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Molecular Pathways: Increased Susceptibility to Infection Is a Complication of mTOR Inhibitor Use in Cancer Therapy

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Abstract

As one of the earliest examples of "chemical biology," the **M**echanistic **T**arget of **R**apamycin (mTOR) protein and its chemical inhibitors have been extensively studied across a spectrum of physiological and pathological processes at the molecular, organismal, and patient population levels. There are several FDA-approved mTOR inhibitors (sirolimus, everolimus and temsirolimus) with indications for cancer treatment and for prevention of solid organ rejection. Dozens of mTOR inhibitors are currently being evaluated in hundreds of on-going clinical trials across a spectrum of diseases, including numerous cancer indications, autoimmune diseases, and a number of congenital disorders. As many of the approved and investigational indications for mTOR inhibitors require long-term treatment, the magnitude and incidence of particular side effects differs from those observed in shorter-term treatments. Here, we focus on the particular increased risk of infections while receiving mTOR inhibitors. While increased infection rates might be expected from a class of drugs approved as post-transplant immunosuppressants, we review reports from clinical, mechanistic, and genetically engineered mouse model (GEMM) studies detailing a much more nuanced view of mTOR inhibitor drug action and target biology.

Background

First isolated in 1975 from the bacterium *Streptomyces hygroscopicus*, rapamycin was characterized as an antifungal and immunosuppressive agent (1) and was later shown to have anti-proliferative properties in tumors (2). Genetic mutation studies using the budding yeast *Saccharomyces cerevisae* identified the requirement of the intracellular receptor FK506-binding protein-12 (FKBP12) for the growth arresting effect of rapamycin and demonstrated that the products of the Tor1 and Tor2 genes were the targets of rapamycin-FKBP12 inhibition (3). Subsequently, several labs identified the mammalian homolog of these genes,

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No potential conflicts of interest were disclosed.

now referred to as the Mechanistic Target of Rapamycin (mTOR, formerly known as FRAP, RAPT-1 and RAFT-1) (4-6).

A serine/threonine protein kinase, mTOR acts as a central regulator of cell growth, metabolism, proliferation and survival. mTOR responds to environmental cues including growth factors, insulin, oxygen, energy and stress by phosphorylating downstream targets, which activate translation, lipid synthesis and ribosome biogenesis while inhibiting autophagy (Fig. 1) (7,8). In this way, mTOR activation functions as a metabolic switch, moving the cell from a catabolic, quiescent state to an anabolic, active state in response to growth factors, cytokines and mitogens when growth conditions are favorable. mTOR inhibition effectively mimics a starved state, and similar to the extended life-spans observed in aging studies with caloric restriction, healthy mice treated with rapamycin long-term (9), and mice with genetically dampened mTOR expression in all tissues have extended life-spans compared to controls (10). In mammals, the cellular pool of mTOR proteins is divided into at least two structurally and functionally distinct complexes with recent evidence suggesting the possibility of yet another mTOR complex (11). mTOR complex 1 (mTORC1) involves RAPTOR (regulatory-associated protein of mTOR) binding and mTOR complex 2 (mTORC2) involves RICTOR (rapamycin-insensitive companion of mTOR) binding. Rapamycin and its analogs (rapalogs) inhibit mTOR kinase activity by disrupting the association between mTOR and RAPTOR. Prolonged exposure inhibits mTORC2 by preventing the formation of RICTOR-mTOR interactions but it cannot disrupt these associations in complexes that have already been formed (Fig. 1) (12).

Activated mTORC1 drives cell anabolism by promoting protein, lipid and nucleotide synthesis as well as mitochondrial and ribosomal biogenesis (7, 13-15). The metabolic activities promoted by mTOR permit the accumulation of biomass necessary for cell division or activation. The kinase activity of mTORC1 deactivates eIF4E (eukaryotic initiation factor 4E) binding protein 1 (4E-BP1), permitting the *protein translation*-inducing activity of eIF4E (15). Another well-characterized target of mTORC1 kinase activity is the ribosomal S6 kinase 1 (S6K1), the activity of which results in *ribosome biogenesis* as well as the promotion of *protein synthesis* (14). *Fatty acid synthesis* is regulated by mTORC1 kinase activity through the transcription factors sterol regulatory element binding protein 1 (SREBP1) and peroxisome proliferator-activated receptor-γ (PPARγ) (16, 17). The shift in anabolism driven by mTORC1 kinase activity under growth favorable conditions includes the inhibition of catabolism. Studies have demonstrated that mTORC1 phosphorylation of a protein complex composed of unc-51-like kinase 1 (ULK1), autophagy-regulated gene 13 (ATG13) and focal adhesion kinase family-interacting protein of 200 kDa (FIP200) inhibits autophagy (18-20). In addition to transitioning cell metabolism from catabolic to anabolic processes upon stimulation (21), mTOR also regulates cell cycle progression (22, 23). Inhibition of mTORC1 by rapamycin has been shown to cause G1 arrest by permitting the accumulation of p27, which is degraded by mTOR activity (22). Furthermore, rapamycin causes the suppression of retinoblastoma protein (RB) phosphorylation by preventing the phosphorylation of 4E-BP1 by mTORC1 (23).

Mutations upstream of mTOR that result in hyperactive receptor tyrosine kinases, PI3K and AKT or mutations causing the loss of PTEN function are frequently observed in human

cancers (24, 25); PTEN is one of the most frequently inactivated tumor suppressors in sporadic cancers and PIK3CA mutations occur in 27% of breast, 13% of colon and 28% of endometrial tumors (26). Additionally, mutations within mTOR itself, although relatively rare, have now also been documented (27). These often activating mutations prevent the inhibition of mTOR activity that would normally occur in the hypoxic tumor microenvironments due to activation of tuberous sclerosis complex (TSC), a key negative regulator of mTOR, by AMP-activated protein kinase (AMPK) and DNA damage response 1 (REDD1) (7). Drugs inhibiting one or more of the constituents of the PI3K/AKT/mTOR pathway have been tested as single agents or in combination in a variety of cancers (28, 29); preclinical and clinical results of several PI3K/AKT/mTOR pathway inhibitors in cancer treatment have been reviewed previously (28, 29).

A key function of mTORC2 in the PI3K/AKT/mTOR pathway is in the full activation of AKT, which requires phosphorylation of serine residues 308 and 473. AKT is a serine/threonine kinase frequently activated in cancer (30). Ser308 is phosphorylated by PDK1, while Ser473 can be phosphorylated by mTORC2 (31). Therefore, mTORC2 functions as an upstream positive regulator of mTORC1 activity in response to growth factors (31). MTORC2 also phosphorylates and activates serum- and glucocorticoid-induced kinase-1 (SGK1) as well as protein kinase C alpha (PKCα) (32, 33). The kinase activity of both AKT and SGK1 toward the forkhead box protein O1 (FOXO1) and FOXO3a transcription factors causes the masking of the nuclear localization signal by 14-3-3 proteins, resulting in the nuclear exclusion of these transcription factors (34). FOXO1 and FOXO3a regulate the expression of genes critical to the development of lymphocytes and will be addressed below.

mTOR inhibition and the immune system

The immunosuppressive capacity of rapamycin, and subsequently the role of mTOR function in the immune system, has garnered immense scientific and clinical interest over several decades. The capability of rapamycin to inhibit T cell proliferation in response to IL-2 stimulation (35) spurred particular focus on the use of rapamycin for preventing rejection in solid organ transplantation. Sirolimus (rapamycin, Rapamune) was first approved as an immunosuppressant for transplant rejection prophylaxis in 1999, followed by everolimus (Zortress, RAD001) in 2010 (36). Rapamycin/sirolimus and its analogs (rapalogs) are allosteric inhibitors of mTOR that predominately inhibit the activity of mTORC1. The preferential activity of rapalogs towards inhibition of mTORC1 is not entirely understood, and prolonged treatment with rapamycin has been demonstrated to inhibit mTORC2 in some instances (12). Rapamycin has been used extensively as a chemical tool for characterizing the role of mTOR in immune responses. Total deletion of mTOR complex subunits in traditional genetically engineered mouse models often results in embryonic lethality, however, conditional knockout (KO) techniques have allowed researchers to generate tissue specific phenotypes of mTOR subunit deficiencies (37, 38). More recently a viable hypomorphic mTOR knock-down (KD) mouse was generated by neo-insertion within *Mtor* exon 12 that partially disrupts transcription in all tissues resulting in only 30% of mTOR expression compared to wild-type (WT) (39). The mTOR KD mice, rapamycin, and conditional knockouts of mTOR, RAPTOR and RICTOR have been used to study the effects of mTOR signaling loss on specific cell types within the immune system

(40). Table 1 highlights the role of mTOR in activation, differentiation and maturation of a number of immune cell types.

B-cell development and function

The role of mTOR in B cell development and function has been reviewed previously (41). Stimulation of B cells by antigen presentation in the absence of functional B cell receptors (BCRs) results in cell death. The rescue of B cell survival by constitutive activation of the PI3K/AKT/mTOR pathway demonstrated the requirement for mTOR signaling in the immune response of B cells (42). Additionally, rapamycin has been shown to impair the differentiation and expansion of B cells stimulated by *Staphylococcus aureus* and CD40 ligand (CD40L), agents that elicit potent stimulation in untreated B cells (43). Surprisingly, specific deletion of the TSC, which results in constitutive mTORC1 activity in B cells, did not increase B cell responsiveness. In fact, TSC KO B cells exhibited impaired maturation and decreased numbers in splenic marginal zones (MZ), where non-circulating B cells would normally mount rapid antibody responses to circulating antigens. The TSC KO B cells also showed a poor ability to form the sites of proliferation, differentiation and maturation known as germinal centers (GC) (44). Deletion of PTEN, another negative regulator of the PI3K/AKT/mTOR axis, in B cells produced significant increases in MZ B cells; however, antibody production in response to both T cell independent and dependent antigens was still reduced (45). B cell development is heavily dependent on the control of gene expression by FOXO1, and the differences observed in these models may be a function of the developmental stage when PI3K/AKT/mTOR signaling is induced (46). In one study, conditional knockout of the mTORC2 subunit RICTOR impaired B cell development, possibly due to an increase in the FOXO1 protein levels (47). The mTOR KD mouse model was shown to have significant B cell alterations including a partial block in B cell development in the bone marrow, altered splenic populations, decreases in proliferation and migration to chemokines, and limited humoral immune responses to T cell-dependent antigens (39). mTOR KO mice were generated by crossing $mTOR^{flox/flox}$ mice with CD19-cre mice (which allowed the specific deletion of mTOR from cells expressing the B cell specific marker CD19) in order to determine the intrinsic function of mTOR in B cells. B cell-specific mTOR KO mice had decreased mature, transitional type 2 (T2) and MZ B cells, impaired GC formation, and reduced antibody production in response to T cell dependent antigens (38). When these mice were challenged with streptococcal infection, antibody responses were impaired and fewer mice survived compared to responses in WT littermates (38). These findings suggest that tight regulation of mTOR activity is critical for B cell development and function. Both constitutively active and repressed or deleted mTOR impaired B cell function either by preventing quiescence required for maintenance of long-lived B cells or by limiting expansion upon antigen recognition (48). Clinical mTOR inhibition does not affect the ability of B cell populations to detect antigens; however, the transduction of BCR stimulation and expansion of B cell populations is limited (42).

T-cell development and function

Regulation of mTOR function is critical in maintaining naïve T cell quiescence and directing T cell differentiation (49, 50). A KD model with disrupted mTOR expression was characterized by reductions in body, organ, and cell size (39). T cells from KD mice had

defects in proliferation, survival, and cytokine production, but had an increased percentage of FoxP3 CD4+ cells after stimulation; these regulatory T cells often develop in situations involving chronic inflammation (39). Other studies have shown enhanced differentiation of naïve T cells to Foxp3+ regulatory cells after rapamycin treatment (51); however, in a separate study, rapamycin was shown to induce immune-stimulatory effects and thereby increased CD8+ memory T cell development (52). When elderly participants (65 years and older) were treated with everolimus prior to influenza vaccination, improved serological responses and fewer CD4+ and CD8+ T cells expressing programmed cell death 1 (PD-1), which is involved in down-regulation of immune function, were observed relative to placebo treated controls (53). These findings suggest that vaccination to common pathogens could provide patients with memory T cells capable of responding to infection during the course of mTOR inhibitor treatment.

Granulocytes, dendritic cells, and mast cells

The migration of neutrophils to sites of infection requires mTOR (50, 54), as does the production of pro-inflammatory cytokines (50, 55, 56). Therefore, the ability of patients taking an mTOR inhibiting drug to mount an innate immune response is likely to be reduced. Furthermore, dendritic cell (DC) maturation is an mTOR-dependent process and rapamycin administration impairs the production of interferon α (IFNα) and IFNβ in DCs in response to microbial stimulation (50, 57). Compromised IFN production allows pathogens to gain a foothold, and likely plays a role in the increased infection observed with mTOR inhibitor administration. mTOR is also required for the expansion and survival of mast cells (58), and low mTOR levels induced by mTOR inhibitors can lead to fewer mast cells and thereby lessen first line responses to pathogens. These defects in innate immunity may be compounded by the effects of mTOR inhibition on stromal cells, which is known to impair wound healing (59), and is likely critical to the development of stomatitis and pneumonitis, which in turn provide opportunities for pathogens to enter the body (60).

Clinical-Translational Advances

There has been incredible interest in developing drugs targeting the PI3K/mTOR pathway for treating cancer. Two allosteric mTOR inhibitors or “rapalogs,” everolimus (Afinitor) and temsirolimus (Torisel) have received FDA approval for single agent indications in various tumor types, including advanced renal cell carcinoma (RCC), progressive neuroendocrine tumors of pancreatic origin (PNET) and subependymal giant cell astrocytoma (SEGA). Although there are patients with tumors that are exquisitely sensitive to mTOR inhibition alone (61, 62), so called “exceptional responders,” it is likely the greatest clinical benefits of targeting mTOR will be seen in the context of drug combination strategies. The clinical benefit of including everolimus in combination strategies for advanced breast cancer has been evaluated in several large Phase III trials, and an indication for everolimus in combination with exemestane in advanced breast cancer was approved in 2012 (63, 64). Numerous combination strategies including mTOR inhibitors are currently being evaluated in on-going clinical trials in dozens of tumor types (clinicaltrials.gov). Although none have yet reached the stage of regulatory approval, there are many second-generation mTOR

inhibitors targeting the ATP-binding site of the mTOR kinase or both PI3K and mTOR under investigation (65).

Due to the important role mTOR plays in major cell processes across numerous cell types, a critical challenge in the development of mTOR inhibitors for cancer treatment has been to devise dosing regimens with anti-tumor efficacy while minimizing adverse events. Adverse events (AEs) reported in cancer patients treated with mTOR inhibitors have included stomatitis (distinct from that seen with some cytotoxics), non-infectious pneumonitis, hyperglycemia, hyperlipidemia, fatigue, and infections (66, 67). The extensive understanding of mTOR biology gained since the discovery of rapamycin provides important insight into the pathology of many of these AEs. Translating this mechanistic understanding into the development of strategies to prevent and/or manage AEs from extended treatment with mTOR inhibitors is critical for the full realization of the long-term benefits in improving survival and quality of life for patients.

As mTOR inhibitors were first approved as immunosuppressants for preventing organ transplant rejection, the increased incidence of infection with mTOR inhibitor use in cancer may not be surprising. A recently reported retrospective case-control study found higher risk of infection associated with treatment by PI3K/AKT/mTOR pathway inhibitors. In this study, of the 366 patients treated with PI3K/AKT/mTOR pathway inhibitors, 27% also received antibiotic treatment for infections compared to 8% of the 100 control patients treated with unrelated drugs in 10 phase I clinical trials (68). Several studies have now identified secondary infection as the most common adverse event in mTOR inhibitor-treated breast cancer (69, 70).

Unlike conventional cytotoxic cancer therapies, which often result in cell death, mTOR inhibition causes growth arrest and often immunosuppression by preventing the expansion of specific immune cell populations (71). The immune cells of a patient treated with an mTORi are restrained in a quiescent state, but remain viable. Impairments of B cell activation, T cell differentiation to effector lineages, expansion and survival of mast cells and DC interferon production are likely causes for the immunological deficits arising from mTOR inhibition. The enhanced memory T cell differentiation, along with the improved responses to immunization observed with mTOR inhibition, suggests that vaccination may be a viable option for reducing incidence of infection (52, 53). Of course patients cannot be immunized against every possible pathogen, but an enhanced prophylactic immunization strategy, antibiotic treatment or a combination may reduce discontinuation of mTOR inhibiting cancer therapies.

The increased rate of infection in patients receiving mTOR inhibitors as a part of their cancer therapy is an intrinsic negative side effect that has not been separated from the anti-proliferative, therapeutic effects of these compounds. Some options for ameliorating the inextricable immunosuppressive side effect of mTOR inhibition in cancer treatment may involve altered dosing regimens. Pulse dosing (72), for instance, may result in better clinical outcomes as the immunosuppression would only occur for discrete periods of time during which exposure to pathogens could be actively prevented. Pretreatment with antibiotics may be advisable for ameliorating the increased risk of infection in mTORi treated patients. The

administration of vaccines prior to treatment is another possible avenue to prevent infections; however, vaccinating against the correct pathogen presents an obstacle for this approach. Nanoparticle or other targeted delivery of mTOR inhibiting drugs (73) might allow the precise targeting of cancer cells. The existence of a hypomorphic mTOR mouse may allow for modeling the effectiveness of antibiotic and vaccine treatments in preventing infections resulting from decreased mTOR activity. mTOR has many diverse roles in the development and function of immune cells, which lead to the complication of immunosuppression in cancer patients treated with mTOR inhibitors. Adopting best practices for treatment of immunocompromised patients could prevent a significant fraction of infections by decreasing exposure to pathogens in mTORi treated patients. A thorough understanding of mTOR's role in the immune system will help us to better protect patients and maximize the benefits of mTOR inhibitors during cancer treatment.

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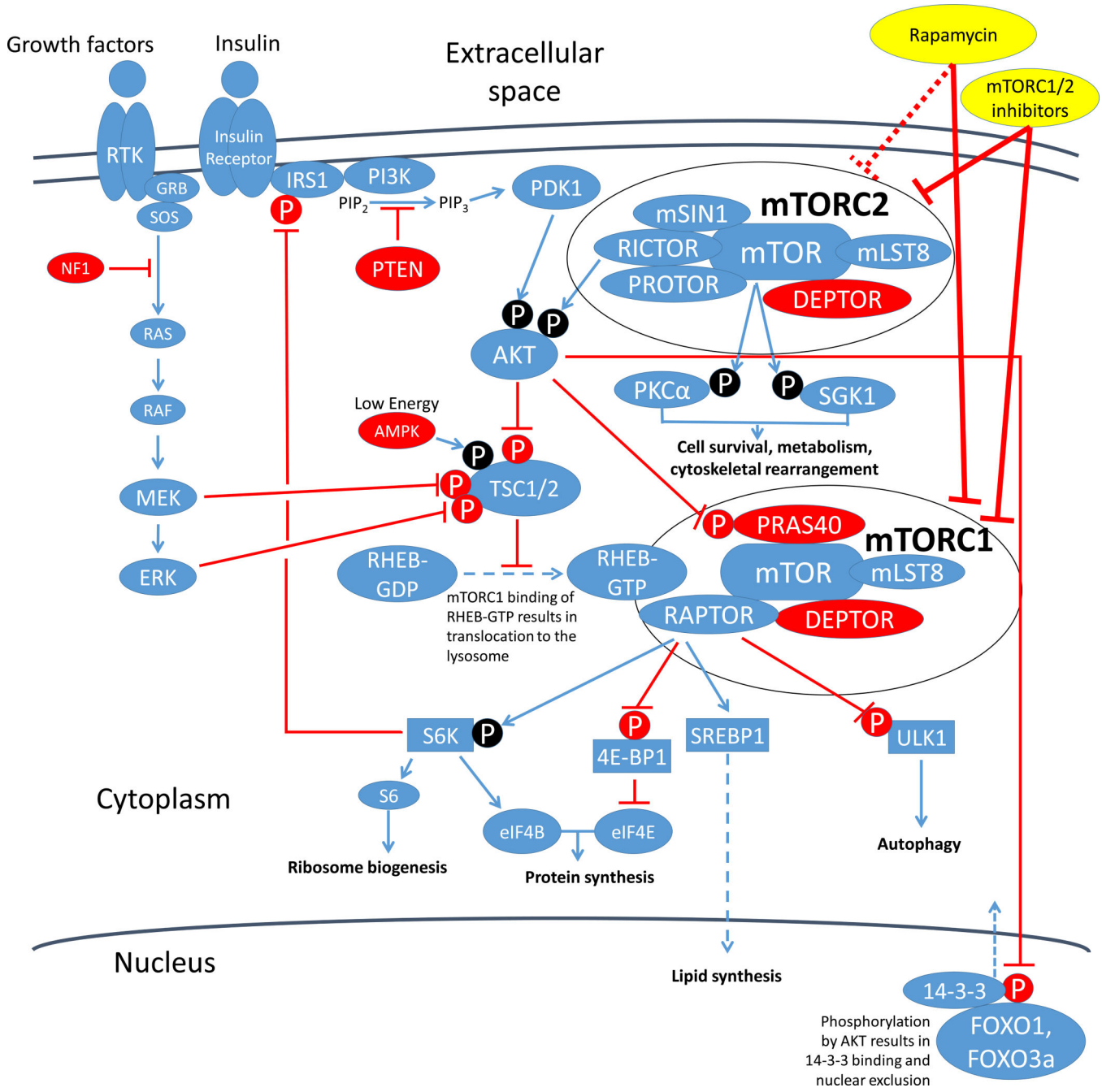

Figure 1. Simplified diagram of the mTOR pathway

The mTOR protein exists in two structurally and functionally distinct complexes; mTOR complex 1 (mTORC1) contains the mTOR kinase, mLST8, RAPTOR and the inhibitory PRAS40 whereas mTOR complex 2 (mTORC2) contains mTOR, mLST8, RICTOR, mSIN1, and PROTOR. DEPTOR inhibits both mTOR complexes and is degraded upon their activation. Upstream of mTOR, the GTPase RHEB is a positive regulator of mTOR activity. Immediately upstream of RHEB is the key negative regulator TSC which inactivates RHEB through its GAP (GTPase activating protein) activity. The activation of mTOR results in protein and lipid synthesis, autophagy, and ribosome biogenesis. Red indicates inhibitory

function and blue and black indicate stimulatory activity with respect to the PI3KAKT-mTOR signaling axis.

Table 1

mTOR is involved in both innate and adaptive immune responses. This table highlights the role of mTOR in activation, maturation and differentiation of a diverse array of cell types.

Cell Type	Role of mTOR	References
Antigen Presenting Cells Dendritic Cells Macrophages	Maturation, Activation, Differentiation, Cytokine (interferon,interleukin) production; Co-stimulatory molecule expression	40, 50, 57
Mast Cells	Proliferation, Activation, Homeostasis, Survival	50, 58
Neutrophils	Activation, Migration	50, 54-56
T Cells	Maturation, Activation, Differentiation, Proliferation, Migration, Survival, Cytokine production	39-40, 49, 51-53
B Cells	Activation, Differentiation, Survival, Maturation, Antibody production	40, 42-48
Stromal Cells	Wound healing	59