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Cyclin-dependent kinases: molecular switches controlling anergy and potential therapeutic targets for tolerance

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Abstract

A large body of research has established the importance of costimulatory signals and proliferation for the generation of productive T cell immune responses. While costimulation and cell cycle progression are each individually necessary for CD4⁺ effector T cell differentiation, it has become clear that neither of these processes alone are sufficient to avoid anergy. This review outlines the links between T cell differentiation, tolerance, and the cell cycle, and highlights recent work that has implicated cyclin-dependent kinases as important regulators and potential targets for modulation of T cell immunity and tolerance.

1. Introduction

T lymphocytes form the basis of immunity to foreign pathogens, but also precipitate autoimmune disease and mediate the rejection of organ transplants. For instance, current estimates suggest that as many as 1 in 20 peripheral T cells are alloreactive, and these cells may expand 20- to 50-fold during an alloimmune response *in vivo* [1]. The proliferation of T lymphocytes from a few clones into a population of antigen-specific effector cells represents a critical and highly-regulated phase of a productive immune response, and the majority of immunosuppressive drugs currently used to ameliorate transplant rejection or autoimmune pathology in experimental models and in the clinic result in inhibition of T cell proliferation.

Growth factors such as IL-2 drive T cell division, clonal expansion, differentiation and oppose tolerance, but the basis for the association between these processes is not known. Does growth factor signaling directly oppose tolerance induction independently of its effects on cell cycle progression, or is tolerance a state that must be 'escaped' by cell division? Likewise, do genes induced by growth factors instruct naïve T cells to develop effector function, or does cell cycle progression influence the expression of genes associated with T helper differentiation by a more indirect mechanism? Specifically how mitogenic signals contribute to the avoidance of tolerance and development of effector and memory T cells has been unclear, and a further understanding of the nature of this relationship may allow T cell responses to be modulated with greater specificity and efficacy.

Recent studies have implicated cyclin-dependent kinases (CDK) as major regulators of T cell immunity and tolerance (reviewed in [2]), and point to these molecules as a potential link between the cell cycle and T cell function. These studies have added to our understanding of

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T cell anergy at the molecular level, but in addition, they have implicated CDK as novel potential targets for therapy in autoimmune disease and organ transplantation. At least four CDK inhibitory drugs are currently at various stages of clinical trials in the treatment of cancer [3], but the activity or efficacy of these drugs as immunosuppressive agents in the context of autoimmunity or transplantation has not been explored.

2. T cell differentiation and anergy are linked to the cell cycle

2.1. Association between the cell cycle and T cell differentiation

Naïve T cells are unable to efficiently produce cytokines such as IFN γ and IL-4, but gain this function several days following their initial activation [4]. A significant step forward in our understanding of T cell differentiation was achieved when several studies demonstrated that the delay in this gain of effector function is not time-dependent, but rather cell division-dependent. Thus, in populations of differentiating Th1 or Th2 cells, only those cells that have undergone several rounds of cell division are able to express the cytokine genes characteristic of these polarized responses [5–7]. This link between cell cycle progression and T cell effector function has been observed in a myriad of studies in a multitude of immunological models, and represents an important clue to the nature of T cell differentiation.

2.2. Association between the cell cycle and T cell clonal anergy

T cells activated in the absence of inflammatory signals fail to produce IL-2 [8], and are rendered hypo-responsive to further stimuli [9,10]. This state, called T cell anergy (reviewed in [11]), is an important mechanism of peripheral tolerance that is associated with altered recruitment of TCR-associated tyrosine kinases, impaired PLC γ -1 activation, defective activation of the ERK and JNK MAP kinase cascades, and results in defective assembly of active transcription factor complexes at the *IL2* promoter [12–19]. In addition, anergic T cells suffer from active, dominant repression of *IL2* gene transcription [20–22] that may involve epigenetic mechanisms [23–25]. Under physiologic conditions, anergy is induced when T cells receive antigenic stimulation in the absence of CD28 costimulation, and appears to involve signaling through Ca²⁺/calcineurin to the transcription factor NFAT [26] (Fig. 1 A). These signals induce the expression and/or activity of anergic, negative regulatory factors, which include E3 ubiquitin ligases and transcriptional repressors (reviewed in [27,28]).

But if anergy is an active and integral consequence of antigen receptor signaling, then how do T cells avoid this fate to induce immunity? The predominant view is that signals from the CD28 costimulatory receptor oppose the expression or activity of negative regulatory factors that are induced by antigen receptor signals (Fig. 1 B), but a molecular basis for this hypothesis has not been well-defined. One idea is that AP-1 activated by CD28 partners with NFAT, diverting NFAT from a program of anergy-associated genes to genes associated with T cell differentiation [26]. However, CD28 costimulation also leads to synthesis of IL-2 and its receptor, and results in cell cycle progression and clonal expansion (Fig. 2). So, an alternative hypothesis is that costimulation promotes anergy avoidance indirectly by driving proliferation, and that processes associated with cell cycle progression then directly oppose the negative regulatory factors induced by the TCR (Fig. 2). The fact that IL-2 is required for the avoidance of anergy [29], and growth factor signals can replace CD28 costimulation in this process [30, 31], argues for this scenario.

Primary T cells that receive optimal antigen and costimulatory receptor stimulation proceed synchronously through early activation and G1 phase progression, but go on to exhibit marked heterogeneity and asynchrony in mitotic behavior [32]. Those T cells that progress through multiple (*i.e.*, 5–8) cell divisions during the primary response exhibit strong IL-2 production, IFN γ production, and proliferation upon restimulation [5–7,18,33]. Conversely, those cells that

did not divide during the primary response fail to produce IL-2 and exhibit growth arrest upon restimulation, despite the fact that these cells were primed in the presence of full TCR and CD28 costimulation [18,33]. These studies have provided support for the idea that CD28 costimulation is not sufficient, while cell cycle progression is necessary, for anergy avoidance. Additional support for the importance of cell cycle progression has come from studies demonstrating that pharmacological inhibition of G1 progression with butyrate [34,35] or IL-2R signaling with rapamycin [36,37] results in induction of anergy in CD4⁺ T cells despite the presence of full TCR and CD28 costimulation. Cell cycle arrest within the G1 phase appears to be important for anergy induction, because a pharmacologic block within S phase using hydroxyurea failed to induce anergy in T cell clones stimulated through the TCR and CD28 [36]. This implies that biochemical events occurring before the G1 to S phase transition are critical for anergy avoidance.

3. Cyclin-dependent kinases as a molecular link between the cell cycle and T cell function

3.1. Anergy is accompanied by dysregulated mitogenic signaling

What aspect of cell cycle progression promote anergy avoidance? Two crucial events that occur before the G1 to S phase transition are the activation of the D-type cyclin-dependent kinases CDK4 and 6, and the E-type cyclin-dependent kinase CDK2. Anergic T cells can induce cyclin D2 and CDK6 expression [38,39], but lack cyclin E expression and CDK1 and 2 activity [39]. One consequence of CDK activity is the induction and activation of the E2F family of transcription factors [40]. Anergic T cells exhibit impaired expression and nuclear localization of the pro-mitogenic family members E2F1 and E2F3, and express moderately elevated levels of E2F4, a family member that inhibits the transcriptional activity of the pro-mitogenic members (our unpublished observations). While all the E2F DNA binding activity in TCR/CD28-activated effector cells exists as free E2F1–4 complexes that are capable of transcriptional activation, at least one-third of the E2F DNA binding activity in anergic T cells is associated with the retinoblastoma (Rb) tumor-suppressor protein in a complex that represses gene expression and helps to keep cells in a quiescent state (our unpublished observations). Another important consequence of CDK activity is the phosphorylation and targeted degradation p27kip1, a critical inhibitor of CDK activity [41]. In several models, anergic cells exhibit elevated expression of p27kip1, and are unable to downregulate this protein in response to restimulation [18,33,39,42]. These studies together suggest that CDK activity is impaired in anergic T cells, and implicate cyclin-dependent kinases as factors that may promote anergy avoidance in T cells.

3.1. CDK1/2 activity opposes anergy induction

The elevated expression of p27kip1 by anergic T cells has led to the hypothesis that p27kip1, and the regulation of CDK1/2 activity by this protein, is necessary for the induction and/or maintenance of T cell clonal anergy. p27kip1 positively regulates the first G0 to G1 to S phase transition during the activation naïve, quiescent CD4⁺ T cells [42]. This is because p27kip1 has been shown in other tissues to serve as a scaffold which facilitates the assembly of cyclin D-CDK4/6 complexes during G1 progression, protecting these complexes from inhibition by the ink4 family members [43]. However, after the first cell division (when cells no longer need to undergo a G0 to G1 transition), p27kip1 acts in T cells to oppose clonal expansion by setting the threshold for the amount of CD28 costimulation and growth factor signaling is required to initiate cell cycle progression (reviewed in [2]). In the absence of p27kip1, CD4⁺ T cells can enter the cell cycle and expand in the absence of CD28 costimulation and in response to small amounts of IL-2, demonstrating that p27kip1 is a crucial intracellular biochemical sensor that 'tells' T cells when they have received the appropriate signals to undergo an immune response. This role for p27kip1 is so crucial that, in the absence of this protein, CD4⁺ T cells activated

without CD28 costimulation fail to undergo the silencing of the *IL2* gene that is the integral hallmark of anergy, and engage in secondary clonal expansion upon re-encounter with antigen [42,44]. This activity is not shared by all CDK inhibitory proteins, because p18ink4c, which inhibits CDK4 and 6 but not CDK1 or 2, is not required for anergy (our unpublished observations). This implies a unique role for p27kip1 and CDK1/2 in anergy avoidance and effector differentiation.

Anergy induction with CTLA-4Ig is associated with an abortive proliferative response [7,32, 33], and genetic elimination of the cell cycle inhibitor p27kip1 renders CD4+ T cells resistant to anergy induced in the absence of CD28 costimulation [2]. The simplest explanation for these results is that the enhanced proliferation afforded by the absence of p27kip1 allows T cells to escape anergy. However, we have found that anergy avoidance by p27kip1^{-/-} T cells can occur in the first 24 hours after stimulation with CTLA-4Ig (our unpublished observations), and therefore does not depend upon cell division. This implies that p27kip1 functions independently of cell cycle regulation to promote anergy in CD4+ T cells, presumably through its opposition of CDK activity. CDK might directly regulate *IL2* transcription, and several factors involved in *IL2* gene expression have been shown in other models to be regulated by these kinases. These include NFκB [45], Sp1 [46], the co-activator and histone acetyltransferase p300/CBP [47], and subunits of the RNA polymerase holoenzyme complex TFIIB and RNAPolα [46]. CDK activity could also promote IL-2 production by inhibiting the activity of negative regulatory factors. This idea is supported by a recent study showing that CDK-mediated inactivation of the transcriptional repressor Smad3 [48] occurs in primed, but not anergic, T cells, and that dysregulated Smad3 activity in p27kip1-deficient T cells is an important component of their anergy-resistant phenotype [44]. Therefore, a revised hypothesis would state that cyclin-dependent kinase (most likely CDK2) activity, and not cell cycle progression *per se*, is crucial for anergy avoidance (Fig. 3). Further molecular studies will be needed to solidify this idea.

3. Cyclin-dependent kinases regulate autoimmune and alloimmune tolerance

3.1. CDK activity can influence self tolerance

While the studies described above demonstrate a very significant role for p27kip1 in limiting effector differentiation and promoting anergy induced either *in vitro* [42] or *in vivo* [44], these studies do not prove whether p27kip1 or cyclin-dependent kinases are important in regulating the more complex array of T cell functions required for functional immunity or tolerance *in vivo*. Mice deficient for the CDK inhibitory proteins p27kip1, p18ink4c and p15ink4b exhibit peripheral lymphoid hyperplasia, however, fail to develop signs of autoimmunity (reviewed in [2]). This indicates that these molecules are not required for self-tolerance. p21cip1 inhibits CDK1 and CDK2, and is involved in p53-mediated cell cycle arrest in response to DNA damage. In two studies, aged p21cip1^{-/-} mice were found to have increased frequencies of activated CD4+ T cells, hypergammaglobulinemia, and anti-dsDNA antibodies reminiscent of a lupus-like syndrome [49,50]. Conversely, deletion of p21cip1 on the lupus-prone BXSB background ameliorated disease, apparently due to enhanced apoptosis of activated autoreactive T cells [51]. Thus, p21cip1 may regulate both the activation and survival of autoreactive T cells, and may therefore either promote or oppose self-tolerance depending on underlying genetic predisposition. CDK2 itself also appears to display this duality in life/death function (reviewed in [2]). While this kinase is clearly important for the proliferation and differentiation of mature T cells, mice genetically deficient in CDK2 exhibit defective negative selection of autoreactive T cells in the thymus. This function of CDK2 is apparently mediated through its ability to promote pro-apoptotic signaling in T cells.

3.2. CDK activity influences acquired alloimmune tolerance

The studies above suggest a role for certain cyclin-dependent kinases in central tolerance, and recent work has also demonstrated that CDK activity can strongly influence the acquisition of peripheral tolerance. This was tested recently in a cardiac allograft model using mice with deregulated CDK1/2 activity due to loss of p27kip1 [52], or deregulated CDK4/6 activity due to loss of p18ink4c (our unpublished data). Mice lacking either p27kip1 or p18ink4c reject fully MHC-mismatched cardiac allografts with the same kinetics as wild-type mice, however, pathological findings in the grafts of p27kip1-deficient recipients suggested stronger mononuclear cell infiltration and the involvement of acute humoral alloimmune responses. Allograft rejection requires the CD28 and CD40 costimulatory pathways (reviewed in [53]), and blockade of both of these receptors in wild-type mice leads to long-term allograft survival and donor-specific tolerance [54]. However, blockade of CD28 and CD40 costimulation in p27kip1-deficient mice results in acute, costimulation-independent cellular rejection associated with massive lymphocyte expansion, a 5-fold increase in the frequency of allospecific IFN γ -producing cells in the periphery, and increased infiltration and proliferation of CD4+ T cells in the cardiac grafts [52]. These results show that E-type cyclin-dependent kinase activity strongly promotes T cell differentiation during a physiologic immune response *in vivo*, and that inhibition of CDK2 and/or CDK1 activity by p27kip1 is required for the development of transplantation tolerance in the absence of CD28/CD40 costimulation.

As with p27kip1-deficient T cells [42], T cells with deregulated CDK4 and 6 activity due to p18ink4c deficiency exhibit enhanced cell division and a reduced costimulatory requirement *in vitro* [55]. However, unlike p27kip1-deficient mice, mice lacking p18ink4c are actually more susceptible to transplantation tolerance than wild-type mice. Blockade of either CD28 or CD40 individually in wild-type mice results in prolonged survival but eventual rejection of allografts. Conversely, blockade of these pathways in p18ink4c-deficient mice results in long-term allograft survival, and p18ink4c^{-/-} T cells exhibit decreased production of proinflammatory cytokines and an increased rate of apoptosis (our unpublished observations). These data suggest that the dominant role for p18ink4c in T cells is to promote survival and differentiation during an immune response. This is also how p18ink4c functions during plasma cell differentiation [56,57], and could explain the disparate roles that p27kip1 and p18ink4c play in the induction of T cell tolerance.

3.3. Do CDK influence regulatory T cell function?

Long-term, donor-specific tolerance to organ transplants requires regulatory T cells [58]. Therefore, while these cyclin-dependent kinase inhibitors clearly influence differentiation and energy in naïve T cells, these molecules could also modulate allograft tolerance through altering regulatory T cell function. Mice lacking either p27kip1 or p18ink4c contain normal numbers of CD4+CD25+Foxp3+ T cells in the periphery, and these 'natural' regulatory T cells function normally in *in vitro* suppression assays ([52] and our unpublished data). Also, allografts rejecting despite CD28/CD40 blockade in p27kip1^{-/-} animals contain large numbers of Foxp3-positive cells, suggesting that uncontrolled, costimulation-independent effector differentiation is the primary reason for the failure of tolerance in animals that lack p27kip1. However, whether normal or dysregulated CDK activity influences *in vivo* regulatory T cell function, or the induction of regulatory T cells from naïve T cell precursors, are issues that requires further study.

4. Cyclin-dependent kinases as therapeutic targets for immune modulation

The studies described above suggest that cyclin-dependent kinases and their regulatory partners have discrete and crucial roles in the regulation of T cell immunity *vs.* tolerance, and imply that CDK may represent novel therapeutic targets for the inhibition of allograft rejection or

autoimmunity. Dozens of compounds have been described that inhibit the activity of various cyclin-dependent kinases [59]. Of these, most are ATP analogs that act as competitive inhibitors of ATP binding to the active site of the kinase. For instance, the CDK inhibitory drugs flavopirdol (HMR 1275, L86-8275) and roscovitine (CYC202, seliciclib) are in human trials for the treatment of malignancy [60]. Roscovitine has been used therapeutically in experimental models of cancer [61], stroke [62], glomerulopathy [63], polycystic kidney disease [64], and pleurisy [65], but CDK inhibitory drugs have not been tested in models of autoimmunity or alloimmunity.

Our studies on the effects of CDK de-regulation on allograft rejection and tolerance provide a conceptual framework for novel immuno-modulatory applications of CDK inhibitory drugs. For instance, the fact that p27kip1, a genetically-encoded inhibitor of CDK1 and CDK2, is required for transplantation tolerance suggests that the use of synthetic CDK1/2 inhibitory compounds could promote allograft survival and alloimmune tolerance. Such drugs could potentially ameliorate immunopathologic T cell responses during autoimmune disease, as well. Conversely, de-regulation of CDK4/6 activity appears to have the opposite effect on T cell-mediated immunity *in vivo*. Therefore, compounds that selectively inhibit CDK4 or CDK6 activity could potentially act as adjuvants to enhance the effects of vaccines or cellular therapies against cancer and infectious diseases. Alternatively, the development of drugs that de-regulate CDK4/6 activity by inhibiting the capacity of ink4 family members to bind to CDK4 or 6 could promote alloreactive or autoreactive T cell apoptosis, thereby forming a therapy for organ transplant rejection and/or autoimmune disease.

5. Summary

Cyclin-dependent kinases are positive regulators of cell cycle progression that are known to act as key regulators of embryonic development and cancer. Recent progress in this field has indicated that these proteins likewise direct cell fate decisions during the development of immune and tolerant T cell responses, and therefore may serve as important targets for immune modulation.

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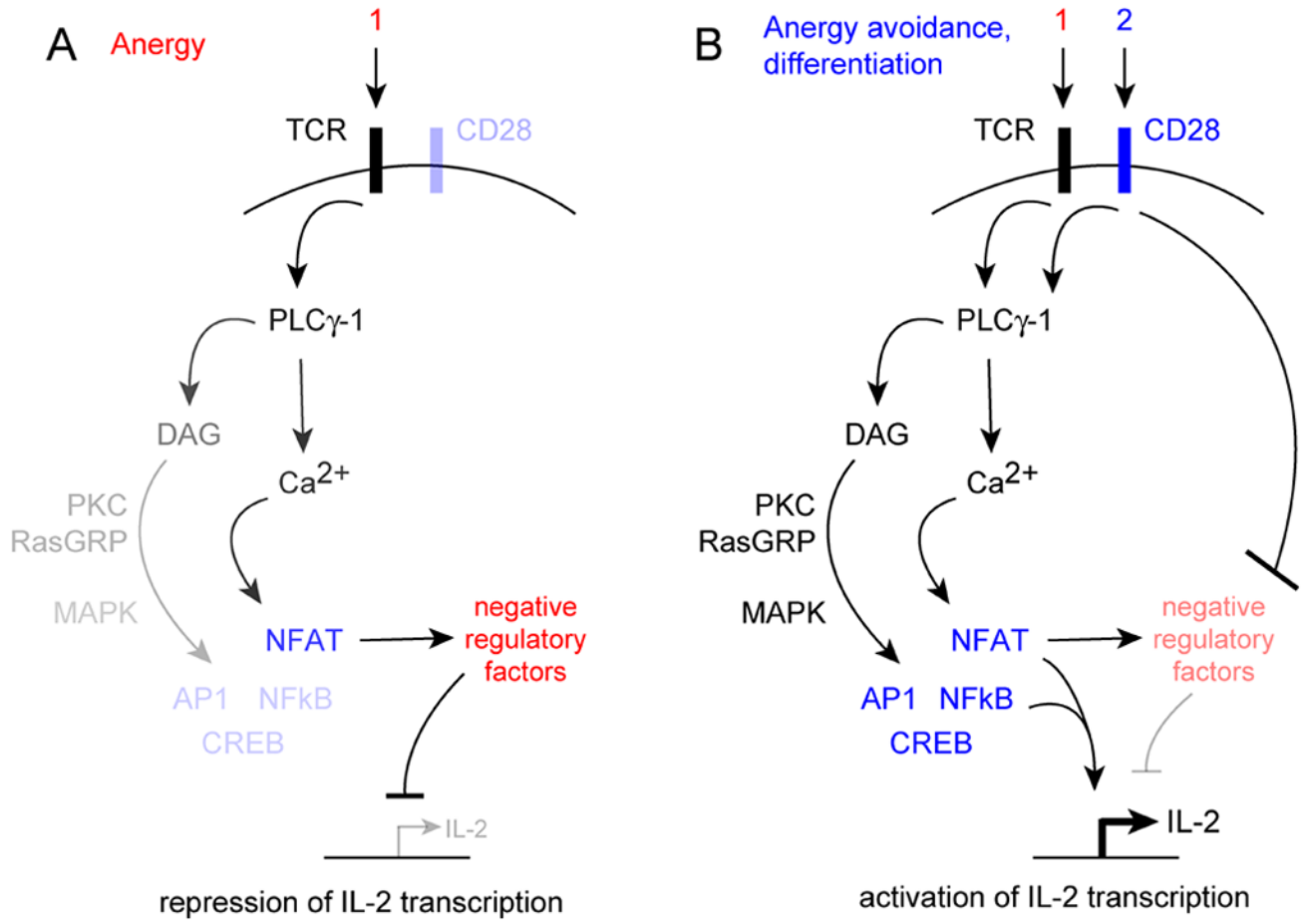


Fig 1. Current molecular view of T cell energy (A) and energy avoidance (B).

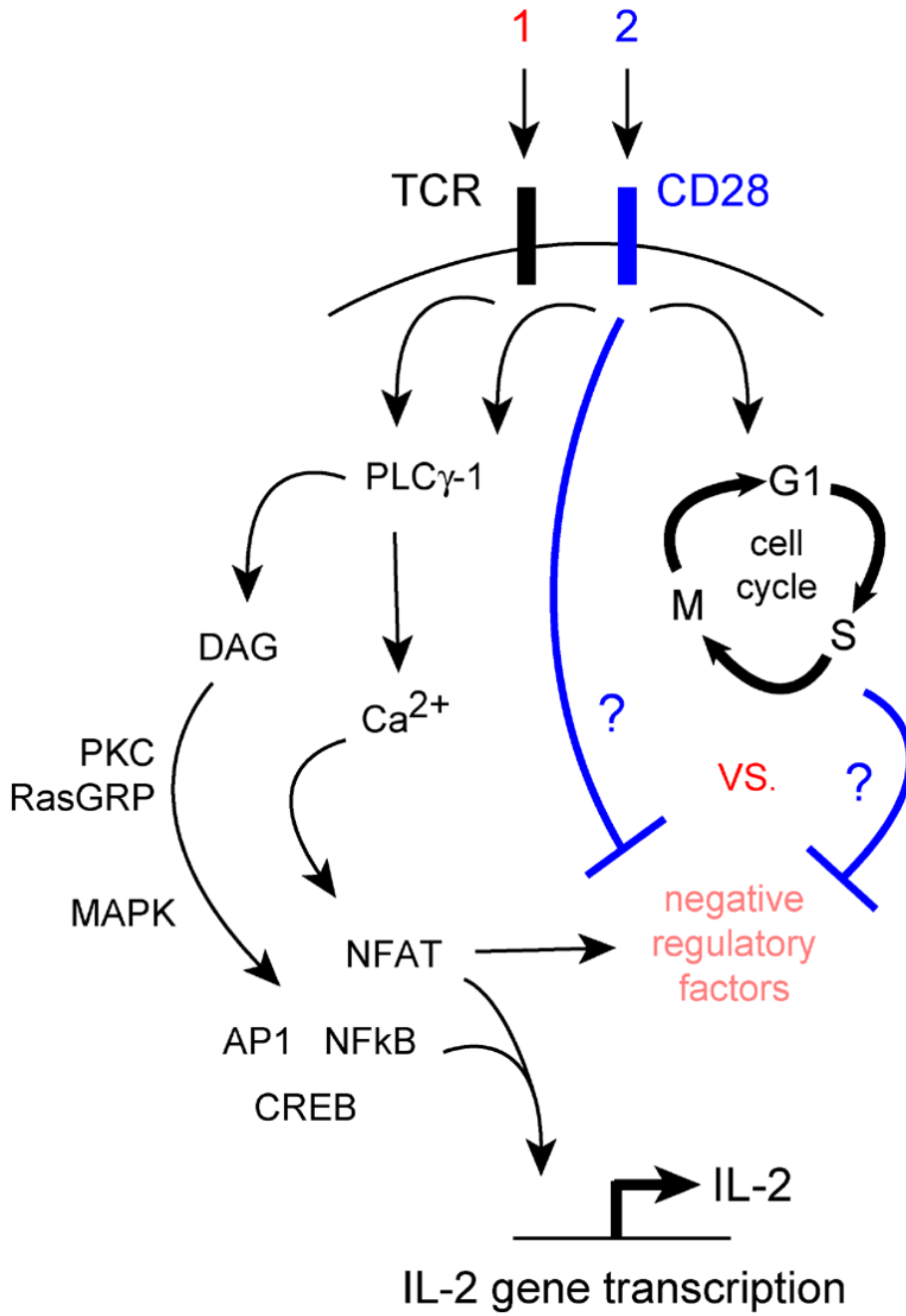


Fig 2. Cell cycle-dependent vs. cell cycle-independent energy avoidance mediated by CD28 costimulation.

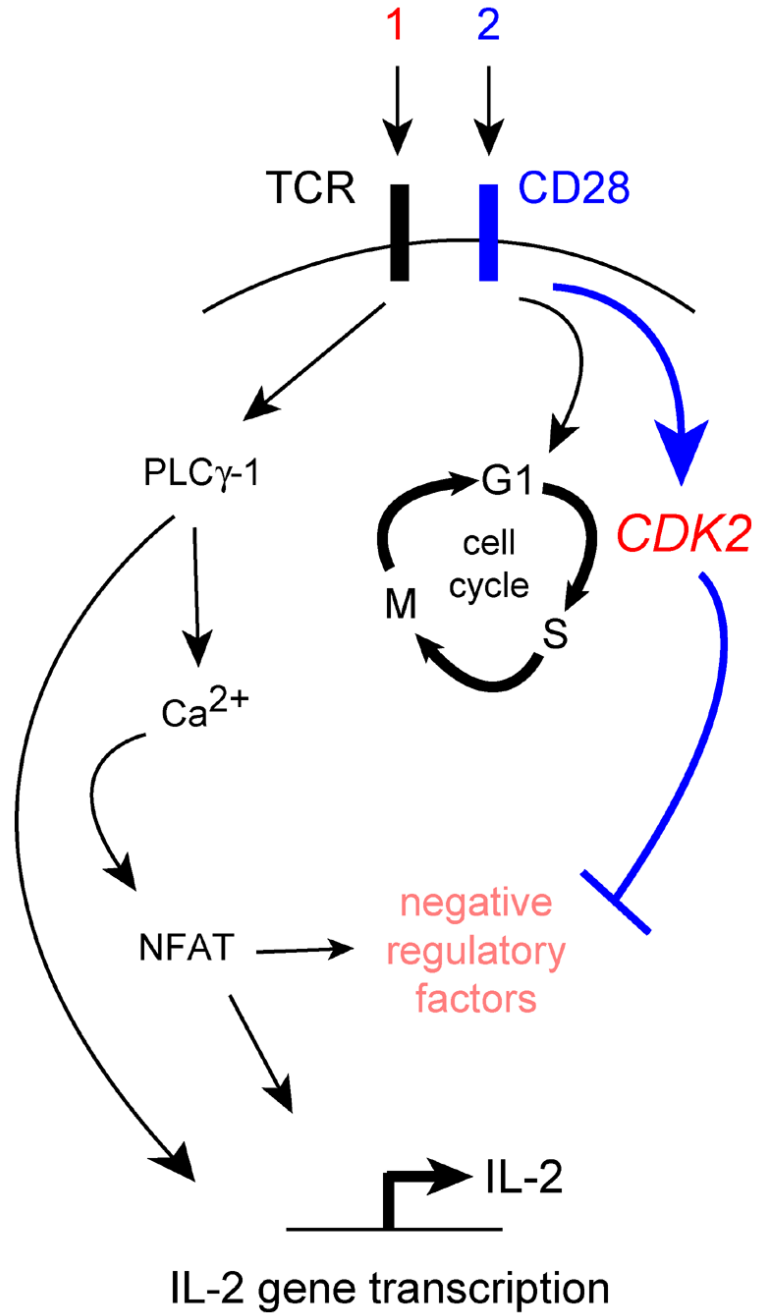


Fig 3.
CDK-dependent energy avoidance.