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Review

Induction and stability of the anergic phenotype in T cells

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ABSTRACT

One of the mechanisms that are in place to control the activation of mature T cells that bear self-reactive antigen receptors is anergy, a long-term state of hyporesponsiveness that is established in T cells in response to suboptimal stimulation. T cells receive signals that result not only from antigen recognition and costimulation but also from other sources, including cytokine receptors, inhibitory receptors or metabolic sensors. Integration of those signals will determine T cell fate. Under conditions that induce anergy, T cells activate a program of gene expression that leads to the production of proteins that block T cell receptor signaling and inhibit cytokine gene expression. In this review we will examine those signals that determine functional outcome following antigen encounter, review current knowledge of the factors that ensure signaling inhibition and epigenetic gene silencing in anergic cells and explore the mechanisms that lead to the reversal of anergy and the reacquisition of effector functions.

1. Introduction

Mechanisms of central and peripheral tolerance exist to eliminate or inactivate self-reactive T cells and prevent dangerous responses against self-tissues. In the thymus, T cells bearing high affinity T cell receptors (TCR) that can recognize self-antigens are deleted through negative selection [1,2]. However, cells that carry the potential to turn into dangerous self-reactive T cells can still escape negative selection and may cause autoimmune reactions. The establishment of successful mechanisms of peripheral tolerance is, therefore, necessary to prevent autoimmunity. Peripheral tolerance involves a balance of multiple control mechanisms that include T cell deletion, cell-mediated suppression and intrinsic inactivation of T cells [3–5]. Deletion of T cells bearing self-reactive TCRs can occur in the periphery by apoptosis, which is prompted by specific antigenic stimulation of self-reactive T cells. Studies using mouse models have shown that signaling through Fas regulates apoptosis of those self-reactive T cells and that proteins of the Bcl-2 family also participate in the modulation of peripheral T cell deletion [6,7]. Active suppression of T cell activity by regulatory T cells (Treg) constitutes a major mechanism of peripheral T cell tolerance [8]. Tregs express the transcription factor Foxp3 and have the capability of suppressing the activation of other T cells in a contact-dependent manner [9–13], although studies have shown that cytokines, such as interleukin (IL-)35, transforming growth factor beta (TGFβ) or IL-10, also participate in Treg-mediated suppression of T cell responses [14–16].

This review will focus on T cell anergy, a mechanism of peripheral tolerance that defines the functional inactivation of T cells following antigen recognition under non-optimal conditions [17]. Classically, anergy is defined as the hyporesponsive state that is established in T cells when they recognized antigen (signal 1) in the absence of costimulation (signal 2), usually provided by CD28 [18–20]. Under those conditions, T cells fail to become fully activated and enter a state of unresponsiveness that prevents cell proliferation and cytokine production in response to antigen re-encounter [17,21]. Recent studies have expanded our understanding of the context in which T cells become anergic. Together with the signals transduced by the recognition of peptide bound to Major Histocompatibility Complexes (MHC), T cells evaluate not only the presence or absence of CD28 engagement but also multiple other environmental cues that ultimately decide T cell fate [22].

2. Signal integration: T cell activation *vs.* T cell anergy

The most important variable that will determine if the fate of a T cell after encountering antigen will be to activate and engage in effector functions or to become anergic is the presence of costimulation provided by binding of CD28 to its ligands, CD80 or CD86, expressed on antigen presenting cells [18,19,23]. However, numerous studies have made it evident that CD28 signaling is not the only information that T cells integrate together with antigen recognition to decide their functional outcome (Fig. 1). One of the main consequences of CD28 engagement is induction of the expression and increased stabilization of the *Il2* mRNA [24,25]. Production of IL-2 constitutes one of the most important mechanisms of anergy

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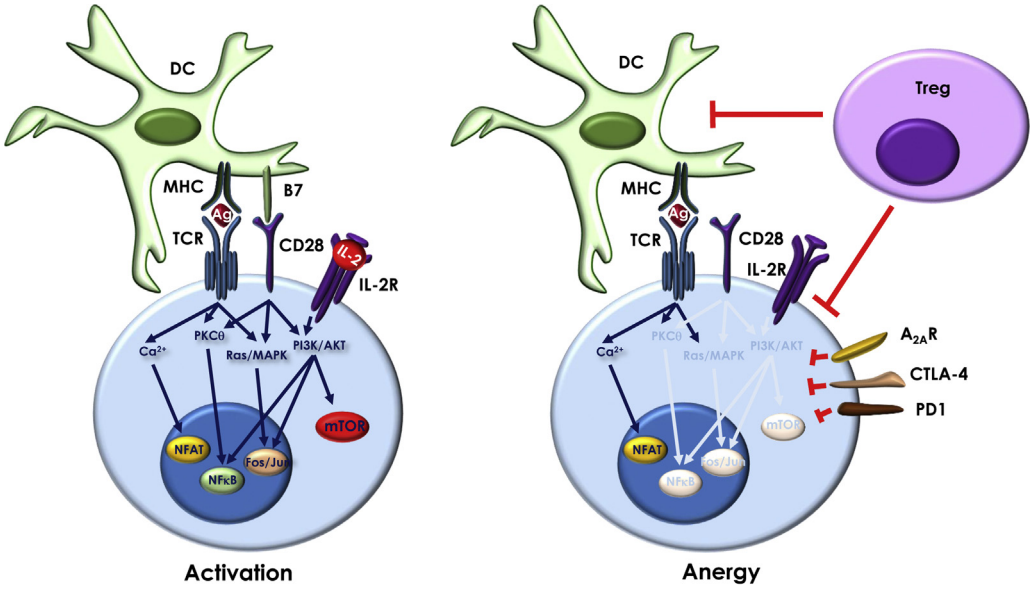

Fig. 1. Signal integration determines T cell fate. Activated T cells integrate signals triggered by recognition of MHC-antigen (Ag) complexes by the TCR, together with those induced by the engagement of CD28 by B7 ligands and by binding of IL-2 to the IL-2 receptor. Those signals translate into the activation of a series of signaling pathways, including increased calcium entry and activation of PKCθ, Ras/MAPKs, PI3K/AKT and mTOR, which allow the T cell to upregulate its metabolism and induce the transcription factors (*e.g.* NFAT, Fos/Jun or NFκB) required to maintain an activation-induced program of gene expression. When TCR engagement occurs in the absence of costimulation and/or the presence of inhibitory signals (*e.g.* effects of Tregs on DCs and effector T cells or engagement of coinhibitory receptors such as CTLA-4, PD-1 or A2aR) an unbalance activation of those signaling pathways leads to the induction of an alternative program of gene expression that will result in anergy.

avoidance induced by CD28 co-engagement, and signaling through the IL-2 receptor has been shown to prevent the induction of anergy even in the absence of co-stimulation [26]. Different targets have been identified downstream of the IL-2 receptor that may explain how this signaling pathway may be responsible for the avoidance of anergy. Engagement of the IL-2 receptor activates the phosphatidylinositol 3 kinase (PI3K)/AKT axis, which, among other targets, induces the degradation of the cyclin-dependent kinase inhibitor $p27^{kip1}$ [27–29]. In the absence of costimulation or IL-2 receptor signaling, $p27^{kip1}$ fails to be degraded and progression through the cell cycle is halted. Consequently, T cells that lack $p27^{kip1}$ become resistant to costimulation blockade-induced anergy [29]. Recently, it has been also shown that engagement of the IL-2 receptor causes repression of the expression of the histone deacetylase sirtuin 1 (Sirt1), which by inhibiting Jun activity, plays an important role in the suppression of activation-induced responses in anergic T cells [30,31]. This effect is also mediated by the activation of PI3K/AKT, which results in the cytosolic sequestration of FoxO3, a transcription factor required for the expression of Sirt1 in anergic cells.

The importance of the mammalian target of rapamycin (mTOR) as a regulator of T cell fate has been brought to light recently in studies that analyzed mouse models deficient for components of the mTOR complexes in T cells [32,33]. Activated AKT downstream of the IL-2 receptor phosphorylates tuberous sclerosis complex proteins (TSC), inhibiting the GTPase activating protein activity that TSC has on the GTP-binding protein Rheb, an mTOR activator. Consequently IL-2 receptor engagement results in increased levels of GTP-bound Rheb and mTOR activation [34,35]. The importance of this pathway for T cell anergy was demonstrated by early studies that showed that activation of T cells in the presence of the mTOR inhibitor rapamycin induced anergy even when cells received full costimulation [36]. Though initially thought that this effect was due to the fact that mTOR was required for T cells to undergo the G1-to-S transition, it was soon proven that inhibition of cell cycle progression through the targeting of other cell cycle regulators did not cause T cells to become anergic following full stimulation, and that transition from G1 to S did not prevent cells from becoming anergic [37,38]. These results suggested that it was mTOR signaling itself what really was necessary to prevent anergy. In fact, it was later shown that it was the role of this kinase in the modulation of T cell metabolism what defined mTOR as a regulator of T cell fate [39]. T cell activation is a highly metabolically demanding process and activation of mTOR is necessary for T cells to adapt to this new high demand. If mTOR activity is not efficiently induced and T cells cannot increase their metabolism during activation, they become anergic. It is interesting to note once anergic T cells are not only unable to proliferate and secrete IL-2 but also to induce the metabolic machinery required to sustain activation, failing to upregulate the expression of glucose, amino acid and iron transporters [40,41]. Inhibition of mTOR with rapamycin during activation, does not only prevent anergy avoidance but also promotes the differentiation of Tregs, cells that are also intrinsically anergic, as they do not produce IL-2 or proliferate when stimulated, unless exogenous IL-2 is provided [32].

Although the presence of costimulatory positive signals is necessary to avoid anergy, T cells also integrate signaling received through the engagement of inhibitory receptors. For instance, coinhibition through the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or the programmed cell death 1 (PD-1) receptors is also an important cue in the regulation of T cell fate, and both coinhibitory receptors have been shown to participate in the regulation of T cell anergy in different contexts [42–48]. Similarly, engagement of the adenosine receptor $A_{2A}R$ causes inhibition of the Ras/Mitogen activated protein kinase (MAPK) signaling pathway and induces anergy even in the presence of costimulation [49]. Recently the role of Tregs in the maintenance of T cell anergy has been proposed. Mice adoptively transferred with self-antigen specific arithrogenic $CD4^+$T cell populations failed to induce anergy in those T cells in the absence of Tregs and could not prevent the development of arthritis [50]. This study supports that signals imparted directly or indirectly by Tregs are also key regulators of the maintenance of peripheral T cell tolerance, not only through suppression of the activity of other cell populations but also by inducing T cell anergy. All data available

indicate, thus, that the induction of T cell anergy is a tightly controlled process that results from the integrated balance of multiple positive and negative signals that constitute a much more complex system than the classical signal1/signal2 model (Fig. 1).

3. Transcriptional regulation of T cell anergy

The engagement of the TCR by MHC-antigen complexes allows the CD4 or CD8 coreceptor-associated src-family lymphocyte-specific protein tyrosine kinase (Lck) to phosphorylate immunoreceptor tyrosine based activation motifs (ITAMS) on the CD3 chains of the TCR, creating docking sites for ζ-associated protein of 70 kDa (ZAP70) that is then phosphorylated by Lck [51–55]. The subsequent phosphorylation of the membrane-associated linker for T-cell activation (LAT) and the SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) by Zap70, induces the recruitment of the Grb2-related adapter downstream of shc (GADS) [56,57]. The new formed docking sites allow phospholipase C-γ1 (PLCγ1) to be activated by the Tec family kinase Itk [58]. PLCγ then cleaves the membrane phospholipid phosphatidylinositol-4,5-biphosphate into inositol 1,4,5-triphosphate (IP^3) and diacylglycerol (DAG). PKC/Ras/MAPK signaling is activated by TCR-induced DAG [59,60], but CD28 engagement is required for optimal activation of those signaling pathways [61]. IP^3 interacts with its receptor and induces the aperture of calcium channels at the endoplasmic reticulum. Depletion intracellular calcium stores is sensed by STIM proteins that then activate store-operated calcium entry (SOCE) channels at the plasma membrane [62]. This process requires only TCR engagement. When T cells are activated without the engagement of costimulation, unbalance calcium signaling in the absence of full activation of PKC/Ras-regulated pathways results in the expression of a specific set of genes encoding for proteins that are responsible for the hyporesponsive state of anergic T cells [63]. Blockade of CD28-regulated pathways by inhibitory receptors would have a similar effect. The importance of calcium signaling in the induction of T cell anergy was evident form early experiments that showed that the calcineurin inhibitor cyclosporine A prevented T cells activated with anti-CD3 without CD28 engagement from becoming anergic [64]. The calcium/calmodulin-dependent phosphatase calcineurin is a major transducer of calcium signaling in T cells. Activation of calcineurin in response to increased intracellular calcium results in the dephosphorylation of members of the nuclear factor of activated T cells (NFAT) family of transcription factors, which are otherwise retained in the cytosol in a highly phosphorylated state [65,66]. Upon dephosphorylation, NFAT proteins undergo a conformational change that exposes their nuclear localization signal and allows their translocation into the nucleus [67]. In activated T cells, NFAT partners with AP-1 and other transcription factors to induce the expression of many activation-induced genes [65,68,69]. Suboptimal activation of T cells would cause to the calcium/calcineurin-induced nuclear translocation of NFAT without full activation of the MAPK-dependent AP-1 transcription complexes. Under these circumstances, NFAT1 can form low affinity homodimers [70,71], instead of the higher affinity NFAT/AP-1 complexes that would assemble in fully activated cells [63,72]. In anergic cells, NFAT homodimers assemble onto symmetric binding sites similar to the canonical κB sites [70,71] to induce a specific program of anergy-associated gene expression (Fig. 2) [72,73]. The central role of NFAT1 in the induction of T cell anergy is supported by the fact that NAFT1-deficient T cells are resistant to stimuli that induce anergy, whereas expression of a constitutively active form of this protein renders T cells hyporesponsive [73].

In addition to NFAT1, other transcription factors also contribute to the activation of the anergy-inducing gene expression program. Expression of early growth response proteins 2 and 3 (Egr-2/Egr-3) is upregulated in an NFAT-dependent manner in anergic CD4+ T cells. Egr proteins control the expression several anergy-associated genes, including those encoding for the ubiquitin ligase Casitas B-lineage lymphoma b (Cbl-b) and diacylglycerol kinase alpha (DGKα) [74–76]. Consequently, Egr2-deficient T cells are resistant to tumor-induced anergy *in vivo*, which permits mice bearing those T cells to mount more efficient and potent antitumor responses [76]. The activity of Egr2 can also be modulated by other proteins. Deltex-1 is also expressed in anergic T cells in an NFAT-dependent way and Deltex1-deficient T cells are hyperactive and resistant to anergizing stimuli [77]. Deltex-1 interacts with Egr-2 to upregulate the expression of *Cblb*, though the mechanism through which this functional synergy occurs is still not known [77].

The hyporesponsive state in anergic T cells responds, therefore, to the activation of an anergy-associated program of gene expression that is controlled by a specific set of transcription factors including NFAT1 and Egr2. As we will discuss below, proteins encoded by those genes are responsible for the blockade of TCR signaling and the inhibition of cytokine expression that defines anergic T cells (Fig. 2).

4. Inhibitory mechanisms engaged in anergic T cells

When integration of the different positive and negative signals received during the interaction with cognate antigen results in anergy, T cells induce the expression of a series of proteins that eventually will prevent the cell from being activated following new encounter with antigen. Functionally, these proteins exert their inhibitory action at two different levels: blocking signaling downstream of the TCR and/or CD28, and directly inhibiting the expression of cytokine genes.

4.1. Signaling blocks in anergic T cells

Initial characterization of signaling pathways in anergic T cells identified a block in the activation of MAPK/Ras that precluded activation following re-stimulation [78,79]. In the last 10 years many of the mediators responsible for this signaling blockade have been discovered and the mechanisms underlying their effects characterized. Several E3 ubiquitin ligases are expressed in anergic T cells that target components of the TCR- and CD28-activated signaling pathways for degradation or inactivation. The RING-type E3 ubiquitin ligase GRAIL is expressed in anergic T cells, and GRAIL-deficient cells show a hyper responsive phenotype with resistance to tolerance induction *in vitro* and *in vivo* [80–82]. Several targets have been proposed to explain the function of this ubiquitin ligase in T cells including CD3, the Rho guanine dissociation inhibitor (RhoGDI), CD40 ligand or the actin-related protein 2/3 subunit 5 (Arp2/3-5). GRAIL-mediated ubiquitination of these proteins causes their inactivation or degradation, which leads to reduced TCR-induced signaling and impaired formation of mature immunological synapses [82–86]. Cbl-b, another RING-type ubiquitin ligase, and Itch, a HECT-type E3 ligase, are also expressed in anergic cells where they regulate the inactivation and degradation of several signaling intermediates including PKCθ, PLCγ-1, the p85 regulatory subunit of the PI3K, Jun or the zeta chain of the TCR [87–91].

Proteolytic cleavage of Vav1 and GADS also occurs in anergic CD4+ T cells. This cleavage is not due to the activity of the ubiquitin/proteasome system but mediated instead by the calcium-regulated activation of caspase 3, and prevents recruitment of those proteins to the plasma membrane, inhibiting effective signaling through the TCR signalosome [92]. Expression of DGKα is also upregulated in $CD4^+$ T cells in response to anergizing stimuli in an NFAT/Egr2-dependent manner [73,76]. DGKα phosphorylates DAG

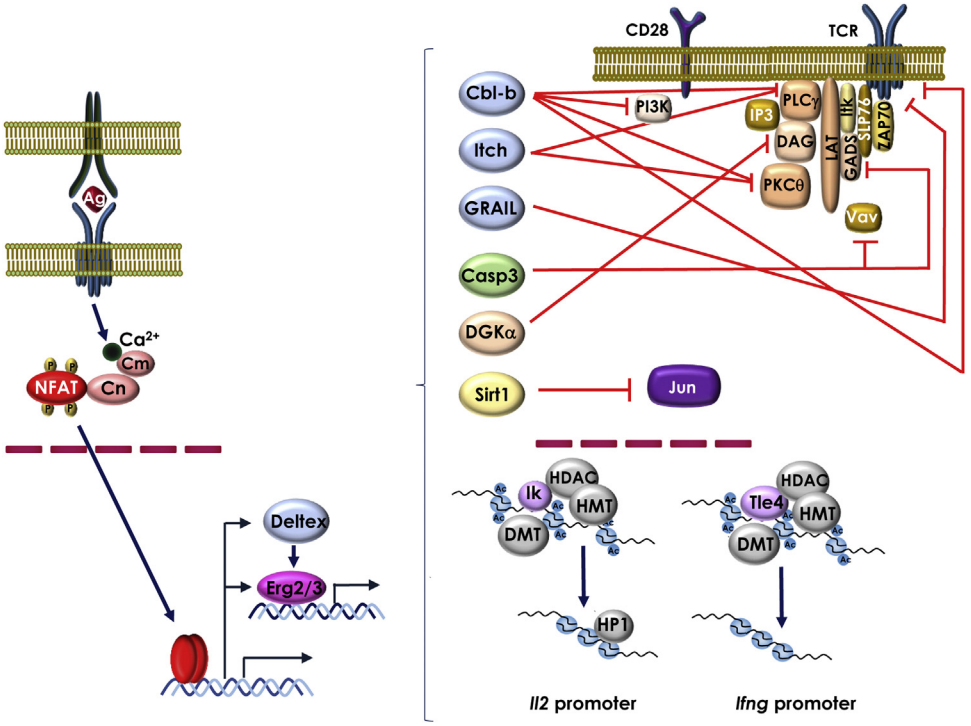

Fig. 2. NFAT proteins activate an anergy-inducing program of gene expression. In response to tolerizing stimuli, preferential activation of the Ca^{2+}-Calmodulin (Cm)-Calcineurin (Cn) signaling pathway leads to the expression of an NFAT-dependent program of gene expression. NFAT containing complexes, including NFAT1 homodimers, and other transcription factors, especially Egr proteins, will induce the expression of a series of proteins thatinhibit T cell activation at different levels. The ubiquitin ligases GRAIL, Itch and Cbl-b, caspase 3 and DGKα, block TCR and CD28 signaling through targeted degradation or inactivation of multiple proteins that form part of the TCR signalosome. Sirt1 directly deacetylates and inactivates Jun. Binding of transcriptional repressors, such as Ikaros (IK) or Tle4, to the *Il2* and *Ifng* loci recruit chromatin remodeling proteins that together with DNA methyl transferases (DMT) induce epigenetic modifications that turn those genes into transcriptionally silenced loci.

to form phosphoric acid, hence blocking DAG-activated signaling pathways. Upregulation of the expression of this kinase to prevent DAG-induced PKC/Ras signaling has been shown to be a crucial inhibitory mechanism required for the induction of T cell tolerance [93,94].

Other mechanisms that cause defective upstream signaling in anergic T cells have also been identified. For instance, defective LAT palmitoylation in anergic T cells results in decreased recruitment of LAT to the plasma membrane and prevent localization of this key adaptor protein into the immunological synapse, though the mechanism responsible for the changes in LAT palmitoylation remain to be identified [95].

4.2. Transcriptional regulation of cytokine expression

Although inhibition of TCR signaling should prevent the expression of cytokines, mechanisms that directly inhibit cytokine gene expression that contribute to maintain an unresponsive state are also engaged in anergic T cells. Recent studies have identified Sirt1, a type III histone deacetylase, as a key factor for the maintenance of T cell tolerance. Sirt1 interacts with and deacetylates c-Jun. That modification inactivates this transcription factor that forms part of the NFAT/AP-1 complexes required to induce *Il2* expression in activated T cells [31]. Furthermore, negative regulators of *Il2* gene expression, including the transducer of ERBB2 1 (Tob1), Smad3 and the cAMP responsive element binding protein/cAMP responsive element modulator complex (CREB/CREM) are also expressed in anergic T cells, where they can bind the *Il2* promoter and directly repress *Il2* gene transcription [96,97].

Inhibition of *Il2* gene expression in anergic T cells is not only controlled by the transient action of transcription factors. In the last few years, several studies have shown that epigenetic imprinting occurs at this locus that silences the expression of the *Il2* gene. Ikaros, the founding member of the Ikaros family of transcription factors, binds to sites in the *Il2* promoter where it recruits histone deacetylases (HDAC) to remove acetyl-groups from the tails of histones H3 and H4 and induce silencing of the *Il2* gene [98,99]. It is interesting to note that two other members of the Ikaros family, Eos and Helios, have been shown to be responsible for the epigenetic silencing of the *Il2* gene in natural Tregs [100,101], suggesting that different Ikaros proteins may regulate the epigenetic silencing of *Il2* transcription in distinct T cell populations that have an anergic phenotype. Histone deacetylation is however not the only chromatin modification that maintains this gene in a repressed state. Histone deacetylation-dependent recruitment of the histone methyl transferase Suv39H1 in anergic $CD4^+$ T cells results in tri-methylation of histone H3 at lysine 9. This modification creates docking sites for the heterochromatin binding protein HP-1, which results in the translocation of the *Il2* locus into silent heterochromatin-rich regions in the nucleus [102]. Changes in DNA methylation are also often associated with transcriptionally active or silent loci. Whereas upregulation of the *Il2* gene transcription in activated T cells correlates with marked DNA demethylation of the *Il2* promoter [103], in an *in vivo* model of superantigen induced T cell anergy, decreased *Il2* expression in T cells was accompanied by increased DNA methylation of the *Il2* promoter, that is likely to contribute to the imprinting of a stable silencing state of this gene in anergic cells [104]. Interestingly, this modification was not only circumscribed to the *Il2* promoter but was also detected in the *Ifng* locus, suggesting that epigenetic silencing of cytokine expression affects not only IL-2 but may also extend to effector cytokines such as IFNγ [104]. Supporting this hypothesis, our lab has recently found that Tle4, a member of the Groucho family of transcription factors, is also expressed in anergic cells, where it binds to an *Ifng*

enhancer, inducing epigenetic marks associated with transcriptional repression, including histone deacetylation and trimethylation of lysine 9 on histone H3 (Bandyopadhyay, S and Macian, F; unpublished observations). A recent study has suggested that alternative check points may be also activated in anergic T cells to prevent expression of cytokines. Using an *in vivo* model of adaptive tolerance, TCR-transgenic CD4$^+$ T cell transferred into a host expressing a soluble version of the cognate antigen became anergic but did not show a significant decreased in their ability to transcribe cytokine genes. These cells instead had a block in cytokine mRNA translation that resulted in greatly diminished production of effector cytokines [105]. Though the mechanisms responsible for the regulation of cytokine mRNA translation in anergic T cells have not been elucidated yet, it is tempting to speculate that post-transcriptional gene regulation by RNA-binding proteins and/or micro RNAs may constitute additional controls on the expression of cytokine in these cells.

The existence of a series of inhibitory mechanisms that target the TCR signaling pathway at multiple levels and block cytokine expression through different mechanisms should ensure that anergic T cells stay unresponsive and autoimmune reactions do not occur. It is likely, though, that distinct subsets of those mechanisms that sustain anergy might be induced in response to different tolerizing environments that deliver specific negative signals to self-reactive T cells.

5. Stability of the anergic phenotype. Plasticity in anergic T cells

Anergy is defined as a long-term state of unresponsiveness, and in many models of T cell tolerance, anergic T cells can be found long after the tolerizing stimulus has been delivered. This long-term nature of the anergic state could respond to epigenetic modifications on cytokine genes, which would lead to stable inhibition of gene transcription [98,99,102,104]. Furthermore, it is also possible that a self-maintaining circuitry may exist. Due to the signaling blocks present in anergic cells, re-encounter with antigen would only produce a suboptimal activation that would reinforce the expression of the genetic program of anergy. This could explain why in some models continuous presence of antigen has been reported to be necessary to sustain anergy *in vivo* [106]. Maintenance of hyporesponsive T cell populations might respond to a need to keep an effective TCR repertoire, as elimination in the thymus or the periphery of all T cells bearing TCRs with any level of affinity for self-antigen could result in dangerously reduced TCR diversity. If this is the case, it would be expected that plasticity should exist in the anergic T cell compartment that would allow reactivation and transformation of anergic cells into effector T cells when required. Co-stimulation alone is not able to reverse tolerance. The presence of blocks that prevent transduction of signals downstream of CD28 explain why anergic T cells do not respond to reencounter with antigen even when costimulation is present. It has been known, however, for some time that signaling through the IL-2 receptor not only prevents the induction of anergy but can also reverse it [107]. Different mechanisms have been proposed to explain how IL-2 receptor engagement can break T cell tolerance. One of the main signaling defects in anergic T cells is the inhibition of the Ras/MAPK pathway, but IL-2 can directly induced activation of MAPK and AP-1, bypassing that signaling block [108]. Similarly, PI3K/AKT activation downstream of the IL-2 receptor can activate mTOR, allowing anergic T cells to upregulate their metabolic output to adapt to the demands raised by activation in otherwise metabolically anergic cells [39,41]. Recently. It has been shown that, also as a result of the activation for the PI3K/AKT pathway downstream of the IL-2 receptor, the transcription factor FoxO3a remains sequestered in the cytosol, preventing it from interacting with Egr proteins to induce the expression of Sirt1, a protein required for the establishment of the unresponsive state in anergic T cells [30,31]. In any case, breaking tolerance through signaling mediated by the IL-2 receptor would allow anergic T cells to become effector cells if cross-recognition of a foreign antigen occurred in the context of an active immune response, where IL-2 would be secreted by other non-tolerant cell populations.

Well characterized in the CD8$^+$ T cell compartment, transfer of anergic T cells into a lymphopenic host leads to homeostatic proliferation that results in a break of tolerance and the activation of effector functions in the transferred population [109]. Expansion of T cells transferred into a lymphopenic host appears to respond to interactions of T cells with MHC complexes presenting self-peptides, and by homeostatic cytokines, such as IL-7 or IL-15, which by signaling through the common gamma chain could contribute to the reversal of tolerance. This process has been shown to have the potential to be a potent tool to improve anti-tumor responses by breaking tumor antigen-induced T cell anergy [110,111]. A recent study has shown, however, that even though transfer of TCR transgenic CD8$^+$ T cells anergic to a specific self-antigen into lymphopenic hosts results in proliferation and regaining of their ability to respond to cognate antigen, reversal of tolerance appears to be transient, and cells reacquired an anergic phenotype after a few months. Surprisingly, this occurs even in hosts that do not express the self-antigen recognized by the TCR-transgenic CD8$^+$ T cells [112]. This study has important implications for our understanding on how T cell tolerance is maintained, as it indicates that the capacity of anergic T cells to regain effector functions after homeostatic proliferation is limited in time, an effect which is likely due to intrinsic cell properties, as it occurs in the absence of tolerizing antigen. Available data clearly indicates that silencing of cytokine genes in anergic cells is maintained through epigenetic mechanisms, which might also include microRNAs. The "anergic" memory described in CD8$^+$ T cells could therefore be a results of epigenetic memory, which would be temporarily overcome under specific situations (*e.g.* inflammatory reaction, homeostatic proliferation) but that would cause the cell to re-establish an anergic program once those stimuli that induce tolerance reversal disappear.

The development of T cells with suppressive capacity occurs concomitant with the generation of hyporesponsive cells in several models of T cell anergy, and signals that induce anergy are also involved in the generation of Tregs [32,49,113–115]. Furthermore, proteins that regulate the induction and maintenance of anergy have also been reported to participate in the generation of Tregs [116–119]. These results may indicate the existence of a common differentiation pathway that would lead to anergy or to the acquisition of regulatory function depending on the specific context in which antigen is encounterd and the presence of specific additional signals. However, it is also possible that anergic T cells might be able to acquire not only effector functions, as described above, but also suppressive capacity, and constitute a reservoir of T cells bearing self-reactive TCRs that can become regulatory T cells.

6. Concluding remarks

In the last decade we have gained a clear insight into the molecular mechanisms that regulate the induction and maintenance of T cell anergy. T cells integrate a series of different cues that extend beyond the presence or absence of costimulation to include the activation of cytokine or coinhibitory receptors or the presence of populations of cells with suppressor activity. Under conditions that result in tolerance, NFAT and Egr transcription factors induce the activation of a program of gene expression that results in the synthesis of proteins that inhibit TCR signaling by targeting different

pathways downstream of the TCR or CD28; and induce epigenetic modifications that result in the stable silencing of cytokine expression. The unresponsive phenotype in anergic cells is, however, not a differentiation end-point. IL-2 receptor signaling or forced homeostatic proliferation can break tolerance. Under these circumstances anergic T cells regain the ability to expand in response to antigen and engage in effector functions. The exact mechanisms that are responsible for anergy reversal are still not fully understood but they need to involve bypassing of signaling blocks and the removal of epigenetic repression. Recent evidence has indicated that reversal of anergy is not a stable but a transient process in $CD8^+$ T cells. We still do not know, though, whether the same holds true for T helper cells. Tight epigenetic regulation of the transcription or translation of genes involved in the maintenance of the anergic status may account for the unstable plasticity of anergic T cells, however the mechanisms responsible for the imprinting of anergic memory in T cells are still not known. The characterization of the cell populations, signaling networks and epigenetic mechanisms that control the induction and reversal of the anergic phenotype in T cells may help us understand better the dynamic nature of anergy and identify new therapeutic targets aimed at promoting tolerance, to prevent autoimmune disease or graft rejection, or activation, to boost anti-tumor immune responses.

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