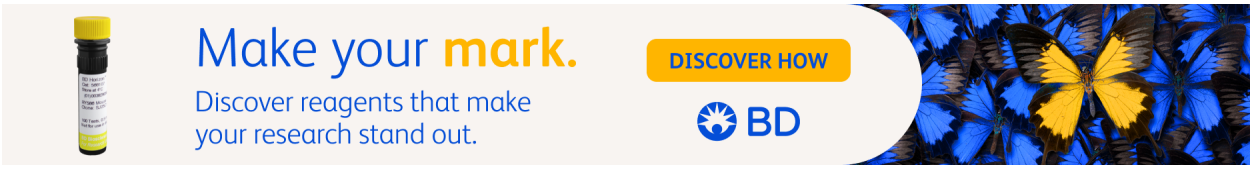

The Journal of Immunology

A Role for Mammalian Target of Rapamycin in Regulating T Cell Activation versus Anergy

This information is current as of August 9, 2022.

Yan Zheng, Samuel L. Collins, Michael A. Lutz, Amy N. Allen, Thomas P. Kole, Paul E. Zarek and Jonathan D. Powell

J Immunol 2007; 178:2163-2170; ;
doi: 10.4049/jimmunol.178.4.2163
http://www.jimmunol.org/content/178/4/2163

References This article **cites 35 articles**, 18 of which you can access for free at:
http://www.jimmunol.org/content/178/4/2163.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2007 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Downloaded from http://www.jimmunol.org/ by guest on August 9, 2022

A Role for Mammalian Target of Rapamycin in Regulating T Cell Activation versus Anergy[1]

Yan Zheng, Samuel L. Collins, Michael A. Lutz, Amy N. Allen, Thomas P. Kole, Paul E. Zarek, and Jonathan D. Powell[2]

Whether TCR engagement leads to activation or tolerance is determined by the concomitant delivery of multiple accessory signals, cytokines, and environmental cues. In this study, we demonstrate that the mammalian target of rapamycin (mTOR) integrates these signals and determines the outcome of TCR engagement with regard to activation or anergy. In vitro, Ag recognition in the setting of mTOR activation leads to full immune responses, whereas recognition in the setting of mTOR inhibition results in anergy. Full T cell activation is associated with an increase in the phosphorylation of the downstream mTOR target S6 kinase 1 at Thr^{421}/Ser^{424} and an increase in the mTOR-dependent cell surface expression of transferrin receptor (CD71). Alternatively, the induction of anergy results in markedly less S6 kinase 1 Thr^{421}/Ser^{424} phosphorylation and CD71 surface expression. Likewise, the reversal of anergy is associated not with proliferation, but rather the specific activation of mTOR. Importantly, T cells engineered to express a rapamycin-resistant mTOR construct are resistant to anergy induction caused by rapamycin. In vivo, mTOR inhibition promotes T cell anergy under conditions that would normally induce priming. Furthermore, by examining CD71 surface expression, we are able to distinguish and differentially isolate anergic and activated T cells in vivo. Overall, our data suggest that by integrating environmental cues, mTOR plays a central role in determining the outcome of Ag recognition. ***The Journal of Immunology,*** **2007, 178: 2163–2170.**

In a two-signal model of immune activation, the ultimate outcome of an immune response is not dictated by the Ag itself, but by the context in which the Ag is encountered. Signal 1 refers to TCR engagement that heralds recognition, whereas signal 2 refers to costimulation and is necessary for the induction of full T cell activation (1). In in vitro models, signal 1 alone leads to anergy and signal 2 can be delivered by the cross-linking of CD28. In vivo, however, signal 2 involves multiple inputs, both activating and inhibitory, from multiple accessory molecules and cytokines. Consequently, when a T cell encounters Ags, it must also sense and integrate environmental cues to respond appropriately.

The mammalian target of rapamycin (mTOR)[3] is an evolutionarily conserved 289-kDa serine/threonine protein kinase that is inhibited by rapamycin (2). In yeast and mammalian cells, TOR integrates environmental cues in terms of nutrients, energy, and growth factors, and directs cell growth and proliferation. Insulin and other growth factors activate mTOR in a PI3K→AKT-dependent fashion. AKT in turn phosphorylates and hence inactivates tuberous sclerosis complex 2, a potent inhibitor of mTOR activity (3). Growth factors also have the ability to activate mTOR through an AKT-independent pathway that involves the activation of phospholipase D and the generation of phosphatidic acid (4). Alternatively, mTOR is inhibited when there is a lack of energy stores. Under these conditions, an increase in the ratio of AMP:ATP leads to the activation of AMP kinase that in turn inhibits mTOR by promoting tuberous sclerosis complex 2 activity (3). Although the list of downstream targets of mTOR is continuously expanding, one of the most studied readouts of mTOR function is the phosphorylation of S6 kinase-1 (S6K-1), which is a translational regulator (5).

Previously, our group and others demonstrated that the mTOR inhibitor rapamycin promoted T cell anergy even in the presence of costimulation (6, 7). Initially, this was thought to be due to the ability of rapamycin to block cell cycle progression. Indeed, multiple investigators have correlated anergy with blockade in the cyclin-dependent kinase cascade (8–11). However, it is becoming increasingly clear that the induction of anergy can be disassociated from cell cycle arrest (12, 13). In this study, we provide further evidence that TCR engagement in the absence of cell cycle progression does not necessarily lead to anergy and cell proliferation does not necessarily reverse anergy. Furthermore, we test the hypothesis that mTOR plays a role in dictating the outcome of TCR engagement. Just as mTOR plays a critical role in integrating environmental cues for regulating growth and differentiation, we propose that mTOR plays a central role in integrating the environmental signals that determine whether Ag recognition will lead to activation or tolerance.

Materials and Methods

Reagents and Abs

Rapamycin (a gift from S. Sehgal, Wyeth-Ayerst, Princeton, NJ) was dissolved in ethanol. For in vivo injection, rapamycin was dissolved in carboxymethylcellulose (Sigma-Aldrich). Sanglifehrin A (SFA; a gift from R. Sedrani, Novartis, Basel, Switzerland) was dissolved in DMSO. Pigeon cytochrome *c* (PCC) peptide (81–104; FAGIKKKAERADLIAYLKQ ATAK) for injection was a gift from H. Yu (Johns Hopkins University,

The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University Medical Institutions, Baltimore, MD 21231

Received for publication May 19, 2006. Accepted for publication November 29, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants; National Institute of Allergy and Infection Diseases Grant 5K22AI1773 and National Cancer Institute Grant RO1CA98109.

[2] Address correspondence and reprint requests to Dr. Jonathan D. Powell, Department of Oncology-Immunology/Hematopoiesis, Johns Hopkins School of Medicine, 1650 Orleans Street, CRB 443, Baltimore, MD 21231. E-mail address: Poweljo@jhmi.edu

[3] Abbreviations used in this paper: mTOR, mammalian target of rapamycin; CSA, cyclosporin A; PCC, pigeon cytochrome *c*; RR-mTOR, rapamycin-resistant mTOR; S6K-1, S6 kinase-1; SFA, sanglifehrin A; WT-mTOR, wild-type mTOR.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00

Downloaded from http://www.jimmunol.org/ by guest on August 9, 2022

Baltimore, MD), and PCC peptide used in in vitro stimulation was purchased from Sigma-Aldrich. CFSE was obtained from Molecular Probes. Total S6K-1 and phospho-S6K-1 (Thr^{421}/Ser^{424}) were obtained from Cell Signaling Technology. Total AKT and phospho-AKT (Ser^{473}) were from Upstate Biotechnology. $p27^{Kip1}$ and cyclin D3 Abs were purchased from Santa Cruz Biotechnology. Actin Ab was obtained from Sigma-Aldrich. Anti-rabbit IgG HRP-linked Ab was from Amersham Biosciences. Conjugated Abs for CD71 and Vβ3 were from eBioscience and BD Pharmingen.

Cell culture and mice

A.E7, a $CD4^+$ Th1 clone specific for PCC peptide 81–104, was maintained as previously described (6). Jurkat cells were obtained from American Type Culture Collection and maintained in RPMI 1640 with 10% FBS (Invitrogen Life Technologies). The 293T cells were purchased from American Type Culture Collection and cultured in DMEM with 10% FBS (Invitrogen Life Technologies). B10.A and 5C.C7 mice were purchased from Taconic Farms. The 5C.C7 mice are TCR transgenic (specific for the $I\text{-}E^k$-restricted PCC 81–104 peptide) on a $Rag2^{-/-}$ B10.A background. The studies have been reviewed and approved by the animal review committee at Johns Hopkins Medical Institutions.

Constructs

Bicistronic lentivirus vector EF.CMV.GFP (a gift from X. Yu, Johns Hopkins University, Baltimore, MD) in which the gene of interest is driven by a human EF1-α promoter, and GFP by a CMV promoter. Wild-type mTOR (WT-mTOR) and rapamycin-resistant mTOR (RR-mTOR) were cut from pCDNA3-WT-mTOR and RR-mTOR vectors (a gift from J. Chen, University of Illinois, Urbana, IL), and subcloned into the bicistronic lentiviral vector to make EF.WT-mTOR-CMV.GFP and EF.RR-mTOR-CMV.GFP constructs.

In vitro anergy model

A total of 5×10^6 A.E7 cells was stimulated with plate-bound anti-CD3 (1 μg/ml, 2C11; BD Pharmingen) to induce anergy, or stimulated with plate-bound anti-CD3 plus soluble anti-CD28 (1/1000 of ascitic fluid containing anti-CD28 mAb 37.51; a gift from J. Allison, Sloan Kettering, New York, NY) to be activated. After 16 h, the cells were washed three times with PBS to remove Abs and rested in medium for 5–7 days. Upon rechallenge, 5×10^4 A.E7 cells were stimulated with 5×10^5 irradiated B10.A splenocytes (served as APCs) and PCC peptide (0–0.5 μM); proliferation was measured by thymidine incorporation after 48 h. To determine IL-2 production upon rechallenge, the treated cells were stimulated with plate-bound anti-CD3 (1 μg/ml) plus soluble anti-CD28 (1/1000), supernatant was collected after 24 h, and IL-2 was measured by ELISA (eBioscience). To study anergy reversal, A.E7 cells were anergized with 1 μg/ml plate-bound anti-CD3 for 16 h, washed, and rested in medium supplemented with either 100 ng/ml IL-2 (BioSource International) or 10 ng/ml IL-7 (BioSource International) for 5–7 days. Then the cells were washed three times with PBS to remove residual cytokines and rechallenged, as described above.

Transfection of Jurkat cells

To transfect Jurkat cells, 6 μg of lentiviral constructs was added to 12×10^6 cells in 300 μl of Jurkat medium without antibiotics and incubated for 15 min at room temperature, and the cells were then electroporated at 300 V, 24 Ω, and 800 μF using BTX EMC600 electroporator.

Infection of 5C.C7 T cells

To make recombinant lentivirus, 293T cells (10×10^6) were seeded onto 150-mm dishes, cultured for 16 h, and transiently transfected with 9 μg of lentivirus construct, 12 μg of pCMVΔR8.91, plus 3 μg of pMDG plasmids using Lipofetamine 2000 (Invitrogen Life Technologies). The virus supernatants were collected at 24, 48, and 72 h, and concentrated using filtration columns (Centricon Plus-70, molecular mass cutoff 100 kDa; Millipore). Viral titer was determined by transduction of 293T cells (2×10^5 cells/well in 6-well plates) with serially diluted virus supernatant plus 8 μg/ml polybrene (Sigma-Aldrich), and the percentage of GFP^+ cells was assessed 3 days later by FACS. To transduce 5C.C7 cells, spleens and lymph nodes were harvested from 5C.C7 mice and activated by PCC peptide (5 μM). Forty-eight hours later, 3×10^6 activated 5C.C7 cells, 8 μg/ml polybrene, and recombinant lentivirus (at multiplicity of infection of 1) were placed in 12-well plates, centrifuged for 3 h at 1800 $\times$ *g*, 32°C, and cultured for another 16 h. Cells were then washed and rested in fresh A.E7 medium supplemented with 1 ng/ml IL-2 and 10 ng/ml IL-7 for 5 days before being sorted by BD FACSAria.

In vivo peptide-induced anergy model

Splenocytes and lymphocytes from 5C.C7 TCR transgenic $Rag2^{-/-}$ mice ($CD4^+V\beta3^+$) were labeled with CFSE, and $4–5 \times 10^6$ cells per mouse were adoptively transferred into syngeneic B10.A hosts by i.v. injection ($8–10 \times 10^6$ cells per mouse for the naive condition to increase recovery, because these cells do not expand). On the second day, the mice received i.v. either 150 μg of PCC peptide (81–104) or HBSS (vehicle). For rapamycin-treated mice, rapamycin was prepared at 2 mg/ml in carboxymethylcellulose, and injected i.p. at a dose of 1.5 mg/kg beginning on the day of adoptive transfer, and continuing daily till the end of the experiment. On day 4, spleens were harvested, and $CD4^+$ T cells were isolated by negative selection using MACS $CD4^+$ T cell isolation kit and MACS LS separation columns (Miltenyi Biotec). Next, CFSE low $V\beta3^+$ clonotypic cells were sorted by BD FACSAria, and 2×10^4 sorted cells were rechallenged with 2×10^5 syngeneic irradiated B10.A splenocytes and 0.5–5 μM PCC peptide. Supernatant was collected 48 h later, and IL-2 was analyzed by ELISA.

Immunoblot analysis

To analyze mTOR activity during anergy induction, A.E7 cells were stimulated with anti-CD3 (1 μg/ml, plate bound) alone, anti-CD3 plus anti-CD28 (1/1000, soluble), anti-CD3 plus anti-CD28 plus SFA (1 μM), or rapamycin (1 μM) for 16 h in A.E7 medium. To analyze mTOR activity during anergy reversal, A.E7 cells were anergized with 1 μg/ml plate-bound anti-CD3 for 16 h, washed, and rested in A.E7 medium alone, or A.E7 medium supplemented with either 100 ng/ml IL-2 or 10 ng/ml IL-7 for 4 days. Next, the cells were washed three times with PBS and cultured for 4 h in the same previous condition in the absence of serum. To compare the effect of IL-2 and IL-7 on primary T cells, splenocytes from 5C.C7 mice were harvested and activated with 5 μM PCC peptide for 48 h, followed by a 5-day expansion in medium supplemented with 1 ng/ml IL-2. These preactivated primary 5C.C7 T cells were then washed with PBS three times and cultured with 100 ng/ml IL-2 or 10 ng/ml IL-7. To make cell lysate, the treated cells were harvested and resuspended in ice-cold lysis buffer containing 50 mM HEPES (pH 7.5), 0.5% Nonidet P-40, 5 mM EDTA, 50 mM NaCl, and 1× protease inhibitor mixture (Roche). After a 15-min incubation, the cells were spun and supernatant was collected for evaluation of protein concentration by bicinchoninic acid protein assay kit (Pierce). Equal amount of extracts was loaded on 10% Bis-Tris minigel (Invitrogen Life Technologies), separated by SDS-PAGE, and followed by transfer to nitrocellulose membrane (Invitrogen Life Technologies). Western blot analysis was performed using Abs listed above and corresponding secondary Abs.

Results

TCR engagement in the absence of mTOR activation induces T cell anergy

It is clear that anergic T cells fail to undergo cell cycle progression; however, whether this is a cause or consequence of anergy remains controversial. Although it has been proposed that TCR engagement in the absence of cell cycle progression causes anergy, recently, we have demonstrated that the novel cyclophilin-binding compound SFA could block cell cycle progression in G_1 without inducing anergy (13). In contrast, we and others have shown that inhibiting cell cycle progression with rapamycin (which also blocks cell cycle progression in G_1) does induce anergy (6, 7). Thus, we wanted to exploit the differences between these two inhibitors and try to dissect the biochemical mechanisms contributing to anergy induction. As seen in Fig. 1*A*, stimulation of the PCC-specific Th1 T cell clone A.E7 with anti-CD3 and anti-CD28 (signal 1 + 2) led to proliferation. Both rapamycin and SFA equally inhibited proliferation. However, as seen in Fig. 1*B*, blocking cell cycle progression with rapamycin (during the initial stimulation or induction phase) led to anergy such that the cells failed to produce IL-2 upon full rechallenge 7 days later. In contrast, inhibition of cell cycle progression with SFA did not result in anergy because these cells produced copious amounts of IL-2 upon rechallenge.

A number of groups have proposed that anergy induction is facilitated in part by cell cycle inhibitor $p27^{Kip1}$ (10, 14). This

Downloaded from http://www.jimmunol.org/ by guest on August 9, 2022

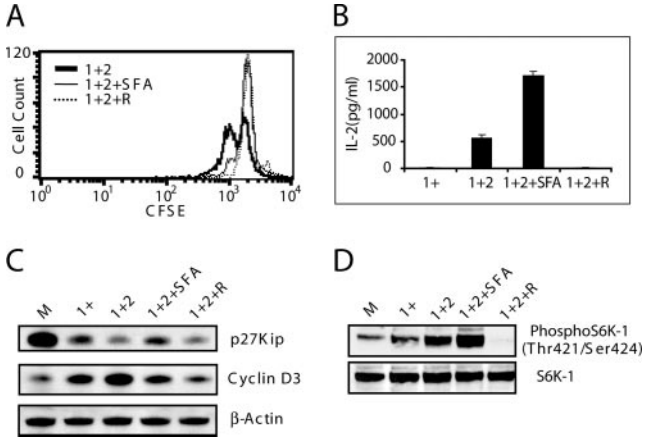

FIGURE 1. TCR engagement in the absence of mTOR activation induces T cell anergy. *A*, Both SFA and rapamycin block T cell proliferation. CFSE-labeled A.E7 cells were stimulated with anti-CD3 plus anti-CD28 (1 + 2), anti-CD3 plus anti-CD28 plus SFA (1 + 2 + SFA), or anti-CD3 plus anti-CD28 plus rapamycin (1 + 2 + R). After 48 h, cell division was analyzed by CFSE dilution. *B*, Rapamycin, but not SFA, induces anergy. A.E7 cells were stimulated 16 h with anti-CD3 alone (1+), anti-CD3 plus anti-CD28 (1 + 2), anti-CD3 plus anti-CD28 plus SFA (1 + 2 + SFA), or anti-CD3 plus anti-CD28 plus rapamycin (1 + 2 + R). Abs and drugs were then removed, and the cells were rested for 5–7 days. Next, the cells were rechallenged with anti-CD3 plus anti-CD28, and evaluated for IL-2. *C*, SFA and rapamycin arrest cell cycle progression with different mechanisms. A.E7 cells were left untreated (M) or stimulated 16 h with anti-CD3 (1+), anti-CD3 plus anti-CD28 (1 + 2), anti-CD3 plus anti-CD28 plus SFA (1 + 2 + SFA), or anti-CD3 plus anti-CD28 plus rapamycin (1 + 2 + R). The expression of $p27^{kip1}$ and cyclin D3 was analyzed by Western blot. The blot was then stripped and reblotted for β-actin as a loading control. *D*, Rapamycin, but not SFA, inhibits mTOR activity. A.E7 cells were stimulated for 16 h with mock (M), anti-CD3 alone (1+), anti-CD3 plus anti-CD28 (1 + 2), anti-CD3 plus anti-CD28 plus SFA (1 + 2 + SFA), or anti-CD3 plus anti-CD28 plus rapamycin (1 + 2 + R). Phospho-S6K-1 was analyzed by Western blot. The blot was then stripped and reblotted for total S6K-1 as a loading control. Data are representative of three independent experiments.

prompted us to examine the effects of both rapamycin and SFA on $p27^{Kip1}$ expression (Fig. 1*C*). In resting A.E7 cells, $p27^{Kip1}$ levels were quite high at baseline (mock), they decreased with anti-CD3 (signal 1) stimulation, and this decrease was enhanced by the addition of costimulation in the form of anti-CD28 (signal 2). Interestingly, SFA was able to inhibit the ability of costimulation to down-modulate $p27^{Kip1}$ expression, whereas rapamycin did not. That is, upon stimulation, $p27^{Kip1}$ levels were higher in the cells treated with SFA than the cells treated with rapamycin. The inability of rapamycin to block the initial reduction of $p27^{Kip1}$ upon activation is consistent with a recent report by Colombetti et al. (15), who also reported that the up-regulation of cyclin D3 is mitigated in anergic cells. In addition to the down-regulation of $p27^{Kip1}$, G_1-S phase transition in T cells is also facilitated by up-regulation of cyclin D3 (15). Thus, we examined the effect of SFA and rapamycin on cyclin D3 expression. As seen in Fig. 1*C*, cyclin D3 expression increased with TCR stimulation and was further up-regulated by the addition of costimulation. Consistent with their ability to block proliferation, both SFA and rapamycin inhibited the up-regulation of cyclin D3. In fact, although rapamycin did not up-regulate $p27^{Kip1}$, rapamycin proved to be a potent inhibitor of cyclin D3 expression. Thus, in our model, anergy induction correlates better with reduced cyclin D3 levels than with increased $p27^{Kip1}$ levels.

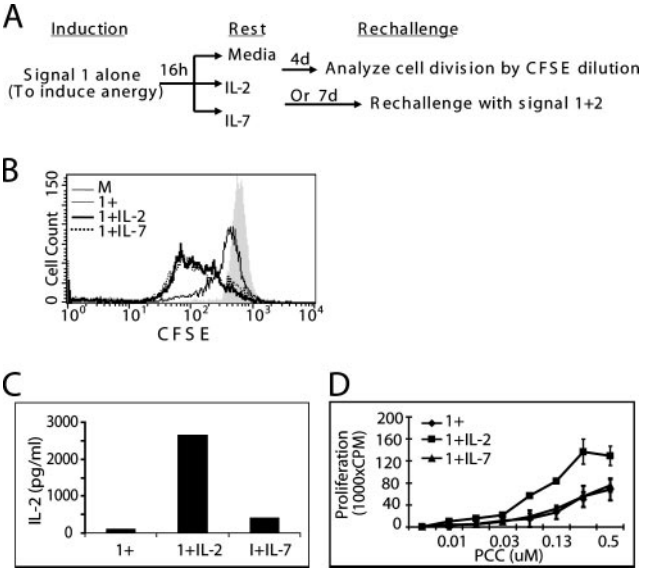

FIGURE 2. Proliferation does not necessarily reverse T cell anergy. *A*, Experimental scheme. *B*, Anergic cells proliferate in both IL-2 and IL-7. CFSE-labeled A.E7 cells were anergized by plate-bound anti-CD3, and rested in medium alone (1+) or medium supplemented with either IL-2 (1 + IL-2) or IL-7 (1 + IL-7). Cell division was analyzed by CFSE dilution. A.E7 cells that were mock stimulated and rested in medium were used as a control (M). *C* and *D*, IL-2, but not IL-7, reverses anergy. A.E7 cells were treated as in *A*. After a 7-day rest, the cells were extremely washed with PBS to remove residual cytokines, followed by rechallenge. IL-2 production (*C*) and proliferation (*D*) were analyzed. Data are representative of three independent experiments.

The expression of the cyclins is regulated by the PI3K-related kinase family member mTOR (15). mTOR has multiple downstream targets. For example, the activation of mTOR is typically measured by the phosphorylation of one of its downstream substrates S6K-1 (5, 16–19). As shown in Fig. 1*D*, and consistent with our previous findings, rapamycin, but not SFA, inhibited mTOR activity, as determined by the phosphorylation of S6K-1 at Thr^{421}/Ser^{424}. There is a basal level of S6K-1 phosphorylation in mock-stimulated cells. TCR stimulation alone induced a modest increase in phospho-S6K-1 levels. In contrast, TCR stimulation in conjunction with costimulation led to a significant up-regulation of phopho-S6K-1, suggesting enhanced mTOR activity. This enhancement was not affected by SFA, but completely inhibited by rapamycin. Overall, these sets of experiments suggest that the induction of anergy might be best correlated with the inhibition of mTOR rather than actual cell cycle progression.

Activation of mTOR reverses T cell anergy

To pursue the potential association between mTOR inhibition and anergy, we next examined mTOR activity during anergy reversal. T cell anergy is not a terminally differentiated state and can be reversed by IL-2. Boussiotis et al. (20) have proposed that signals from common γ-chain ILs regulate T cell activation vs anergy. IL-7R shares the common γ-chain with the IL-2R and has been implicated in regulating cell size and glucose metabolism in a PI3K-mTOR-dependent manner (21). Thus, we wanted to determine whether IL-7 could reverse anergy.

CFSE-labeled A.E7 cells were treated by anti-CD3 alone overnight to induce anergy (Fig. 2*A*). Next, the cells were rested in medium alone, or medium supplemented with IL-2 or IL-7, and proliferation was analyzed by CFSE dilution 4 days later. The anergic cells rested in medium alone did not proliferate (Fig. 2*B*).

Downloaded from http://www.jimmunol.org/ by guest on August 9, 2022

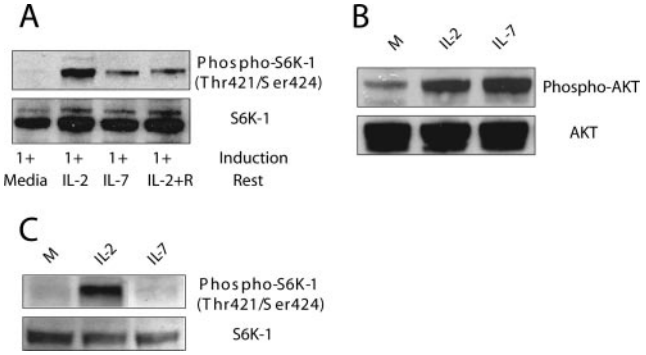

FIGURE 3. *A*, mTOR activity is associated with anergy reversal. A.E7 cells were anergized by plate-bound anti-CD3 and rested in medium alone (1+) or medium supplemented with either IL-2 (1 + IL-2), IL-7 (1 + IL-7), or IL-2 plus rapamycin (1 + IL-2 + R). After 96 h, mTOR activity was analyzed by phosphorylation of S6K-1. The blot was then stripped and reblotted for total S6K-1 as a loading control. *B*, Both IL-2 and IL-7 activate AKT. Preactivated 5C.C7 T cells were left untreated (M) or treated for 30 min with IL-2 or IL-7 at the same doses used in anergy reversal (Fig. 2); phosphorylation of AKT Ser^{473} was assessed. The blot was then stripped and reprobed for total AKT. *C*, IL-2, but not IL-7, phosphorylates S6K-1 at Thr^{421}/Ser^{424}. Preactivated 5C.C7 T cells were left untreated (M) or treated for 24 h with IL-2 or IL-7 at the same doses as in *B*; phosphorylation of S6K-1 Thr^{421}/Ser^{424} was assessed. The blot was then stripped and reprobed for total S6K-1. Data are representative of two independent experiments.

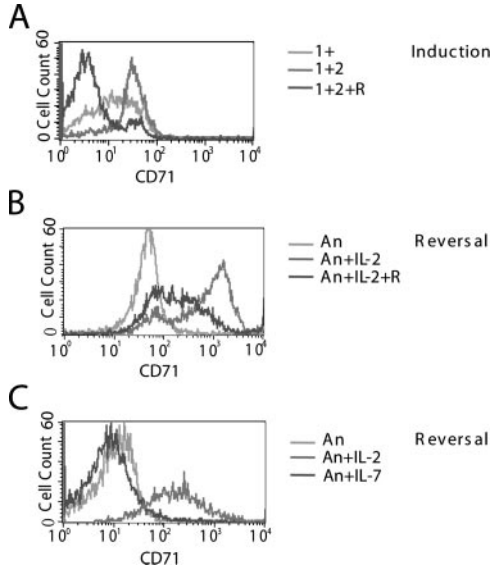

FIGURE 4. CD71 surface expression correlates with mTOR activity in T cells. *A*, The cell surface expression of CD71 increases upon activation. A.E7 cells were stimulated with anti-CD3 alone (1+), anti-CD3 plus anti-CD28 (1 + 2), or anti-CD3 plus anti-CD28 plus rapamycin (1 + 2 + R), and the cell surface expression of CD71 was analyzed 16 h later by FACS. *B* and *C*, The surface expression of CD71 increases when anergy is reversed. Anergic A.E7 cells were rested in medium alone (An), medium with IL-2 (An + IL-2), or medium with IL-2 plus rapamcyin (An + IL-2 + R), and CD71 surface expression was analyzed 24 h later (*B*). Alternatively, anergic A.E7 cells were rested in medium alone (An), medium with IL-2 (An + IL-2), or medium with IL-7 (An + IL-7) for 7 days, and CD71 surface expression was examined (*C*). Data are representative of three independent experiments.

In contrast, the anergic cells in either IL-2 or IL-7 proliferated vigorously. Thus, with regard to the ability to promote proliferation, both IL-2 and IL-7 were equally effective. Next, we compared the ability of IL-2 and IL-7 to reverse anergy (Fig. 2, *C* and *D*). The anergic cells that were rested in medium alone failed to produce IL-2 or proliferate upon rechallenge; they remained anergic. In contrast, anergy was reversed when the cells were rested in IL-2 because upon rechallenge they produced IL-2 and proliferated. Interestingly, although the cells rested in IL-7 proliferated to the same extent as the cells with IL-2, these cells were still anergic when they were rechallenged.

The observation that IL-7 supported proliferation, but did not reverse anergy clearly demonstrates that proliferation alone does not necessarily lead to anergy reversal. Next, we wanted to examine the status of mTOR activation in the anergic cells that proliferated to IL-2 or IL-7. As shown in Fig. 3*A*, there was no phospho-S6K-1 (Thr^{421}/Ser^{424}) in the anergic cells that were rested in medium. In contrast, the addition of IL-2 led to an increase in S6K-1 phosphorylation, which was inhibited by rapamycin. In contrast, IL-7 failed to fully phosphorylate S6K-1, and the levels of phospho-S6K-1 were equivalent to that induced by IL-2 plus rapamycin. Although proliferation alone is not sufficient to reverse anergy, it is the ability of IL-2 to promote mTOR activation as determined by phospho-S6K-1 (Thr^{421}/Ser^{424}) that is associated with anergy reversal.

The inability of IL-7 to activate mTOR as measured by phospho-S6K-1 (Thr^{421}/Ser^{424}) is in contrast to previous reports that IL-7 stimulation activated AKT, which is upstream of mTOR (22, 23). To address this issue, we first wanted to insure that our findings were not idiosyncratic to anergic T cell clones. To this end, primary T cells from 5C.C7 mice were tested. Thus, T cells from 5C.C7 mice were harvested and activated with PCC peptide for 7 days to create previously activated cells. The preactivated 5C.C7 T cells were then left untreated or treated with IL-2 or IL-7. As seen in Fig. 3*B*, consistent with previous reports, both IL-7 and IL-2 activate AKT (22, 23). Interestingly, at later time points (for example, 24 h), IL-7-induced AKT phosphorylation was nearly back to baseline, whereas IL-2-induced phosphorylation was still present (data not shown). Importantly, in these primary T cells, although IL-7 augmented phospho-AKT expression, it did not promote mTOR activation as measured by phosphorylation at Thr^{421}/Ser^{424} (Fig. 3*C*).

Thus, whereas IL-7 promotes the phosphorylation of AKT (Fig. 3*B*) and can induce the phosphorylation of S6K-1 at Thr^{389} (data not shown) (24), unlike IL-2, IL-7 does not promote mTOR-induced phospho-S6K-1 at Thr^{421}/Ser^{424}, which is associated with the reversal of anergy.

Transferrin receptor expression correlates with mTOR activity in T cells

mTOR is known to control a wide range of growth- and proliferation-related cellular events. It has been reported recently that the loss of cell surface transporters for glucose, amino acids, low-density lipoproteins, and iron transferrin resulting from growth factor withdrawal occurs in an mTOR-dependent fashion (25). Among these transporters, the iron transferrin receptor CD71 has been shown to be up-regulated on activated T cells (26). As such, CD71 represents a potential cell surface marker for mTOR activation in T cells, and thus a means of distinguishing activated from anergic T cells. We undertook a series of experiments to examine the cell surface expression of CD71 during anergy induction and full activation.

We first analyzed the cell surface expression profile of CD71 during anergy induction (Fig. 4*A*). A.E7 cells were stimulated with anti-CD3 and anti-CD3 plus anti-CD28 in the absence or presence of rapamycin, and cell surface expression of CD71 was analyzed

Downloaded from http://www.jimmunol.org/ by guest on August 9, 2022

by FACS. Although the cell surface expression of CD71 is clearly detectable on the cells undergoing anergy induction (anti-CD3 alone), when the cells were stimulated with anti-CD3 plus anti-CD28 the cell surface expression of CD71 was markedly increased. That is, the cell surface expression levels of CD71 could distinguish cells stimulated with signal 1 alone vs signals 1 + 2. The cell surface expression of CD71 is mTOR dependent, as demonstrated by the fact that treatment with rapamycin inhibits its expression. Thus, the relative cell surface expression of CD71 can distinguish T cells in which TCR engagement occurs in the context of costimulation vs those in which Ag recognition occurs in the absence of costimulation.

Because mTOR activity is associated with anergy induction as well as anergy reversal, the cell surface expression of CD71 during anergy reversal was also examined (Fig. 4*B*). Anergic A.E7 cells exhibited relatively low levels of CD71, which was quickly up-regulated in the presence of IL-2. Just as rapamycin inhibited IL-2-mediated anergy reversal, the IL-2-induced CD71 cell surface expression was also down-regulated by rapamycin. Consistent with our findings that IL-7 does not reverse anergy (Fig. 2, *C* and *D*) and does not promote mTOR activation leading to the phosphorylation of S6K-1 at Thr^{421}/Ser^{424} (Fig. 3, *A* and *C*), CD71 levels remained low on the anergic cells rested in IL-7 (Fig. 4*C*). Taken together, these data demonstrate that the cell surface expression of CD71 not only correlates with mTOR activity in T cells, but also the functional phenotype with regard to anergy.

Rapamycin promotes T cell anergy by inhibiting mTOR

Thus far, we have been able to demonstrate an association between mTOR activity leading to the phosphorylation of S6K-1 at Thr^{421}/Ser^{424} and full T cell activation. We next wanted to directly test the ability of mTOR activation to prevent anergy induction. Consistent with the role of mTOR in promoting full T cell activation is the observation that inhibiting mTOR with rapamycin promotes T cell anergy even in the presence of costimulation. To prove that rapamycin promotes anergy by inhibiting mTOR, we transfected T cells with a RR-mTOR mutant.

Rapamycin associates with mTOR through the FKBP12-rapamycin binding domain. Ser^{2035} within this domain is critical for the interaction, and mutations at this site eradicate the ability of rapamycin to bind mTOR such that the mutant mTOR is resistant to rapamycin-induced inhibition (27). Jurkat cells were transfected with an empty lentivirus vector construct, WT-mTOR, or RR-mTOR. As demonstrated in Fig. 5*A*, in the absence of rapamycin, the constitutive mTOR activity in Jurkat cells (which is a lymphoma cell line) was equivalent in all the transfected cells (*top row*). In contrast, rapamycin at a concentration of 30 nM inhibited phosphorylation of S6K-1 in the Jurkat cells transfected with empty lentivirus vector or WT-mTOR (*middle row*). In contrast, even in the presence of 100 nM rapamycin, phospho-S6K-1 could still be detected in the cells expressing RR-mTOR (*bottom row*). If the ability of rapamycin to promote T cell anergy was in fact due to its ability to inhibit mTOR, then we would expect that T cells expressing the RR-mTOR would be resistant to rapamycin-induced anergy. To test this hypothesis, T cells from 5C.C7 TCR transgenic $Rag2^{-/-}$ mice were infected with WT- or RR-mTOR recombinant lentivirus, and stimulated with anti-CD3 plus anti-CD28 plus rapamycin at 0, 30, and 100 nM. After a 5-day rest, the infected cells were sorted and rechallenged with APCs plus PCC peptide. As seen in Fig. 5, *B* and *C*, rapamycin promoted anergy in the WT-mTOR-expressing cells in a dose-dependent fashion such that upon rechallenge (in the absence of any rapamycin), these cells proliferated less and produced less IL-2. Alternatively, the cells that were transfected with the RR-mTOR construct proliferated and produced IL-2 equivalently to their nonrapamycin-treated counterparts. These data indicate that it is the ability of rapamycin to inhibit mTOR activation that is responsible for promoting anergy in the presence of costimulation. Therefore, mTOR is required for T cell activation, and TCR engagement in the setting of mTOR inhibition results in anergy.

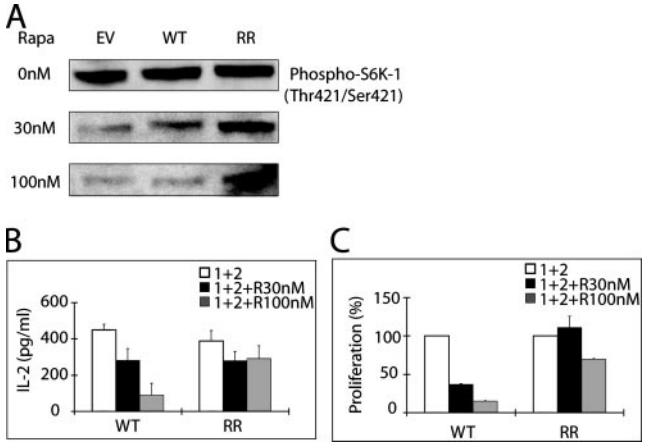

FIGURE 5. Rapamycin promotes T cell anergy by inhibiting mTOR. *A*, Overexpression of RR-mTOR leads to S6K-1 phosphorylation at Thr^{421}/Ser^{424} even in the presence of rapamycin. Jurkat cells were transfected with empty lenti-vector (EV), lenti-WT-mTOR (WT), or lenti-RR-mTOR (RR), and then treated with rapamycin at 0, 30, and 100 nM for 2 h. Phosphorylation of S6K-1 was analyzed by Western blot. *B* and *C*, Overexpression of RR-mTOR prevents rapamycin-induced anergy. The 5C.C7 T cells were infected with WT- or RR-mTOR lentivirus, and stimulated with anti-CD3 plus anti-CD28 (1 + 2), or anti-CD3 plus anti-CD28 plus rapamycin (1 + 2 + R, 30 or 100 nM). After a 5-day rest, the cells were rechallenged with APCs plus PCC peptide. IL-2 production (*B*) and proliferation (*C*) upon rechallenge were assessed. Data are representative of three independent experiments.

TCR engagement in the absence of mTOR activation leads to anergy in vivo

In vitro, signals 1 and 2 can be precisely controlled. In vivo, however, the induction of T cell activation vs tolerance is most likely determined by the integration of multiple positive and negative accessory signals. To test the hypothesis that mTOR activation plays a critical role in integrating these signals in vivo, we adopted a well-established in vivo peptide-induced anergy model (28). Briefly, CFSE-labeled 5C.C7 T cells ($CD4^+V\beta3^+$) were adoptively transferred into syngeneic B10.A hosts (day 0). The next day, the mice were challenged with soluble PCC peptide (to induce anergy; tolerant) or PCC peptide plus LPS (to promote activation; primed). Also, a cohort of the primed mice received daily rapamycin beginning on day 0 (primed plus R). On day 4, T cells were harvested, and expansion of the transferred clonotypic 5C.C7 cells was analyzed by CFSE dilution (Fig. 6*A*). First, in the absence of Ag (naive), there was very little proliferation of the clonotypic cell in vivo (R1 gate). In contrast, there was marked proliferation of the cells transferred into the primed mice. Importantly, even though there was less proliferation, essentially all of the clonotypic cells in the tolerant and primed plus R mice proliferated at least once (R2 gate), indicating that essentially 100% of the cells had seen Ag. As seen in Fig. 6*B*, when equal numbers of clonotypic cells were rechallenged in vitro with APCs plus PCC peptide, the cells from mice treated with peptide alone (tolerant) were hyporesponsive compared with the cells from peptide plus LPS-treated mice (primed). Additionally, as is the case with our in vitro model, the

Downloaded from http://www.jimmunol.org/ by guest on August 9, 2022

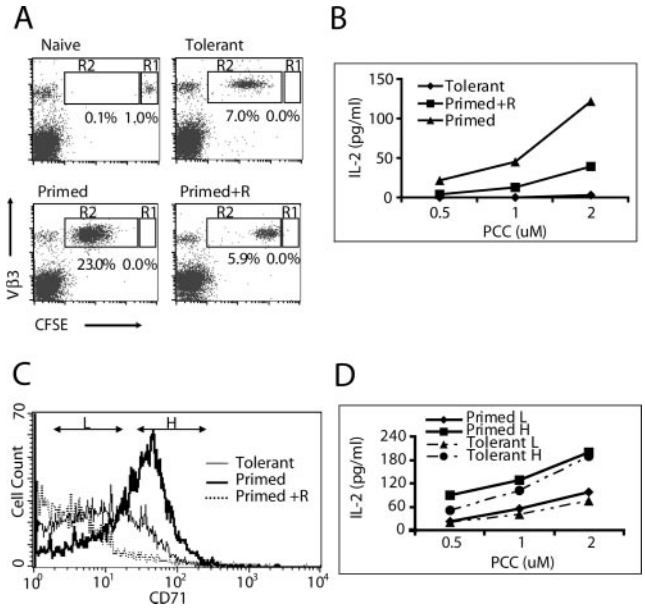

FIGURE 6. TCR engagement in the absence of mTOR activity leads to anergy in vivo. *A*, Adoptively transferred 5C.C7 T cells encountered Ags in vivo. CFSE-labeled 5C.C7 T cells ($CD4^+V\beta3^+$) were adoptively transferred into syngeneic B10.A hosts. The mice were then challenged with soluble PCC peptide (Tolerant), PCC peptide plus LPS (Primed), or PCC peptide plus LPS plus rapamycin (Primed + R). Mice that received 5C.C7 cells only (Naive) were used as controls. Division of the transferred 5C.C7 cells was analyzed by CFSE dilution 4 days later. R1 represents clonotypic 5C.C7 cells that did not proliferate in vivo (CFSE high). R2 represents 5C.C7 cells that expanded in response to the Ag challenge in vivo (CFSE low). Percentages of the cells in each gate are listed. *B*, Rapamycin induces anergy in vivo. CFSE low 5C.C7 T cells from the conditions depicted above were sorted and rechallenged in vitro with APCs plus PCC peptide, and IL-2 production was assessed. *C*, CD71 surface expression is up-regulated in activated T cell in vivo. The cell surface expression of CD71 on 5C.C7 CFSE low T cells (gate R2) was analyzed by FACS. *D*, CD71 surface expression distinguishes activated from anergic cells. The 5C.C7 CFSE low T cells (gate R2) from the tolerant and primed mice were sorted based on the surface expression levels of CD71, as indicated in *C* (labeled as Primed H, Primed L, Tolerant H, and Tolerant L, respectively), and rechallenged in vitro with APCs plus PCC peptide; IL-2 production was analyzed. Data are representative of three independent experiments.

cells that were given full activation in the presence of rapamycin (primed plus R) were rendered anergic. These data support the notion that in vivo TCR engagement in the setting of mTOR inhibition also leads to anergy.

Next, we wanted to determine whether we could use the cell surface expression of CD71 as a means of identifying cells that were rendered anergic in vivo. As seen in Fig. 6*C*, cell surface expression of CD71 was highest in primed cells and was decreased in cells isolated from tolerant and primed plus R mice. Clearly, there was overlap of CD71 surface expression between the activating and tolerizing conditions. We interpret these observations as indicating that in the tolerizing conditions, some of the cells encountered Ag in the context of mTOR activation. Likewise, in the priming conditions, some of the cells encountered Ag in the absence of mTOR activation. In other words, even under the priming condition some of the cells were anergized, and conversely, even under the tolerizing condition some of the cells were fully activated. To test this hypothesis, we sorted the clonotypic 5C.C7 cells from tolerant or primed mice based upon the expression levels of CD71 (tolerant L, tolerant H, primed L, and primed H), and rechallenged these cells in vitro with APCs plus PCC peptide. As can be seen in Fig. 6*D*, CD71 high expressing cells produced higher levels of IL-2 upon rechallenge regardless of whether the cells were derived from primed or tolerant mice. Likewise, CD71 low expressing cells consistently produced lower levels of IL-2 regardless of whether they were derived from primed or tolerant mice. That is, cells that were activated in vivo in the setting of decreased mTOR activity (as marked by CD71 surface expression) behaved as if they were anergic when rechallenged under fully activating conditions in vitro. Inasmuch as the CD71 surface expression levels reflect mTOR activation, these data support the notion that in vivo TCR engagement in the setting of high mTOR activity leads to full activation, whereas TCR engagement in the setting of decreased mTOR activity leads to tolerance.

Discussion

The relationship between cell cycle blockade and anergy induction is complex. Several groups have proposed that the cell cycle inhibitor $p27^{Kip1}$ plays a role in promoting anergy, whereas in other models, including our own, this does not appear to be the case (9, 10, 12, 14, 29, 30). Using the novel cyclophilin ligand SFA, we showed that G_1 phase arrest does not necessarily lead to anergy (Fig. 1) (13). Furthermore, IL-7-mediated proliferation of anergic cells did not reverse anergy, suggesting that cell cycle progression alone is not sufficient for anergy reversal either. Interestingly, at the biochemical level, it is the activity of mTOR as measured by phosphorylation of S6K-1 at Thr^{421}/Ser^{424} that correlates best with the T cell function in term of activation vs anergy. In this regard, it will be of interest to determine whether mTOR activity is inhibited in models of anergy for which $p27^{Kip1}$ plays an important role.

The fact that T cells overexpressing RR-mTOR were resistant to anergy induction provides evidence that the underlying mechanism by which rapamycin promotes anergy is dependent on its ability to inhibit mTOR. This pharmacogenetic approach clearly identifies mTOR activity as playing a role in the decision between full activation vs tolerance. In addition, using a well-established mouse anergy model, we demonstrated that mTOR inhibition by rapamycin also promotes anergy in vivo and that by using cell surface expression of CD71 as a surrogate for mTOR activity we can isolate cells that underwent productive stimulation vs nonproductive stimulation in vivo.

The outcome of Ag recognition is not dictated by the nature of peptide-TCR interaction (signal 1), but rather the context in which this interaction occurs. In the inflammatory milieu, APCs will upregulate costimulatory molecules, which in turn promote full T cell activation. However, even in this setting, the outcome of TCR engagement will be influenced by cytokine and chemokine stimulation as well as the concomitant engagement of negative receptors such as CTLA-4, PD-1, and the potential presence of regulatory T cells (31). Therefore, just as TOR plays a critical role in sensing signals from growth factors, nutrients, and energy, we propose that mTOR senses the environment for the presence and absence of danger (Fig. 7). In this fashion, mTOR plays a central role in integrating both stimulating and inhibitory signals to execute the appropriate T cell response. We propose that signal 2 represents the net sum of positive and negative signals, which is integrated by mTOR. Based on this model, we predict that signaling pathways that promote mTOR activation (as measured by phosphorylation of S6K-1 at Thr^{421}/Ser^{424}) will also promote T cell activation, whereas those that inhibit mTOR will promote anergy.

Although our data define a role for mTOR in the decision between T cell activation and anergy, the precise downstream substrates responsible for this regulation have yet to be elucidated. The phosphorylation of S6K-1 is routinely used as a measure of

Downloaded from http://www.jimmunol.org/ by guest on August 9, 2022

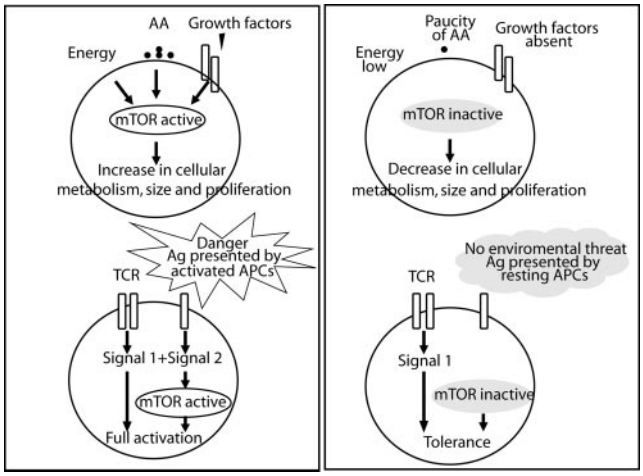

FIGURE 7. A model for the role of mTOR in regulating T cell activation vs tolerance. In mammalian cells, mTOR integrates environmental cues in terms of nutrients, energy, and growth factors, and directs cell growth and proliferation. In the presence of these factors, mTOR is activated and promotes an increase in protein synthesis, cell size, and proliferation (*upper left*). In contrast, in the absence of these factors, mTOR is inactive, leading to a decrease in protein synthesis, cell size, and proliferation (*upper right*). By analogy, we propose that in T cells, mTOR integrates environmental cues in terms of the presence or absence of danger. In the setting of danger such as an infection, Ags are presented by activated APCs (*lower left*). In this context, TCR engagement occurs concomitant with mTOR activation, and hence, a full immune response ensues; signal 1 plus mTOR activation leads to full T cell activation. Alternatively, if Ags (perhaps self peptides) are presented by resting APCs in the absence of danger (*lower right*), mTOR remains inactive. As a result, the T cells are rendered tolerant; signal 1 in the absence of mTOR activation leads to tolerance.

mTOR function (5, 16–19). S6K-1 has multiple phosphorylation sites; however, we have determined that phosphorylation at Thr^{421}/Ser^{424} best corresponds with mTOR activity as it relates to T cell function. In contrast, phosphorylation at Thr^{389} of S6K-1 is more promiscuous, occurring in response to a wide variety of activating stimuli, including IL-7 (24) (data not shown). This highlights the point that the downstream consequences of mTOR activation are dependent upon the inducing stimulus. For example, it has been shown that the phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (another downstream target of mTOR activation) at specific sites can be differentially regulated depending on whether mTOR is activated via insulin or amino acid (32). Interestingly, it has been reported that mTOR does not phosphorylate S6K-1 directly, but rather it acts indirectly by preventing its dephosphorylation by protein phosphatase 2A (33). Of interest, protein phosphatase 2A has been shown to associate with CD28 and CTLA-4 and negatively regulate T cell activation (34).

Finally, our findings have practical implications in terms of developing novel strategies to treat autoimmune disorders and the prevention of graft rejection. Unlike cyclosporin A (CSA) and FK506, rapamycin does not inhibit TCR signaling, and therefore is not a very potent single agent immunosuppressant. However, the ability of rapamycin to promote tolerance makes it a potentially powerful component of an immunosuppressive regimen. Currently, solid organ transplant patients must remain on CSA for life because, although CSA prevents rejection, it also prevents tolerance induction. In experimental models, CSA blocks rapamycin-induced tolerance (35). However, regimens combining noncalcineurin inhibitors (such as costimulatory blockade) with mTOR inhibitors might be able to promote tolerance such that patients are not destined to lifelong immunosuppressive therapy (35, 36). To this end, we have used rapamycin in a mouse model of stem cell transplantation to promote long-term tolerance in the absence of long-term immunosuppression (37).

Acknowledgments

We thank Drs. Xiaobing Yu and Jie Chen for the lentivirus and mTOR constructs; James Flook for high-speed flow sorting; and Dr. Jun O. Liu, Hyam I. Levitsky, Charles G. Drake, Christopher J. Gamper, and Christian F. Meyer for critical review of the manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Schwartz, R. H. 1992. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell* 71: 1065–1068.
2. Hay, N., and N. Sonenberg. 2004. Upstream and downstream of mTOR. *Genes Dev.* 18: 1926–1945.
3. Tokunaga, C., K. Yoshino, and K. Yonezawa. 2004. mTOR integrates amino acid- and energy-sensing pathways. *Biochem. Biophys. Res. Commun.* 313: 443–446.
4. Chen, J. 2004. Novel regulatory mechanisms of mTOR signaling. *Curr. Top. Microbiol. Immunol.* 279: 245–257.
5. Harris, T. E., and J. C. Lawrence, Jr. 2003. TOR signaling. *Sci. STKE* 2003: re15.
6. Powell, J. D., C. G. Lerner, and R. H. Schwartz. 1999. Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation. *J. Immunol.* 162: 2775–2784.
7. Vanasek, T. L., A. Khoruts, T. Zell, and D. L. Mueller. 2001. Antagonistic roles for CTLA-4 and the mammalian target of rapamycin in the regulation of clonal anergy: enhanced cell cycle progression promotes recall antigen responsiveness. *J. Immunol.* 167: 5636–5644.
8. Gilbert, K. M., and W. O. Weigle. 1993. Th1 cell anergy and blockade in G_1a phase of the cell cycle. *J. Immunol.* 151: 1245–1254.
9. Powell, J. D., D. Bruniquel, and R. H. Schwartz. 2001. TCR engagement in the absence of cell cycle progression leads to T cell anergy independent of $p27^{Kip1}$. *Eur. J. Immunol.* 31: 3737–3746.
10. Boussiotis, V. A., G. J. Freeman, P. A. Taylor, A. Berezovskaya, I. Grass, B. R. Blazar, and L. M. Nadler. 2000. $p27^{kip1}$ functions as an anergy factor inhibiting interleukin 2 transcription and clonal expansion of alloreactive human and mouse helper T lymphocytes. *Nat. Med.* 6: 290–297.
11. Wells, A. D., M. C. Walsh, D. Sankaran, and L. A. Turka. 2000. T cell effector function and anergy avoidance are quantitatively linked to cell division. *J. Immunol.* 165: 2432–2443.
12. Colombetti, S., F. Benigni, V. Basso, and A. Mondino. 2002. Clonal anergy is maintained independently of T cell proliferation. *J. Immunol.* 169: 6178–6186.
13. Allen, A., Y. Zheng, L. Gardner, M. Safford, M. R. Horton, and J. D. Powell. 2004. The novel cyclophilin binding compound, sanglifehrin A, disassociates G_1 cell cycle arrest from tolerance induction. *J. Immunol.* 172: 4797–4803.
14. Jackson, S. K., A. DeLoose, and K. M. Gilbert. 2001. Induction of anergy in Th1 cells associated with increased levels of cyclin-dependent kinase inhibitors $p21^{Cip1}$ and $p27^{Kip1}$. *J. Immunol.* 166: 952–958.
15. Colombetti, S., V. Basso, D. L. Mueller, and A. Mondino. 2006. Prolonged TCR/CD28 engagement drives IL-2-independent T cell clonal expansion through signaling mediated by the mammalian target of rapamycin. *J. Immunol.* 176: 2730–2738.
16. Chung, J., C. J. Kuo, G. R. Crabtree, and J. Blenis. 1992. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell* 69: 1227–1236.
17. Kim, D. H., D. D. Sarbassov, S. M. Ali, J. E. King, R. R. Latek, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini. 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 110: 163–175.
18. Kim, D. H., D. D. Sarbassov, S. M. Ali, R. R. Latek, K. V. Guntur, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini. 2003. GβL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. *Mol. Cell* 11: 895–904.
19. Stromberg, T., A. Dimberg, A. Hammarberg, K. Carlson, A. Osterborg, K. Nilsson, and H. Jernberg-Wiklund. 2004. Rapamycin sensitizes multiple myeloma cells to apoptosis induced by dexamethasone. *Blood* 103: 3138–3147.
20. Boussiotis, V. A., D. L. Barber, T. Nakarai, G. J. Freeman, J. G. Gribben, G. M. Bernstein, A. D. D'Andrea, J. Ritz, and L. M. Nadler. 1994. Prevention of T cell anergy by signaling through the γc chain of the IL-2 receptor. *Science* 266: 1039–1042.
21. Rathmell, J. C., E. A. Farkash, W. Gao, and C. B. Thompson. 2001. IL-7 enhances the survival and maintains the size of naive T cells. *J. Immunol.* 167: 6869–6876.
22. Pallard, C., A. P. A. Stegmann, T. van Kleffens, F. Smart, A. Venkitaraman, and H. Spits. 1999. Distinct roles of the phosphatidylinositol 3-kinase and STAT5 pathways in IL-7-mediated development of human thymocyte precursors. *Immunity* 10: 525–535.

Downloaded from http://www.jimmunol.org/ by guest on August 9, 2022

23. Barata, J. T., A. Silva, J. G. Brandao, L. M. Nadler, A. A. Cardoso, and V. A. Boussiotis. 2004. Activation of PI3K is indispensable for interleukin 7-mediated viability, proliferation, glucose use, and growth of T cell acute lymphoblastic leukemia cells. *J. Exp. Med.* 659–669.
24. Brown, V. I., J. Fang, K. Alcorn, R. Barr, J. M. Kim, R. Wasserman, and S. A. Grupp. 2003. Rapamycin is active against B-precursor leukemia in vitro and in vivo, an effect that is modulated by IL-7-mediated signaling. *Proc. Natl. Acad. Sci. USA* 15113–15118.
25. Edinger, A. L., and C. B. Thompson. 2002. Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake. *Mol. Biol. Cell* 13: 2276–2288.
26. Pattanapanyasat, K., and T. G. Hoy. 1991. Expression of cell surface transferrin receptor and intracellular ferritin after in vitro stimulation of peripheral blood T lymphocytes. *Eur. J. Haematol.* 47: 140–145.
27. Chen, J., X. F. Zheng, E. J. Brown, and S. L. Schreiber. 1995. Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. *Proc. Natl. Acad. Sci. USA* 92: 4947–4951.
28. Pape, K. A., E. R. Kearney, A. Khoruts, A. Mondino, R. Merica, Z. M. Chen, E. Ingulli, J. White, J. G. Johnson, and M. K. Jenkins. 1997. Use of adoptive transfer of T-cell-antigen-receptor-transgenic T cell for the study of T-cell activation in vivo. *Immunol. Rev.* 156: 67–78.
29. Rowell, E. A., M. C. Walsh, and A. D. Wells. 2005. Opposing roles for the cyclin-dependent kinase inhibitor $p27^{kip1}$ in the control of $CD4^+$ T cell proliferation and effector function. *J. Immunol.* 174: 3359–3368.
30. Verdoodt, B., T. Blazek, P. Rauch, G. Schuler, A. Steinkasserer, M. B. Lutz, and J. O. Funk. 2003. The cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p21^{Cip1}$ are not essential in T cell anergy. *Eur. J. Immunol.* 33: 3154–3163.
31. Ichikawa, M., and L. Chen. 2005. Role of B7-H1 and B7-H4 molecules in down-regulating effector phase of T-cell immunity: novel cancer escaping mechanisms. *Front. Biosci.* 10: 2856–2860.
32. Wang, X., A. Beugnet, M. Murakami, S. Yamanaka, and C. G. Proud. 2005. Distinct signaling events downstream of mTOR cooperate to mediate the effects of amino acids and insulin on initiation factor 4E-binding proteins. *Mol. Cell. Biol.* 25: 2558–2572.
33. Peterson, R. T., B. N. Desai, J. S. Hardwick, and S. L. Schreiber. 1999. Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycin associated protein. *Proc. Natl. Acad. Sci. USA* 96: 4438–4442.
34. Chuang, E., T. S. Fisher, R. W. Morgan, M. D. Robbins, J. M. Duerr, M. G. Vander Heiden, J. P. Gardner, J. E. Hambor, M. J. Neveu, and C. B. Thompson. 2000. The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A. *Immunity* 13: 313–322.
35. Larsen, C. P., E. T. Elwood, D. Z. Alexander, S. C. Ritchie, R. Hendrix, C. Tucker-Burden, H. R. Cho, A. Aruffo, D. Hollenbaugh, P. S. Linsley, et al. 1996. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* 381: 434–438.
36. Hale, D. A., R. Gottschalk, T. Maki, and A. P. Monaco. 1998. Determination of an improved sirolimus (rapamycin)-based regimen for induction of allograft tolerance in mice treated with antilymphocyte serum and donor-specific bone marrow. *Transplantation* 65: 473–479.
37. Powell, J. D., C. Fitzhugh, E. M. Kang, M. Hsieh, R. H. Schwartz, and J. F. Tisdale. 2005. Low-dose radiation plus rapamycin promotes long-term bone marrow chimerism. *Transplantation* 80: 1541–1545.

Downloaded from http://www.jimmunol.org/ by guest on August 9, 2022