T helper cell differentiation: on again, off again

Jane L Grogan* and Richard M Locksley†

Recent studies raise the possibility that T helper (Th) polarization may be attributable to generalized activation and regulated silencing rather than regulated activation of target cytokine genes. The binding of transcription factors GATA-3 or T-bet to specific enhancers does recruit transcription factors such as NFAT-1 to IL-4 or IFNγ promoters, respectively; however, GATA-3 also intrinsically suppresses T-bet and vice versa. Silencing of GATA-3/T-bet, which is influenced by factors such as cytokines, is associated with irreversible Th polarization. For the first few divisions (perhaps reflecting the situation in lymph nodes), naive Th cells retain pluripotency; after further cell divisions (perhaps under the influence of an inflammatory cytokine milieu) they may become polarized appropriately to respond to the specific environment.

Addresses
Howard Hughes Medical Institute, University of California San Francisco, 521 Parnassus Avenue, C-443, San Francisco, CA 94143-0654, USA
*e-mail: jgrogan@itsa.ucsf.edu
†e-mail: locksley@medicine.ucsf.edu

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Abbreviations
CNS-1 conserved noncoding region 1
IL-12R IL-12 receptor
Th T helper

Introduction

Mechanisms by which naive CD4+ T cells achieve their effector fates continue to provide provocative twists of much interest to the understanding of immunity and broader issues of cell differentiation. Emphasis remains focused on the differentiation of naive cells to committed Th (T helper)1 and Th2 cells, which secrete the canonical cytokines IFNγ and IL-4, respectively. Observationally, Th1 cells orchestrate responses to pathogens that have overcome epithelial barriers and attack internal tissues. Conversely, Th2 cells arm epithelial and mucosal sites to make life difficult for pathogens attempting to prosper there. Since naive CD4+ T cells can adopt either of these two fates, a central conundrum remains: how do MHC–peptide complexes convey sufficient information to newly activated T cells to enable them to acquire the appropriate effector function in the periphery? Distinct dendritic cell populations may play a role in relaying information that allows naive T cells to anticipate what is to come [1,2]. Alternatively, the inflammatory milieu in the peripheral tissue may set the final effector repertoire by a combinatorial mix of signals assimilated through cytokine, costimulatory and chemokine receptors [3–5].

Current paradigms envision a major role for the cytokines IL-4 and IL-12 in providing permissive signals that favor polarization by directly activating the cytokines IL-4 and IFN-γ, respectively, while at the same time reinforcing expression of the canonical master regulators GATA-3 and T-bet (Figure 1). Here, we update information published during the past year suggesting that effector T cells may acquire their restricted cytokine repertoire by selective silencing of activated genes rather than by activation of distinct expression patterns. The factors that turn cytokine gene expression on and off at different stages of differentiation and that open up or close down gene accessibilty will be detailed.

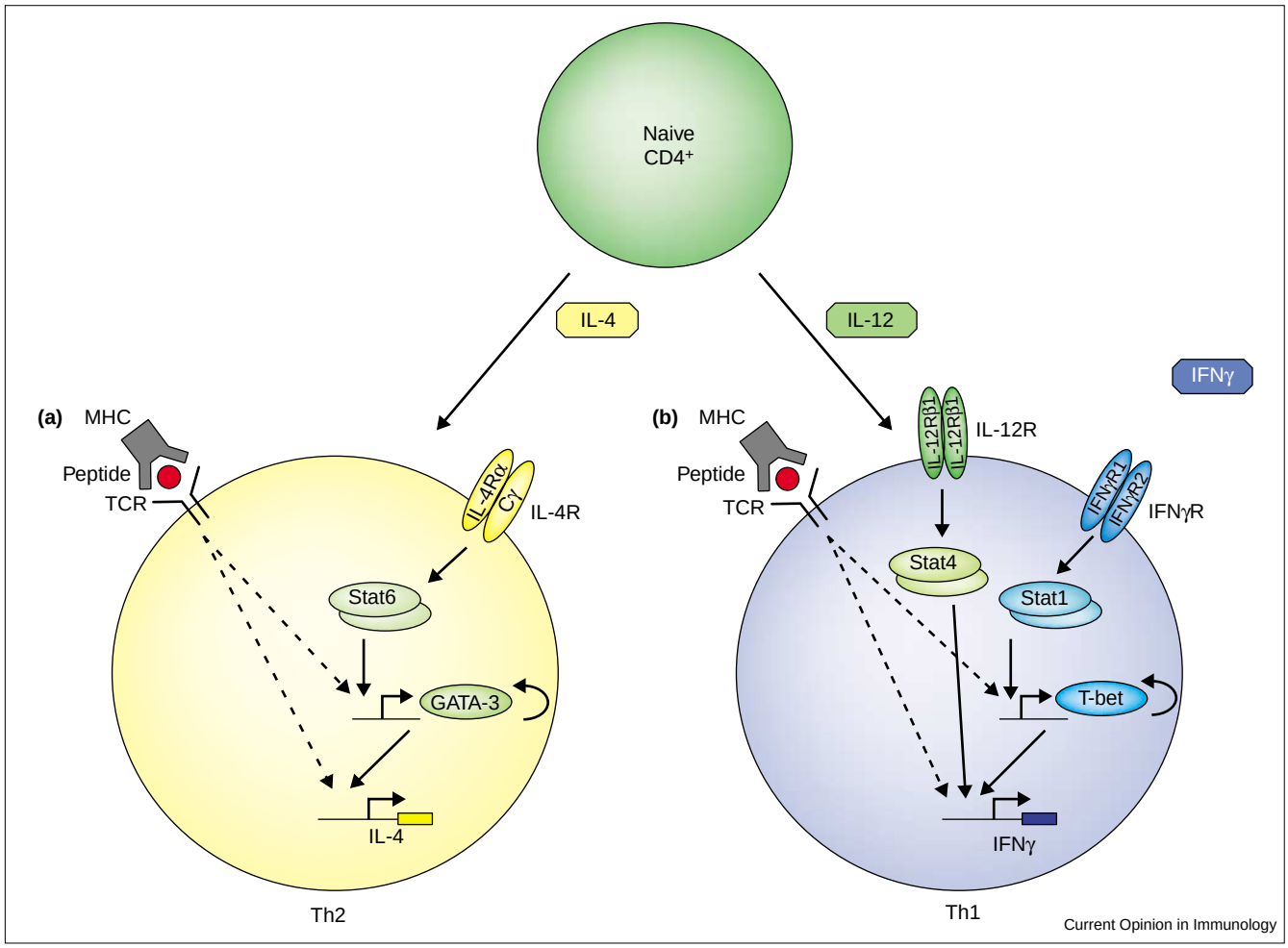
Opening up

Current evidence for Th differentiation supports a model whereby progressive chromatin remodeling and DNA demethylation render otherwise quiescent cytokine loci accessible to activation-induced transcription factors that ultimately define lineage fate.

Kinetic studies reveal specific DNase I hypersensitivity sites within 2 days and gene demethylation after 4–7 days at the respective cytokine loci following polarization of naive CD4+ T cells [6,7]. Using a minilocus construct linking Th2-induced hypersensitivity sites to the IL-4 promoter and a luciferase reporter, multiple transgenic mice were analyzed to establish the relative hierarchy of the sites in conferring IL-4 expression in differentiated Th2 cells [8••]. The clear result was that a combination of all the sites most resembled the pattern of endogenous IL-4 expression. The hypersensitivity site between the IL-4 and IL-13 genes, which encompasses a 400-basepair element highly conserved in mammals and is termed 'conserved noncoding region 1' (CNS-1) [9], was the most potent IL-4 enhancer, whereas the intronic and 3′ distal enhancers conferred Th2-specificity. At least part of the latter was explained by marked augmentation of expression in the presence of GATA-3, supporting the capacity of this transcription factor to confer lineage fate. Gene-targeted deletion of the intergenic CNS-1 element confirmed its importance in regulating expression from the type 2 cytokine genes. Transcription of IL-4 was delayed in the absence of CNS-1 and Th2 development was impaired in various *in vivo* models [10].

GATA-3 was demonstrated, using chromatin immunoprecipitation in Th2 cells, to bind to the distal 3′ enhancer. The distal positioning of GATA-3 was associated with recruitment of the general T cell transactivator, NFAT-1, to the IL-4 promoter and the 3′ enhancer [11•]. GATA-3 was recruited to the distal enhancer in c-maf-deficient mice, indicating a downstream role for c-maf during inducible IL-4 expression rather than in mediating more

Figure 1



Helper T cell differentiation. According to current models, activation of naive T cells becomes influenced highly by cytokine signals delivered through Stat6 and Stat4. **(a)** During Th2 differentiation, activation of GATA-3 by the TCR is amplified by Stat6, and GATA-3 works across the IL-4 locus to sustain gene expression initiated through TCR-delivered signals. Auto-amplification of GATA-3 is crucial to the polarizing nature of the process. **(b)** In similar fashion, activation of T-bet during TCR activation leads to downstream activation of IFNγ initiated during TCR-delivered signals. Although Stat4 serves to amplify activation at the IFNγ locus, T-bet is amplified by Stat1 signals generated in response to IFNγ. Again, auto-amplification of T-bet serves to drive the inherent polarizing nature of the differentiative process. See text for further details.

global aspects of Th2 competence. Conversely, Th1 conditions induced recruitment of NFAT-1 to the IFNγ promoter, where it was demonstrated to contribute to gene activation [12]. Together, these studies demonstrate how lineage-specific factors can target more general transcription factors to subsets of cytokine genes. The presence of GATA-3 sites throughout the type 2 cytokine cluster [13–15], its capacity to autoregulate its own expression [16•] and its ability to induce chromatin remodeling at the locus [16•,17] — akin to activities of GATA-1 in other hematopoietic lineages [18] — emphasize its central role in regulating Th2 differentiation *in vivo* and *in vitro* [19].

The role of T-bet, a T-box family transcription factor, in coordinating Th1 differentiation has also been examined. T-bet was initially described as a gene induced under Th1 conditions that was sufficient to induce IFNγ expression even within Th2-differentiated cells [20]. T-bet induction was attenuated in the absence of Stat4 [21••], but this effect is indirect and reflects the capacity of IFNγ to induce T-bet expression in a Stat1-dependent manner [22••]. The findings that T-bet autoregulates itself, similar to GATA-3, and induces expression of the IL-12 receptor (IL-12R)β2 chain are consistent with an early role in lineage commitment, although it remains unclear whether these effects are IFNγ/Stat1-dependent [23••]. Regardless of the precise mechanism, T-bet induces chromatin accessibility at the IFNγ gene that stabilizes the capacity of Stat4 to recruit a histone acetyltransferase, CREB-binding protein, thus potentiating gene expression [23••].

Initial studies that linked cytokine expression with cell division in naive helper T cells [6,24] provoked a series of observations scrutinizing early events in Th differentiation.

Unexpectedly, transcripts for both canonical polarizing cytokines IL-4 and IFNγ were detected within hours of TCR+CD28-mediated activation by a mechanism independent of Stat4- or Stat6-mediated signals [21••]. Indeed, T cells with a knockin bicistronic IL-4–GFP reporter were used to demonstrate that 30%–50% of cells activated under Th2 conditions expressed GFP by 36 hours and prior to cell division [25]. These findings will require mechanistic reconciliation with studies demonstrating that cytokine genes, while physically occupying areas of euchromatin in the nucleus [21••], exist in basal states characterized by DNA methylation and a relatively inaccessible chromatin structure [6,7]. Discriminating causal roles for chromatin and DNA modulation in gene transcription — as opposed them being used as simple marks for transcribed genes — remains problematic. However, T cells with targeted deletion of the maintenance DNA methyltransferase gene, *Dnmt1*, demonstrated globally enhanced cytokine expression after TCR ligation, indicating that basal methylation of cytokine genes limits their expression [26•].

Closing down

The presence of GATA-3 mRNA in naive T cells [27] and induction of T-bet mRNA after cell activation, even in the absence of Stat1-mediated signals [22••], are consistent with the early transcription of IFNγ and IL-4 after stimulation [21••]. Cells activated in the absence of either IL-12 or IL-4 express both transcriptional activators [28•]. Although GATA-3 and T-bet were identified on the basis of their capacity to activate canonical cytokines in developing Th subsets, in each case these transcription factors powerfully silence 'opposing' cytokines. Importantly, the cytokine-silencing capacity of the regulatory transcription factor is cell-intrinsic; for example, expression of T-bet in IFNγR1-deficient Th2 cells caused comparable suppression of IL-4 production as in wild-type cells [20]. Thus, despite the role of IFNγ/Stat1-signaling in T-bet induction, T-bet-mediated silencing of IL-4 expression was IFNγ-independent. Similarly, GATA-3 suppresses IFNγ production in the absence of IL-4/Stat6-mediated signaling by a cell-intrinsic mechanism [29,30].

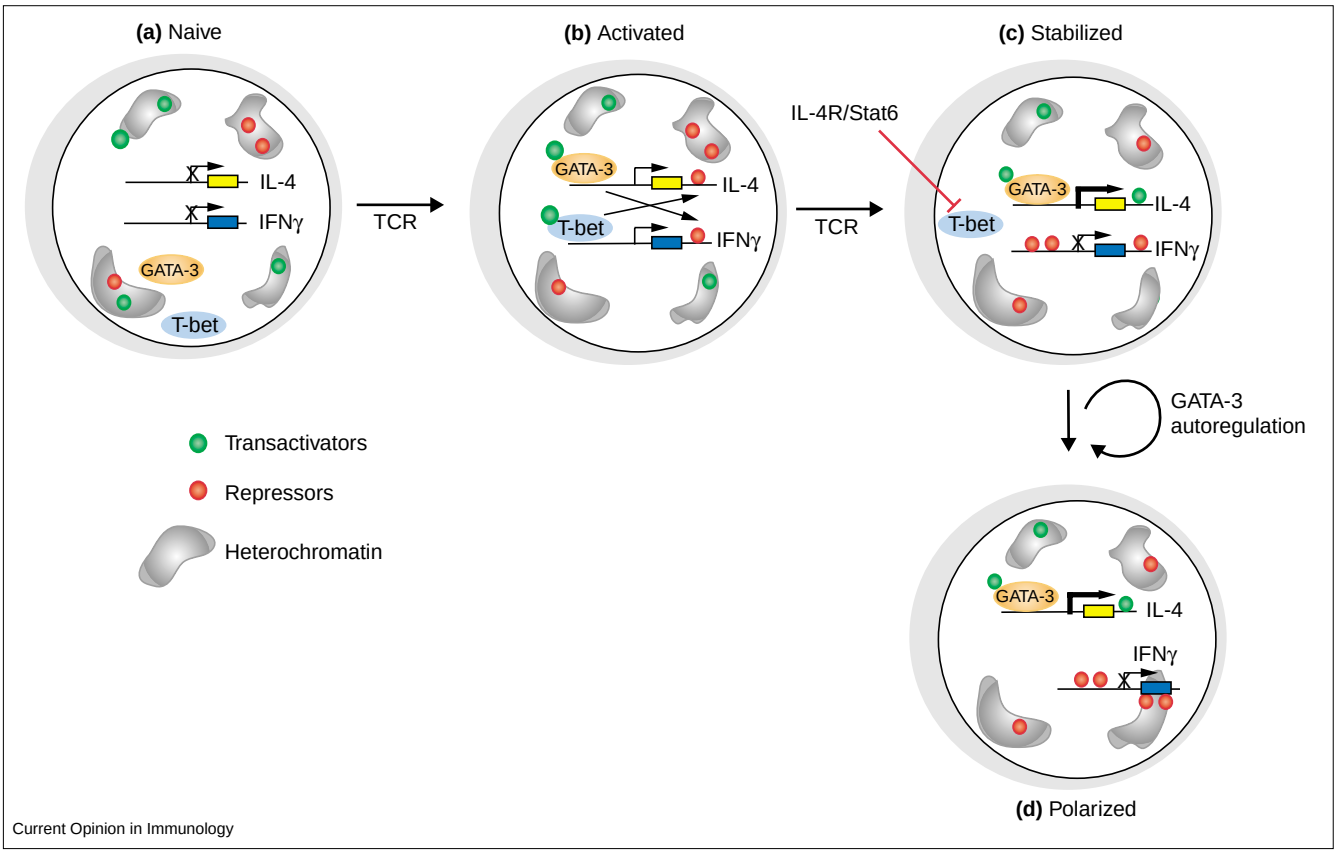
Such findings force reconsideration of the ways in which the cytokine environment mediated through Stat4 and Stat6 signals so profoundly affects Th polarization. Since T-bet and GATA-3 operate intrinsically to suppress opposing cytokines, these transcription factors themselves represent critical targets of the cytokine milieu. Accordingly, the Th2-inducing factor GATA-3 is extinguished in Th1 cells by IL-12/Stat4-mediated signaling [29]. Conversely, the Th1-inducing factor T-bet is extinguished in Th2 cells by IL-4/Stat6-mediated signaling [23••]. With loss of one of these master regulators, the capacity to sustain downstream cytokine gene expression at the discrete genes becomes lost and lineages become established in response to the remaining regulator, resulting in terminal differentiation into polarized T helper subsets. Although these Stat-mediated pathways remain the best-characterized, other cytokine receptors and pathways may contribute, potentially by targeting these transcriptional activators [31–35].

The dissociation between cytokine activation and silencing followed earlier observations demonstrating that terminal differentiation of Th lines was acquired after multiple rounds of polarized stimulation [36]. Sorting of cells by division after their initial activation demonstrated that the capacity to reactivate opposing cytokine genes was division-dependent; cells variably lost their ability to express alternative patterns after 3–5 divisions under polarizing conditions [21••]. Further analysis revealed that pluripotent fates were dependent on the presence of the transcriptional activators T-bet and GATA-3, which were lost by the fifth cell division. Careful dissection using inhibitors of cell division to investigate terminal cell fates demonstrated that lineage activators, although induced prior to entry into the cell cycle, acquired the ability to mediate chromatin-modifying lineage commitment only after DNA replication and that this correlated with their capacity to become heritably silenced [28•]. The coming year will undoubtedly bring further insights into silencing mechanisms, with potential targets including interacting proteins that affect function [37,38] or the genes themselves, which contain endogenous silencing elements [39,40]. Basal GATA-3 induction and Th2 differentiation were reduced in naive T cells deficient in *mel-18*, a homolog of a component of the *Drosophila* Polycomb repressive complex 1 [41•].

An additional component of silencing was suggested by the repositioning of nonexpressed cytokine loci to areas of centromeric heterochromatin within polarized Th cell nuclei [21••]. The higher-order chromatin structure imposed at mammalian centromeres serves to silence genes in physically apposed compartments. Ikaros family members comprise at least one group of proteins that recruit silenced genes to heterochromatin through direct recognition of Ikaros-binding sites within centromeric DNA repeats [42]. Repositioning to heterochromatin may confer heritable silencing on cytokine genes, but silencing is unlikely to require repositioning. Binding of two Ikaros family proteins, Ikaros and Aiolos, to the λ5 promoter was sufficient to silence expression without repositioning [43].

The finding that most cytokine genes, including IFNγ [23••] and IL-4 [44,45], can be expressed monoallelically has invited comparison to genes, such as immunoglobulin loci, that become activated monoallelically [46]. The immunoglobulin alleles, like imprinted genes, reside in distinct nuclear compartments revealed by asynchronous replication during late S phase and reposition early after activation [46,47]. Cytokine alleles invariably reside in euchromatic nuclear domains [21••] and, as assessed using a sensitive knockin IL-4 reporter, both IL-4 alleles are expressed in most cells during T cell activation [25]. These findings need to be reconciled with observations using heterozygous knockout mice suggesting that

Figure 2



A hypothetical model for T helper subset differentiation. **(a)** GATA-3 and T-bet in the nucleus of resting naive $CD4^+$ T cells are unable to bind target genes while critical transactivators (green balls) remain sequestered in heterochromatic domains of the nucleus (gray shaded areas). **(b)** In TCR-activated cells, dispersed transactivators interact with GATA-3 and T-bet and enhance their inherent binding activities, fostering target gene interactions. Conversely, these factors facilitate the cross-targeting of repressors (red balls) to distal sites that serve to limit efficiency of overall transcription rates. Such activated cells may represent the status of T cells as they leave lymph nodes and traffic to sites of inflammation. **(c)** In inflammatory sites, Stat-activating cytokines (i.e. IL-4, signaling via IL-4R and Stat6 in this example) work in stabilized cells to inhibit alternative cytokine pathways. Under the conditions shown, IL-4-mediated Stat6 signals inhibit T-bet and its displacement allows occupancy of the site by repressors and the release of partial repression on IL-4 transcription. GATA-3, left to autoactivate its expression, facilitates spreading of activators across the type 2 cytokine locus, resulting in high-level gene expression (thick arrow at the IL-4 locus). **(d)** The coalescence of repressors on the IFNγ gene promotes repositioning into heterochromatin, which terminally silences expression and restricts the cytokine repertoire available in polarized memory T cells. Similar pathways might occur through IL-12-mediated Stat4 signals to inhibit GATA-3, leading to the creation of polarized Th1 cells (not shown). Although interactions are depicted by lines, these pathways remain highly stylized and are likely to be indirect, via intermediates.

cytokine gene activation is a poorly efficient and stochastic process [23••,45]. On the basis of above considerations, however, monoallelic cytokine expression in Th clones might be explained more readily by inhomogeneous silencing of comparably activated alleles rather than by biased activation. Because of the tight linkage of activation with silencing, both mechanisms may contribute under varying conditions depending on the target gene and the strength of signal imparted by combinations of TCR avidity, costimulation and/or cytokine milieu.

Cell division may facilitate the rapid dispersal of activators and repressors among these nuclear compartments for interactions with newly accessible target genes. Relocalization of a general component of a transactivating complex from pericentromeric chromatin to euchromatin permitted interaction with a lineage-specific component to complete a functional complex that mediated activation of globin genes during erythroid differentiation [48]. If similar mechanisms apply to cytokine gene regulation, lineage-specific silencing of the cell-specific components of transactivating complexes would ablate gene expression and potentially result in reorganization of genes into pericentromeric domains. The creation of stable memory cells with highly enforced patterns of cytokine expression and biologic phenotypes [49] might result.

Conclusions

The shift from a paradigm of constrained expression to generalized activation and constrained silencing invites speculation regarding how such mechanisms are established

(Figure 2). The observation that cells that have undergone few cell divisions retain pluripotent Th fates [21••,28•] suggests that cells initially activated within lymph nodes may remain uncommitted. Indeed, cells recovered after cytokine-neutral rounds of division can be re-expanded to become either canonical Th1 or Th2 effectors [50–52]. The induction of CTLA-4 may function to arrest initial expansion and thus limit the terminally differentiated state [53].

Our speculation is that most antigenic challenges are ferried in relatively low copy number to the lymph nodes, where naive precursors undergo clonal expansion under generally bland cytokine conditions. After a relatively low number of divisions [54,55], cells exit lymph nodes and traffic to tissues, where higher antigen loads and fixed and recruited inflammatory cells conspire to enable a second wave of proliferation. In the presence of an innate cytokine stew reflecting the tissue context and the antigenic challenge [56,57], pluripotent cells with cytokine genes activated indiscriminately into states 'poised' for rapid transcription receive highly polarizing signals that silence inappropriate responses, leaving auto-amplifying positive feedback loops unimpeded. The result is extremely efficient polarization that matches the appropriate cytokine response with the type of pathogenic invader. Although few *in vivo* models exist, IL-4 expression among $CD4^+$ T cells was unaffected by the absence of Stat6 in the draining lymph nodes, but profoundly altered in tissue due to the inability to stabilize IL-4 gene expression and a failure of Th2 cells to accumulate [25,58]. Additionally, mice without suppressor of cytokine signaling-1 (SOCS-1) develop fulminant hepatitis that is relieved by either IFNγ/Stat1- or IL-4/Stat6-deficiency, suggesting that both canonical cytokines are left over-expressed in the absence of appropriate downregulation, at least in NKT cells [59]. Indeed, the inherent auto-enforcing nature of polarized Th responses may underlie the difficulty in treating dysregulated states that occur in autoimmune and atopic disorders.

Update

Two recent publications report the phenotype of T-bet-knockout mice. As predicted by the arguments presented here, T-bet-deficient $CD4^+$ T cells spontaneously develop into Th2 effectors in response to environmental respiratory antigens, resulting in a natural model for allergic airways diseases like asthma [60••]. Unexpectedly, T-bet, although necessary for IFNγ expression by $CD4^+$ T cells and NK cells, was not required for IFNγ expression by $CD8^+$ T cells [61]. This observation cautions that roles for GATA-3 and T-bet may differ in non-$CD4^+$ T cells. Indeed, IL-4/Stat6-mediated signals did not downregulate T-bet in γδ T cells, perhaps explaining the proclivity of these cells to produce IFNγ, but not IL-4 [62].

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