC differentiation inhibits the upregulation of CD86 expression induced by Toll-like receptor (TLR) ligands (such as LPS or CpG DNA) or CD40-specific

monoclonal antibody[36]. mTOR inhibition also decreases nitric oxide (NO) production by LPS-stimulated macrophages[37], an effect attributed, in part, to impaired secretion of IFNβ, which is an autocrine co-factor for NO production. Furthermore, the inactivation of mTOR decreases IL-2 secretion upon stimulation of DCs through the C-type lectin receptor Dectin 1[38], indicating that cytokine production through C-type lectin receptor activation involves mTOR.

Immature DCs undergo differentiation in response to hypoxia and over-express hypoxia-inducible factor 1α (HIF1α) and its downstream target genes, including vascular endothelial growth factor and glucose transporter-1[39]. mTOR inhibition attenuates these responses, and rapamycin can suppress hypoxia-induced inflammation through inhibition of the HIF-1α pathway[39]. Whereas these observations all relate to the effects of soluble rapamycin, there are recent reports that the delivery of rapamycin through biodegradable nano- or microparticles enhances its inhibitory effects on the maturation and T-cell stimulatory function of mouse and human DCs[40-42].

Antigen uptake and presentation

DCs use several pathways for antigen uptake: phagocytosis, constitutive macropinocytosis **[G]** and mannose receptor-mediated endocytosis. Rapamycin impairs the macropinocytosis and endocytosis of antigens by cultured mouse immature bone marrow-derived DCs[43]. Rapamycin also inhibits endocytosis *in vivo* in mouse splenic DCs[34]. Human monocyte-derived DCs differentiated in the presence of rapamycin have decreased expression of antigen uptake receptors (such as CD205, CD32, CD46 and CD91) and decreased receptor-mediated and fluid-phase endocytosis and phagocytosis of bacteria and apoptotic cells[44] **(**FIG. 2A**)**. Although the mechanisms by which rapamycin inhibits endocytosis are unclear, the inhibition of mTOR by short-term exposure to rapamycin abolishes the translation of Rho-associated kinase 1 (ROCK1) in mouse macrophages, which results in the inhibition of phagocytosis and chemotaxis[45].

Unlike calcineurin inhibitors **[G]** (such as cyclosporin A [CsA] and FK506), rapamycin does not inhibit the presentation of MHC-restricted antigens (such as ovalbumin [OVA]) or of minor histocompatibility antigens **[G]** to T cells by mouse bone marrow-derived DCs[46-48]. This might reflect the inhibitory effects of calcineurin inhibitors on early events (such as cytokine production) in DC-induced activation of naïve T cells, whereas rapamycin inhibits subsequent events. There is, however, recent evidence that mTOR inhibition can suppress the transient aggregation of ubiquitylated cytoplasmic proteins during lipopolysaccharide (LPS)-induced DC maturation[49, 50]. These 'aggresomes' (DC aggresome-like structures; DALIS **[G]**) **(**FIG. 2A**)** might function as depots of antigen that can be cross-presented by MHC class I molecules, and they might allow DCs to coordinate their maturation and antigen-presenting functions during migration from the periphery to secondary lymphoid tissue.

Cross presentation of antigen is essential for the generation of cytotoxic T-cell responses against certain tumour cells and viruses. The fact that calcineurin inhibitors, but not rapamycin, inhibit the cross presentation of an exogenous antigen by mouse DCs[47] might explain why, in mice bearing an allograft and a tumour, CsA inhibits allograft rejection but promotes tumour growth (through inhibiting the cross-presentation of tumour-specific antigens), whereas rapamycin suppresses both graft rejection and tumour growth[51].

Autophagy **[G]** is a constitutive process in DCs[52] in which autophagosomes sequester intracellular contents (such as damaged organelles and macromolecules) and target them for lysosomal degradation. This process is thought to be involved in the MHC class II antigen presentation pathway leading to self tolerance, and in innate and adaptive immunity[53, 54]. TOR1 and TOR2 are involved in repression of autophagy in *Saccharomyces cerevisiae*[55]. Rapamycin induces autophagy in yeast[56]. and also in mouse macrophages and DCs, enhancing

their Ag presenting ability[57]. Further studies are required to determine the extent to which autophagy influences the peptide–MHC class II repertoire, and how this might be affected by mTOR inhibition. Overall, rapamycin can interfere with antigen uptake in DCs and can modulate events associated with antigen presentation, although the mechanistic basis of these effects requires clarification.

DC survival

DC survival is important for the induction of immune responses, for example to viruses and tumours[35, 58]. Blocking of granulocyte/macrophage colony-stimulating factor (GM-CSF) signalling by rapamycin induced the apoptosis of both human monocyte-derived DCs and DCs derived from $CD34^+$ precursor cells, but not of monocytes or macrophages. By contrast, the frequency of apoptotic or dead cells was consistently <10% in rapamycin-conditioned, bone marrow-derived mouse DC cultures, as well as for *in vivo*-generated DCs from rapamycin-treated mice[34]. As pointed out by these authors, pro- and anti-apoptotic effects of rapamycin have been reported for different cell types, possibly reflecting differential sensitivity of mTORC2 to disruption in these cells. Interestingly, no increase in cell death was observed following mTOR inhibition by rapamycin in virus-infected, FLT3L-derived DCs[38].

DC migration

By increasing expression of the chemokine receptor CCR7, rapamycin increases the migration of human DCs in response to CCL19 *in vitro* and mouse DC migration to lymph nodes *in vivo*[59]. Similarly, functional expression of CCR7 is retained on mouse rapamycin-conditioned DCs[36, 60], which promotes their homing to secondary lymphoid tissue during inflammatory responses following allogeneic haematopoietic cell transplantation[60]. Intact *in vivo* migration of syngeneic rapamycin-conditioned DCs to secondary lymphoid tissue has also been reported in a heart allograft model[61]. Retention of this *in vivo* migratory ability is probably crucial for the immune-regulatory properties ascribed to rapamycin-conditioned DCs, to allow these cells to reach appropriate T-cell areas in the lymphoid tissue.

Cytokine production by DCs and pDCs

The coordinated secretion of pro- and anti-inflammatory cytokines is essential for effective immunity[62]. An unexpected finding is that mTOR suppresses caspase-1 activation (the molecular basis of which is unclear) and therefore the production of bioactive IL-1β[38]. Both *in vitro* and *in vivo*, mTOR inhibition elicits de novo production of IL-1β by otherwise phenotypically immature, mouse bone marrow-derived DCs[63]. Moreover, IL-1β production by rapamycin-conditioned DCs promotes overexpression of the transmembrane form of the IL-1R family member, IL-1R-like 1 (also known as ST2)[63] **(**FIG. 2B**)**, the recently identified receptor for IL-33, which promotes T helper 2 (T_H2)-cell responses[64]. ST2 has also been implicated as a potent negative regulator of TLR signalling in macrophages[65]. In keeping with this function, IL-1β-induced ST2 expression suppresses responses of rapamycin-conditioned mouse DCs to TLR and CD40 ligation, an effect that is absent in $ST2^{-/-}$ DCs[63]. So, by inducing IL-1β production, with consequent upregulation of ST2 expression, mTOR inhibition impedes DC maturation and their ability to stimulate effector T cell responses. By contrast, these rapamycin-conditioned DCs favour the differentiation of potent, alloantigen-specific $CD4^+CD25^+FOXP3^+$ T_{Reg} cells (see later)[36].

PI3K negatively regulates TLR-mediated IL-12 production by mouse DCs[66], a feedback mechanism that might prevent excessive T_H1-cell polarization. Recently, it has been shown that two other kinases, mTOR and GSK3, differentially regulate IL-12 production by mouse DCs[67]. Blocking mTOR increases IL-12 production by LPS-stimulated DCs, an effect that is mediated by inhibition of negative feedback through autocrine IL-10, and activation of mTOR (by transduction of a constitutively active form of Rheb) inhibits IL-12 production. By contrast,

GSK inhibition attenuates IL-12 production, but increases IL-10 production by LPS-stimulated DCs. In related studies, the activation of mTOR in mononuclear phagocytes increased the activity of signal transducer and activator of transcription 3 (STAT3) and the production of IL-10, but decreased the production of pro-inflammatory molecules (such as IL-12) and nuclear factor-κB (NF-κB) activation; mTOR inhibition had reciprocal effects[67]. Similarly, rapamycin increased IL-12 production and decreased IL-10 production by human DCs stimulated with LPS or *Staphylococcus aureus*[68]. Such findings imply that mTOR and GSK3 pathways might regulate the T_H1–T_H2-cell balance through modulation of IL-10 and IL-12 production by DCs **(**FIG. 2C**)**. Rapamycin-exposed monocytes induce the polarization of T_H1 cells and T_H17 cells[68], and rapamycin greatly increases the expression of IL-12/IL-23 p40 (p40) and IL-23 p19 (p19) mRNA and IL-23 protein in human macrophages induced by *Mycobacterium tuberculosis*[69]. Collectively, these observations identify the GSK3–mTOR pathway as a key regulator of innate immune homeostasis.

The mTOR pathway has recently been implicated in regulation of the production, by plasmacytoid (p)DCs[70], of type-1 IFNs (IFN-α/β) that are crucial for anti-viral immunity. mTOR associates with the scaffold protein MyD88 to allow activation of IFN regulatory factor 5 (IRF5) and IRF7, which are master transcription factors for type-1 IFN genes[38, 71]. Thus, inhibition of mTOR signaling during pDC activation through TLR9 blocks the interaction between TLR9 and MyD88 and the subsequent phosphorylation and nuclear translocation of IRF7, resulting in impaired IFNα/β production[72] **(**FIG. 2D**)**. Decreased IFNα levels in serum and decreased production of IFNα by pDCs in response to stimulation with CpG or a viral vaccine, leads to impaired adaptive $CD8^+$ T-cell-mediated immune responses[72].

In summary, rapamycin exerts multiple effects on DC differentiation, maturation and function. It interferes with antigen uptake and might modulate antigen presentation; its differential effects on cytokine production and chemokine receptor expression regulate interactions between innate immunity and adaptive T-cell responses.

mTOR in effector and regulatory T cells

T cells have a fundamental role in host responses to invading pathogens and tumours. T_{Reg} cells exert control over the reactivity of effector T cells and their important role is emphasized by the severe pathological conditions associated with T_{Reg}-cell deficiency[73]. As our understanding of the fine balance between T cells and T_{Reg} cells becomes clearer, the influence of mTOR inhibition on their activity is providing surprising insights.

Thymocyte development

In rodents, blockade of mTOR activity causes profound thymic involution, associated with decreased T-cell output[74]. Recent reports have shown that prolonged rapamycin administration blocks the conversion of thymocytes from double-negative ($CD4^-CD8^-$) to double-positive T cells[75]. Although the absolute number of T cells is decreased, rapamycin administration does not alter the proportion of $CD4^+$ single positive cells that up-regulate expression of FOXP3 in the thymus[76]. This indicates that the ontogeny of natural T_{Reg} cells is unaffected by mTOR blockade. This is surprising in view of recent evidence that FOXP3 expression is negatively regulated by the PI3K–AKT–mTOR axis; thymocytes expressing a constitutively active form of AKT were unable to up-regulate FOXP3 expression[77]. Thus, it might be expected that, by blocking mTOR (which lies downstream of AKT), a higher proportion of $CD4^+$ cells would up-regulate FOXP3. Absence of such an effect in the thymus suggests that thymic T_{Reg} cell generation is based on the initiation of a genetic program[78] controlled by AKT signaling but independent of mTOR activity.

T-cell activation and anergy

T-cell activation in the periphery is a complex process requiring progress through two major checkpoints **(**FIG. 3A**)** that limit entrance into the cell cycle[79]. Progression from G0 to G1 of the cell cycle is the first checkpoint. Detailed analysis of the molecular processes that underlie the two-signal T-cell activation model **[G]** has shown that, in addition to TCR-driven activation of NFAT and MAPK, integration of both TCR and CD28 signals is required for PI3K and AKT activation. As discussed earlier, mTOR is a target of AKT and, through its activity in the TORC1 and TORC2 complexes, can stimulate four pivotal processes that regulate progression from G0 to G1: increased mRNA translation, increased glycolysis and consequent ATP accumulation, degradation of the cyclin-dependent kinase (Cdk) inhibitor $p27^{Kip1}$ and promotion, in combination with the transcriptional regulators NFAT and AP1, of IL-2 and IL-2 receptor α (IL-2Rα) expression. The increase in protein production as a result of mTOR activity allows the T cells to reach the critical mass required for successful division, a process that requires the energy made available through increased ATP. Initial progression from G0 to G1 is then regulated by the activity of CdK proteins, which drives the cell to the second checkpoint: the transition from the G1 to S phase of the cell cycle. Successful progression through this transition requires sustained stimulation through TCR and CD28, together with autocrine signalling by the IL-2 produced during progression through the first checkpoint. The PI3K–AKT–mTOR pathway is also involved in transmission of this IL-2R signal[79]. It now seems that mTOR activity at this phase of the cell cycle is in the form of a new complex with the proteins Survivin (an inhibitor of apoptosis family protein) and Aurora B (a serine/threonine kinase)[80]. The expression and activity of both Survivin and Aurora B depends on CD28 stimulation and IL-2 signalling. The mTOR–Survivin–Aurora B complex has target specificity similar to TORC1, with an additional Aurora-regulated capacity to control Rb phosphorylation, cyclin A expression, and Cdk1 and Cdk2 activity, all of which are involved in G1- to S-phase progression[79]. Interestingly, similar to TORC1, this new mTOR complex is susceptible to inhibition by rapamycin.

An additional immunosuppressive effect of mTOR blockade during T-cell activation has been recently identified. IL-2–IL2R signalling in differentiating effector T cells results in down-regulation of the transcription factor Kruppel-like factor 2 (KLF2), which controls expression of the homing molecules, SP1, CD62L, CCR7 and other chemokine receptors. This decrease in KLF2 levels depends on PI3K activation and involves mTOR activation[81]. The presence of rapamycin during IL-2 signalling prevents the down-regulation of KLF2 and consequently, the modulation of expression of the aforementioned homing receptors necessary for the egress of effector T cells from lymph nodes. So, in addition to blocking cell-cycle progression during T-cell activation, rapamycin-mediated mTOR inhibition could sequester activated T cells in lymphoid tissues and prevent them from reaching the target tissue.

mTOR activation is a key process in preventing T-cell anergy **[G]**. Treatment of antigen-stimulated T cells with rapamycin slows cell-cycle progression to the G1 phase and prevents the down-regulation of genes involved in the development of anergy[82]. In addition, it is now clear that IL-2–IL-2R signalling has a direct role in the avoidance of anergy[83]. An excess of IL-2 can reverse anergy through an mTOR-dependent, rapamycin-sensitive signalling pathway[84, 85]. These observations support the view that pro-anergy factors are induced early after TCR stimulation, and are subsequently degraded or suppressed in response to IL-2R signalling. This indicates that conventional mTORC1 activity is necessary for T-cell activation, but is insufficient to reverse the program of anergy induction. A separate, IL-2-specific signalling event is required during the G1 phase to preserve antigen responsiveness, and might involve the aforementioned Survivin–Aurora B–mTOR complex. The activity of this complex may underlie the recent observations regarding the involvement of the PI3K–AKT–mTOR axis in the induction of FOXP3 expression in the periphery (see below).

Rapamycin is most effective at preventing T-cell division under conditions of low IL-2 availability[79]. In the presence of optimal autocrine IL-2 secretion and/or exogenous IL-2, rapamycin delays but cannot prevent later cell division[86]. This alternative regulation of late cell-cycle progression by IL-2 seems to depend on the recently discovered STAT5-dependent expression and activity of the serine/threonine kinase phosphatidylinositol mannoside 2 (PIM2)[87]. PIM2 can maintain nutrient uptake and ATP synthesis at a high level and, similarly to AKT, regulates cell survival during blastogenesis. mTOR-induced signalling might regulate the rate of initial cell-cycle entry and the integration of sequential signals dictated by TCR–CD28 engagement and IL-2 production, but it becomes dispensable later, when significant quantities of IL-2 are available[86].

T_{Reg}-cell homeostasis, function and proliferation

Rapamycin does not affect the function and homeostasis of T_{Reg} cells to the same extent as 'conventional' T cells, as recently reviewed[88]. *In vitro* exposure of mouse or human $CD4^+CD25^+$ T_{Reg} cells to rapamycin does not impair their ability to suppress effector T-cell proliferation[89], an effect that is lost using the calcineurin inhibitor CsA[90, 91]. Additionally, prolonged *in vivo* rapamycin administration results in a pronounced, relative increase in the number of T_{Reg} cells compared with $CD4^+$ T cells in all lymphoid organs, although the absolute numbers of all T cells are decreased, as in the thymus[76, 92]. A similar finding has been made in kidney transplant recipients[92, 93]; patients treated with rapamycin have a markedly increased frequency of $CD4^+CD25^+FOXP3^+$ T cells over bulk $CD4^+$ T cells. This effect is reversed in patients given calcineurin inhibitors. In a TCR-transgenic mouse model, rapamycin was used *in vitro*, in combination with IL-2, to selectively expand cells with regulatory activity from a starting population of bulk $CD4^+$ T cells[94]. This observation was then confirmed for human T_{Reg} cells[89, 95, 96], which could be expanded selectively *in vitro* from both healthy donors and patients with autoimmune disease.

The suppressive activity and proliferative capacity of T_{Reg} cells depend on TCR engagement and IL-2 availability. Differential susceptibility to rapamycin inhibition suggested that the TCR signalling pathway in T_{Reg} cells differed from that in conventional T cells. The observation that T-cell stimulation in the presence of rapamycin favored upregulation of FOXP3 expression and acquisition of a regulatory phenotype[97], offered the alternative explanation that the observed increase in T_{Reg}-cell frequency after prolonged rapamycin administration could derive from peripheral conversion of T cells into T_{Reg} cells (see next section). However, our current understanding is that both processes can occur. Mouse T_{Reg} cells are defective in their ability to activate phospholipase C-γ and generate the downstream signals that result in NFAT, NF-κB and Ras–ERK–AP1 activation[98]. In parallel, T_{Reg} cells are defective in phosphorylation of AKT in response to stimulation, and restoration of AKT activity impairs their suppressor function[99]. This has been further clarified by a report that T_{Reg} cells express high levels of the negative PI3K regulator, phosphatase and tensin homolog (PTEN)[100, 101]. In contrast to conventional T cells, PTEN expression is maintained in T_{Reg} cells after TCR stimulation. Targeted depletion of PTEN does not affect the suppressive capacity of T_{Reg} cells, but increases their sensitivity to rapamycin. These data indicate that T_{Reg} cells do not rely on the conventional PI3K–AKT–mTOR activation pathway, and explain their lower susceptibility to rapamycin inhibition.

The above observations do not clarify the mechanism of T_{Reg}-cell-specific activation in the presence of rapamycin. The IL-2R–STAT5 pathway is essential for T_{Reg}-cell homeostasis and activity[102, 103]. It has been reported recently that FOXP3 expression is associated with the induction of PIM2 expression[104]. Notably, PIM kinases have been implicated in conferring T-cell resistance to rapamycin-mediated inhibition, when STAT5-signalling cytokines (including IL-2) are present in adequate amounts[87]. It is therefore possible that T_{Reg} cells can integrate

TCR and IL-2 signalling in a PIM-dependent pathway that allows them to progress through the cell cycle despite mTOR inhibition **(**FIG. 3B**)**.

Induction of FOXP3 expression

Natural T_{Reg} cells arise in the thymus, but $CD4^+FOXP3^+$ T cells with regulatory capacity can also be generated in the periphery (induced T_{Reg} cells) by antigen-driven conversion of naïve T cells under certain conditions[105]. In particular, *in vitro* TCR stimulation of $CD4^+CD25^-$ T cells in the presence of transforming growth factor-β (TGFβ) and IL-2 induces FOXP3 expression. The observation that rapamycin favors this conversion has focussed much attention on the involvement of mTOR in regulation of FOXP3 expression **(**FIG. 4A**)**. The induction of FOXP3 expression by TGFβ depends on activation of the transcription factor mothers against decapentaplegic homolog 3 (SMAD3)[106] which, in combination with TCR-induced NFAT, binds to the enhancer region of the *FOXP3* gene and promotes chromatin remodeling necessary for translation. Interestingly, signaling through the AKT–mTOR pathway can inhibit activation of SMAD3[107], providing an explanation for the observation that mTOR inhibition through rapamycin favours FOXP3 up-regulation.

T cells with constitutively active AKT have impaired up-regulation of FOXP3 in response to activation in the presence of TGFβ[77]. Interestingly, when rapamycin is present at the time of transduction of constitutively active AKT, the inhibitory effect is lost. This implies that a rapamycin-sensitive signal involving AKT activation (and consequently, mTOR) is responsible for the control of peripheral FOXP3 induction. This effect is not restricted to FOXP3, and extends to approximately 50% of the genes modulated in response to TGFβ[77], which confirms that conversion to a regulatory phenotype involves complex genetic re-programming[78]. However, important differences remain between natural T_{Reg} cells and induced $FOXP3^+$ T cells. Expression of a constitutively active form of AKT in thymocytes decreases thymic development of T_{Reg} cells, without affecting T-cell ontogeny[77], confirming the ability of the AKT–mTOR pathway to prevent T_{Reg}-cell development. Expression of FOXP3 in already differentiated circulating T_{Reg} cells, however, is not affected by AKT transduction, supporting the proposed model that a specific genetic program is implemented in T_{Reg} cells during their development.

A process of FOXP3 up-regulation, independent of TGFβ signalling, has begun to be characterized **(**FIG. 4B**)**. Premature interruption of *in vitro* TCR–CD28 stimulation (after 18h) is associated with the upregulation of FOXP3 expression in approximately 10% of T cells[108]. Addition of PI3K inhibitors or rapamycin after the interruption of TCR stimulation increased this fraction to up to 75% of T cells. The effect did not involve TGFβ signalling, as neutralizing antibodies specific for TGFβ and SMAD kinase inhibition did not affect FOXP3 induction promoted by inhibition of PI3K and mTOR. Interestingly, this effect was closely associated with the timing of interruption of TCR–CD28 stimulation and the addition of PI3K and mTOR inhibitors. Early addition of the inhibitors blocked the necessary T-cell activation, whereas prolongation of TCR–CD28 stimulation beyond 18 hours markedly decreased FOXP3 up-regulation. In addition, following initial TCR–CD28 stimulation, histone modifications (that grant genes accessibility to transcription factors) at the *FOXP3* locus were observed and subsequently lost after continuous TCR–CD28 signalling. These findings suggest that rapamycin administration establishes conditions for the initiation of a two-step process of FOXP3 up-regulation, similar to that described for thymic T_{REG} development[109, 110]. TCR–CD28 signalling causes chromatin remodeling that predisposes towards expression of the *FOXP3* gene. This first step probably involves PI3K–AKT–mTOR signalling, as the addition of inhibitors at the same time as TCR–CD28 stimulation prevents the necessary T-cell activation. After prolonged TCR–CD28 stimulation, the accessibility of the *FOXP3* gene is then restricted in a process that involves a second round (or continuation) of PI3K–AKT–

mTOR activity. It is reasonable to imply that, by blocking this second round of signalling, the pre-activated cell can then respond differently to endogenous cytokine (such as IL-2 and IL-15) stimulation. This promotes activation of the STAT5 pathway, which is involved in both intrathymic T_{Reg}-cell development and the modulation of FOXP3 expression in peripheral T cells[102, 103, 111]. This model of FOXP3 induction might explain the recent observation that a combination of histone/protein deacetylase (HDAC) inhibition and rapamycin augments $FOXP3^+$ T_{Reg} cells *in vivo*[112].

On the whole, these observations delineate differences between T cells and T_{Reg} cells that shed light on previously unappreciated effects of mTOR inhibition. The observation that rapamycin causes a generalized increase in the frequency of $FOXP3^+$ cells is now interpreted as the sum of two effects — the ability of T_{Reg} cells to proliferate in the presence of rapamycin, and the promotion of FOXP3 expression in peripheral T cells that are then converted into modulators of immune reactivity.

Implications of mTOR inhibition for therapeutic immunosuppression

The remarkable inhibitory action of rapamycin on DCs and effector T cells, but not T_{reg} cells, singles out mTOR inhibition as a promising therapeutic strategy in transplantation and autoimmune disease.

Transplant tolerance

In renal transplantation, rapamycin is a powerful anti-rejection agent when used judiciously with other immunosuppressants. It has unique anti-atherogenic and anti-neoplastic properties that distinguish it from other anti-rejection drugs, and can promote tolerance and decrease the incidence of chronic allograft nephropathy[113]. Rapamycin was used initially with CsA, but it is also effective when combined with other immunosuppressants. However, the effects of calcineurin inhibitors (such as CsA) and rapamycin in transplantation seem to be markedly different. In rodents, rapamycin, but not CsA, permits activation-induced death of the large number of alloreactive effector T cells and favours tolerance induction [114, 115]; in addition, rapamycin enhances, whereas CsA prevents, the tolerance-promoting ability of costimulation blockade[116, 117]. In transplant recipients, calcineurin inhibitors, but not rapamycin, decrease the proportion of $CD4^+CD25^+FOXP3^+$ T_{Reg} cells, a presumed negative effect of the former agents[93]. Recently, use of FOXP3 reporter mice has shown that rapamycin promotes the de novo (TGFβ-dependent) conversion of alloantigen-specific $CD4^+$ T_{Reg} cells under tolerizing conditions, whereas CsA abrogates this process[118]. Converted T_{Reg} cells were more resistant to apoptosis than conventional T cells, and adoptive transfer of the former potently suppressed the rejection of donor but not third-party skin grafts. The ability of rapamycin plus IL-10 to induce T regulatory type-1 (Tr1) cells **[G]** that mediate stable, alloantigen-specific tolerance in pancreatic islet cell transplantation has also been reported[119]. Collectively, these data provide further evidence that the differential effects of rapamycin on effector T cells and T_{Reg} cells (both naturally-occurring and induced), favour its ability to promote tolerance and support its use in tolerance-promoting protocols.

Recent reports suggest that mTOR inhibition, together with targeting of other key molecules involved in immune regulation, can promote transplant tolerance in mice. The chemokine receptor CXCR3 and its ligands (CXCL9, CXCL10 and CXCL11) constitute an important pathway for effector T-cell recruitment. When combined with a sub-therapeutic regimen of rapamycin, CXCR3-specific monoclonal antibody induces indefinite (>100 day) survival of heart or islet allografts[120]. A second finding concerns HDAC9 inhibition and consequent increases in FOXP3 expression, as well as the production and function of T_{Reg} cells[112]. When HDAC inhibition is combined with a short course of low-dose rapamycin, permanent, donor-specific, T_{Reg}-cell-dependent heart and pancreatic islet allograft survival is achieved[112].

T_{Reg} cells also seem to be important in mediating the tolerogenic effect of minimally effective rapamycin in combination with monoclonal antibodies specific for T-cell immunoglobulin domain and mucin 1 (TIM1), which is associated with a T_H1- to T_H2-type cytokine switch in experimental heart transplantation[121].

Tolerogenic cell therapy in transplantation

The capacity of rapamycin to deplete effector T cells, but to spare the growth and function of T_{Reg} cells, can be exploited in the design of novel and safe protocols for cell therapy of allograft rejection and other T-cell-mediated disorders. Rapamycin-conditioned APCs are poor stimulators of allogeneic $CD4^+$ effector T-cell proliferation, but enrich for potent $FOXP3^+$ T_{Reg} cells[36]. Such rapamycin-conditioned murine DCs pulsed with donor alloantigen then adoptively transferred to organ allograft recipients, in combination with a short post-operative course of low-dose rapamycin, induce indefinite (>100 day) graft survival[36] Also, rapamycin-conditioned DCs of recipient genotype inhibit GVHD after allogeneic haematopoietic cell transplantation, consistent with their tolerogenic phenotype[60]. CCR5, CCR7 and CD62L expression on rapamycin-conditioned DCs is not affected by mTOR inhibition, which allows them to traffick to secondary lymphoid tissues, where their immunoregulatory function is required[59-61]. Such observations might, in part, explain the recently reported beneficial effects of rapamycin on the incidence of GVHD after haematopoietic cell transplantation in the clinic[122, 123]. As an example of an alternative mechanism, alloantigen-specific $CD4^+CD25^+$ T_{Reg} cells expanded *in vitro* in response to immature allogeneic DCs, then infused into rapamycin-conditioned heart allograft recipients without use of a T-cell-depletion strategy, induce indefinite graft survival, in association with T-cell anergy[124].

Autoimmune disease

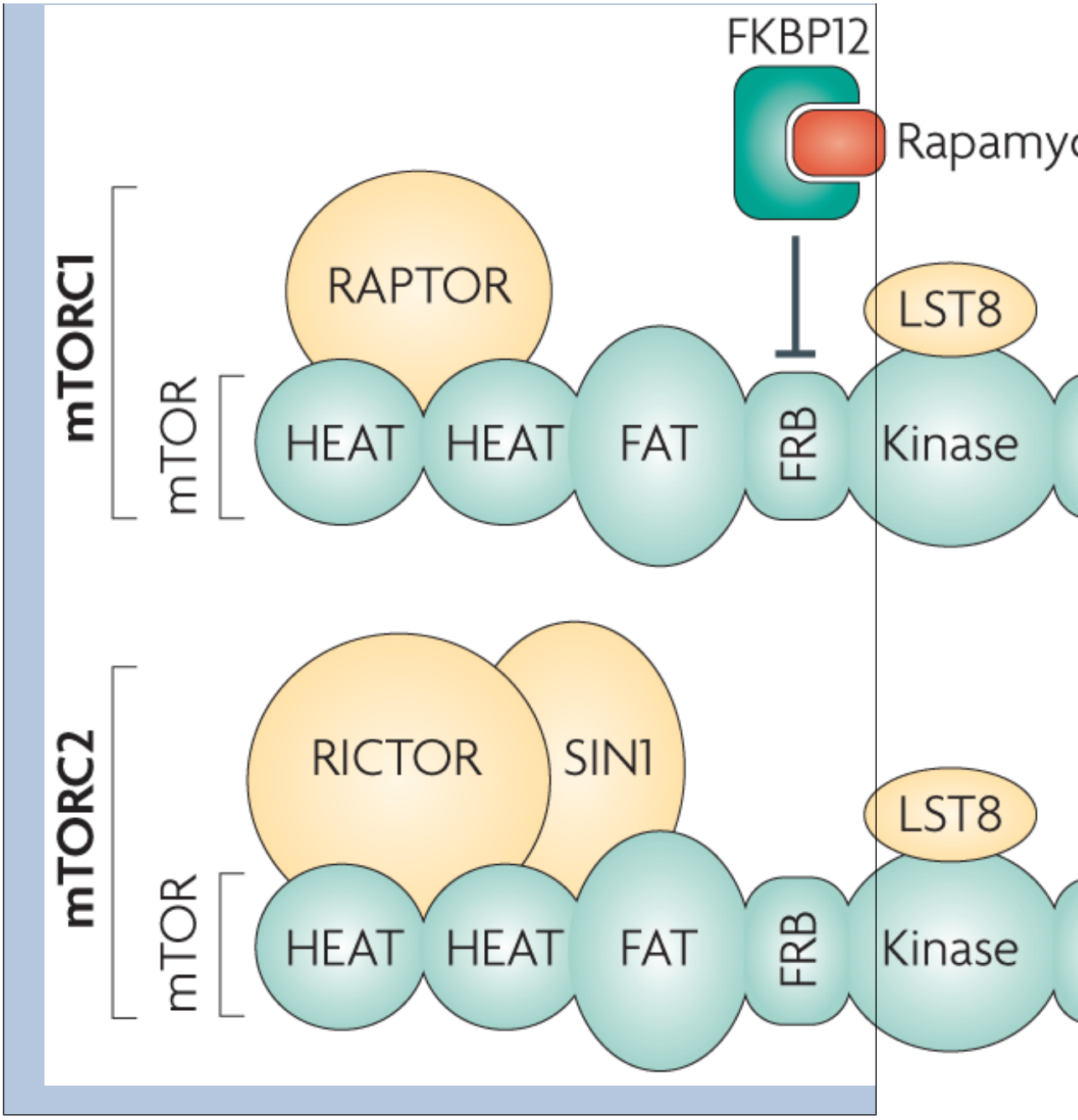
There are numerous reports that mTOR inhibition can suppress experimental autoimmune diseases, in particular type-1 diabetes, lupus nephritis and adjuvant arthritis[125]. Rapamycin combined with IL-10 blocks the incidence of type-1 diabetes and induces long-term tolerance without chronic immunosuppression in diabetes-prone, non-obese diabetic (NOD) mice[126]. In this model, rapamycin mediates the accumulation of suppressive $CD4^+CD25^+FOXP3^+$ T_{Reg} cells in the pancreas, preventing diabetes. By contrast, when rapamycin is combined with CD3-specific antibody, it exerts a detrimental effect on disease outcome in NOD mice[127]. As discussed by the authors, rapamycin may interfere with restoration of IL-2 production (defective in NOD mice) by anti-CD3, and thus prevent a crucial role for this cytokine in maintenance of tolerance in these diabetes-prone animals. In patients with type-1 diabetes, rapamycin promotes the *ex vivo* proliferation of functional T_{Reg} cells ($CD4^+CD25^+FOXP3^+$) [89]. Moreover, naturally occurring T_{Reg} cells from patients with type-1 diabetes on rapamycin monotherapy have restored ability to suppress proliferation of $CD4^+CD25^-$ effector T cells compared with T_{Reg} cells before treatment[128]. In NZB × NZW F1 female mice (a model of systemic lupus erythematosus), administration of rapamycin (from 12–37 weeks of age) decreases the production of autoantibodies, glomerular deposits of immunoglobulins and the development of proteinuria, and prolongs survival[129]. Also in this model, rapamycin attenuates the severity of established nephritis through reduced lymphoproliferation, decreased CCL5 expression and decreased lymphoinfiltration of the kidneys[130]. Rapamycin is also effective for the treatment of de novo autoimmune hepatitis after human liver transplantation[131], and has recently been used to treat a case of refractory Crohn's disease[132]. Interestingly, rapamycin might be a clinically effective and safe therapeutic option in IPEX (immune dysregulation, polyendocrinopathy, enteropathy and x-linked syndrome) **[G]** and IPEX-like patients[133], in whom naturally occurring $FOXP3^+$ T_{Reg} cells are absent, resulting in severe autoimmune disease.

Conclusions

In summary, whereas the importance of mTORC1 in regulation of innate and adaptive immunity is now well-recognized, the role of mTORC2 has yet to be clarified. New evidence that mTOR regulates cytokine production by APCs in response to inflammatory stimuli suggests a pivotal role for this molecule in determining the nature of T-cell responses. The mechanisms by which rapamycin suppresses immunity have been expanded from inhibition of T-cell proliferation, to blockade of DC maturation and support of T_{Reg} cells, including their *de novo* induction. Ongoing and future areas of enquiry, which are likely to further elucidate the role of mTOR in the regulation of immunity and tolerance, include investigation of the role of the newly-identified mTOR–Survivin–Aurora B complex in T-cell activation (and in other immune cells, including DCs), clarification of the role of PIM1 and PIM2 in determining T_{Reg}-cell resistance to mTOR inhibition, and in-depth understanding of TGFβ-dependent and -independent mechanisms of FOXP3 up-regulation and their physiological role (which will be relevant to therapeutic application, as they are both affected by mTOR modulation). Insight is also needed into the role of mTOR in memory T cells.

Box 1 | Two distinct mTOR-containing complexes and their interaction with rapamycin

Mammalian target of rapamycin (mTOR) is a large (~289 kDa), atypical kinase, which, like other members of the phosphatidylinositol kinase-related kinase (PIKK) family, contains a carboxy-terminal serine/threonine protein kinase domain[14, 18, 19]. Also consistent with other PIKKs, mTOR contains a FRAP-ATM-TTRAP (FAT) domain, and a carboxy-terminal FAT domain (FATC) that might have a role in mTOR structure and stability[134]. Mammalian LST8 (mLST8, also known as GβL) associates with the kinase domain of mTOR and is thought to facilitate mTOR signalling, but its precise functional role is yet to be defined[135, 136]. In addition, the large mTOR protein with its multiple domains seems to be involved in various protein–protein interactions that determine the physiological functions of mTOR. It has been established that mTOR exists in at least two distinct complexes, defined as mammalian target of rapamycin complex 1 (mTORC1) and mTORC2. The mTOR kinase and mLST8, together with the regulatory associated protein of mTOR (Raptor) form mTORC1. Raptor is essential for mTORC1 activity and is proposed to interact with mTOR through shared HEAT (Huntington, Elongation Factor 3, PR65/A, TOR) domains[19]. Rapamycin binds to the immunophilin FK506-binding protein 1A, 12 kDA (FKBP12) to form a drug–receptor complex that specifically and effectively blocks mTORC1 activity. The FKBP12–rapamycin complex binds just amino-terminal to the kinase region of mTOR (in the FKBP12–rapamycin-binding domain, FRB)[136] and disrupts the *in vitro* and *in vivo* activity of mTORC1, potentially by disrupting the interaction between Raptor and mTOR[14]. mTORC2 also contains mLST8, but instead of Raptor, associates with rapamycin-independent companion of mTOR (Rictor) and possibly stress-activated MAP kinase-interacting protein 1 (mSIN1; also known as MAPK-associated protein 1)[19, 137]. Unlike mTORC1, mTORC2 is resistant to direct inhibition by rapamycin. It is unknown what prevents the interaction between the FKBP12–rapamycin complex and the FRB on mTORC2[14].



Box 2 | Effect of mTOR inhibition on B cells, NK cells, neutrophils and mast cells

In addition to its influence on antigen-presenting cells and T cells, rapamycin exerts direct effects on other immune cells that impair their proliferation and function. mTOR inhibition thus exerts a broad spectrum of inhibitory effects on immune effector cells.

B cells

Phosphatidylinositol 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) are required for B-cell proliferation. Early and late B-cell receptor (BCR) signals are blocked by mTOR inhibition[138]. Thus, rapamycin inhibits BCR-induced S6K1 activation and DNA

synthesis in mouse B cells[139]. B-cell responses to lipopolysaccharide are also sensitive to rapamycin[140]. mTOR inhibition also suppresses mouse splenic B-cell activation through CD40[141], resulting in decreased proliferation and differentiation to $IgM^{hi}IgD^{low}$ cells. Rapamycin inhibits CD40-specific antibody-mediated prevention of apoptosis induced by BCR cross-linking and suppresses human B-cell proliferation after CD40 ligation in the presence of B-cell-activating cytokines, accompanied by increased apoptosis[142]. Moreover, rapamycin potently inhibits the number of IgM,- and to a lesser extent, IgG-producing B cells. When *Staphylococcus aureus* and CD40L are used as stimulants, rapamycin decreases IL-2-dependent and -independent human B cell proliferation, as well as IL-2-dependent differentiation to antibody-secreting cells[143]. Rapamycin also suppresses cytokine-induced proliferative responses and IgM production by pre-activated human B cells[144].

NK cells

Rapamycin inhibits the proliferation of rat primary natural killer (NK) cells and of the RNK-16 NK-cell line, by blocking progression from the G1 to S phase of the cell cycle[145], but it does not affect interferon-γ secretion by primary NK-cell lines. NK-cell-mediated killing of conventional YAC-1 target cells is modestly decreased by mTOR inhibition. *In vivo*, rapamycin administration decreases the number of circulating NK cells in rat liver allograft recipients[145], but does not seem to influence NK-cell cytotoxic activity in rats receiving hamster skin xenografts[146].

Neutrophils

Rapamycin potently suppresses human neutrophil chemotaxis and chemokinesis elicited by granulocyte/macrophage colony-stimulating factor (GM-CSF), and inhibits responses to IL-8[147, 148]. Rapamycin inhibits the increase in S6K1 activity induced by GM-CSF, and also inhibits GM-CSF–induced actin polymerization, a marker of leukocyte migration. mTOR complex 1 participates in Toll-like receptor 2- and 4-induced neutrophil activation and acute lung injury[149].

Mast cells

Rapamycin has little effect on histamine release from lung mast cells, but antagonizes the inhibitory effect of FK506 on anti-IgE-induced histamine release from human mast cells[150].

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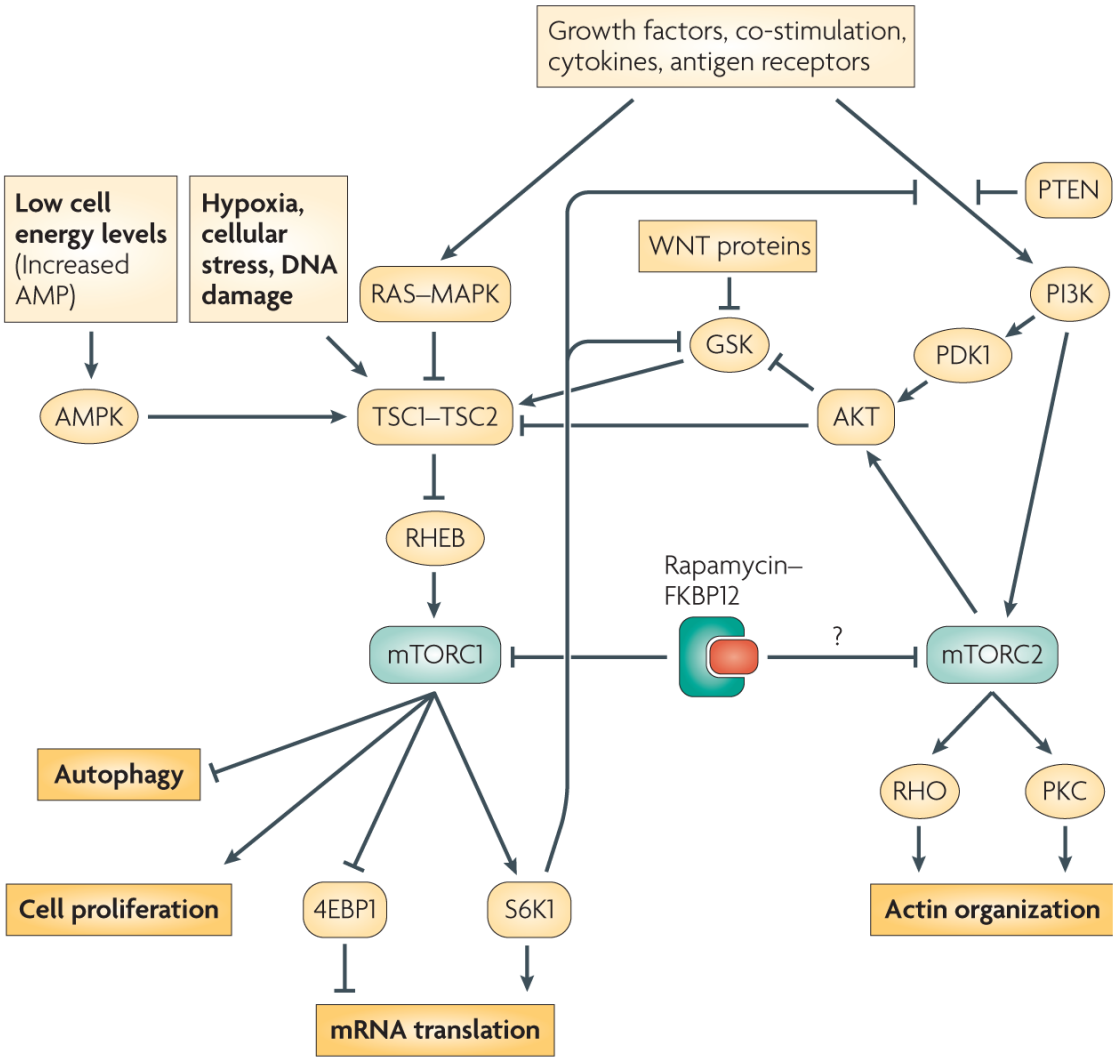

Figure 1. mTORC1 (mammalian target of rapamycin complex 1) and mTORC2 signalling pathways

mTORC1 is the direct target of the rapamycin–FK506-binding protein 1A, 12 kDa (FKBP12) complex and regulates cell growth and size by controlling translation, ribosome biogenesis and autophagy. Diverse signals, arising from growth factors, such as insulin and Fms-like tyrosine kinase 3 ligand (FLT3L), various cytokines, ligated costimulatory molecules and antigen receptors, WNT (Wingless and Integrase-1) proteins, and the relative cellular energy and oxygen levels, determine mTORC1 activity as a result of their effects on the tuberous sclerosis complex 1 (TSC1)–TSC2 complex, which is the main negative regulator of mTORC1. Activation of Ras–MAPK (mitogen-activated protein kinase) and phosphatidylinositol-3-kinase (PI3K)–AKT results in inhibitory phosphorylation of TSC2 and removes repression of Rheb (Ras homologue enriched in brain), which is the mTORC1 stimulator. Activation of AKT by PI3K is negatively regulated by phosphatase and tensin homologue (PTEN). Activated mTORC1 promotes translation through stimulating S6K1 (p70 ribosomal protein S6 kinase 1) and inhibiting 4EBP1 (eIF4E binding protein 1). Activated S6K1 can also feed back to negatively regulate input from PI3K-AKT by facilitating the degradation of signaling intermediates between surface receptors (such as the insulin receptor) and PI3K. Low energy and nutrient levels, as well as hypoxic conditions, increase TSC1–TSC2-mediated inhibition of mTORC1 through input from GSK3 (glycogen synthase kinase 3) and AMPK (AMP-

activated protein kinase). mTORC2 is not inhibited directly by rapamycin, but long-term rapamycin administration disrupts its assembly in some cells. mTORC2, activated by PI3K, directly phosphorylates AKT. mTORC2 also regulates cytoskeleton dynamics. PDK1,= phosphoinositide-dependent kinase 1.

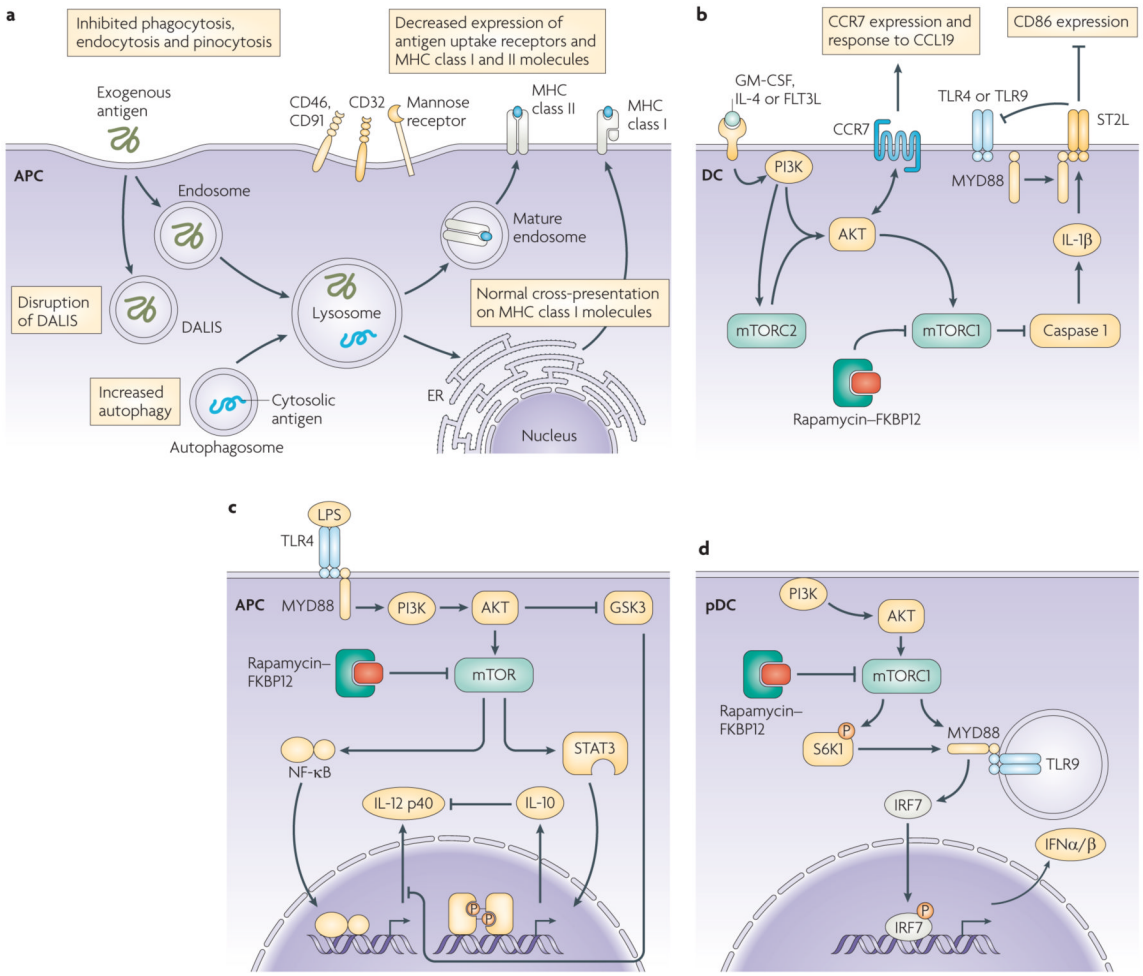

Figure 2. mTOR and rapamycin regulate APC function

a | Several changes are observed in antigen acquisition and presentation by antigen–presenting cells (APCs) exposed to or differentiated in rapamycin. Rapamycin inhibits endocytosis and phagocytosis, and the expression of antigen uptake receptors and MHC class II molecules, and disrupts dendritic cell aggresome-like structures (DALIS), although cross-presentation of exogenous antigen on MHC class I molecules might not be affected. Rapamycin facilitates autophagy, an evolutionarily conserved process and could thereby influence self-antigen presentation. Thus, mTOR has a role in regulation of immune responses at an early stage, influencing how exogenous and endogenous antigen is acquired, processed and presented. **b** | Interruption of mTOR signalling in monocytes, macrophages and dendritic cells (DCs) during Toll-like receptor (TLR) ligation results in increased IL-12 production by decreasing IL-10 production and derepressing NF-κB. This indicates that mTOR is a crucial mediator of T-cell polarization and immune responses. **c** | In plasmacytoid DCs (pDCs), coordinated signalling through TLR9 and phosphatidylinositol-3-kinase (PI3)K–mTOR is needed to drive type-1 IFN-α/β production. S6K phosphorylation by mTORC1 promotes the interaction of MyD88–TLR9–IFN regulatory factor (IRF)7 and the subsequent translocation of phosphorylated IRF7 to the nucleus to initiate transcription of the genes encoding type-1 IFN. mTOR has been reported to interact with MyD88 and positively regulate cytokine production mediated by IRF5 and IRF7. **d** | Differentiation of DCs in rapamycin generates DCs with weak T-cell stimulatory capacity, but intact/improved homing to CCL21. mTOR is a negative regulator of caspase-1 and treatment with rapamycin therefore promotes IL-1β production by caspase 1. IL-1β

subsequently induces expression of ST2, which sequesters MyD88 and negatively regulates TLR4 and TLR9 signalling. Failure of rapamycin to impede AKT signalling might allow/promote CCR7 activity and DC migration to CCL21.

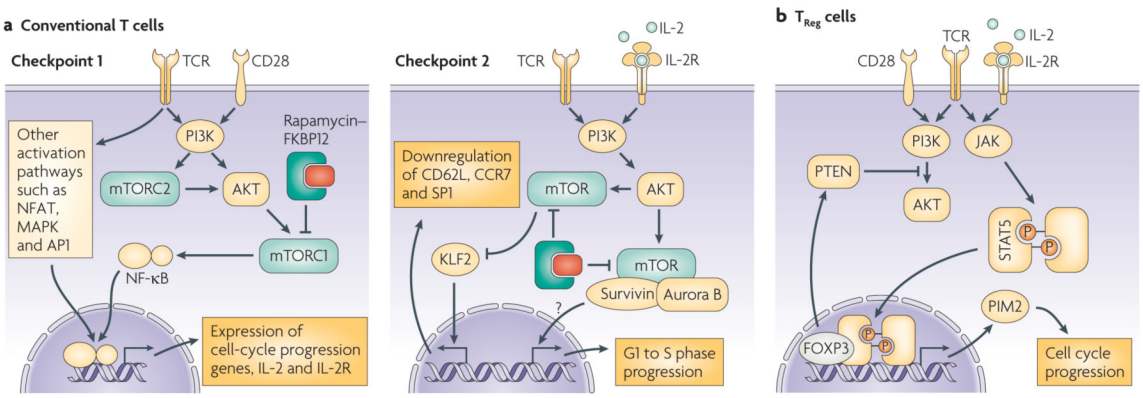

Figure 3. Proposed molecular mechanisms responsible for T-cell susceptibility or resistance to rapamycin

a | In conventional T cells, mTOR integrates the TCR and CD28 signals that are necessary to pass two checkpoints of T-cell activation. The transition from G0 to G1 phase of the cell cycle (checkpoint 1) requires activation of NFAT, MAPKs and NF-κB. The activity of NF-κB is controlled by the PI3K–AKT–mTOR pathway. mTOR works as part of the complexes TORC1 and TORC2, of which the former is susceptible to inhibition by rapamycin. The coordinated activity of NFAT, AP1 and NF-κB regulates multiple genes involved in cell-cycle progression and the expression of IL-2 and its high-affinity receptor. IL-2R signals through the PI3K–AKT-mediated activation of mTOR complexed with survivin and aurora B, which regulates G1- to S-phase progression (checkpoint 2). In addition, mTOR activity is involved in rapamycin-sensitive down-regulation of the transcription factor Kruppel-like factor 2 (KLF2), which controls expression of lymphoid tissue-homing molecules to ensure that activated T cells can exit the lymph nodes. **b** | The ability of T_{Reg} cells to proliferate when stimulated in the presence of rapamycin seems to be connected to two effects. First, T_{Reg} cells do not down-regulate phosphatase and tensin homologue (PTEN) expression after TCR engagement, which impedes the activation of the rapamycin-susceptible PI3K–AKT–mTOR pathway. Second, FOXP3 drives expression of phosphatidylinositol mannoside (PIM)2, reinforced by IL-2- and TCR-mediated activation of signal transducer and activator of transcription (STAT)5, which compensates for AKT inactivity and promotes cell-cycle progression.

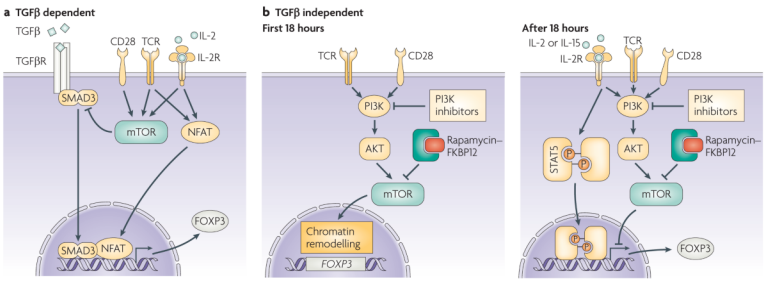

Figure 4. Mechanisms of FOXP3 induction in naïve T cells

a | Stimulation of FOXP3^{-} T cells through TCR and CD28 in the presence of TGFβ promotes expression of the *FOXP3* gene through the cooperation of NFAT and mothers against decapentaplegic homologue 3 (SMAD3). This process is counteracted by mTOR activation, which explains the increased expression of FOXP3 when stimulation takes place in the presence of rapamycin (which inhibits mTOR activation). **b** | Limited TCR–CD28 stimulation (<18 hours) promotes a PI3K–AKT–mTOR-mediated re-organization of chromatin that includes increased accessibility to the *FOXP3* gene. Prolonged TCR/CD28 stimulation prevents, again through activation of the PI3K–AKT–mTOR pathway, the expression of FOXP3 which would otherwise probably be induced by signal transducer and activator of transcription (STAT)5-activating cytokines generated during the initial stimulation. This two-step model rationalizes the opposing effects of rapamycin administration during T-cell activation that have been observed, which probably depend on the timing of mTOR inhibition.