



Searching for the real function of mTOR signaling in the regulation of PD-L1 expression

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ABSTRACT

The mammalian target of rapamycin (mTOR), via forming two important complexes: mTOR complex 1 (mTORC1) and complex 2 (mTORC2), plays an important role in the regulation of immunity in addition to exerting many other biological functions. Beyond its regulatory effects on immune cells, the mTOR axis also regulates the expression of programmed death-ligand 1 (PD-L1) in cancer cells; accordingly, inhibition of mTOR alters PD-L1 levels in different cancer cell types. However, the currently published studies on mTOR inhibition-induced PD-L1 alteration have generated conflicting results. This review will focus on summarizing current findings in this regard and discussing possible reasons for the discrepancies and their potential implications for PD-L1 modulation in cancer therapy.

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Introduction

Programmed death-ligand 1 (PD-L1) expression on cancer cells is a critical mechanism contributing to immunosuppression and immune escape through inactivation of immune cells such as T-cells, NK cells and macrophages via interacting with program death-1 (PD-1) on these immune cells [1–3]. Accordingly, immunotherapy targeting the PD-1/PD-L1 immune checkpoint has shown great benefit against several types of cancer and has changed the landscape of cancer therapy [4–6]. However, the

promise of clinical benefit can be achieved only in some patients. In fact, a majority of patients do not benefit from monotherapy using PD-1/PD-L1 blockade [5,7,8].

The mammalian target of rapamycin (mTOR), via forming two distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), critically regulates various biological functions such as cell proliferation and differentiation, metabolism, survival and immunity [9–11]. mTORC1 contains the key partner protein raptor and mainly activates p70 S6 kinase (p70S6K) and suppresses eIF4E-binding protein 1 (4EBP1) function via

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phosphorylation, regulating cap-dependent translation initiation, an essential process for synthesizing many oncogenic proteins such as cyclin D1, c-Myc and Mcl-1 involved in controlling growth, survival and progression of cancer cells [10,11]. mTORC2 has rictor as its key partner protein, and has less defined biological functions compared with mTORC1 other than regulation of cell cytoskeleton and survival primarily by phosphorylating Akt, PKC and serum and glucocorticoid inducible kinase [12]; although mTORC2 is involved in the positive regulation of cancer development [13–15]. Furthermore, both mTORC1 and mTORC2 are involved in the post-translational regulation of proteins, i.e., protein degradation. While mTORC1 signaling regulates proteasomal degradation of some important proteins such as DEPTOR [16–18] and Lipin1 [19], mTORC2 also positively regulates the stabilities of several oncogenic proteins including cyclin D1, Mcl-1, SREBP1 and Snail as we recently demonstrated [20–23]. Therefore, dysregulation of the mTOR axis in cancers has emerged as an attractive cancer therapeutic target [9,10,24].

mTOR inhibitors include conventional rapamycin (sirolimus) and its analogues (rapalogs) and ATP-competitive mTOR kinase inhibitors (TORKinibs). The former are specific allosteric inhibitors of mTOR and preferentially inhibit mTORC1/p70S6K signaling, while the latter suppress both mTORC1 and mTORC2. Some rapalogs (e.g., everolimus/RAD001 and temsirolimus/CCI779) are approved drugs for the treatment of certain cancers including metastatic renal cell carcinoma [25–27], pancreatic neuroendocrine tumors [28], and postmenopausal hormone receptor-positive advanced breast cancer [29]. The new generation of TORKinibs have been tested in clinical trials as potential anticancer drugs [30–32], but have not proven to be effective for the treatment of cancer in the clinic [32,33].

Rapalogs as immunosuppressants and beyond

Rapamycin has long been regarded as a potent immunosuppressant used in organ transplantation. However, there are many studies suggesting that the effects of rapamycin on immune function, particularly T cell activation, are highly dose dependent [34–37]. It has been shown that lower doses of rapamycin actually stimulate memory CD8+ T cell generation and enhance the memory T cell response to viral infection or cancer in mice [38]. Similarly, RAD001 at low doses (e.g., 0.5 mg/daily, po) improves immune function in elderly volunteers, as assessed by their response to influenza vaccination [39]. A human study in renal cancer patients has shown that the therapeutic efficacy of rapalogs is associated with modulation of antitumor T-cell immunity. Patients receiving rapalogs showed better clinical responses if they presented a shift toward decreased Tregs levels and high expansion of antitumor Th1 or activated CD8+ T cells [40]. Consistently, several studies have shown that rapalogs enhance response to various modalities of immunotherapy including adoptive cell therapy and cancer vaccines, among others [41–44]. Hence, rapalogs may exert dual functions in the regulation of immunity depending on the doses used.

Effects of mTOR inhibition on PD-L1 expression: conflicting results

One early study by Wang et al. in 2013 reported that treatment of human graft endothelial cells with rapamycin elevated levels of PD-L1, consistent with its immunosuppressive effect on organ rejection [45]. The same study further showed that knockdown of mTOR or raptor, but not rictor, substantially elevated the levels of PD-L1 in endothelial cells, suggesting PD-L1 upregulation as a consequence of mTORC1 inhibition [45]. In agreement, a subsequent study by Hirayama et al. found that treatment of human 786-O and mouse RENCA renal cell carcinoma (RCC) cell lines with RAD001 upregulated PD-L1 expression. This upregulation was also observed in RENCA tumor xenografts exposed to RAD001 [46]. However, a study reported by Lastwika et al. showed that inhibition of PI3K/mTORC1 signaling using LY294002, rapamycin, AZD8055 (a TORKinib) or raptor knockdown decreased PD-L1 levels in a panel of human non-small cell lung cancer (NSCLC) cell lines [47], reaching the opposite conclusion.

Three recent publications have examined mTOR inhibition and PD-L1 expression in different types of cancer cells, including one from our own

group [48–50]. While working on a novel mTORC1/2 inhibitor named MTI-31 (LXI-15029) against NSCLC cells, Zhang et al. reported that treatment with MTI-31 or knockdown of both raptor and rictor decreased PD-L1 levels in some EGFR- and ALK-driven NSCLC cells [49]. Interestingly, another study from a different group by Zhang et al. reported that treatment of RCC cells with either rapamycin or Torin 1 increased PD-L1 protein levels including cell surface PD-L1 detected by flow cytometry, although this study focused on the role of transcription factor EB (TEFB) in mediating immune evasion and resistance to mTOR inhibition of RCC [48]. In our study dedicated to demonstrating the impact of mTOR inhibition on PD-L1 expression [50], we used comprehensive approaches to definitively address the effect of mTOR signaling inhibition on PD-L1 expression in NSCLC and other types of cancer cells. Our major findings were as follows [50]: 1) Treatment with either rapamycin or TORKinibs (INK128, Torin 1 and AZD8055) elevated PD-L1 levels, including cell surface PD-L1, in a panel of NSCLC cell lines along with cell lines from cancers of other histology. This trend was particularly evident in those cell lines expressing detectable basal levels of PD-L1; 2) Genetic knockdown of raptor, but not rictor, increased PD-L1 levels in several cancer cell lines; 3) Inhibition of p70S6K, a well-known substrate of mTORC1, with two structurally distinct inhibitors (FRI00705 and PF4708671) also elevated PD-L1; 4) Other inhibitors including BEZ235 (a dual PI3K/mTOR inhibitor), BKM120 (a pan-PI3K inhibitor) and MK2206 (an AKT inhibitor), having the common feature of suppressing p70S6K, robustly increased PD-L1 levels; and 5) PD-L1 positivity was significantly associated with p-S6 negativity based on immunohistochemical staining of these two markers in a cohort of 194 cases of NSCLC tissue specimens, indicating that PD-L1 expression is negatively correlated with p-S6 staining. Hence, our findings strongly indicate that inhibition of mTORC1/p70S6K signaling elevates PD-L1 levels in cancer cells.

Possible reasons behind the conflicting results

As discussed above, several publications have generated conflicting results in terms of mTOR regulation of PD-L1. Understanding the causes of this discrepancy and determining the actual role of mTOR in PD-L1 regulation are important in defining the potential implications for PD-L1 modulation in cancer therapy.

Among these studies, 4 used Western blotting to detect PD-L1 protein levels [47–50], whereas two studies detected only cell surface PD-L1 levels with flow cytometry [45,46]. It is crucial to note that one major technical issue among these studies is that they all used different sources of anti-PD-L1 antibodies in their Western blotting (Table 1). The anti-PD-L1 antibody (#ab58810; Abcam) used in the paper by Lastwika et al. [47] was not validated in the users' lab and is no longer available due to a self-reported failure in meeting the company's quality criteria (<http://www.abcam.com/pd-l1-antibody-ab58810.html>). The antibodies used in other two studies were from Sino Biological (no catalog number provided) [49] and ProteinTech (#17952-1-AP) [48], respectively, and were not validated in the users' labs. However, the anti-PD-1 antibody from ProteinTech (#17952-1-AP) was validated by the vendor via gene knockdown and interferon γ (INF γ) induction (<https://www.ptglab.com/products/CD274-Antibody-17952-1-AP.htm#validation>). The rabbit anti-PD-L1 monoclonal antibody (#13684/clone E1L3N; Cell Signaling Technology, Inc) used in our study [50] has been widely reported in the research community (https://www.citeab.com/antibodies/2043262-13684-pd-l1-e1l3n-xp-rabbit-mab?utm_campaign=Widget+All+Citations&utm_medium=Widget&utm_source=Cell+Signaling+Technology) and was validated in a previous study [51]. The specificity of this antibody was also confirmed in-house with PD-L1 knockdown, PD-L1 overexpression and INF γ or EGF induction by our group [50]. Therefore, we strongly believe that the application of different anti-PD-L1 antibodies is likely to be the main cause of the conflicting results on mTOR regulation of PD-L1. Only validated antibodies can yield reliable and accurate findings.

Another factor that may affect the outcome of a study is the concentration of mTOR inhibitor tested. While most studies used mTOR inhibitors (e.g., rapamycin) at a concentration range of ≤ 100 nM, a

Table 1
PD-L1 antibodies used in different studies to detect PD-L1 by Western blotting in cells with mTOR inhibition.

Study	mTOR inhibition	PD-L1 change	Cell type	Antibody source	Antibody validation ^a	Antibody availability
Lastwika et al. (2016)	Rapamycin AZD8055	Decrease	NSCLC	Abcam (ab58810)	No	Discontinued
Zhang Q et al. (2019)	Raptor KD MTI-31 Rapamycin	Decrease	NSCLC	Sino Biological	No	Yes
Zhang C et al. (2019)	Raptor KD Rictor KD Rapamycin Torin 1	Increase	Renal cell carcinoma	ProteinTech (#17952-1-AP) ^b	No	Yes
Deng et al. (2019)	Rapamycin INK126 AZD8055 BEZ235 BKM120	Increase	NSCLC/ Breast cancer/colon cancer/prostate cancer	Cell Signaling Technology (#13684)	Yes	Yes

^a Via gene knockdown (KD), overexpression and INF γ and EGF induction in user's lab.

^b Validated by company with KD and INF γ induction.

typical concentration range used in the mTOR research field, the study by Zhang et al. [49] used 1 μ M rapamycin and 1–5 μ M MTI-31 for studying their impact on modulation of PD-L1 expression, although the IC₅₀ of MTI-31 is around 0.5 μ M against the growth of NSCLC cells. This factor should be considered. Of course, the context-dependent regulation of PD-L1 by mTOR under a given condition can also not be excluded.

Mechanisms underlying PD-L1 upregulation mediated by mTOR inhibition

Since most studies support the upregulation of PD-L1 upon mTOR inhibition, we will primarily discuss potential mechanisms accounting for mTOR inhibition-induced PD-L1 elevation. The first question is which mTORC inhibition causes PD-L1 elevation. The study by Wang et al. reported that knockdown of mTOR and raptor, but not rictor, increased cell surface PD-L1 in endothelial cells as rapamycin did [45]. Our study showed that knockdown of raptor, but not rictor, in general elevated PD-L1 levels in several cancer cell lines. Moreover, inhibition of p70S6K with small molecule inhibitors increased PD-L1 levels as well [50]. Thus, it appears that inhibition of mTORC1/p70S6K signaling causes PD-L1 elevation. This can also explain why rapalogs effectively increase PD-L1 levels [45,46,48,50], because they primarily exert potent inhibitory effect against mTORC1.

In RCC cells, it was shown that TFEB, a transcription factor, bound to the PD-L1 promoter and inhibition of mTOR led to enhanced TFEB nuclear translocation and PD-L1 expression [48]. This suggests a mechanistic model for transcriptional regulation of PD-L1 involving TFEB-mediated gene transcription. In our study primarily with human NSCLC cell lines, transcriptional regulation of PD-L1 expression seems to play a limited role in PD-L1 elevation induced by mTORC1 inhibition because the mTOR inhibitors tested did not increase PD-L1 mRNA levels in every tested NSCLC cell line. Rather, mTOR inhibitors, p70S6K inhibitors and raptor knockdown all slowed down PD-L1 degradation, suggesting that PD-L1 elevation caused by inhibition of mTORC1/p70S6K primarily occurs at the post-translational level through suppressing PD-L1 protein degradation [50]. β -TrCP has been suggested to mediate PD-L1 degradation [52]. Our study has also demonstrated that mTORC1/p70S6K inhibition enhances β -TrCP degradation, reducing the levels of β -TrCP [50], a previously undiscovered finding. Since there is a tight correlation between β -TrCP reduction and PD-L1 elevation in our study, it is logical to propose that mTORC1/p70S6K signaling negatively regulates PD-L1 levels through stabilizing β -TrCP protein and enhancing PD-L1-mediated degradation; inhibition of this signaling pathway will facilitate β -TrCP degradation, resulting in increased PD-L1 stability and levels (Fig. 1).

Potential implications of PD-L1 modulation during mTOR-targeted cancer therapy

Although there is a strong scientific rationale for targeting mTOR as an attractive cancer therapeutic strategy, major success has not been achieved, particularly with TORKinibs that inhibit both mTORC1 and mTORC2. Since PD-L1 expression on cancer cells is a critical mechanism contributing to immunosuppression through its interactions with PD-1 on immune cells, it is logical to speculate that PD-L1 induction by mTORC1 inhibition may contribute to the modest anticancer activity of rapalogs or limited anticancer activity of TORKinibs via encouraging the immune escape of cancer cells. This may represent another important mechanism accounting for the limited success of targeting mTOR for cancer therapy.

Theoretically, the elevation of PD-L1 in cancer cells might increase the availability of epitopes for anti-PD-L1 antibody to bind, which could enhance therapeutic efficacy. In the clinic, tumors expressing high levels of PD-L1 responded well to anti-PD-L1 therapy [53] and even to anti-PD1 therapy [54]. A study has shown that CDK4/6 inhibition elevates PD-L1 levels and significantly sensitizes cancer cells to PD-1 blockade therapy [55]. Therefore, mTOR inhibition-induced elevation of PD-L1 in cancer cells may constitute a strong scientific rationale for combining mTOR inhibition with PD-1 or PD-L1 blockade immunotherapy as an effective strategy to enhance therapeutic efficacy. Indeed, the combination of RAD001, CCI779 or rapamycin with PD-L1 blockade significantly enhanced antitumor activity compared with each single agent treatment against RCC and oral cavity cancer, including enhancement of the cytotoxic functions of tumor-infiltrating cytotoxic T lymphocytes [46,48,56]. The combination of rapamycin with anti-PD-1 antibody also enhanced the reduction in lung tumor burden in a mutant *KRAS*-driven mouse lung cancer model, even based on the rationale that rapamycin decreased PD-L1 levels [47].

As discussed above, many studies in the past decade have suggested that rapalogs (particularly at low dosage) or mTOR inhibition activate memory CD8+ T cells, leading to enhanced vaccine responses to viral antigens and immune responses against cancer [34,35,57,58]. This may provide another scientific rationale for co-targeting mTOR and PD-1/PD-L1 checkpoint. In this regard, selection of the most appropriate doses for different mTOR inhibitors should be considered.

INF γ is the primary cytokine released by cytotoxic T cell that upregulates PD-L1 transcription primarily through activation of the JAK/STAT3 signaling pathway [59,60]. Although INF γ plays a pivotal role in antitumor host immunity by inducing Th1 polarization, cytotoxic T cell activation, and dendritic cell tumoricidal activity, its induction of PD-L1 in cancer cells can also impair local tumor immunity, causing immune escape of cancer cells [59,60]. Our study showed that mTOR inhibition (e.g., with rapamycin) and INF γ combination further enhanced PD-L1 levels in some

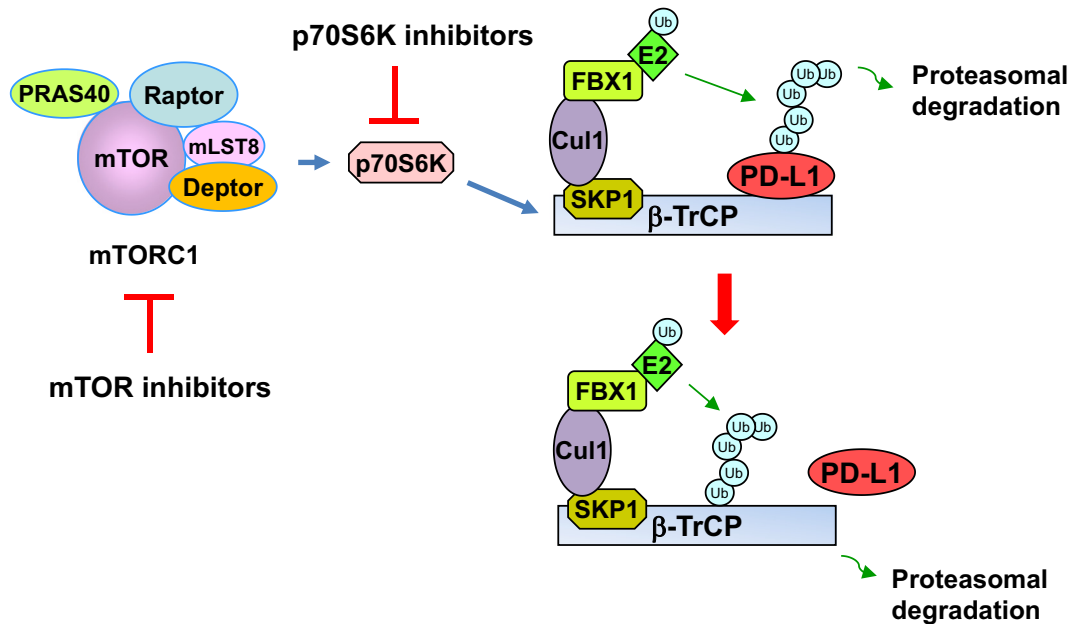


Fig. 1. A working model for mTORC1/p70S6K signaling regulation of β -TrCP-mediated PD-L1 degradation. mTORC1/p70S6K signaling stabilizes the E3 ubiquitin E3 ligase, β -TrCP, resulting in destabilization of PD-L1. Upon inhibition of this signaling pathway with either an mTOR or p70S6K inhibitor, β -TrCP will be degraded via the proteasome, leading to PD-L1 stabilization. The red arrow indicates the consequences of mTOR or p70S6K inhibition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NSCLC cell lines expressing basal level of PD-L1 [50]. Therefore, co-targeting mTOR and PD-1/PD-L1 checkpoint may also raise a concern on accordingly increasing adaptive resistance to PD-L/PD-L1 blockade immunotherapy. This may be considered by optimizing treatment schedules such as the intermittent one with mTOR-targeted therapy first followed by PD-L/PD-L1 blockade immunotherapy. Nonetheless, further investigation in this direction is warranted.

Perspectives

It appears that mTOR inhibition has a direct effect on the modulation of PD-L1 expression in cancer cells, albeit with conflicting results from different studies. Nonetheless, fully elucidating the effects of mTOR or mTOR inhibition on the regulation of PD-L1 expression in cancer cells including the underlying mechanisms will be very helpful for us to wisely utilize mTOR inhibitors for the treatment of human cancers, including in combination with immunotherapy.

The majority of studies primarily tested the effects of rapalogs combined with a PD-L1/PD-blockade against the growth of different tumors. In addition to mTORC1, mTORC2 inhibition also enhances the generation of CD8 + memory cells [61] based on the recent finding that mTORC2 is involved in the critical regulation of CD8 + T cell memory [58]. Rictor deficiency favors memory formation and increases IL-2 secretion capacity without dampening T effector cell functions. Moreover, mTORC2-deficient memory T cells mount more potent recall responses [62]. Consistently, selective inhibition of mTORC2 activity in activated dendritic cells augments their pro-inflammatory and T cell stimulatory profile, in association with their enhanced capacity to promote protective CD8 + T cell responses in vivo, leading to slowed tumor progression [63]. It has been shown that inhibition of SREBPs, critical transcription factors that regulate lipid metabolism, in T cells enhances generation of CD8 + T memory cells [64]. Interestingly, our recent data show that mTORC2 inhibition suppresses SREBP1 through promoting its degradation [22], further supporting the role of mTORC2 in the regulation of T cell memory. Together, these data support the scientific premise that inhibition of mTORC2 together with PD-1/PD-L1-targeted immunotherapy or even other target-oriented immunotherapies may also be an effective strategy for cancer therapy. TORKinibs are potent mTORC1 and mTORC2 inhibitors with promising preclinical efficacies against different

types of cancer. However, the potential activity of TORKinibs combined with immunotherapy such as PD-1/PD-L1 blockade is under-researched and should be considered as a future direction. In support of this, a previous study showed that AZD8055 combined with a CD40 agonist antibody exerted enhanced antitumor activity in a murine metastatic RCC xenograft model with increased infiltration, activation, and proliferation of CD8(+) T cells and natural killer cells in liver metastatic foci [65].

One interesting finding in our study was that PD-L1 expression is negatively correlated with p-S6 staining in human NSCLC tissue specimens: i.e., tissues with positive p-S6 staining are likely to have negative or low PD-L1 expression [50]. Given that PD-L1 positivity is a predictive biomarker for guiding PD-1/PD-L1 immunotherapy [66], whether p-S6 staining can be used as a predictive biomarker for this purpose should be considered. We predict that tumors negative for p-S6 staining are likely to respond better to PD-1/PD-L1 immunotherapy.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contribution

Shi-Yong Sun: Literature search and manuscript writing.

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