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New Insights into the Molecular Basis of T Cell Anergy: Anergy Factors, Avoidance Sensors, and Epigenetic Imprinting

Andrew D. Wells

The vertebrate immune system has evolved to deal with invasive pathogens, but this adaptation comes at the expense of immunopathology. Among a number of mechanisms that coevolved to control adaptive immunity is anergy, the functional inactivation of T lymphocytes that respond to Ag in the absence of inflammation. In this review, I highlight a series of intracellular proteins in quiescent T cells that function to integrate signals from Ag, costimulatory, and growth factor receptors. These factors ensure that cells that fail to engage all three pathways are shunted into an alternative transcriptional program designed to dissuade them from participating in subsequent immune responses. Recent studies indicate that anergy is the combined result of factors that negatively regulate proximal TCR-coupled signal transduction, together with a program of active transcriptional silencing that is reinforced through epigenetic mechanisms. The Journal of Immunology, 2009, 182: 7331–7341.

Anergy refers to a state of reduced function in which a viable, Ag-specific T cell is unable to respond to an immunogenic stimulus. Anergy can be induced under a number of circumstances that, for the most part, can be categorized as resulting from either a normal antigenic stimulus received in the absence of costimulation or from an altered and/or chronic TCR stimulus (1). A central hallmark of anergy, as first described in CD4⁺ Th1 clones, is the failure to synthesize the major T cell growth factor IL-2, resulting in an abortive proliferative response (2); but anergy in primary CD4⁺ and CD8⁺ T cells is also characterized by defective production of inflammatory cytokines such as IFN-γ and TNF-α (3). Foxp3⁺ regulatory T cells (Treg), whose function is to control rather than mediate inflammatory responses, also fail to express inflammatory cytokine genes and can therefore be categorized as anergic.

CD28 costimulation is required for efficient TCR-induced transcription of the il2 gene

Engagement of the T cell Ag receptor is not sufficient for full activation and induction of il2 gene expression but requires a second signal provided by the CD28 costimulatory receptor (4, 5). Coligation of TCR and CD28 leads to activation of phospholipase Cγ (PLCγ)-1, Ras, and protein kinase Cθ (PKCθ), which in turn activate the MAPK, JNK, PI3K/Akt, and IkB kinase (IKK) pathways and induce the mobilization of intracellular calcium (Fig. 1). These signal transduction pathways activate multiple transcription factors, among which NFAT, AP-1, CREB, and NFκB are particularly important for transactivation of the il2 gene (6, 7).

TCR engagement in the absence of CD28 costimulation does not efficiently activate the MAPK, PI3K/Akt, and IKK pathways (reviewed in Ref. 8), which results in reduced AP-1 and NFκB activity, defective transactivation of the CD28 response element (CD28RE) in the il2 promoter, and induction of anergy. This situation as been modeled experimentally by inducing a blockade or a genetic deficiency in the ligands for CD28 (B7-1 and B7-2), but it is thought to occur physiologically when T cells meet their cognate Ag on APCs that have not received the inflammatory signals necessary for up-regulation of costimulatory ligands. Chronic exposure to superantigens, high-dose soluble Ags, or parenchymal Ags also induces T cell anergy (2), but it is not clear whether or not this is a consequence of limiting costimulatory ligand availability. Another physiologic situation that can result in T cell anergy is the existence of high levels of extracellular adenosine. Adenosine released by tumor cells or regulatory T cells can induce anergy in conventional CD4⁺ T cells (9, 10). Engagement of A2A receptors by adenosine leads to elevation of intracellular cAMP, an immunosuppressive secondary messenger that can also be generated by other G protein-coupled receptors expressed by T cells.
Because CD28- and cAMP-coupled signaling pathways antagonize each other, a shift toward cAMP/protein kinase A signaling would favor anergy.

Why is there such tight control over expression of the \textit{il2} gene? The immune system has evolved multiple layers of control over the expression of this T cell growth factor; however,
IL-2 is not absolutely required for T cell proliferation. There are several functions of IL-2 aside from its role as an autocrine growth factor that would be problematic if not stringently controlled. For instance, IL-2 promotes CD8+ T cell memory (12), and drives the efficient induction of IFN-γ and IL-4 during Th1 and Th2 differentiation (13). The requirement for both antigens and costimulatory signals for the induction of IL-2 therefore assures that CD4+ T cell help will only occur under the appropriate conditions (e.g., inflammation and/or infection). These circumstances also initiate a negative feedback loop, as IL-2 potently primes T cells for activation-induced cell death and mediates Treg homeostasis (reviewed in Ref. 14). Therefore, IL-2 represents a potent and complex factor influencing the balance between tolerance and immunity. Finally, as described below, IL-2 signaling through the IL-2R is crucial for licensing T cells to progress beyond the anergic checkpoint and participate in an immune response.

**Signals from IL-2R are required for T cells to escape anergy induction**

CD28 costimulation is not sufficient for anergy avoidance, because anergy can be induced in the face of active CD28 signaling if IL-2 activity is neutralized or if IL-2R-coupled signal transduction is inhibited (reviewed in Ref. 15). In fact, CD28 is not necessary for T cells to escape anergy, because exogenous IL-2 can compensate for a lack of costimulation to allow anergy avoidance. Therefore, CD28 promotes anergy avoidance through its requisite role in driving transcription of the il2 gene. This also illustrates that anergy is actually an integral consequence of TCR/CD28 stimulation, while IL-2 signaling through IL-2R transduces the signal that allows T cells to escape anergy. IL-2 binding to the IL-2R induces activation of the Jak3-Stat5 and MAPK pathways (Fig. 1), but current evidence suggests that the anergy avoidance signal is transduced through a pathway leading from PI3K to mammalian target of rapamycin (mTOR) and cyclin-dependent kinases (CDKs). The first line of evidence came from studies showing that mTOR activity is inversely correlated with anergy in CD4+ T cells (16) and that inhibition of mTOR activity in the face of otherwise normal TCR/CD28/IL-2R costimulation results in anergy (17). These data show that IL-2R-coupled mTOR activity is required for T cells to escape anergy. A second line of evidence comes from studies showing that PTEN (phosphatase and tensin homologue deleted on chromosome 10), a negative regulator of PI3K activity, and p27kip1, a CDK inhibitor, are both required for the induction of T cell anergy (18, 19). p27kip1 is expressed in quiescent T cells, and TCR activation in the absence of costimulation leads to further cAMP-dependent induction (20). Conversely, CD28- or IL-2R-dependent MAPK and Akt activity targets p27kip1 for proteolytic degradation, leading to derepression of CDK and cell cycle progression. In this sense, p27kip1 has been described as an “anergy factor” (20) or, perhaps more accurately, as an “anergy avoidance sensor” (19) in that it acts as an intracellular sensor of IL-2, the signal that opposes anergy. Ectopic expression of p27kip1 has been shown to induce anergy (20), whereas CD4+ T cells that lack p27kip1 are resistant to anergy induced by a costimulatory blockade in vitro (19). Moreover, p27kip1-deficient mice are unable to develop donor-specific tolerance to cardiac allografts in response to a costimulatory blockade in vivo (21). Some modes of anergy induction do not require p27kip1. Anergic T cells from superantigen-treated mice lose expression of the common γ-chain (22) and therefore lack IL-2R signaling. Similarly, CD4+ T cell lines are energized in vitro with immobilized anti-TCR Ab exhibit defective mTOR activity (16). These forms of anergy do not require p27kip1 (23, 24), most likely because the anergy avoidance signal in these cells has been cut off upstream of p27kip1 (see Fig. 1).

How does p27kip1 promote anergy? This protein is perhaps best known for its role in binding to and inhibiting the activities of CDK1 and CDK2, so the most straightforward interpretation of the available data is that these CDK are involved in anergy avoidance and that p27kip1 promotes anergy by inhibiting this function of CDK. However, p27kip1 has other CDK-independent activities that could contribute to its role in regulating T cell anergy. For instance, p27kip1 inhibits actin polymerization and focal adhesion by binding to RhoA through its C-terminal portion (25). The implications of this function of p27kip1 for anergy are 2-fold. First, anergic T cells exhibit defective integrin-mediated adhesion (26, 27); therefore, inhibition Rho/Rac function could contribute to this defect. Second, RhoA co-operates with Rap1, a GTP binding protein involved in CAMP signaling and anergy (28); therefore, p27kip1 could influence anergy induction through modulation of Rap1 function. p27kip1 also binds to Jab1, a co-activator of Jun, and inhibits gene transactivation by AP-1 and other Jun-containing transcription factors (29). Recent studies using T cells expressing a deletion mutant of p27kip1 that lacks the CDK binding domain have suggested that the anergy-promoting activity of p27kip1 is mediated solely through CDK inhibition (30). However, this mutant also lacks the serine 10 motif required for the proper subcellular localization and inhibition of Jun activity. Given the important role of Fos/Jun heterodimers in il2 transcription and anergy, these studies do not exclude that p27kip1 may contribute to anergy through inhibiting the transcriptional activity of Jun. Although existing evidence is consistent with a role for CDK in anergy avoidance, the establishment of a clear role for distinct CDKs will require further research.

**Transcriptional repressors as anergy avoidance sensors at the il2 promoter**

How does a T cell, or specifically the il2 gene in a T cell, know whether costimulatory signals have been received? Reduced NF-κB/AP-1 binding at the CD28 response element may be indicative of a lack of costimulation; however, this is actually not sufficient to keep the il2 gene transcriptionally inactive. Conventional CD4+ T cells harbor several molecular mechanisms that oppose transactivation of the il2 gene in the absence of CD28 costimulation, and Treg cells exhibit additional mechanisms that maintain anergy even in the presence of costimulatory and mitogenic signals.

*Ikaros*. Recent studies have revealed a mechanism that operates directly on the il2 promoter in quiescent T cells that keeps the il2 gene silenced until signals from CD28 are received. This mechanism involves Ikaros, a zinc finger DNA binding protein previously recognized for its requisite role in lymphocyte development (31). Ikaros has been shown to promote the differentiation of hematopoietic progenitors by recruiting complexes that regulate the expression of genes involved in lymphocyte development; but how this transcription factor functions in mature peripheral T cells has just begun to be addressed. Ikaros is expressed in resting, naive CD4+ T cells, where it is bound to the endogenous il2 promoter at the TCEd/NFIL-2C element,
as well as 4 bp from the first TATA box (32). In this quiescent state, hypomethylated nucleosomes are positioned throughout the promoter region and the transcriptional start site of the il2 locus that block access to transcription factors (Fig. 2A; reviewed in Ref. 33). However, in naive T cells that lack Ikaros or express a mutant form of Ikaros that cannot bind DNA, the histones at the il2 promoter are constitutively acetylated, resembling a transcriptionally “poised” locus (32). Consequently, the il2 gene no longer requires signals from CD28 for full expression in these cells, and instead of being rendered anergic, Ikaros-mutant CD4+ T cells undergo a Th1 effector differentiation program in the absence of costimulatory signals from CD28 (32). Ikaros therefore enforces quiescence through epigenetic modification of the il2 locus and acts as a specific, gene-proximal sensor of CD28 costimulation in naive CD4+ T cells.

p50, ZEB1, Blimp-1, Tob, and Smads. Other transcriptional repressors may cooperate with Ikaros to maintain silence at the il2 promoter in naive T cells (Fig. 2A). Specifically, p50-p50 NFκB homodimers are present in the nuclei of resting CD4+ T cells, bind to the TCE4/NFIL-2C element in vitro, and suppress il2 promoter-luciferase activity when overexpressed (34). These homodimers can recruit histone deacetylases (HDACs) to other NFκB-responsive promoters and have been shown to be involved with superantigen-induced anergy (35).

What is the sensing mechanism? Given the data outlined above, it is remarkable to note that the reduced transcriptional activity of AP-1, CREB, and NFκB induced by TCR signaling alone is still sufficient to transactivate the il2 gene if not opposed by active silencing mechanisms (Fig. 2). The fact that CD28 costimulation functions not only to activate factors that positively regulate il2 transcription but also to oppose mechanisms that silence il2 gene expression in quiescent T cells represents a new insight into the regulation of T cell activation and anergy. But how do repressive factors such as Tob, p50, and Ikaros “sense” costimulatory and mitogenic signals? One potential mechanism could be competition between negative and positive transcription factors for binding to regulatory elements in the il2 promoter. For instance, repressive p50 homodimers or Ikaros could be inactivated by CD28 and/or IL-2R-coupled kinases (e.g., PI3K, PKB/Akt, mTOR, IKK, and CDK). In this way, costimulatory and mitogenic signaling could derepress the il2

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**FIGURE 2.** A. The il2 promoter-enhancer in quiescent T cells is maintained in a closed conformation by HDAC activity recruited by Ikaros (Ik), ZEB1, p50-p50, and Tob-Smad. B. TCR and CD28 costimulation not only induces transactivators but also opposes the activity of corepressors. C and D, Coactivators (e.g., the CREB-binding protein CBP) are recruited to the derepressed promoter acetylate local nucleosomes, creating docking sites for chromatin remodeling complexes (Brg/Brm) and rendering the TCEd, CD28RE, ARRE-1, and TATA elements accessible to induced and basal transcription factors (C). This creates a molecular scaffold for the assembly of the il2 enhanceosome, a structure that stabilizes and directs transcription by the RNA polymerase (RNAPol) holoenzyme (D).
gene through direct inactivation of negative regulators. An example of this may be the inactivation of Smad3 by CD28 or IL-2R-stimulated CDK2 activity in T cells (30). The evidence for this is that a mutant of Smad3 that cannot be phosphorylated by CDK2 was able to inhibit IL-2 production in Ag-stimulated CD4+ T cells. Importantly, in addition to acting downstream of CD28, Ikaros is also a sensor of IL-2R signal transduction, as CD4+ T cells with reduced Ikaros DNA binding activity are also insensitive to anergy induced by a blockade of IL-2/IL-2R (32). This establishes Ikaros as a central factor regulating il2 gene expression in response to not only TCR/CD28 signaling but also to anergy avoidance signals transduced by IL-2 itself, and it suggests that, like Smads, Ikaros may also function downstream of CD28- and IL-2R-coupled kinases (Fig. 1).

An alternative program of gene expression as a basis for the anergic state

Anergy induction is an active process that requires new protein synthesis and can be blocked by drugs that interfere with TCR-coupled calcium mobilization (39, 40). Indeed, a hyporesponsive state resembling anergy can be induced in CD4+ T cells by artificially elevating intracellular calcium levels (41, 42). This has led to the concept of an “anergy factor” that is actively induced by calcium-dependent signals and enforces dominant inhibition of TCR/CD28-induced il2 gene expression. This concept has served as a valid and useful framework for research into the molecular nature of anergy, but our current understanding suggests that anergy is likely a consequence of a program of coordinately regulated factors as opposed to a single factor.

Induction of anergy-associated genes by NFAT1

When T cells receive antigenic signals in the context of costimulation, NFAT activity induced by TCR-coupled Ca2+ influx cooperates with Fos/Jun dimers (AP-1) to induce activation-associated genes. An example of this is at the Ag receptor response element (ARRE)-2 site of the il2 promoter, where NFAT and AP-1 bind cooperatively to adjacent DNA elements (Fig. 1). In the absence of costimulatory signals, AP-1 activity is not induced, but activation of NFAT can still be achieved because of its low threshold for intracellular Ca2+. An important advance in our molecular understanding of anergy came from a study by Rao and colleagues showing that a “TCR-only” energizing signal can be simulated by TCR/CD28 costimulation in the presence of a mutant of NFAT1 that does not pair with AP-1 (42). Remarkably, this uncoupling of NFAT from AP-1 results not only in failed il2 gene expression but also in the induction of a separate set of NFAT-responsive genes whose expression is not dependent upon AP-1 (Fig. 3A). Therefore, NFAT1 functions as a highly perceptive sensor of TCR engagement and as a biochemical switch that drives two distinct patterns of gene expression, depending upon whether a costimulatory pathway (leading to AP-1) has been initiated. The set of anergy-induced genes identified in this study can be divided into two subsets of factors whose role in anergy is basically understood: 1) inhibitors of proximal receptor signaling; and 2) inhibitors of gene transcription.

NFAT-induced proximal TCR signaling defects in anergic cells

Anergic CD4+ T cells exhibit defective TCR-coupled signal transduction (reviewed in Refs. 1 and 2), with reported defects that include altered coupling of TCRζ to Fyn instead of Lck, failed coupling of ZAP70 and LAT (linker for activation of T cells) to the TCR, reduced activation of PLCγ-1 and blunted Ca2+ mobilization, and defective activation of PKCθ and Ras. In general, however, anergic T cells suffer from a defect in either the coupling of LAT and PLCγ-1 to the TCR or in the coupling PKCθ and PLCγ-1 to downstream pathways. Regardless of the precise location of the defect, this leads to blocked activation of the Ras, ERK, and JNK pathways and represents an essentially universal aspect of T cell anergy (Fig. 3A). Similar proximal defects in TCR signaling have been observed in Foxp3+CD4+ regulatory T cells, which likewise fail to produce IL-2 and IFN-γ in response to TCR ligation (43, 44).

A more recent advance was the realization that these molecular defects may result from negative regulators of TCR signaling induced by NFAT1 activity during a tolerogenic stimulus (Fig. 3A). Diacylglycerol kinase (DGK) is an NFAT1-induced enzyme (42) that inhibits Ras activation by depletion of the secondary messenger diacylglycerol. Two recent studies showed that DGKα and the related enzyme DGKζ contribute to the Ras-MAPK block in anergic T cells and that this enzyme is required for the induction of anergy (45, 46). T cells rendered anergic by multiple stimuli also overexpress enzymes involved in the proteolytic turnover of signaling molecules. The E3 ubiquitin ligases Itch and Cbl-b, which can target PKCθ and PLCγ-1 for proteosome-mediated degradation, are necessary for CD4+ T cell anergy (reviewed in Ref. 47). Similarly, caspase 3 is elevated in anergic cells, where it promotes anergy by degrading GADS (Grb2-related adaptor of downstream of Shc) and Vav (48). The action of this battery of negative regulators likely explains the failed coupling of PKCθ and PLCγ-1 to downstream pathways exhibited by anergic cells (Fig. 3B).

Gene related to anergy in lymphocytes (GRAIL) and TNFR-associated factor (TRAF) are two additional E3 Ub ligases whose activity is required for anergy induction in CD4+ T cells, but the mechanism by which these enzymes function is less clear. TRAF6 functions in the TRANCE (TNF-related activation-induced cytokine) signaling pathway, and its expression is not specific to anergic cells (49). Whether TRAF6 functions in a manner analogous to Itch and Cbl-b, i.e., by target signaling molecules for degradation, is not known; however, TRAF6-deficient CD4+ T cells express less Cbl-b than wild-type T cells. Therefore, TRAF6 may promote anergy indirectly by regulating Cbl-b gene expression. GRAIL is a RING finger E3 ubiquitin ligase specifically expressed upon anergy induction. A dominant-negative form of GRAIL inhibits anergy induction, whereas forced expression of functional GRAIL induces an anergy-like state in the presence of full TCR/CD28 costimulation (50). How GRAIL functions to promote anergy is not known, but may involve ubiquitination of surface receptors and endosomal signaling molecules.

NFAT-induced repressors of gene transcription

NFAT1 activity in the absence of AP-1 also induces a network of repressive transcription factors that actively inhibit cytokine gene transcription (Fig. 3A). Egr2 and Egr3 are expressed specifically in anergic cells, where they oppose expression of the il2 gene and are required for anergy in several models (51, 52). Ikaros is a transcriptional repressor of the il2 gene that is absolutely required for the induction of anergy in response to calcium ionophore, CD28 blockade, and IL-2R blockade (32, 53). Ikaros is not strictly an anergy-specific factor, as it is also expressed in naive cells at levels sufficient to repress the il2 gene in the absence of costimulatory signals (32). However, in Ag-experienced Th1
cells, productive activation through the TCR and CD28 down-
regulates Ikaros (C. Chen and A. Wells, unpublished observa-
tions), whereas treatment with ionomycin results in elevated
Ikaros levels (42, 53). This differential regulation of Ikaros in
naive vs primed T cells may influence the susceptibility of naive
vs Ag-experienced T cells to anergy induction and may have

FIGURE 3. A, Signals from the TCR alone do not lead to derepression of the il2 promoter but allow transcription of NFAT1-responsive genes. B, Anergy-induced E3 ubiquitin (Ub) ligases and caspase 3 (Casp3) target proximal TCR- and CD28-coupled enzymes for proteolysis, while DGKα interrupts diacylglycerol-dependent
signaling. In addition, transcriptional repressors such as Ikaros, CREM, Egr2, and Egr3 are recruited to the
il2 gene, leading to further silencing through a mechanism
that may involve DNA and histone methylation.
larger implications for T cell memory. The inhibitory cAMP response element binding protein CREM (cAMP response element modulator) is an isoform of CREB that is induced by T cell activation. Unlike CREB, this isoform lacks a transactivation domain and can bind cooperatively with NFAT1 to the distal AP-1 site at −180 bp of the il2 promoter-enhancer (54). In anergic CD4⁺ T cell clones CREB/CREM heterodimers are preferentially bound to the −180 AP-1 site, and mutation of this site leads to reduced CREM binding and resistance to energy induction (55). Thus, binding of NFAT1 with AP-1 vs CREM could act as a biochemical switch between induction vs energy induction (55). This is supported by the fact that binding of NFAT1 with AP-1 vs CREM could act as a biochemical switch between induction vs repression of il2 transcription. These factors, when recruited to the il2 promoter, most likely synergize with transcriptional repressors already present in the quiescent cells to actively suppress the il2 gene in anergic T cells (Fig. 3B).

Foxp3 depends upon NFAT transcription factors for its expression and function. Regulatory T cells are a subset of CD4⁺ T cells that are anergic, i.e., they do not produce cytokines like IL-2 or IFN-γ in response to TCR/CD28 costimulation and, in addition, they can actively inhibit the capacity of neighboring conventional CD4⁺ and CD8⁺ T cells to produce these cytokines. These cells specifically express the Forkhead winged helix transcription factor Foxp3, which is necessary and sufficient for the development and basic functional aspects of this lineage (reviewed in Ref. 56). NFAT2 cooperates with Smad3 to induce expression of the foxp3 gene in response to signals from the TGFβ and IL-2Rs (57). Thus, NFAT is required for the generation of “induced” Treg from conventional CD4⁺ precursors, although its role in the development of Treg lineage cells in the thymus is less clear.

In cells that have adopted the Treg lineage, the inability to produce IL-2 and IFN-γ appears to be a consequence of direct transcriptional repression by Foxp3. Foxp3 binds to the regulatory regions of the endogenous il2 and ifnγ loci in natural Treg (58–60), and binding of ectopically expressed Foxp3 to these loci in conventional CD4⁺ T cells is sufficient to inhibit activation-induced IL-2 and IFN-γ production (59, 61). Interestingly, although Foxp3 constitutively occupies the endogenous ctl4a, cd25, and girt promoters (genes that are constitutively expressed in Treg), binding of Foxp3 to the il2 promoter requires active signaling from the TCR (59, 61) and is blocked by the calcineurin inhibitor cyclosporin A (59). This is likely because the Ca²⁺/calcineurin-dependent transcription factor NFAT1 cooperates with Foxp3 for binding to composite NFAT-Forkhead elements in the ARRE-2 region of the il2 promoter-enhancer (58) (Fig. 4). As described earlier in this review, NFAT in conventional T cells binds at the ARRE-2 site of the il2 promoter to a composite NFAT-AP-1 element. In Treg, Foxp3 inhibits AP-1 DNA binding and takes its place with NFAT1 at the ARRE-2 (58, 62). In this way, Foxp3 simulates the distal consequences of a lack of CD28 costimulation by directly uncoupling NFAT1 from AP-1 cooperativity. Foxp3+ Tregs also exhibit enhanced expression of NFAT1 target genes such Egr2, Egr3, and several E3 ubiquitin ligases (63), but whether this is a direct consequence of NFAT-Foxp3 cooperativity or is mediated by Foxp3 per se is not known.

Thus, from the point of view of the il2 promoter, Foxp3 acts as a sensor that actively represses il2 (and ifnγ) gene expression in response to antigenic and costimulatory signals. Foxp3 integrates these signals in the opposite manner as Ikaros, which responds to CD28 and IL-2R signaling by allowing il2 gene expression in conventional CD4⁺ T cells. This suggests that an important role for Foxp3 in regulatory T cells is to specifically counteract anergy avoidance signals, an activity that may be reinforced through the recruitment of epigenetic modifiers to Foxp3-responsive genes. This issue will be discussed further below.

Epigenetic imprinting of the anergic state

Anergic CD4⁺ T cells suffer from the active, dominant repression of the il2 gene that can be mapped to distinct sequences in the promoter region (55, 64, 65) and can behave as a trait that is inherited from a naive precursor to its daughter cells over several rounds of cell division (66, 67). These data suggest that gene-proximal mechanisms of stable transcriptional repression are operative in anergic T cells.
Histone deacetylation at the \textit{il2} and \textit{ifn} loci in anergic CD4$^+$ T cells. The tissue-specific and signal-dependent expression of the \textit{il2} gene depends upon a locus control region located ~8 kb upstream of the transcription start site, suggesting a specific role for local chromatin context (68). Cells capable of producing IL-2 exhibit stimulus-independent dimethylation on lysine 4 of histone H3 (dimethylH3K4), a relatively stable histone modification associated with nonsilenced and transcriptionally active genes, within this region (69). Upon productive T cell activation, the promoter/enhancer region of the \textit{il2} locus exhibits rapid histone acetylation and nucleosome remodeling mediated by CREB-binding protein and the Swi-Snf/Brg-Brm class of ATP-dependent remodeling complexes (Fig. 2) (reviewed in Ref. 33). Induction of histone acetylation and nucleosome remodeling at the \textit{il2} promoter/enhancer in naive CD4$^+$ T cells requires TCR engagement, but antigenic signals are not sufficient for these changes. Like IL-2 production, physicochemical remodeling of the \textit{il2} promoter/enhancer is dependent upon signals from the CD28 costimulatory receptor (70). Naive CD4$^+$ T cells stimulated in the presence of CTLA4-Ig, which induces anergy by blocking B7-CD28 costimulatory interactions, completely fail to acetylate and remodel nucleosomes at the \textit{il2} promoter (70) (Fig. 3A). Likewise, CD4$^+$ T cells energized with immobilized anti-TCR Abs or with calcium ionophore, stimuli that simulate TCR signaling in the absence of costimulation, also exhibit histone hypoacetylation at the \textit{il2} promoter (53). This same epigenetic signature is observed in CD4$^+$ T cells rendered anergic in vivo by superantigen stimulation, where histone hypoacetylation also extends to the \textit{ifn} locus (71). In addition to hypoacetylation, the nucleosomes at the \textit{il2} promoter in anergic cells also exhibit trimethylation of lysine 9 of histone H3 (our unpublished observations), an epigenetic modification that tags loci for higher level silencing and the formation of heterochromatin (Fig. 3B).

Several lines of evidence suggest that the lack of histone acetylation in anergic cells is not a passive consequence of failed signaling. For instance, histone acetyltransferase (HAT) activity in general is intact in anergic T cells, as promoters for constitutive genes such as \textit{cd3e} exhibit strong histone acetylation (53, 70, 71). Also, induction of anergy in previously primed T cells, which exhibit increased histone acetylation at the \textit{il2} promoter under resting conditions as compared with naive or anergic cells (70), results in active histone deacetylation (53), whereas full TCR/CD28 restimulation fails to induce histone acetylation at the \textit{il2} promoter in anergic cells (53). Also, pharmacologic inhibition of HDAC activity during anergy induction restores histone acetylation at the \textit{ifn} and \textit{il2} loci in multiple models (Ref. 53; C. Chen and A. Wells, our unpublished observations). These data suggest that HDAC activity maintains nucleosomes in a hypoacetylated state at cytokine gene loci in anergic T cells. Indeed, these epigenetic signatures appear to be targeted to the \textit{il2} locus by the same DNA binding proteins that contribute to transcriptional repression in anergic T cells, and the closed chromatin conformation of the \textit{il2} promoter in anergic CD4$^+$ T cells is determined in large part by Ikaros. Ikaros is known to interact with corepressor complexes such as NURD (nucleosome remodeling and deacetylase), CtBP, and Sin3 (31), which contain histone deacetylases and remodeling complexes that wrap DNA tightly around nucleosomes and induce chromatin condensation. Consistent with this, Ikaros-\textit{il2} promoter complexes in naive and anergic CD4$^+$ T cells are enriched for HDAC1, HDAC2, and hypoacetylated histone H3 and H4 (32, 53). Unlike normal CD4$^+$ T cells, cells with reduced Ikaros function fail to maintain histone hypoacetylation at the \textit{il2} promoter in response to anergy-inducing stimuli and fail to silence expression of the \textit{il2} gene. Conversely, overexpression of Ikaros in effector CD4$^+$ T cells induces histone deacetylation at the \textit{il2} promoter and reduces the capacity of these cells to produce IL-2 in response to TCR/CD28 costimulation (32, 53). The anergy factor CREM recruits HDAC1 activity to gene promoters, and inhibition of CREM expression in activated CD4$^+$ T cells leads to increased histone acetylation at the \textit{il2} promoter and enhanced IL-2 production (72). It is likely that other factors that contribute to repression of \textit{il2} transcription in anergic cells (e.g., Tob, Smad3, p50, Egr2, and Egr3) also contribute the maintenance of a closed chromatin structure at the \textit{il2} promoter/enhancer (Fig. 3B); however, direct evidence for this has not been demonstrated.

Histone deacetylation at the \textit{ifn} locus in anergic CD8$^+$ T cells. CD8$^+$ T cells primed in the absence of CD4$^+$ T cells expand normally, produce IFN-\gamma and other proinflammatory cytokines, gain cytolytic function, and are able to clear pathogen infections during the initial effector phase of the response. However, such “unhelped” CD8$^+$ T cells exhibit defective survival and IFN-\gamma production compared with helped CD8$^+$ cells during the memory phase and are unable to protect against subsequent pathogen challenge (reviewed in Ref. 73). In this sense, this state is similar to activation-induced nonresponsiveness in CD8$^+$ T cells (3); however, unlike CD8$^+$ T cell anergy induced by costimulatory or cytokine blockade, this defective memory phenotype does not appear to result from decreased TCR-proximal signaling, as no defect in MAPK activation could be detected and the anergic phenotype could not be reversed through the bypassing of proximal TCR-coupled events with phosphor ester and ionomycin (74). Instead, anergy in unhelped CD8$^+$ T cells is strongly associated with changes in chromatin structure at the \textit{ifn} locus. CD8$^+$ T cells activated during lymphocytic choriomeningitis virus infection in a CD4-sufficient environment exhibit strong histone acetylation at the \textit{ifn} promoter and intronic enhancer (74). These epigenetic modifications are stably maintained into the memory phase months after virus has been cleared and the remaining virus-specific cells have returned to a quiescent state. Conversely, CD8$^+$ T cells activated in the absence of CD4 help failed to acetylate the \textit{ifn} locus. This defect was already observable at the effector phase, was maintained for months in the absence of antigenic stimulation, and correlated with the inability of these cells to mount an efficient secondary IFN-\gamma response. Thus, the acquisition of long-term memory to this virus is impaired on the \textit{ifn} locus in CD8$^+$ T cells by CD4 help at an early stage of the response. Epigenetic modification of Foxp3 target genes in regulatory T cells. Foxp3$^+$CD4$^+$ regulatory T cells differentially express thousands of genes compared with conventional, Foxp3-negative CD4$^+$ cells, and Foxp3 binds directly to a large proportion of these genes (58–61). Although some genes are repressed as a consequence of Foxp3 occupancy, other genes are induced, and the epigenetic modifications present at these genes correlate with their transcriptional activity (59, 60). For instance, binding of Foxp3 to the genes encoding CTLA-4 and CD25 induces epigenetic modifications that promote transcription, such as acetylation of histone H3 on lysine 9 and methylation on lysine...
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4 (59, 60). Conversely, Foxp3 binds to and represses genes such as pde3b, il2, and ifnγ, which is accompanied by marked histone deacetylation and methylation of lysine 27 at these loci (59, 60) (Fig. 4). Foxp3 actively recruits HDAC activity to the il2 and ifnγ loci, as Foxp3-mediated deacetylation of these genes is blocked by the HDAC inhibitor trichostatin A (our unpublished observations). These epigenetic modifications appear to oppose chromatin remodeling in Treg, as even very strong mitogenic signals are unable to induce DNase sensitivity at the il2 promoter in these cells (75), and inhibition of HDAC activity in Treg results in increased expression of ifnγ and several other Foxp3-responsive genes (76). This dual function of Foxp3 in directing either histone acetylation or deacetylation in the context of different loci is consistent with the ability of Foxp3 to associate with HATs, HDACs, and components of the Swi/Snf (switch/sucrose nonfermentable) chromatin remodeling complex in cell extracts (77, 78). Inhibiting the interaction between Foxp3 and HATs opposes Foxp3 transcriptional activity (78), and mutation of lysines in Foxp3 that are the targets of acetylation abrogates Foxp3 binding to the il2 promoter, repression of il2, and suppression of conventional CD4+ T cell responses (76).

Is local chromatin structure directly involved in cytokine gene repression in anergic cells? From the studies described above, it is clear that transcriptional repression of cytokine genes during anergy is tightly associated with epigenetic mechanisms, including histone deacetylation; however, this does not establish a causal link per se. As mentioned above, small molecule HDAC inhibitors can restore histone acetylation at the ifnγ and il2 loci during anergy induction; however, whether this is sufficient to restore the expression of the genes in anergic T cells is less clear. In ionomycin-induced anergy, global inhibition of HDAC activity during the induction phase using trichostatin A led to increased il2 gene expression; however, this model appears to be unique in this respect. In regulatory T cells and conventional CD4+ T cells rendered anergic by costimulatory blockade, HDAC inhibitors do not restore IL-2 production (our unpublished observations), even though these drugs increase histone acetylation at the promoter. Indeed, a number of studies have established that histone deacetylase inhibitors actually inhibit activation-induced il2 gene expression (79, 80). The basis for the requirement for HDAC activity is not well characterized, but appears to involve a role for MEF2 and HDAC4 in the positive regulation of il2 transcription (81). This makes it difficult to interpret studies that use global approaches to modulate HDAC or HAT activity, and a clear answer awaits further characterization and specific modulation of the enzymes that act at the il2 locus.

HDAC activity clearly opposes acetylation and expression of the ifnγ gene during CD4+ T cells differentiation (reviewed in Ref. 82), but whether HDAC or histone acetylation directly opposes IFN-γ production in anergic cells is not known. This issue has been addressed in CD8+ T cells where, activation in the absence of CD4 help results in long-term anergy. Activation of CD8+ T cells in the absence of CD4+ T cells either in vitro or in vivo results in histone hypoacetylation at the ifnγ locus (74, 83), but treatment of unhelped CD8 cells with trichostatin A can fully restore acetylation (83). Importantly, HDAC inhibition promotes not only histone acetylation but also the development of memory CD8+ T cell function. In this model, both helped and unhelped CD8 cells expand and contract during primary stimulation, but only helped cells are able to achieve long-term homeostasis, produce IFN-γ upon restimulation, and protect mice against a lethal pathogen challenge (83). However, if unhelped cells are primed in the presence of trichostatin A, these cells exhibit survival capacity and IFN-γ recall responses comparable to those of CD8 cells that received CD4 help and are likewise able to mediate immunity to an intracellular pathogen. Therefore, in this form of T cell anergy, HDAC activity is required to repress unlicensed CD8+ T cell function and IFN-γ-dependent immunity and, remarkably, reduction of HDAC activity in CD8+ T cells can completely replace CD4 help for the development of immune function.

An important caveat of all the studies cited above is that, while experiments such as these indicate a causal link between HDAC activity and gene expression, they do not prove that HDACs are acting directly at the il2 or ifnγ loci. Effects of global modulation of HDAC activity could be indirect by effecting the expression or the activity of factors that in turn regulate these cytokine genes. Much of the conceptual framework for the regulation of the il2 gene (and probably the ifnγ gene) by chromatin remodeling comes from the fact that the regulatory regions of these loci appear to function like an “enhanceosome,” an extended structure that orchestrates chromatin remodeling and the recruitment and stabilization of RNA polymerase in a promoter region (see Ref. 33 and Fig. 2D). Like other defined enhanceosomes, the il2 promoter contains a positioned nucleosome that opposes transcription factor binding and is acetylated and remodeled following activation, but how these events are orchestrated and to what degree they are codependent has not been established for the il2 gene. Thus, it currently remains difficult to establish more than a strong correlation between local chromatin structure and transcription at these cytokine genes. DNA hypermethylation at the il2 locus reinforces the anergic state. Another epigenetic mechanism that effects cytokine gene expression by T cells is DNA methylation. Global inhibition of DNA methylation during T cell activation under many circumstances leads to increased and promiscuous cytokine production (84, 85). In naive CD4+ and CD8+ T cells, roughly one-third of the CpG dinucleotides within the il2 and ifnγ promoters are methylated (70, 71, 74, 86, 87), although some groups have reported higher methylation levels at the il2 promoter-enhancer (88, 89). Productive activation leads to DNA demethylation within these regulatory elements at both the il2 and ifnγ loci in CD4+ T cells (particularly during Th1 differentiation) and in memory CD8+ T cells (70, 71, 74, 88, 90), which correlates with the augmented production of IFN-γ by these cell types. However, induction of anergy is associated with the specific retention of DNA methylation at the il2 locus, but not the ifnγ locus. Anergic CD4+ and CD8+ T cells exhibit a 2- to 3-fold increase in methylation at each of the four CpG sites within the il2 promoter-enhancer compared with effector cells (70, 71, 74). Methylation of each of these sites has been shown to contribute to the transcriptional repression of il2 promoter-reporter constructs (88), and methylation of the CpG located in the ARRE-2 element directly blocks Oct1/NFAT binding and negatively regulates transcription of the endogenous IL2 gene in human CD4+ T cells (89). Therefore, increased methylation at these CpG sites would be expected to contribute to reduced IL-2 production by anergic T cells; however, more studies are required to fully establish this link.
 NFAT1 induces an array of anergy-associated genes including erodimers, NFAT1 actively promotes T cell activation and differentiation, and effector cell fates. When partnered with active AP-1 heterodimers involved in signal transduction or gene transcription (Fig. 1 and 2).

Conclusions

In this review, I outline a current view of how T cells use extracellular signals to decide between anergy and differentiation and how the anergic state is established and maintained at the molecular level. The decision between anergy and productive T cell activation is made by a series of intracellular proteins that actively oppose induction of the il2 gene but are inactivated by signals from CD28 and/or the IL-2R. Ikaros, Tob, NFAT1, and p27kip1 (and potentially p50 and ZEB1) fulfill this role, as these factors are present in naive T cells (i.e., their expression is not anergy specific) and are required for the induction of anergy. In the absence of either Tob or Ikaros, quiescent T cells are able to produce IL-2 and differentiate in response to signals from the TCR alone. T cells lacking p27kip1 still require CD28 costimulation for IL-2 production during the primary stimulus, but during restimulation they behave as though they received an anergy avoidance signal. Ikaros is also involved in decoding signals from the IL-2R, as T cells with reduced Ikaros activity fail to produce IL-2 when restimulated. Ikaros is also involved in decoding costimulation for IL-2 production during the primary stimulus, rather than resulting in the loss of function characteristic of anergy. Ikaros is also required for the induction of anergy factors involved in signal transduction or gene transcription (Fig. 1 and 2).

Disclosures

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