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Genetic and biochemical regulation of CD4 T cell effector differentiation: insights from examination of T cell clonal anergy

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Abstract

The two-signal model of T cell activation states that antigen recognition by TCR provides a tolerogenic signal (termed Signal 1) unless the T cell receives simultaneous costimulation (Signal 2) that permits antigen recognition to prime activation. Our efforts to characterize genetic and biochemical factors resulting from Signal 1 alone have identified signaling molecules, transcription factors, and an epigenetic regulator that each contribute to the anergic phenotype observed. However, our most striking finding is that the same factors identified using anergy to model T cell activation versus tolerance also participate in determining the outcome of the effector phenotype of fully activated T cells. We summarize our own findings and other recent advances in the genetic and biochemical understanding of T cell activation, tolerance, and plasticity in this review.

Keywords

CD4 T cell; Anergy; Adenosine receptor; Early growth response gene; DNA methyltransferase

The two-signal hypothesis for T cell activation

Each T cell carries a unique TCR that dictates which peptide/MHC complex may be recognized as an antigen. However, the outcome of antigen recognition (termed Signal 1) is dramatically influenced by the local context in which it occurs. Experiments stimulating Th1 clones with formaldehyde fixed APC plus peptide found that cells stimulated in this fashion did not proliferate or secrete IL-2 upon subsequent rechallenge with activated APC plus peptide [1]. This process, termed T cell clonal anergy by Jenkins, Schwartz and colleagues, was found to be inhibited by addition of cyclosporine A or cyclohexamide to the induction culture; thus, it is dependent on TCR-induced calcium signaling and new protein biosynthesis. Conversely, anergy could be reversed by addition of exogenous IL-2 to the induction cultures [1]. It was subsequently shown that a critical element of “Signal 2” during T cell activation came from CD28 stimulation by B7 family molecules on the APC and that ligation of CD3 and CD28 on the T cell with stimulatory antibodies could mimic a full activating stimulus in vitro [2].

Anergic cells are hypoproliferative upon rechallenge despite the fact that some in vivo models of anergy are expected to expose T cells to APC expressing costimulatory B7 molecules. Based on this observation, Jenkins proposed that Signal 2 might be more broadly considered to include any stimulus that leads to T cell proliferation [3]. Thus, anergy might result from T cell activation in the presence of cell-cycle blockade, and proliferation due to costimulation would

act to dilute anergic factors that were synthesized after TCR stimulation. In support of such a model, it was observed that blockade of T cell cycle in G1 with rapamycin in the presence of Signal 1 + 2 does result in anergy while cell cycle arrest in S with hydroxyurea does not [4]. However, subsequent experiments using a different G1 inhibitor, sanglifehrin A, demonstrated that it is not G1 arrest per se that is associated with anergy induction but rather a costimulatory signal transmitted via the mTOR signaling pathway that is necessary for full T cell activation [5].

Hypothesis-based analysis of microarrays to identify genes associated with anergy

Collectively, the findings described earlier implied that a set of calcium-induced genes in T cells might participate as “anergic factors” and contribute to the stable inhibition of T cell function seen in anergic cells in vitro and in vivo. Rao et al. [6] showed that NF-AT proteins are critical elements of the calcium-dependent TCR signaling that promotes expression of genes that control anergy. Interestingly, however, efforts to identify genes transcriptionally upregulated by Signal 1 + 2 versus Signal 1 alone in T cells only found a small subset of genes that display transcriptional regulation by costimulatory signals [7,8]. Such data greatly influenced our laboratory’s approach to identify potential anergic factors by microarray analysis. In order to identify genes associated with anergy, we did not compare Signal 1 versus 1 + 2. Rather, using a hypothesis-based analysis of microarrays (HAM) [9], we compared transcripts from resting cells to cells stimulated with Signal 1 alone, Signal 1 + CSA or the PKC inhibitor Go6976 (both of which inhibit induction of anergy), and Signal 1 + a combination of a MEK inhibitor, a p38 inhibitor, and rapamycin (all of which promote anergy). Comparison of these five conditions significantly enriched for identification of genes upregulated in association with anergy induction [10]. Select genes identified by this technique and further evaluated are summarized below in Table 1.

Genes associated with anergy also regulate T cell effector differentiation and function

One observation that immediately stood out was that many genes that were transcriptionally upregulated under anergizing conditions had already been shown to have effects on regulating cellular differentiation in other contexts. Thus, it was not surprising when several of the genes that we characterized as inhibitors of the T cell activation program have subsequently been shown to control T cell effector differentiation as well. Schematically, this is depicted in Fig. 1. Prime examples are Egr-2 and Egr-3. Egr-2 had previously been shown to play an essential role in normal myelination of neurons (reviewed in [23]); and more recently, it was shown to cooperate with the co-activator/co-repressor NAB-2 to repress neutrophil-specific genes and promote macrophage differentiation [24]. In T cells, Egr-2 and Egr-3 participate in upregulation of Fas-L following TCR stimulation [11,12]. We observed that Egr-3 is transiently expressed while Egr-2 expression is prolonged in anergic cells. Overexpression of either is sufficient to inhibit IFN- γ and IL-2 secretion by T cells and enhance expression of the E3 ligase Cbl-b that is critical to regulation of T cell tolerance/anergy [10]. Furthermore, T cells lacking Egr-2 and Egr-3 have enhanced proliferation and IL-2 production, while T cells with transgenic overexpression of Egr-3 are hypoproliferative and secrete less IL-2 [14]. This correlates with the enhanced ability of Egr-3 KO T cells and inhibited ability of Egr-3 TG T cells to elicit autoimmune pneumonitis compared to WT T cells [14]. This correlation between Egr-2 and Egr-3 expression by T cells and immune tolerance is supported by data from murine models of spontaneous and induced lupus. In these models, Egr-2 and Egr-3 expression in T cells decreases following development of autoimmune disease, but it is increased after treatment of mice with a peptide derived from the CDR1 of an anti-DNA antibody. Such treatment with the

CDR1 peptide and increased Egr-2/Egr-3 expression correlates with decreased disease [25]. Conditional KO of Egr-2 in murine T cells was recently described and demonstrates that T cell expression of Egr-2 is necessary for normal immune tolerance. Conditional KO mice spontaneously develop a lupus-like autoimmune disease with anti-nuclear antibodies, infiltration of T cells into multiple organs, glomerulonephritis, and increased numbers of IFN- γ - and IL-17-producing CD4 T cells [26].

While these data support a critical role for Egr-2/Egr-3 in regulating a genetic program essential to anergy, more recent work has begun to identify roles for these transcription factors in controlling expression of genes differentially regulated in T cell effector subsets. Yamamoto and colleagues recently reported that Egr-2 expression is significantly elevated in a CD4⁺CD25⁺FoxP3⁺LAG3⁺ regulatory T cell population that expresses large amounts of IL-10 upon activation and is hypoproliferative. Retroviral overexpression of Egr-2 in naïve CD4 T cells is sufficient to convert cells into the IL-10 and LAG-3 expressing phenotype, and naturally occurring or retrovirally generated cells both have the capacity to prevent T cell-mediated colitis in RAG KO recipient mice [27]. Furthermore, our laboratory has observed that Egr-3 TG T cells are not only hypoproliferative and secrete less IL-2, but they unexpectedly have enhanced secretion of IL-17 in the absence of deliberate Th17 skewing (R. Parkinson and J. Powell, unpublished observation). Experiments to characterize the mechanism of this spontaneous Th17 skewing are underway. Together, these recent data illustrate the potential of anergy-related genes to influence other outcomes of T cell effector programming.

Another example of this relates to the signaling from the adenosine A2A receptor in T cells. A2AR stimulation with agonist acts to increase intracellular cyclic AMP that acutely inhibits activation of T cells. Such inhibition in T cells and other immune cells limits excessive inflammatory responses [28]. Our microarray screen identified upregulation of A2AR in a cyclosporin inhibitable manner consistent with a potential role in anergy. Consistent with this hypothesis, culture of both a CD4 T cell clone and primary cells with an A2AR agonist during full activation (Signal 1 + 2 + agonist) results in decreased proliferation and IL-2 production when the cells are rechallenged (Signal 1 + 2) after several days of rest in the absence of any further A2AR agonist. Functionally, the cells are anergic, and biochemically they show inhibition of Erk phosphorylation and AP-1 activation. This is clinically relevant since pharmacologic or genetic blockade of A2AR signaling in T cells enhances mortality in a model of autoimmune pneumonitis [15].

However, as we explored the mechanism of the A2AR-mediated inhibitory program in T cells, we found decreased production of IL-6 and enhanced TGF- β when cells are activated in the presence of A2AR agonist. This results in the expected decrease in Th1 and Th17 effector cell generation and promotion of FoxP3⁺ and LAG3⁺ Treg cells [15]. Such an effect would be predicted to occur within a tumor due to high local adenosine concentrations in the hypoxic microenvironment and could contribute to the tumor's ability to suppress anti-tumor immune responses. Such a hypothesis is supported by evidence that A2AR selective antagonists enhance T cell effector cytokine expression and augment anti-tumor responses [29].

An additional target identified in our microarray screen was the *Spry1* gene encoding Sprouty1, an inhibitor of receptor tyrosine kinase signaling. Choi et al. have shown that Sprouty1 is a TCR-induced gene and demonstrated that overexpression of Sprouty1 in Th1 clones or previously activated primary CD4 T cells inhibits proliferation and IL-2 secretion. Mechanistically, Sprouty1 appears to interfere with TCR-induced activation of both the calcium-dependent NF-AT pathway and the Ras-MAPK pathway proximal to AP-1 activation [20]. Our own unpublished data confirms that overexpression of Sprouty1 inhibits TCR-mediated calcium signaling and Ras-MAPK activation, and recently this was shown to be related to inhibition of phosphorylation of LAT following recruitment of Sprouty1 to the

immunologic synapse [21]. While these data would place Sprouty1 in the category of an anergy-related gene, the Schwartz laboratory also examined the effects of overexpression and siRNA-mediated inhibition of Sprouty1 in naïve CD4 T cells and observed an enhancement of both proliferation and IL-2 expression [20]. Such findings emphasize that assignment of a particular function to TCR-induced genes is dependent on the activation and differentiation state of the T cell.

A finding consistently noted for the anergic phenotype is that it remains stable over time after removal of the initial anergizing stimulus. One contributor to stable patterns of gene expression in tissues is regulation of chromatin configuration by epigenetic modification. In particular, genes with altered histone methylation, decreased histone acetylation, and increased DNA cytosine methylation are incorporated into condensed nucleosomes termed heterochromatin and remain inaccessible to transcriptional machinery [30]. In T cells, there is evidence that the *Il2* gene promoter undergoes demethylation at the time of T cell activation [31], and *Il2* promoter methylation remains higher in anergic cells than in cells activated with costimulation [32]. In light of these data, we were particularly intrigued by the selective upregulation of DNMT3a, a member of the DNA methyltransferase gene family that catalyzes de novo DNA methylation, in our microarray screen [22].

Prior analysis of immune function of mice deficient in DNMT3a was hampered by the perinatal lethal phenotype of mice globally deficient in this enzyme [33]. However, our own analysis of lymphocytes from runted DNMT3a KO pups found a decrease in total lymphoid cellularity consistent with the overall diminished body size of the runts but similar proportions of T cells and initial T cell proliferation and cytokine proliferation to healthy WT littermates [22]. Using this model, we noted that KO CD4 T cells develop an unusual IFN- γ and IL-4 co-expressing phenotype after undergoing multiple rounds of stimulation with anti-CD3+ irradiated WT APC. These observations suggested that DNMT3a is essential for normal polarized expression of either IFN- γ or IL-4. Since changes in DNA methylation marks at cytokine genes have been extensively characterized for Th1 and Th2 subsets, we sought to analyze the effects of DNMT3a deficiency on deliberate skewing of naïve CD4 T cells into these populations. We took advantage of the conditional DNMT3a allele generated by Li et al. [34] and crossed these mice with CD4-Cre transgenic mice to obtain T cell conditional DNMT3a KO mice. Such mice are healthy and their cellularity and resting immunophenotype is similar to their WT littermates [22]. Sorted naïve CD4 T cells from these mice are able to achieve normal Th1 and Th2 skewing under standard culture conditions in vitro, and Th17 and Treg skewing is similarly unaffected (unpublished observation). Strikingly, Th2 cells from conditional KO mice are devoid of de novo methylation at the *Ifng* promoter, despite having similar expression of IL-4 and GATA3 to their WT Th2 counterparts. The effect of this absence of promoter methylation is that KO Th2 cells express significantly more IFN- γ when subsequently placed into Th1 culture conditions in vitro; thus, the absence of methylation correlates with increased plasticity in the cytokine expression phenotype.

mTOR activation, an essential requirement for effector differentiation

In parallel with our efforts to characterize genes upregulated by Signal 1 alone involved in anergy, we sought to identify how signals arising from costimulation modify this anergic gene program. As noted earlier, full activation of Th1 cells in the presence of rapamycin (which inhibits mTOR TORC1 complex formation) leads to development of anergy upon rechallenge. This observation led us to propose that mTOR plays an important role in integrating environmental cues in T cells. To explore this hypothesis, we utilized a genetic approach, deleting mTOR selectively in T cells using conditional knockout technology. Interestingly, when naïve T cells are activated in the absence of mTOR, they fail to become effector cells but rather develop into regulatory T cells [35]. This intrinsic Treg skewing does not require

the addition of exogenous TGF- β during naïve T cell activation and is not reflective of a problem with induction of IL-2 expression or proliferation during priming of naïve, mTOR deficient T cells. Even when naïve mTOR KO T cells are cultured under Th1-, Th2-, or Th17-skewing culture conditions, they fail to develop appropriate effector phenotypes and become FoxP3+ Tregs instead [35]. Strikingly, T cells from mice selectively deficient in TORC1 or TORC2 mTOR complexes show differential effects on skewing to different CD4 T cell effector subsets (G. Delgoffe and J. Powell, unpublished data). Thus, in Th1 cells, TCR stimulation in the absence of mTOR activation leads to anergy; while in naïve T cells, TCR stimulation in the absence of mTOR activation blocks differentiation to effector Th1, Th2, and Th17 cells and results in generation of Foxp3+ T regulatory cells.

Future directions: rational efforts to shape T cell plasticity

The use of T cell clonal anergy as a model system to understand the genetic and biochemical balance between T cell activation versus tolerance has proven to be fruitful in identifying multiple pathways that influence the ultimate outcome of TCR antigen recognition. For instance, it has yielded the adenosine A2A receptor as a target amenable to stimulation with small molecule agonists or antagonists to blunt or strengthen antigen-specific T cell responses, respectively. But these efforts have also highlighted that, to a degree, anergy as measured by one parameter may in fact be the induction of effector function as measured by a different parameter. That is, a T cell that is hypoproliferative and has diminished capacity to secrete IL-2 may have actually obtained an increased capacity to express other proinflammatory (e.g., IL-17A) or immune suppressive (e.g., LAG-3) proteins that are not directly being measured in the model system being tested.

The implication of this is that for any given immunologic problem under examination, from the excessive immune response of graft versus host disease after BMT to the ineffective anti-tumor immune responses after many cancer vaccines, a relevant set of effector functions must be interrogated to see which targets may be amenable to manipulate for therapeutic benefit. Immune activation is not likely to be simply on or off in a particular context, but deviated from the correct axis required for the desired therapeutic result.

In order to redirect an already inappropriate type of immune response, it is assumed that there must be some plasticity to modify the response toward a more desirable one. Initial data examining Th1 and Th2 cells suggested that their cytokine expression phenotypes are eventually fixed, particularly after repeated antigenic stimulation [36]. This was interpreted to be an outcome of not only genetic but also epigenetic modification of cytokine gene loci with changes in histone marks and DNA methylation during Th1 and Th2 differentiation (recently reviewed in [37]). However, more recent data supports some plasticity of effector function. Culturing in vitro generated Th17 cells in IL-12 or IL-23 is able to induce T-bet and IFN- γ expression [38], and adoptive transfer of hen egg lysozyme-specific Th17 cells into mice expressing this antigen in their eyes results in ocular inflammation expressing not only IL-17 but also IFN- γ within the eyes of recipients [39]. The clinical relevance of such a shift in phenotype is apparent in the requirement for melanoma-specific TCR transgenic Th17 cells to retain the capacity to produce IFN- γ to efficiently protect against advanced metastatic melanoma [40].

Additionally, inhibition by regulatory T cells is often felt to be a primary contributor to failure of protective Th1 immune responses to eradicate tumor. While generation of inducible regulatory T cells appears to be a stable process and thus is often interpreted as an irreversible change in T cell phenotype, more recent data suggest this may not be the case. Comparison of global mapping of H3K4me3 and H3K27me3 chromatin marks using CHIP-Seq between naïve, Th1, Th2, Th17, iTreg, and nTreg cells found that Tbx21 encoding T-bet was marked

by both activating and repressive histone modifications in the inducible and natural Treg populations, a condition associated with genes “poised” for reexpression [41]. This led the Zhao laboratory to sort FoxP3-GFP⁺ cells to high purity and culture them under Th1 conditions resulting in induction of T-bet expression and generation of modest numbers of FoxP3-IFN- γ ⁺ cells.

The most compelling data regarding reprogramming of Th1 and Th2 cells has been achieved by manipulating regulators of epigenetic modification through genetic interference with DNA methylation (DNMT3a, DNMT1, and MBD2 KO T cells) and chemical inhibitors of DNA methyltransferases and histone deacetylases. Our findings using DNMT3a conditional KO Th2 cells demonstrate that selective loss of *Ifng* methylation permits IFN- γ reexpression in the context of IL-12 stimulation. T cells lacking DNMT1 (deficient in maintenance of DNA methylation after DNA replication) [42] or MBD2 (deficient in interpretation of DNA methylation marks) [43] have more profound dysregulation with more promiscuous cytokine expression, including coexpression of IFN- γ and IL-4. The HDAC inhibitor butyrate can promote both IFN- γ and IL-4 expression during priming of naïve T cells, and culture of Th2 clones with the DNMT inhibitor 5-azacitidine leads to IFN- γ reexpression [44], suggesting that use of such agents in the context of a vaccination may assist in reshaping T cell cytokine expression patterns in vivo.

Antigen recognition by a T cell in the periphery is the initial step of activation but the outcome of recognition is influenced by the sum of multiple positive, negative, and differentiation signals provided from local cytokines, metabolites, and contact dependent signals from the APC. While anergy is one outcome seen in antigen experienced T cells (in most cases strictly defined as Th1 cells) following insufficient costimulatory input to activate essential mTOR signaling, the sum of the positive and negative biochemical signals triggered by the T cell’s surroundings can also be integrated to help choose among the growing number of diverse functional effector programs that dictate how its daughter cells will interact immune and non-immune tissues. Understanding how these initial choices are controlled at the biochemical and genetic level through the study of factors regulating anergy has provided an opportunity for clinicians to harness the incredible diversity and specificity of adaptive immunity to our best advantage in tackling the challenging problems of autoimmune disease, control of infection, and development of effective anti-tumor immunity.

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Fig. 1.

Th1 cells stimulated with TCR engagement in the absence of costimulation via CD28 (*Th1 Cell-Anergic Conditions*) upregulate expression of many genes, a subset of which encode inhibitors of the full T cell activation program (indicated by "X") resulting in decreased proliferation and IL-2 secretion termed anergy. Upon full activation including CD28 costimulation, activation of mTOR leads to the net expression/activation of activating proteins (indicated by "O") outweighing inhibitory genes, permitting normal cytokine expression and proliferation (*Th1 Cell-Activating Conditions*). In the context of full activation of a naïve CD4 T cell (*Naïve T cell*), integration of multiple costimulatory signals including CD28 costimulation, cytokine expression, and sensing of available nutrients collectively activate mTOR. The result is expression of many proteins that overlap those involved in inhibition and activation of differentiated Th1 cells (indicated by "X" and "O"); but in this cellular context, the gene products participate in commitment of the cell to a differentiated Th effector fate

Table 1

Select genes associated with anergy by hypothesis-based analysis of microarrays

Gene	Function	Effect on anergic phenotype
Egr-2/Egr-3	Transcription factors	Overexpression inhibits T cell proliferation [10]. Bind to <i>FasL</i> promoter and enhance transcription [11,12] but bind <i>Il2</i> promoter and repress transcription [10]. Egr-3 KO T cells resistant to peptide-induced anergy [10]
NAB2	Transcriptional coactivator/corepressor	Transcription upregulated by TCR signaling but protein expression enhanced by costimulation. Transcription repressed by Egr-3 overexpression. Enhances Egr-1-mediated IL-2 transcription [13,14]
A2AR	Transmembrane receptor for extracellular adenosine	A2AR stimulation during full T cell activation (Signal 1 + 2) results in anergy. A2AR agonists promote T cell tolerance to self-antigen and upregulate expression of LAG-3. A2AR deficient T cells are resistant to self-antigen induced tolerance [15]
LAG3	Inhibitory cell surface coreceptor	Expression is enhanced in anergic cells. Lag-3 inhibitory antibodies or genetic KO enhances T cell proliferation and homeostasis and diminishes regulatory T cell function [16–19]
SPRY1	Tyrosine kinase inhibitor	Expression is enhanced in anergic cells. Overexpression inhibits TCR-induced NFAT and AP-1 activation [20,21] (and Collins and Powell, unpublished data)
DNMT3a	De novo DNA cytosine methyltransferase	Transcription upregulated by TCR stimulation but protein expression attenuated by costimulation [22]. Preferentially localizes at <i>Il2</i> promoter under anergizing conditions (Agoston and Powell, unpublished data)