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# Review: mTOR at the crossroads of T cell proliferation and tolerance

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## Abstract

Several events control the activation, proliferation, and the continued Ag responsiveness of naïve and memory T lymphocytes. Here we review the individual contributions of TCR, CD28, and IL-2-driven signaling to T cell proliferation and anergy avoidance. The role of mTOR as a rheostat capable of integrating extracellular, plasma membrane-associated, and intracellular signals with relevance to T cell priming and tolerance is discussed.

## Introduction

Mature, naïve CD4+ T cells traffic between secondary lymphoid organs, constantly scanning resident or recently migrated dendritic cells (DC) for the presence of a high affinity peptide/MHC ligand. Recognition of such a complex by their Ag receptor (TCR) leads to their activation. Nevertheless, 'activation' is an imprecise term that fails to convey either the context of the Ag-recognition event, or the dynamic nature and eventual outcome of the response. During infection, the recognition of new peptide/MHC complexes on DC elicits an aggressive expansion of the clone and its differentiation to an effector cell phenotype best suited to promoting the clearance of the pathogen via help for CD8+ T cell-dependent cellular cytotoxicity and/or B cell-dependent antibody production. Such adaptive immunity also provides for the generation and maintenance of memory after clearance of the pathogen, with an increased frequency of Ag-experienced CD4+ T cells maintaining a heightened level of Ag-responsiveness again homing between secondary lymphoid organs. In stark contrast, naïve CD4+ T cell Ag recognition in the absence of infection or tissue injury predicts an autoreactive TCR specificity and is viewed by the immune system as a risk for immunopathology. Consequently, T cell activation in this context allows for only an abortive clonal expansion and differentiation of the clone, and instructs daughter T cells to lose their Ag-responsiveness, a state referred to here as clonal anergy.

Thus, a strong correlation exists between the degree of activation and extent of clonal expansion following a primary recognition of Ag, and the level of Ag responsiveness retained by daughter cells at the conclusion of the response. In this review, we will consider in depth the relationship between T cell activation, clonal expansion, and the avoidance of clonal anergy. Furthermore, we will discuss the individual contributions of the TCR, CD28, and IL-2 receptors to this regulation. Finally, we will suggest that the mammalian target of rapamycin (mTOR) has the capacity to integrate these discrete T cell activation events and control the long-term outcome of Ag recognition.

## CD28 regulation of TCR-proximal events

At the onset of infection, tissue-resident DC are in a position to endocytose pathogen-derived proteins and process them into peptides within an MHC class II-containing vesicle. Pathogen-associated molecular patterns (e.g., lipopolysaccharides or unmethylated CpG-containing DNA) expressed by an increasingly abundant infecting agent also bind to Toll-like receptors on the DC and trigger a signaling cascade that: a) puts a halt to further Ag processing and presentation, thus locking the pathogen-derived peptide/MHC complexes on their surface, b) induces the expression of the B7 family costimulatory ligands CD80 and CD86, and c) leads to the CCR7-dependent migration of the DC away from the infection and toward the draining lymph node [1].

The Ag-specific naïve T cell has the ability to recognize a remarkably small number of relevant peptide/MHC complexes on the surface the DC within secondary lymphoid organs. TCR ligation, followed by the internalization of that particular TCR/CD3 complex, allows for serial TCR and peptide/MHC engagements [2]. Over a period of minutes to hours, the T cell will scan MHC molecules on one or more Ag-bearing DC, keeping count of the number of TCR internalization events. When a threshold is reached, the T cell undergoes a growth response and enters the cell cycle (G0->G1 phase). Importantly, the simultaneous recognition of B7 family costimulatory ligands along with the relevant peptide/MHC complex on these highly activated DC can lower the threshold for G0->G1 commitment and speed the rate of cell growth. Once committed to undergoing this blastogenesis, the T cell forms a tight association with the Ag-bearing DC, and there begins an accumulation of TCR complexes and other associated signaling molecules within an area of plasma membrane immediately adjacent to the Ag presenting cell (APC) called the immunological synapse [3-5].

TCR (and CD4) ligation by relevant peptide/MHC class II complexes is sufficient to trigger a proximal tyrosine kinase cascade including Lck and ZAP70, with resultant tyrosine phosphorylation of key TCR-associated signaling proteins including TCRζ, LAT, and phospholipase C-γ (PLC-γ) [6]. One consequence of early TCR-dependent signaling is the rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) [7-9]. Rising IP3 levels provoke a release of $Ca^{2+}$ from intracellular stores and trigger an influx of calcium ions through calcium release-activated calcium (CRAC) channels. High intracellular [$Ca^{2+}$] then leads to an increase in the activity of the calcineurin protein phosphatase and promotes the dephosphorylation and nuclear mobilization of the nuclear factor of activated T cells (NF-AT) [10]. The accumulation of DAG and $Ca^{2+}$ adjacent to the immunological synapse also promotes the binding and activation of the Ras-GRP guanine nucleotide exchange factor for p21Ras [11]. As a consequence, nearby Ras molecules are preferentially GTP-loaded and bound by Raf, with resultant activation of the downstream MEK1 and ERK mitogen-activated protein kinases (MAPK) [12].

GTP-loaded Ras may also have some modest capacity to directly regulate phosphatidylinositol 3-kinase (PI3K) activity in the absence of CD28 ligands [13]. However, CD28 ligation and signaling strongly reinforces proximal TCR-induced signaling events through a feed-forward loop involving the recruitment of the p85 PI3K regulatory domain [14-16]. Following Ag recognition, tyrosine kinase activation leads to phosphorylation of the membrane proximal YMNM motif within the cytoplasmic domain of CD28 and the subsequent SH2-dependent recruitment and activation of the full p85/p110 PI3K holoenzyme. The CD28 YMNM also serves as a docking site for the Grb2 and Gads adapter proteins [17]. Assembly of this signaling cluster (PI3K, Grb2, GADS) at ligated CD28 molecules then leads to a Vav-1- and CDC42/Rac1-dependent reorganization of the cytoskeleton that hastens the movement of kinase- and lipid-rich raft microdomains into the nascent immunological synapse [18,19]. The endpoint of this CD28-dependent membrane and signaling protein trafficking appears to be the

development of a mature immunological synapse with a characteristic T cell plasma membrane topological organization: TCR and CD28 molecules enriched within a central supramolecular activation cluster (c-SMAC) bordered by a peripheral ring of integrin molecules (p-SMAC) [3,4].

The function of this mature immunological synapse with its c-SMAC and p-SMAC molecular organization remains controversial [20,21]. Nevertheless, the appearance of tight associations between T cells and DC in vivo within a similar time frame, together with the observations in vitro of recruitment and activation of pleckstrin homology domain (PH)-containing proteins such as protein kinase C-θ (PKC-θ), Vav-1, and Akt to the PI3K-generated phosphatidylinositol 3,4,5-trisphosphate (PIP3) phospholipid, and close association with lipid raft-associated signaling proteins such as PDK1 and CARD11 at the c-SMAC [22,23], have led us to speculate that the PI3K-dependent formation of the SMAC architecture facilitates both a more durable TCR and CD28 signal transduction across the plasma membrane via as well as a broadened repertoire of downstream activation events. Through this mechanism, CD28 YMNM motif-dependent costimulatory signals enhance the activation of PKC-θ and Akt [24,25]. Perhaps in an analogous manner, these CD28 costimulatory signals synergize with the TCR to promote the activation of c-Jun N-terminal kinase (JNK) and p38 [26,27].

## CD28 regulation of CD4+ T cell commitment to cell cycle

What then are the relevant endpoints of this TCR- and CD28-signal transduction to the commitment of T cells to enter the cell cycle? Within the first few minutes of stimulation, activated Akt molecules directly phosphorylate TSC2 (tuberin) reducing its GTPase activity toward Rheb, a GTP-binding protein that regulates the activity mTOR [28-32]. The end result is an increase in the protein kinase activity of the mTOR/raptor complex (mTOR complex 1, or mTORC1) for the translation initiation factor 4E-binding protein 1 (4E-BP1) and for p70S6K (Fig 1.). Phosphorylation of 4E-BP1 abrogates its ability to bind and inhibit the eukaryotic initiation factor 4E, and facilitates the translation of 5’ cap-dependent mRNAs in preparation for cell division. Likewise, mTORC1-dependent phosphorylation and activation of p70S6K and its downstream S6 ribosomal protein component promotes the translational of polypyrimidine tract-containing mRNAs. Such rapid up-regulation of new protein translation is a hallmark of cell cycle entry and subsequent blastogenesis in preparation for cell division, and both activities are sensitive to inhibition with Rapamycin [33,34]. Examples of this regulation of protein translation include a number of nutrient receptors (e.g., CD71 for iron, CD98 for amino acids) that are up-regulated on the T cell surface via the activation of PI3K and mTOR [35]. Consistent with a role for mTORC1 in this process, Rapamycin prevents the growth factor-dependent expression of both CD71 and CD98, and a constitutively active mTOR mutant supports the expression of CD98 even in the absence of growth factors [36-38].

In contrast to mTORC1, an activated mTORC2 complex containing mTOR and rictor together with an SIN1 regulatory molecule is relatively insensitive to Rapamycin, and can directly phosphorylate Akt to increase its activity against a number of select substrates as a form of positive feedback regulation [39-41]. Once Akt is fully activated in this manner, it can phosphorylate the forkhead box nuclear factor FOXO1 and cause its translocation away from the nucleus, its association with 14−3−3, and its proteasomal degradation [31,42]. In the absence of nuclear FOXO1, transcription of the *Cdkn1b* gene fails and the level of the cyclin dependent kinase (Cdk) inhibitor p27Kip1 protein becomes dependent solely on its rate of turnover. Akt also stimulates T cell synthesis of the Glut1 glucose transporter and promotes glycolysis, maintaining a high level of ATP even in the face of decreased mitochondrial oxidative metabolism and increased fatty acid and amino acid anabolic metabolism [43]. Increased ATP and, consequently, reduced availability of AMP limits the activity of AMP-

activated protein kinase (AMPK). In the absence of CD28-dependent costimulatory signals and Akt-induced glycolysis, rising AMP levels trigger AMPK to directly phosphorylate TSC2, increasing its capacity to repress mTOR [44,45].

Rapid induction of *Fos* and *Jun* mRNA and protein expression is another prerequisite for cell cycle entry following Ag recognition. TCR ligation is generally sufficient to activate ERK and induce a phosphorylation of Elk-1 at the *Fos* gene that initiates transcription [46,47]. On the other hand, CD28- and Vav-1-dependent p38 activation is necessary for optimal phosphorylation of a second *Fos*-associated transcription factor called CREB, and for the secondary recruitment of the p300 and CBP histone acetyltransferases to this nuclear factor [27,48]. Heterologous reporter gene studies have also suggested that CD28 signaling can enhance the function of a c-terminal domain within CBP [47]. Consistent with these results, TCR ligation in the presence of CD28 costimulatory signals is associated with a hyperacetylation of nucleosomal histone H4 at the *Fos* promoter and increased association with RNA polymerase II. Increased accessibility of nuclear factors to hyperacetylated *Fos* chromatin together with more efficient RNA polymerase II-dependent transcript elongation appear responsible for the enhanced transcription of *Fos* mRNA observed in the presence of costimulatory signals [47]. Finally, CD28-dependent activation of JNK can ensure the optimal phosphorylation of c-Jun and increase its capacity to transactivate [26]. Thus, the combination of CD28-costimulated *Fos* gene expression and the phosphorylation of c-Jun can lead to both enhanced AP-1 abundance and function.

Once active AP-1 heterodimers accumulate in the nucleus, they can be expected to bind to the *Ccnd1* gene promoter and up-regulate the transcription of cyclin D1 [49]. *Ccnd3* mRNA may also undergo translational up-regulation as a consequence of mTORC1 activity, given its inhibition during T cell activation in the presence of Rapamycin [38,50]. Newly expressed cyclin D molecules bind and activate Cdk4 and Cdk6. Active cyclin D/Cdk4 and cyclin D/Cdk6 protein kinase complexes then phosphorylate the nuclear repressor retinoblastoma (Rb), causing its release of the E2F transcription factor and allowing for the transactivation of *Ccne1*. Cyclin D/Cdk4 and cyclin D/Cdk6 also sequester p27Kip1 and prevent it from antagonizing the function of the newly produced cyclin E/Cdk2 protein kinase complexes. Cyclin E/Cdk2 then has the opportunity to phosphorylate p27Kip1 and induce its proteasomal degradation prior to the end of G1 phase. Each of these cyclin-dependent events is enhanced in the presence of CD28 costimulatory signals and is sensitive to inhibitors of both PI3K and MAPK pathways [51].

Within one hour of initial stimulation, TCR and CD28 signaling events synergize for the induction of IL-2 synthesis. Successful PI3K-dependent recruitment of PKC-θ and PDK1 into the c-SMAC appears to be accompanied by the assembly of a Bcl10/MALT1/CARD11 signaling complex capable of binding and activating the multi-subunit IκB kinase [23,24]. Increased Cot kinase activity in the setting of CD28 costimulation and Akt activation also promotes the phosphorylation and ubiquitin-mediated proteolytic degradation of IκB, the release of sequestered p65 NF-κB, and the translocation of NF-κB to the nucleus where it can bind to the 5' *Il2* enhancer/promoter in association with NF-AT and AP-1 [52]. During CD28 costimulation, increased JNK and p38 activities (in conjunction with ERK) facilitate the transcription of the *Il2* gene by inducing increased Fos and Jun (AP-1) protein expression, and through augmentation of transactivation by both c-Jun itself as well as members of the p300/CBP family of nuclear co-activator proteins (as described above). Finally, CD28 costimulatory signals mediated by a C-terminal proline-rich motif (PYAP) that can bind SH3-containing proteins (e.g., Lck, Fyn, Itk and Grb2) promote IL-2 synthesis and proliferation by reducing the turnover of *Il2* mRNA molecules, perhaps as a consequence of increased JNK activity [25,53].

Consistent with an important role for increased autocrine IL-2 production in the regulation of blastogenesis, *Il2*-deficient CD4+ T cells demonstrate slowed G0->G1 cell cycle entry in vitro [50]. Therefore, it is likely that CD28 signaling from within the c-SMAC ensures an optimal rate of IL-2 synthesis during the recognition of Ag, and IL-2 binding to its receptor can overcome a checkpoint for the entry of T cells into cell cycle. It should be noted that IL-2 is not absolutely required for clonal expansion in vivo; nevertheless, *Il2*−/− T cells still require CD28 ligands to achieve their optimal proliferative response [54]. Whether another cytokine is available in vivo that can substitute for IL-2 in this response remains unknown. Regardless, one can expect that after several hours of Ag stimulation and the up-regulation of IL-2Rα molecules in response to TCR and CD28 costimulation, binding of IL-2 to the high affinity IL-2R (including IL-2Rβ and γc chains) activates STAT5, PI3K and Ras, thus sustaining the downstream activation of Akt, mTOR, NF-κB, and AP-1.

## Control of G1->S phase cell cycle progression

Like cell cycle entry, the progression of CD4+ T cells from G1->S phase is also carefully regulated following the recognition of Ag by both CD28 and the IL-2R [55]. Once cell growth is sufficient to ensure the survival of daughter cells following cell division, replication of chromosomal DNA can begin. Progression beyond the G1/S phase checkpoint depends on the successful degradation of p27Kip1 by cyclin E/Cdk2-mediated phosphorylation and targeting for ubiquitin-mediated proteasomal degradation [56]. Unopposed cyclin E/Cdk2 kinase activity further phosphorylates Rb, thus reinforcing the ability of E2F to transactivate for cyclin A expression. Once synthesized, cyclin A/Cdk2 appears to direct the progression of cells beyond the G1/S phase checkpoint, and DNA replication initiates, with subsequent rapid movement through the cell cycle (S, G2, and M phase) and then return to G1 where the levels of cyclins and Cdk inhibitors are re-assessed [57,58].

In T cells, mTOR has also been identified as one downstream target of IL-2-dependent signaling that is linked to cycle progression because of its role in Cdk2 and Cdc2 kinase activation, p27Kip1 down-regulation, and G1/S checkpoint transition [59,60]. A direct role for mTOR in progression beyond G1 and cell division is further supported by its physical association with newly synthesized aurora B kinase and the inhibitor of apoptosis family protein survivin [61]. The expression of both aurora B and survivin depends on CD28 and PI3K, and can also be triggered by IL-2 binding to its receptor. Interestingly, the kinase activity of this mTOR/aurora B/survivin complex has target specificity similar to mTORC1 and is similarly sensitive to inhibition by Rapamycin. Based on results obtained with both CD28−/− T cells and with T cells transfected with aurora B mutant constructs, hyperphosphorylation of Rb, expression of cyclin A, and the activation of cyclin A/Cdk2, as well as G1->S phase cell cycle progression, require the function of mTOR in this complex with aurora B and survivin.

The investigation of G1->S phase cell cycle progression in T cells has been greatly accelerated by the development of the flow cytometric CFSE dye-dilution method [62]. Gett and Hodgkin took advantage of the technique in vitro and confirmed that CD28 costimulatory signals decrease the threshold for entry into cell cycle, resulting in a shortened time to first cell division [63]. Nevertheless, they found no evidence that the CD28 molecule regulates the rate of subsequent cell divisions. We, too, found an inverse correlation between the strength of TCR and CD28 signaling and the average time necessary to enter into the cell cycle (and by extension, the time to first cell division) [64]. However, blasting CD28−/− T cells also demonstrated a decreased rate of cell cycle progression in our experiments. This pattern of slowed cell division was also seen with the addition of either a PI3K inhibitor or Rapamycin, even when their addition was delayed for 24 hrs following initial Ag stimulation [55]. Likewise, cell division by Ag-stimulated T cells could be aborted even 24 hours into the response by the neutralization of IL-2 and either Ag/MHC complexes or CD80/CD86. These data have led us

to the hypothesis that throughout a clonal expansion, T cells remain sensitive to the level of TCR/CD28 and IL-2R signaling.

This notion of late control over the rate of cell division in CD4+ T cells is controversial. It is well established that CD8+ T cells exposed to Ag for less than 24 hours can be induced to undergo a very durable clonal expansion response both in vivo and in vitro [65,66]. Similar 'programming' of CD4+ T cells for durable proliferation, independent of ongoing Ag recognition, has also been suggested [67]. However, experiments designed to control the levels of an experimental transgenic Ag in vivo using a tetracycline-inducible promoter have indicated that CD4+ T cells remain aware of the presence of Ag throughout their clonal expansion [68]. We have observed a similar abortion of the cell division response within 24 hrs following mAb neutralization of the relevant peptide/MHC complex, both in vivo (unpublished observation), and in vitro in cases where IL-2 secretion is functionally impaired [50,55]. Taken together, these results support the hypothesis that CD4+ T cells remain aware of the presence of Ag, B7 molecules, and cytokines, throughout their clonal expansion. Only when CD4+ T cells detect the continued presence of these ligands can G1->S phase progression continue at an optimal rate.

We have recently reported that in the presence of optimal autocrine IL-2 secretion and/or exogenously provided IL-2, Rapamycin delays, but cannot prevent, later cell divisions [50]. In fact, Rapamycin appears most effective for preventing cell divisions that are induced under conditions of relatively low IL-2 availability [50,56]. One additional key feature of this late regulation of cell cycle progression now appears to be new STAT5-dependent activation of *Pim2* gene expression [69-71]. Synthesis of this constitutively active PIM-2 serine-threonine kinase can support continued blastogenesis even when mTOR activity wanes, through its capacity to independently maintain nutrient uptake and ATP synthesis at a high level. Like Akt, PIM-2 also regulates cell survival during blastogenesis with its ability to phosphorylate and counter-regulate the proapoptotic protein BAD. Nevertheless, this mTOR-independent proliferation cannot be sustained in the absence of IL-2R-induced PI3K activity. Thus, it is reasonable to suggest that mTOR-induced signaling regulates the rate of initial cell cycle entry and progression beyond the first G1/S phase checkpoint as dictated by TCR/CD28 engagement, but becomes dispensable later on once significant quantities of IL-2 have been synthesized [50,64]. In either case (mTOR- or PIM-2-dependent proliferation), CD4+ T cells only continue their high rate of cell division and clonal expansion if Ag and CD28 ligands are present on APC, or IL-2 remains readily available.

## Induction of T cell anergy as a mechanism for peripheral tolerance

As discussed above, complete activation of the T cell via the TCR and CD28 leads to an IL-2-dependent cell growth, multiple rounds of mitotic cell division, and eventually differentiation into effector and memory lymphocytes (Fig. 2). In contrast, suboptimal T cell activation elicits only an abortive clonal expansion, and favors the induction of a state of T cell unresponsiveness called clonal anergy. This term was originally used by Nossal and Pike to describe B cell tolerance in vivo [72], and later on was adapted by Schwartz and co-workers to describe a state of unresponsiveness in cloned murine T cells that could be induced by costimulation-defective APC in the presence of Ag [73,74]. Chronic engagement of the TCR or the CD3 complex in the absence of CD28-driven costimulation, suboptimal TCR engagement by altered peptide ligands (APL), or low density ligands, and inhibition of cell cycle progression and cell division in properly activated T cells [75-80] have all been shown to favor the establishment of clonal anergy. Anergic cells are characterized by the inability to transcribe and translate the *Il2* gene and to proliferate in response to Ag re-encounter [74,81]. Ag unresponsiveness has also been described in vivo in peripheral lymphoid organs, and implicated in the regulation of peripheral T cell tolerance [82-85]. The molecular events dictating T cell anergy in vivo have similarities

to those described in vitro; however, several differences have been described [86]. For this reason the anergic phenotype found in vivo has been referred to as adaptive tolerance [87].

Among the various models of Ag unresponsiveness, the clonal anergy state that is induced by the chronic engagement of the TCR or of its CD3 subunit in the absence of costimulatory receptor occupancy has been the best characterized [74,81]. Several aspects of this form of T cell anergy can be mimicked by treatment of T cells with a calcium ionophore (Ionomycin), which elicits the Ca2+-dependent activation of calcineurin and downstream NF-AT-dependent gene regulatory events. Consistent with this, the induction of clonal anergy can be interrupted in the presence of Cyclosporine A, an inhibitor of calcineurin [9,88]. Inhibitors of new protein synthesis also prevent the development of unresponsiveness, suggesting that anergy-favoring conditions result in the transcription and translation of anergy-associated genes (anergy factors) with the capacity to antagonize TCR-dependent signaling, transcription, and/or cell cycle progression [89]. The possibility that anergy factors are induced during the course of T cell activation, and can interfere with T cell activation in a dominant fashion is further supported by the finding that the fusion of anergic and non-anergic T cells results in a T cell hybrid with an anergic phenotype [90].

Work by Rao and coworkers has confirmed that the translocation of NF-AT to the nucleus in the absence of an activation of other transcription factors such as AP-1 or NF-κB leads to the induction of numerous putative anergy-related proteins [91]. Among these are several phosphatases capable of counteracting TCR-induced kinase activities, the diacylglycerol kinase α (DKGα) that is involved in the metabolism of the second messenger diacylglycerol, as well as genes encoding proteases and E3 ubiquitin ligases [91]. Likewise, mRNA microarray analyses comparing the gene expression profiles of resting T cells, and of cells activated by optimal TCR and CD28 ligation in the presence or absence of Cyclosporine A, have identified an upregulation of the Egr2 and Egr3 zing finger transcription factors in anergic T cells, and have shown that these anergy factors can inhibit T cell activation at least in part through the transactivation of counter-regulatory proteins such as Cbl-b [92-94]. These data strongly support the hypothesis that TCR occupancy drives the expression of anergy-related gene products that act to limit the responsiveness of T cells to Ag. They also predict that in some manner the accumulation or function of these anergy factors is normally opposed during a fully immunogenic stimulation in the presence of CD28/IL-2-dependent signals.

## T cell anergy implementation and maintenance

One function of such anergy-related proteins appears to be a dampening of proximal receptor-associated signaling during Ag re-encounter. Anergic T cells have several alterations in TCR-associated early signaling events consistent with this possibility. Increased constitutive Fyn tyrosine kinase activity has been observed in anergic T cells together with decreased TCR/CD28-induced Lck and ZAP-70 tyrosine kinase activities, thus leading to unique patterns of protein tyrosine phosphorylation (e.g., CD3ζ, LAT, c-Cbl) both at rest and upon Ag re-challenge [95-97]. Such alterations in upstream activation events likely contribute to impairments in the activation of Ras, ERK and JNK reported in anergic T cells [98-101].

Tyrosine phosphatases such as SHP-1 and SHP-2 [102], as well as the E3 ubiquitin ligases c-Cbl, Cbl-b, GRAIL, and Itch (reviewed in [103]) are suitable candidates as anergy factors as they are able to dampen TCR activation and are induced under anergy-favoring conditions. Over-expression of E3 ligases found in anergic T cells has revealed a capacity of these molecules to promote the ubiquitin-mediated degradation of key signaling molecules during T cell activation, such as CD3ζ, PLC-γ, PKC-θ, and Ras-GAP, resulting in premature dissolution of the immunological synapse [104]. Likewise, preferential activation of c-Cbl by Fyn in anergic T cells has correlated with a recruitment of Raf protein kinase to Rap1 rather

than Ras, possibly blocking signal transduction to the MAPK [105]. Anergy-associated overexpression of the lipid kinase DGKα also favors the conversion of diacylglycerol to phosphatidic acid, limiting the ability of RasGRP to load Ras with GTP and interfering with the activation of PKC-θ [106]. Such TCR-proximal counter-regulatory events, therefore, are likely to contribute to the defects in AP-1 and NF-κB activation that have been observed in anergic cells [101,107]. Without effective binding of NF-κB and AP-1 nuclear factors together with NF-AT at the 5' *Il2* enhancer/promoter, transcription of *Il2* cannot proceed efficiently. The preferential binding of transcriptional repressors (CREB-CREM, Tob-Smad, Ikaros) to the *Il2* gene in anergic T cells has also been described and may directly interfere with its transcriptional activation [108,109].

The suboptimal generation of TCR/CD28-dependent intracellular signaling events may account for the failure to degrade p27Kip1 and the G1 cell cycle arrest that has been observed in anergic T cells responding to an Ag rechallenge [110-112]. Without sufficient induction of AP-1 complexes during Ag rechallenge, the stimulation of cyclin D up-regulation in early G1 may be suboptimal and lead to an ineffective sequestration of p27Kip1. We have observed an additional defect in the phosphorylation and activation of Akt in anergic T cells (unpublished observation), and this may also predispose to uncontrolled FOXO1-mediated transactivation and increased p27Kip1 protein synthesis. A failure to down-regulate p27Kip1 in the absence of strong TCR/CD28 and/or IL-2R signaling may play an important role in the induction of T cell tolerance; however, it seems unlikely to fully account for the maintenance of the anergic state. T cells lacking normal p27Kip1 molecules demonstrate uncontrolled phosphorylation of Smad3 by cyclin E/Cdk2 complexes and anergy induction cannot occur in the absence of this counter-regulatory nuclear factor. Nevertheless, in both responsive and Ag unresponsive anergic T cells p27Kip1 is found to be similarly degraded upon TCR occupancy [113,114]. Furthermore, optimal CD3 and CD28 mAb stimulation of anergic T cells induces them to undergo several rounds of cell division, yet retain their unresponsive phenotype [114]. Thus, the data indicate that p27Kip1 participates in the induction of an anergic phenotype, but increased p27Kip1 cannot in itself account for blocked IL-2 production and proliferation.

In contrast to the findings with p27Kip1, forced expression of a constitutively active Ras is unable to prevent the induction of an anergic phenotype [115], yet is sufficient to restore MAPK signaling and overcome the block in IL-2 production in T cells made anergic in vivo or in vitro [106]. Likewise, pharmacological inhibition of the DGKα that is present at higher levels in anergic T cells and appears responsible for the inhibition of the Ras/MAPK pathway, restores the capacity of the T cells to produce IL-2 on Ag re-challenge [106]. These data support the hypothesis that multiple counter-regulatory mechanisms are put into place in anergic T cells to dampen both proximal and distal signals induced by TCR and CD28 that lead to IL-2 production and cell cycle entry, as well as to inhibit anergy reversal pathways.

## Factors controlling the reversal of anergy

The critical event in Ag-stimulated T cell activation that is most important for the avoidance of clonal anergy remains to be determined. Remarkably, treatment of Ag-stimulated T cells with either Rapamycin or the histone deacetylase inhibitor n-Butyrate slows cell cycle progression at the G1a phase and promotes the development of T cell anergy [79]. In contrast, drugs that interfere with G0->G1 cell cycle entry, or cell cycle progression at the G1b or S->G2 phases do not impede Ag responsiveness [80]. Although it was originally proposed that CD28-derived signals would directly antagonize the induction of anergic factors by TCR signals, it is now clear that anergy can also be induced in CD28-costimulated T cells by interfering with IL-2- and IL-2R-driven cell division [78]. The finding that a provision of exogenous IL-2 at the time of anergy induction, or even later after the establishment of T cell anergy, preserves and restores Ag responsiveness, respectively, further supports a direct role

for IL-2/IL-2R-generated signaling in the avoidance of clonal anergy [78,116,117]. Like the avoidance of anergy during Ag stimulation, the reversal of clonal anergy relies on an IL-2-driven, mTOR-dependent, and Rapamycin-sensitive signaling pathway [114]. Anergy reversal was originally attributed to the ability of IL-2 to promote cell division; however, IL-2-dependent reversal of clonal anergy can occur independently of mitosis [118], suggesting that cell cycle per se is not sufficient for anergy reversal. Likewise, forced proliferation of anergic cells does not restore Ag responsiveness [114], which instead relies on signaling through the common gamma chain of the IL-2R [117]. These results have led us to the hypothesis that anergy factors are induced by the TCR early following Ag-recognition, and are subsequently degraded or inhibited in response to IL-2R signaling when the T cell cycles through late G1 phase.

## mTOR in T cell priming and tolerance induction

While dispensable for cell expansion, mTOR appears to be critical for the regulation of Ag responsiveness in CD4+ T cells. Indeed, mTOR inhibition by rapamycin drives properly activated cloned Th1 T cells into clonal anergy [80,118], and prevents IL-2-mediated anergy reversal [114]. Interestingly, both TCR/CD28 and IL-2 elicit Rapamycin-sensitive signaling in anergic T cells. However, while either type of stimulus drives cell cycle progression, only the latter is able to reverse clonal anergy [50,114]. This is best explained by the observation that mTOR is only suboptimally induced by CD3/CD28 engagement in anergic T cells as compared to normal effector T cells (unpublished data). Furthermore, proliferation and anergy reversal appear to require a different threshold of mTOR signaling. While cell cycle progression may require only a relatively low level of mTOR activation, anergy avoidance/reversal may require much more intense or prolonged mTOR signaling that can only be achieved by IL-2/IL-2R. Alternatively mTORC1 activity is necessary but not sufficient to reverse clonal anergy, and a separate IL-2-specific signaling event is required during G1 phase to preserve Ag-responsiveness.

Whether mTOR directly antagonizes the expression or activity of any anergy gene remains to be established. One suitable candidate is the transcription factor Smad3. In T cells, the transcriptional activity and the nuclear localization of Smad3 are controlled by Cdk2 and cdc2-mediated phosphorylation. These are activated upon p27Kip1 degradation [111], which in turn is sensitive to mTOR activity [119]. Thus, mTOR signaling might control Cdk2- and Cdc2-dependent Smad3 phosphorylation and inactivation by driving p27Kip1 degradation. mTOR-signaling also suppresses Smad3 activation induced by TGFβ [119]. Thus, proper mTOR activation might be needed to counter-act TGFβ-mediated Smad3 activation. In addition to Smad3, Egr might also be a suitable anergy-related factor controlled by mTOR, as its expression is induced by TCR-mediated events, inhibited by Cyclosporine A, and favored by Rapamycin [93]. Further studies are currently needed to verify these possibilities and unequivocally identify whether a target for mTOR-dependent phosphorylation is directly involved in the induction and maintenance of clonal anergy.

It should be noted that naïve T cells are relatively resistant to anergy induction and generally require chronic Ag exposure in the absence of infection or adjuvant to develop unresponsiveness [120]. Brief encounters of naïve T cells with Ag under conditions of mTOR inhibition may instead modulate T cell differentiation. Indeed, we have found that the activation of naïve T cells by Ag-bearing APC or by anti-CD3/CD28 mAb in the presence of Rapamycin results in a delayed but sustained cell division, but in the absence of Th1 or Th2 phenotype polarization and acquisition of effector function (unpublished data). Recent experiments have suggested that repeated Ag encounter in the absence of proper mTOR signaling results in the induction of T lymphocytes with suppressive activity [121,122]. Thus, rather than simple inhibition of T cell clonal expansion or the induction of Ag unresponsiveness, the

immunosuppressive effect that Rapamycin can exert on naïve T cells might be attributed to a block in Ag-driven T helper cell differentiation paralleled by the acquisition of suppressive function. The finding that mTOR regulates histone acetylation, [123,124] supports a possible involvement for this pathway in cell differentiation.

Acquisition of such a T regulatory cell phenotype may further reinforce the development of clonal anergy in nearby T responder cells that are undergoing chronic Ag stimulation. Regulatory T cell function is associated with the induction of indoleamine 2,3-dioxygenase (IDO) within the APC, leading to a degradation and depletion of available tryptophan [125]. During blastogenesis, an accumulation of uncharged tRNAs as a consequence of amino acid deprivation leads to an activation of the GCN2 kinase, the phosphorylation of eIF2α, and the interruption of mRNA translation initiation for many genes important to cell cycle progression. In yeast, high TOR kinase activity promotes the phosphorylation of GCN2 and desensitizes this kinase to uncharged tRNA, thus antagonizing this counter-regulatory pathway [126,127]. Nevertheless, the depletion of tryptophan that results from the induction of IDO by regulatory T cells appears sufficient to increase the activity of GCN2 within Ag-stimulated T cells and cause their poor proliferation [128]. Taken together with the observation in vivo that CD25+ Foxp3+ regulatory T cells can facilitate a development of unresponsiveness following chronic exposure to Ag [129], these results predict that the phosphorylation of GCN2 is one mechanism by which mTOR signaling protects against clonal anergy development and promotes its reversal.

## Conclusions

Recent data indicate that the degree and quality of the initial T cell activation events profoundly influence the ability of the T cells to respond to a second Ag encounter. We would propose that mTOR functions as a rheostat capable of integrating information from the microenvironment where Ag recognition is taking place, both in the form of ligands for surface cytokine and costimulatory receptors, as well as nutrients necessary to meet the high metabolic demands of a proliferating cell. Weak mTOR kinase activity in the setting of limited costimulatory signals or poor amino acid availability might be sufficient to drive at least a modest T cell clonal expansion in response to Ag, but will also allow for the accumulation and activation of anergy genes, and thus result in the establishment of T cell anergy or of a suppressive phenotype. Because of defective TCR-dependent proximal signaling, re-encounter with Ag might further impede mTOR signaling in anergic T cells, and both contribute to the proliferative unresponsiveness of the T cell as well as prevent the reversal of the anergic phenotype. In contrast, optimal mTOR induction by IL-2R signaling at the time of T cell activation or once anergy has been established limits the expression and/or activity of the anergy-related genes and serves to prepare the T cell for a re-encounter with its Ag. A thorough in vivo dissection of the molecular mechanisms involved in the induction and reversal of Ag unresponsiveness is now needed to correctly evaluate the immunomodulatory activity of Rapamycin.

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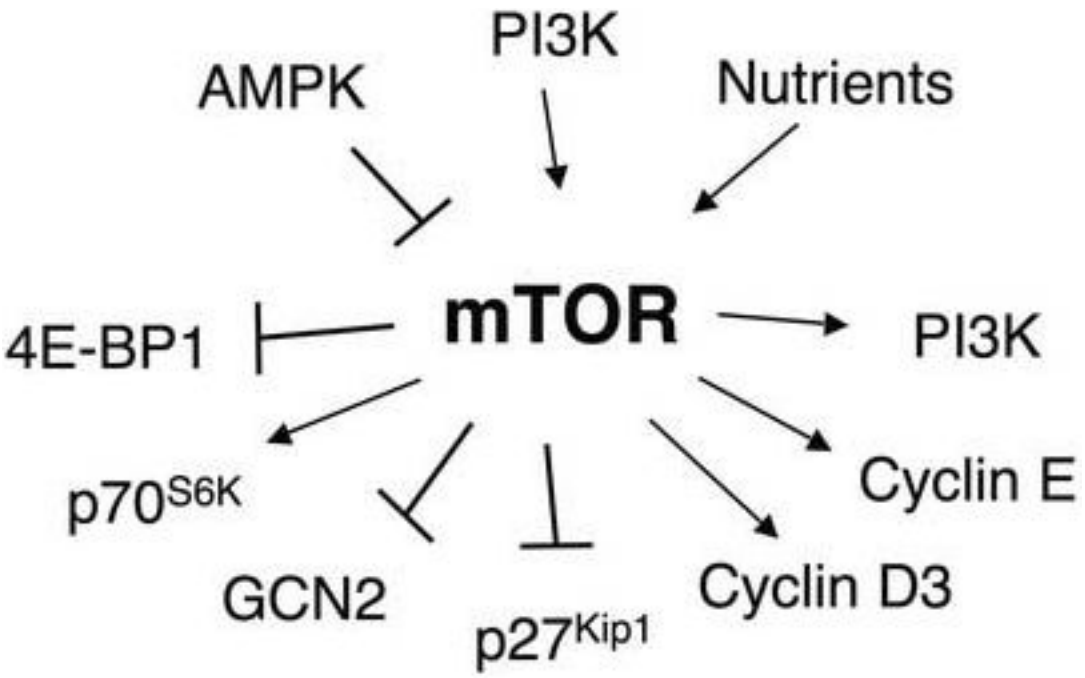


**Figure 1.**
mTOR relationships in T cells. Arrows are stimulatory interactions, perpendicular lines indicate inhibitory interactions.

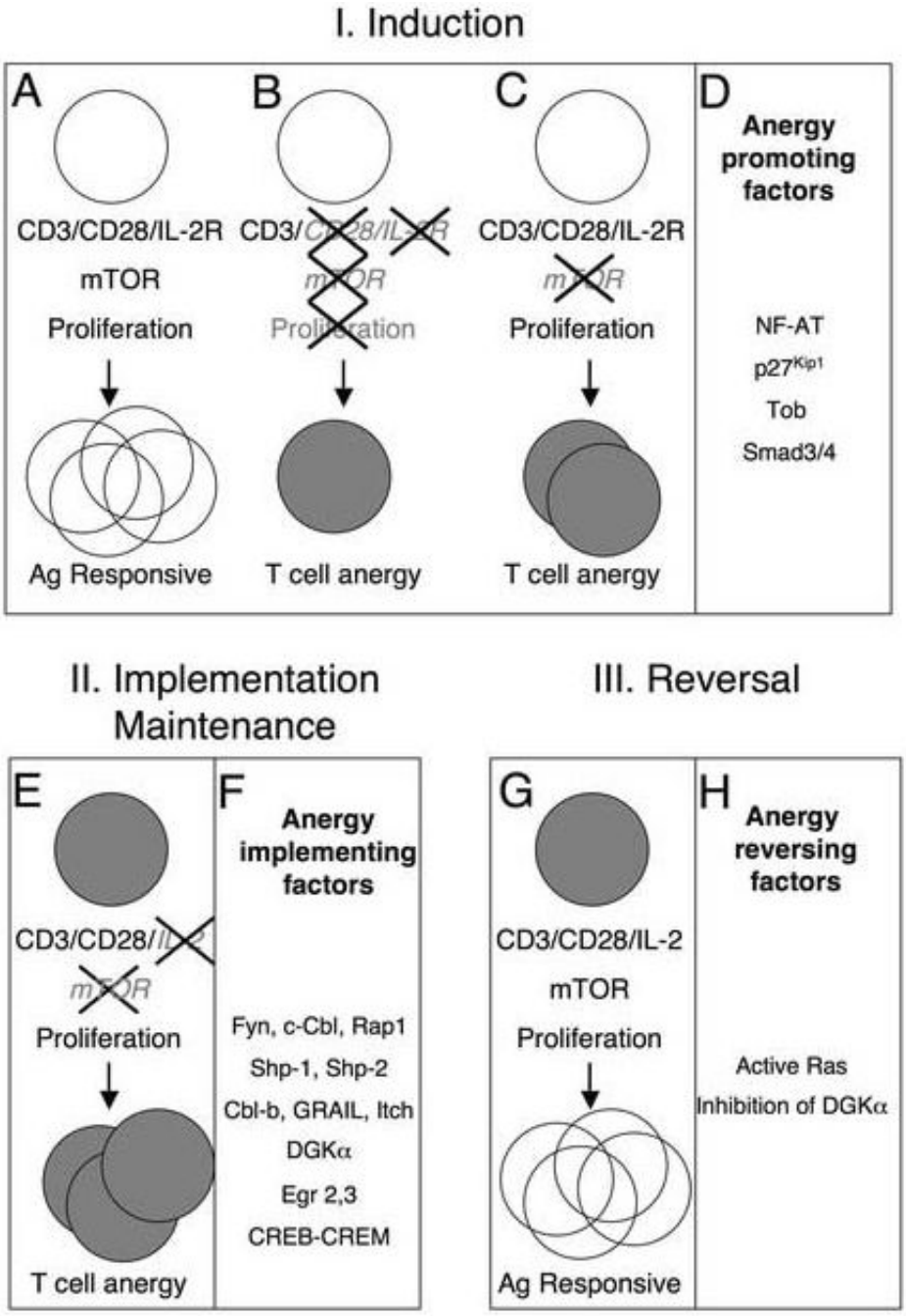


**Figure 2.**

The three phases of anergy: induction, implementation/maintenance and reversal. Depicted are the molecules and signaling events critical for the regulation of T cell proliferation and avoidance of unresponsiveness. I) Induction phase. A) Proper activation via the CD3, CD28, and the IL-2R mediates activation and proliferation of the cells and avoidance of anergy. B) Inhibition of CD28 and IL-2R leads to poor mTOR activation, prevents T cell clonal expansion, and favors anergy. C) Proper activation via CD3, CD28, and IL-2R molecules in the presence of mTOR inhibition (Rapamycin) favors anergy, but only slows cell cycle progression. D) Factors shown to promote anergy induction. II) Implementation and maintenance. E) Once anergy is established, intense activation via CD3 and CD28 mAbs promotes T cell proliferation,

but fails to activate mTOR and restore antigen responsiveness. F) Shown are molecules associated with the interruption of *IL2* expression in anergy (anergy factors). III) Reversal. F) Stimulation of anergic T cells via CD3, CD28, and IL-2R activation activates mTOR and reverses the anergic state. H) Molecular strategies shown sufficient to successfully overcome or reverse established anergy. Proper mTOR signaling is needed to prevent the induction of T cell anergy (A), and to revert the anergic phenotype (G). Likewise deficient mTOR signaling contributes to the maintenance of T cell anergy (E). Crosses, represent blocked signaling. Shading indicates an anergic phenotype.