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# Induction of T cell anergy: integration of environmental cues and infectious tolerance

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## Abstract

Anergy is a state of long term hyporesponsiveness in T cells that is characterized by an active repression of TCR signaling and IL-2 expression [1]. Several forms of anergy have been described and the last few years have brought to light an increasing number of “anergic factors” involved in the induction and the active maintenance of the state in lymphocytes. The role of mTOR and other related metabolic sensors and regulators has recently emerged as of particular importance in broadening our view of anergy-inducing signals. We will discuss the role of these molecules in regulating the choice between anergy and activation, a decision faced by all T cells undergoing TCR stimulation. We will then explore the relationship between the induction of anergy and the induction of regulatory T cells as well as the potential crosstalk responsible for the phenomenon of infectious tolerance.

## Introduction: TCR and CD28, the two signal paradigm of anergy

The state of T cell clonal anergy was initially described *in vitro* as the result of T cell activation through its antigen-specific receptor, referred to as signal 1, in the absence of a second costimulatory signal mediated by CD28 ligation [2–4]. This hyporesponsive state is characterized by a block in the Ras/MAP kinase pathway [5], mediated by an elevation in diacylglycerol kinase alpha (DGKα) [6], which could be reversed by IL-2 or OX40 signaling (reviewed in [1]). Careful subsequent molecular studies have characterized the need for a joint TCR/CD28 signaling to fully recruit the transcription factors involved in IL-2 gene transcription: NFAT, AP1, and NF- B. In the presence of both signals 1 and 2, IL-2 is then transcribed and its subsequent signaling through the IL2R complex serves to fully activate the downstream PI3K/AKT-mTOR pathway leading to p27kip1 degradation and entry into the cell cycle (reviewed in [7•]).

On the other hand, T cell activation involving only signal 1 leads to deficient IL-2 transcription and induction, via the Calcineurin (CaN)/NFAT pathway, of an anergic gene expression profile characterized by the augmentation of several key E3 ubiquitin ligases, such as Cbl-b, GRAIL, Itch and the more recently described, Deltex 1 [8]. These molecules impair TCR signaling in anergic T cells by negative feedback. For example, GRAIL has recently been shown to promote

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CD3 ζ ubiquitinylation and subsequent targeting of the TCR/CD3 complex to the proteasome for degradation following T cell activation [9•]. In addition, transcription factors such as, Ikaros, Egr2/3 and cAMP response element modulator (CREM), are induced, which are involved in the maintenance of the anergic state and the active repression of the IL-2 gene locus (reviewed in [7•]).

## Environmental cues that regulate T cell activation versus anergy induction: extending the signal 2 paradigm

Activation of the AKT-mTOR pathway is also critically required to induce a switch in the T cell metabolic machinery from catabolism to anabolism, through stimulation of glycolysis and up-regulation of key nutrient transporters [10]. This establishes the basis for the optimal activation, proliferation and differentiation of the T cell (reviewed in [10–12]). Anergic T cells have been shown to be blocked at the G1/S checkpoint of the cell cycle and, as recently coined by Powell and colleagues, are in a metabolically anergic state characterized by a failure to up-regulate nutrient transporters, such as CD71, CD98 and Glut1, and to switch to an anabolic state of metabolism [13••].

### mTOR and AMPK: integrating environmental cues beyond CD28 and IL2

Initial studies using the mTOR inhibitor rapamycin had shown that blocking mTOR activation was sufficient to induce anergy in T cells following full activation with anti-CD3 and anti-CD28 [14]. This effect was subsequently shown to go beyond the simple inhibition of cell proliferation. Drugs like Cyclosporine A (CSA) and FK506, which inhibit the CaN/NFAT pathway, or Sanglifehrin (SFA), which like rapamycin induces an arrest at the G1/S checkpoint without affecting $Ca^{2+}$ responses, did not induce subsequent anergy in the T cells [15,16]. Full anergy induction required the activation of the CaN/NFAT pathway with concomitant repression of mTOR activation [17]. Thus, mTOR, downstream of the IL-2R pathway, appeared as a major regulator of anergy versus activation.

Interestingly, not only is mTOR downstream of CD28 and IL-2 signaling pathways but it also serves downstream of several energy and nutrient-sensing pathways in eukaryotic cells [18]. Based on this, recent studies have extended the signal 1 + 2 model by pointing out the essential role of such environmental cues in the anergy induction process. For example, activation in the presence of a leucine or glucose antagonist could actively induce anergy in T cells, even in the presence of the classical signal 1 and 2, as well as normal IL-2 production at the time of initial activation [13••]. In the same study, mTOR regulation was also shown to extend to energy sensing via the heterotrimeric kinase complex AMPK. AMPK is activated by a decrease in ATP in the cell leading to an increase in AMP that specifically binds to the subunit of the AMPK complex, leading it to inhibit mTOR activation [12] [18]. Thus, AMPK serves as a direct sensor of ATP deprivation and hypoxia upstream of mTOR and, similar to nutrient deprivation, direct activation of AMPK using aminoimidazole carboxamide ribonucleotide (AICAR) readily induces anergy in T cells [13••].

These experiments demonstrate the ability of the AKT/mTOR pathway to integrate various extracellular and intracellular signals to control T cell responses and dictate T cell choice between activation and tolerance. In addition to its direct role in regulating the metabolic switch induced by T cell activation, recent studies have demonstrated that mTOR can also act as a facilitator of T cell activation by modulating the expression of anergy maintaining genes through direct transcriptional repression [19] or indirect regulation of their degradation [20]. For example, activated mTOR directly induces the epistatic regulator of GRAIL, Otub1, whose expression results in endogenous GRAIL degradation and removal of an active block in T cell proliferation [20].

### GCN2 and A2AR: Extending anergy regulation to mTOR-independent pathways

The capacity of extracellular signals to actively regulate T cell activation versus anergy induction extends beyond the simple competition with CD28 and IL2 signaling at the level of mTOR activation and involves other nutrients and energy sensing pathways. The first hint of this came from the work of Munn and colleagues in trying to decipher the molecular mechanism of proliferation arrest induced by tryptophan catabolism by Indoleamine 2,3-Dioxygenase (IDO)-expressing APCs. This effect could not be fully recapitulated by the action of rapamycin and mTOR inhibition [21]. This led them to focus their attention on the second known amino acid sensing pathway involving the GCN2 kinase. GCN2 specifically binds and becomes activated by uncharged forms of transfer RNA (tRNA), thus acting as a sensor of amino acid deprivation for the T cell. Its activation leads to a downstream stress response program known as the integrated stress response (ISR), which regulates entry into the S phase of the cell cycle [22]. Careful examination of this process in CD8 T cells *in vitro*, as well as *in vivo*, showed that $GCN2^{-/-}$ T cells are fully resistant to $IDO^{+}$ DC-induced tolerance, and that triggering this pathway in the cell induces a clonal anergy like phenotype reversible by IL-2 treatment [21]. The role of this pathway in CD4 T cells, however, is less clear. Similar studies with $GCN2^{-/-}$ T cells responding in the context of amino-acid starvation, revealed a more prominent action on the overall survival of activated CD4 T cells and induction of regulatory T cells [23,24••]. Nonetheless, the CD8 study provides a good example of a TCR/CD28/mTOR-independent pathway that can control anergy induction in T cells upon otherwise full activation.

In addition to amino acid sensing pathways, the role of hypoxia and adenosine, the metabolite of ATP, should also be mentioned for their ability to create an active local immunosuppressive environment (reviewed in [25] and [26]). For T cells, adenosine signals through the A2A receptor (A2AR), and stimulates adenyl cyclase and increases intracellular cAMP. Mimicking this effect using the A2AR agonist, CGS-21680 (CGS), Zarek and colleagues demonstrated that this results in decreased signaling through the Ras-MAP-Kinase pathway as well as reduced recruitment of active AP-1 to the nucleus. This ultimately led to the promotion of T cell anergy both *in vitro* and *in vivo* [27•]. Such results further extend the set of extracellular cues responsible for signal 2, beyond the initial CD28/IL2 pathway and its regulation of mTOR activation (Figure 1).

## From anergy to suppression: a role for TGF-β and dedicated APCs in regulating anergy versus regulatory T cell commitment?

In addition to long term hyporesponsiveness of the T cell, various models of anergy have described the acquisition of suppressive functions by a subpopulation of those T cells. This extends an initially cell intrinsic decision to the level of regulating a population in a process initially coined by Gershon and Kondo as *"Infectious Tolerance"* [28]. As such, the two most studied regulatory T cell populations, Tr1 and $Foxp3^{+}CD4^{+}$ T cells, were initially described as having all of the characteristics of an anergic T cell population *in vitro* (reviewed in [1]). One question that still hasn't been answered, however, is whether differentiation to a regulatory T cell is a linear process in which anergy induction is the first step and acquisition of suppressive function follows subsequently or if those two events represent two independent choices the cell can make, putting Tregs on the same level as other T helper populations in the choice between anergy versus activation and differentiation.

### The AKT/mTOR pathway and environmental cues regulating $Foxp3^{+}$ T cell peripheral development

In the case of $Foxp3^{+}$ regulatory T cells, peripheral induction from mature naïve T cells has now been documented both *in vivo* and *in vitro* [29,30]. These cells are currently referred to as iTregs. Initial observations on their *in vivo* peripheral induction emphasized the importance

of a low level of TCR stimulation in the absence of strong co-stimulation [29,30]. Mimicking observations done in clonal anergy models, recently published data have demonstrated an essential role for classical anergy signature genes such as the E3 ligases Cbl/b [31] or Itch [32] in regulating Foxp3$^+$ T cell induction, or GRAIL [9,33] in regulating the acquisition and/or maintenance of the suppressive properties of such cells.

Even more interesting, in the context of this review, are recent studies demonstrating the key role played by the AKT/mTOR pathway in regulating effector versus regulatory T cell lineage commitment in the periphery [34–36]. In this regard, T cells expressing a constitutively active AKT fail to up-regulate Foxp3 [34•]. mTORC1-deficient T cells also exhibit blunted responses to IL-12, IFN-γ, IL-4 or IL-6, revealing a role for mTORC1 in regulating T helper lineage differentiation, potentially through direct regulation of the various STAT pathways [36••]. In addition, both GCN2, in response to IDO-mediated tryptophan degradation [23,37], and A2AR signaling pathways have been implicated in positive regulation of Foxp3 expression [27•]. This further emphasizes an important role for what we describe as an “anergy-inducing” environment in the regulation of Foxp3$^+$ iTreg differentiation, and highlights the similarity in the induction of the two states.

## TGF-β and a major role for dedicated APC populations?

From pioneering *in vitro* experiments, the key molecule responsible for Foxp3 expression has been postulated to be TGF-β [38]. On a molecular level, TGF-β seems to divert the cell from the classical pathway of anergy induction by activating Smad3, which will then cooperate with NFAT to induce Foxp3 [39,40]. Subsequently, Foxp3 has been shown to form a complex with NFAT, and other transcription factors, to induce the Treg-signature [41,42].

When delivered in an excess amount to T cells *in vitro*, TGF-β is sufficient to drive Foxp3 expression in the presence of strong TCR stimulation and this process is further enhanced by costimulation and IL-2 signaling [43]. Such *in vitro* data clearly differ from the classical anergy induction conditions and the initial in vivo generated data [29,30]. However, as levels of TGF-β are probably limiting *in vivo*, Treg induction might be more tightly regulated and dependent on the actual environment in which the T cell is being triggered. This was nicely demonstrated by recent studies on the role of mTOR in iTreg differentiation. In a general context of transient TCR stimulation, and thus low mTOR activation, TGF-β seemed to be dispensable for Foxp3 induction [35•]. Alternatively, as revealed by hyperactive Smad3 in mTOR deficient T cells, minute amounts of TGF-β might be sufficient to drive a default differentiation to the Treg lineage under such conditions [36••].

In addition to this, TGF-β levels can be regulated locally; a role that seems to be mostly undertaken by the professional antigen-presenting cells ([44] and reviewed by [45]). Using DEC-205 antibody-coupled peptide administration *in vivo*, regulatory T cell induction by foreign antigen was initially postulated to require low antigenic doses directly targeted to DEC-205$^+$ dendritic cells (DCs) in the spleen under steady state conditions [30]. Such results were further confirmed *in vitro* where CD8$^+$ DEC-205$^+$ DCs could induce Foxp3 expression in the presence of low doses of antigen and this process was further shown to be entirely dependent on their endogenous secretion of TGF-β [46]. This property was then extended to lamina propria CD103$^+$ DCs in a model of oral tolerance induction, with again an essential role for TGF-β in combination with the metabolite of vitamin A, retinoic acid (RA) [47,48]. A non-redundant role of RA in promoting Foxp3$^+$ T cell differentiation has also recently been described for a specific population of migrating dermal DCs [49•].

The exact role of RA is still a matter of debate [50–52]. RA has been postulated to act on nearby pre-activated/memory T cells, preventing expression and secretion of IFN- γ or other negative regulators of Foxp3 induction [51]. It has also been reported to directly act on naïve T cells,

mediating enhanced TGF-β-induced Foxp3 expression [52]. In this manner, RA might act similarly to mTOR inhibition [53], amino-acid deprivation [37] or adenosine [27], illustrating the potential synergistic action of an "anergy-inducing" environment and TGF-β in the regulation of iTreg differentiation and the generation of a dominant form of tolerance.

## Crosstalk between Treg-mediated suppression and anergy induction: Tregs as regulators of environmental cues?

Numerous mechanisms have been proposed for regulatory T cell-mediated suppression such as TGF-β and IL-10 secretion, modulation of DC maturation and function, IL-2 consumption from the extracellular environment or even direct killing of target cells through granzyme B dependent mechanisms (reviewed in [54]). It is interesting, however, to note that several knockout models, shown to be resistant to anergy induction, are also resistant to Treg-mediated suppression. Examples include E3 ubiquitin ligases, Cbl-b [55,56] and TRAF6 [57,58], and the molecular inducer of most anergic factor genes, NFAT1, in combination with NFAT4 [59]. Data are currently missing for molecules such as GRAIL or Itch as well as for the various transcriptional repressors involved in anergy, but it is nonetheless striking that classical anergy markers seem to be playing a non-redundant role in regulating the subsequent activation of T cells undergoing Treg-mediated suppression. It is thus possible that Tregs work, at least in part, as inducers of T cell anergy. Two recently described and/or revived molecular mechanisms, CTLA-4/IDO and CD39/CD73/adenosine, might be playing an important role in this task.

### CTLA-4 and IDO: targeting DCs and inducing infectious tolerance through the consumption of essential amino acids

CTLA-4 was noted in early studies as being expressed on Tregs and was thus postulated to have an important role in their function *in vitro* [60]. This has now been clearly demonstrated by various groups using targeted deletion in $Foxp3^+$ T cells resulting in the impairment of both *in vitro* and *in vivo* suppressive function of the Tregs [61–64]. CTLA-4 expressed on Tregs has been shown to directly target B7 ligands expressed on activated $Foxp3^-$ T cells and to induce CREM expression ending in attenuated IL-2 transcription [65]. However, the major role of CTLA-4 in Treg-mediated suppression is regulating DC maturation and expression of CD80, CD86 [61•] as well as inducing their expression of IDO [66]. This would lead to decrease co-stimulation as well as tryptophan deprivation, resulting in down-regulation of T cell responses, as discussed earlier.

Again, this extends far beyond simply regulating tryptophan levels in the extracellular milieu. In a very interesting study, Cobbold and colleagues describe *in vivo* and *in vitro* evidence that both $Foxp3^+$ iTregs and Tr1 cells can induce expression in DCs of a large variety of amino acid-consuming enzymes including arginase 1 (Arg1), iNOS, Histidine decarboxylase (Hdc), L-amino acid oxidase (IL4i1), Branched chain aminotransferase (Bcat1), Threonine dehydrogenase (Tdh), Tryptophan hydroxylase (Tph) and of course IDO (Indo) [24••]. Each of these enzymes seems to be differentially regulated by the action of CTLA-4, TGF-β or IL-10 expressed by the various Treg populations studied. Specific depletion of any one of the targeted amino acids was sufficient to suppress naïve T cell activation.

### CD39/CD73 and the regulation of T cell activation by Treg-induced adenosine

Finally, Treg-mediated suppression might extend beyond simply inducing tolerogenic DCs. Tregs have recently been postulated to promote a hypoxic environment via their expression of both the 5′-ectonucleotidase CD73 and the ATPase/ADPase CD39 [67•]. We have already mentioned the role of the adenosine receptor in inducing anergy [25,27]. Here, CD39, by hydrolyzing ATP to AMP, and CD73, by converting AMP into adenosine, are postulated to work in tandem to induce an immunosuppressive environment by targeting the A2AR [68].

The genes coding for CD39 and CD73 are both targets of direct transcriptional activation by Foxp3 [69,70] and the proteins are expressed at high frequency on $Foxp3^+$ T cells in both mouse and human [68,70]. Interestingly, CD39 consumption of ATP *in vitro* is only detected on TCR-activated Tregs, adding a level of antigen specificity to the mechanism [70].

In both examples described in this section, it is interesting to note that the regulation of the environment, i.e. amino acid deprivation and adenosine generation, should directly induce anergy in T cells undergoing activation, provided that Tregs do not induce at the same time a block in the CaN/NFAT pathway (Figure 2).

## Conclusion

Overall, it has become increasingly clear that signal 2 in the classical anergy model extends far beyond simple CD28/IL-2 signaling and is likely the result of active integration of multiple environmental cues received by the T cell undergoing activation (Figure 1). Although the CD28 and IL-2 pathways, being specific to the immune system, remain central in explaining how a T cell makes the choice between activation or tolerance in a favorable environment, the role of an extended set of extracellular cues could help to explain various observations of T cell tolerance *in vivo* under what were thought to be optimal activation conditions. In addition, it seems that, when triggered by a similar set of extracellular cues in the presence of TGF-β, the classical “anergy inducing” program can be diverted to induce the differentiation of iTregs with additional suppressive functions. These Tregs in turn act in various ways to modulate the T cell environment and favor tolerance/anergy induction rather than full activation (Figure 2). Additional studies on the fate of T cells undergoing Treg- mediated suppression could help link the fields of anergy and regulatory T cells and would further enhance our global understanding of T cell tolerance.

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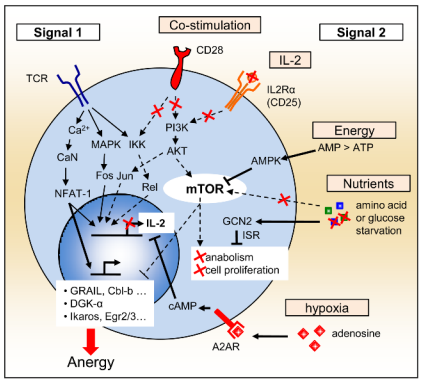


**Figure 1. Extending the signal 2 paradigm**

induction of anergy in T cells was initially described as the result of TCR (signal 1) without concomitant CD28 and IL-2R signaling (signal 2). Recent studies have further demonstrated that the T cell actively sense its microenvironment, through mTOR dependent and independent mechanisms, for available energy and nutrients, as well as additional negative cues such as adenosine. This regulates the T cell commitment to switch its metabolic machinery and enter the S phase of the cell cycle. Interestingly, failing to positively commit to full T cell activation in such cases induces anergy and long-term tolerance in the T cell. Full lines represent active pathways and dashed lines represent blunted pathways during the induction of T cell anergy in an anergic environment.

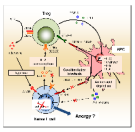

**Figure 2. Infectious tolerance by creating an "anergy-inducing" environment**

It has recently been shown that Tregs can influence the microenvironment in which a naïve T cell gets activated. First, Tregs can, via CTLA-4 expression and IL-10 and TGF-β secretion, induce various amino acid-consuming enzymes, as well as down-regulate CD80 and CD86 expression, in DCs. Second, Tregs, via their constitutive expression of the high affinity chain of the IL2R (CD25), can actively consume IL-2 in the extracellular milieu. Finally, Tregs express the 5′-ectonucleotidase CD73 and the ATPase/ADPase CD39 and activated Tregs have the potency to hydrolyze extracellular ATP into adenosine, thus favoring an hypoxic environment. Full lines represent active pathways and dashed lines represent blunted pathways during active suppression of naïve T cell activation by Tregs.