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Complex Interactions of Transcription Factors in Mediating Cytokine Biology in T Cells

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Summary

T-helper (Th) cells play critical roles within the mammalian immune system, and the differentiation of naive CD4⁺ T cells into distinct T-helper subsets is critical for normal immunoregulation and host defense. These carefully regulated differentiation processes are controlled by networks of cytokines, transcription factors, and epigenetic modifications, resulting in the generation of multiple CD4⁺ T-cell subsets, including Th1, Th2, Th9, Th17, Tfh, and Treg cells. In this review, we discuss the roles of transcription factors in determining the specific type of differentiation and in particular the role of interleukin-2 (IL-2) in promoting or inhibiting Th differentiation. In addition to discussing master regulators and subset-specific transcription factors for distinct T-helper cell populations, we focus on signal transducer and activator of transcription (STAT) proteins and on the cooperative action of interferon regulatory factor 4 (IRF4) with activator protein 1 (AP-1) family proteins and STAT3, in the assembly of complexes that broadly influence T-cell differentiation.

Keywords

transcription factors; cell differentiation; T cells; Th1/Th2/Th17 cells; cytokines; gene regulation

Introduction

CD4⁺ T cells critically control the normal mammalian immune response, serving to orchestrate adaptive immune responses and maintain host defense. Following the activation of naive CD4⁺ T cells by antigen-presenting cells (APCs), signaling via the T-cell receptor (TCR) in the presence of specific cytokines promotes the differentiation of these cells into distinct populations of T-helper (Th) cells with different biological functions (1–4). More than 20 years ago, Mossman and Coffman (5, 6) presented a model of CD4⁺ T-cell differentiation in which proliferating helper T cells could develop into two major types of effector T cells denoted as Th1 and Th2 cells. Th1 cells are important in host defense against intracellular pathogens, predominantly producing interferon- γ (IFN- γ), interleukin-2 (IL-2),

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and tumor necrosis factor- α (TNF α)(3), whereas Th2 cells mediate host immune responses against multicellular helminthes, mainly producing IL-4, IL-5, IL-10, and IL-13 (1). Subsequent to the original Th1/Th2 paradigm, additional populations of Th cells were identified, including Th9, Th17, induced regulatory T (iTreg) cells, and T-follicular helper (Tfh) cells (Fig. 1).

To better comprehend the complex mechanisms underlying CD4⁺ T-cell differentiation, it is necessary to understand transcription factor binding profiles during the differentiation process. Enormous advances in DNA high-throughput technologies, such as chromatin immunoprecipitation coupled with sequencing (ChIP-Seq) (7), now make it possible to efficiently characterize transcription factor binding occupancy, the transcriptome, and epigenetic modifications during CD4⁺ T-cell differentiation. By examining lineage specific transcription factors, master regulators and cytokine expression, our ability to comprehensively investigate complex transcriptional programs has been dramatically enhanced. These studies have revealed many cross-regulatory mechanisms that determine final lineage commitment. In this review, we discuss the roles of transcription factors in T-cell differentiation, including STAT proteins, master regulatory factors, and the BATF/JUN/IRF4 complex. We focus on biochemical complexes and gene/protein networks of interest to both immunological and computational readers.

Transcriptional networks in T-cell differentiation

During CD4⁺ T-cell differentiation, there are distinct transcription factors and cytokines that act to determine the type of T-helper cells that are produced (Fig. 1). Classically, differentiation of each type of cell was believed to be dependent on a single master regulator, but subsequent studies indicated that the interplay of multiple co-expressed lineage specific transcription factors, rather than a single dominant determinant, is responsible for driving the differentiation of CD4⁺ T cells both *in vivo* and *in vitro* (2, 8–10). Over the past few years, the transcription factors determining the differentiation and actions of these T-cell populations have been intensively studied.

Th1 differentiation

As noted above, Th1 cells dominantly produce IFN- γ to provide host defense against intracellular pathogens, including viruses, and the differentiation of these cells is dependent on stimulation with IL-12 in the presence of TCR stimulation (3). The T box transcription factor T-bet is considered to be a master regulator for Th1 cell differentiation, promoting the expression of IFN- γ , while suppressing Th2 differentiation (11, 12). During Th1 differentiation, two important transcription factors, STAT1 and STAT4, are activated by IFN- γ and IL-12, respectively, resulting in the induction of the *Tbx21* gene, which encodes the T-box protein T-bet. T-bet in turn drives Th1 differentiation, thus providing an example of a positive feedback loop. In contrast to its promotion of Th1 differentiation, T-bet antagonizes Th2 and Th17 differentiation by inhibiting the function of GATA-3 and ROR γ t, respectively (13, 14). IL-2 has broad actions in regulating T cell differentiation (15). It plays an important role in the initial steps leading to Th1 commitment by inducing the expression of the IL-12R β 2 chain, which is a component of the IL-12 receptor, thereby enhancing

responsiveness to IL-12 (16). IL-2 also upregulates expression of *Tbx21*, thereby stabilizing Th1 lineage commitment (16). Interestingly, T-bet can form complexes with other transcription factors that further enhance Th1 gene expression. For example, BCL6 can form repressive complexes with T-bet to promote the development of the Th1 cell fate by negatively regulating expression of *Socs1*, *Socs3*, and *Tcf7* (17). In addition, runt-related transcription factor 3 (RUNX3) can cooperate with T-bet to induce *Ifng* expression while silencing *Il4* expression in Th1 cells (18–20). HLX, a homeobox protein, is induced by and genetically interacts with T-bet to promote IFN- γ production in Th1 cells (21); however, whether the two factors physically interact remains to be determined. Moreover, T-bet interacts with RUNX1, thereby blocking the association of RUNX1 with ROR γ t and inhibiting Th17 differentiation (14). Interestingly, in contrast to CD4⁺ T cells, in CD8⁺ T cells, a different TBX family member, Eomesodermin (Eomes), is the major regulator of IFN- γ production (22).

Th2 differentiation

Th2 cells are involved in allergic reactions and host defense to helminthes (1). Th2 differentiation is induced by TCR stimulation in the presence of IL-4, and at least *in vitro*, Th2 differentiation is enhanced by blocking the actions of IL-12 and IFN- γ . STAT6, which is potentially induced by IL-4, drives Th2 differentiation and also contributes to the maintenance of the Th2 phenotype (23, 24). However, IL-2-induced STAT5 signaling plays a critical role in Th2 differentiation (25–27). IL-2 acts at the initiation phase of Th2 differentiation by mediating TCR-induced IL-4R α expression (27). STAT5A and STAT5B binding to the *Il4ra* locus are evident within 8 h of Th2 differentiation *in vitro* (27). By inducing IL-4R α expression, IL-2 increases IL-4 responsiveness, resulting in an IL-2-to-IL-4 signaling cascade (15, 27). In addition, IL-2 promotes STAT5A and STAT5B binding at multiple sites within the *Il4/Il13* Th2 cytokine gene locus, including at well-characterized hypersensitive sites as well as to the locus control region B and C elements in the *Rad50* gene, thereby augmenting the production of Th2 cytokines (27). STAT6 and/or STAT5 can also induce expression of the Th2 master regulator, GATA-3, which then drives transcription of the hallmark Th2 cytokine, IL-4, while inhibiting transcription of the hallmark Th1 cytokine, IFN- γ , both by suppressing STAT4 expression and inhibiting RUNX3-mediated *Ifng* expression. Other transcription factors are also involved in Th2 differentiation. For example, GATA-3 induces expression of c-MAF, which stimulates IL-4 and promotes Th2 differentiation, and JUNB cooperates with c-MAF to augment *Il4* expression (28). Interestingly, the transcription factor DEC2 is expressed in Th2 cells and enhances *Gata3* expression by binding to its promoter (29). Interferon regulatory factor 4 (IRF4) modulates *Il4* gene expression by cooperating with NFATc2 (30). Growth factor independent 1 (GFI-1) is an IL-4-induced STAT6-dependent transcription factor that promotes Th2 cell expansion by enhancing the proliferation of GATA-3^{high} cells (31), while simultaneously suppressing the differentiation of other helper T cells (32, 33). Moreover, chromodomain helicase DNA-binding protein 4 (CHD4) can form a complex with GATA-3 in Th2 cells, which activates Th2 cytokine transcription and represses production of IFN- γ (34). As is evident, Th2 differentiation involves the interaction of multiple transcription factors and signaling pathways that collectively re-enforce this phenotype.

Th9 differentiation

Th9 cells are a subset of helper T cells that produce IL-9 (35–37), which has actions on multiple lineages but is best associated with allergic and inflammatory diseases. Th9 differentiation is induced *in vitro* by TCR stimulation in the presence of IL-4 and transforming growth factor- β (TGF- β). Several studies have implicated transcription factors PU.1 and IRF4 as vital for Th9 differentiation. PU.1 is an ETS family transcription factor that appears to promote Th9 development by repressing the Th2 program (38), whereas IRF4 can directly bind to and transactivate the *Il9* promoter and may additionally contribute to the development and function of Th9 cells (39). Previously, IL-2 was reported to be critical for normal IL-9 production (40), and recently the IL-2-JAK-STAT5 signaling pathway was shown to be vital for Th9 differentiation, with critical STAT5 binding sites at the *Il9* promoter (41). Interestingly, expression of transcription factor BCL6, which is induced by IL-21, inversely correlates with Th9 differentiation, and IL-21-induced BCL6 acts as a negative regulatory factor of Th9 differentiation (41). BCL6 binds in close proximity to many binding sites for STAT5 and STAT6, including at the *Il9* promoter (41), suggesting a possible binding competition for these factors at the *Il9* promoter, given the opposing apparent actions of BCL6 vs. STAT5/STAT6 in Th9 differentiation. Given that Th9 cells are differentiated in the presence of TGF- β and IL-4 and that IL-4 is also the key driver of Th2 differentiation, several transcription factors that are critical for Th2 differentiation also play important roles in Th9 differentiation, including STAT6, GATA-3, and IRF4 (35, 36, 42). IL-4-mediated STAT6 activation promotes Th9 development, based on its repressing the expression of FOXP3 and T-bet (42), which can inhibit IL-9 production. STAT6 also promotes expression of IRF4, which as noted above promotes Th9 differentiation (42). PARP-14 functions as a transcriptional switch and regulates STAT6-dependent activation by increasing the binding efficiency of STAT6 to cognate promoter elements (43) and promotes production of IL-9 (42). TGF- β -activated SMAD2/3 (SMAD transcription factor 2 and 3) can physically interact with IRF4 and cooperatively transactivate the *Il9* promoter (44). Finally, an AP-1 family member, BATF, is enriched in Th9 cells and is required for the expression of IL-9 (37).

Th17 differentiation

Th17 cells represent a population of cells that produce the hallmark cytokine IL-17A, as well as IL-17F and IL-22, contributing to the development of inflammatory immune responses including psoriasis and inflammatory bowel disease (45). Th17 differentiation is induced *in vitro* by stimulation by TCR stimulation, IL-6, and TGF- β , with IL-21 and IL-23 serving important accessory roles. These cytokines activate STAT3 and induce expression of IL-21R and IL-23R, thereby promoting a cytokine signaling cascade (46–48), analogous to the IL-2 to IL-12 cascade for Th1 differentiation and the IL-2 to IL-4 cascade for Th2 differentiation (16, 27), as noted above. During Th17 differentiation, STAT3 activation augments the expression of ROR γ t, the master regulator for Th17 cells (49). IL-17 production is diminished in ROR γ t-deficient mice, but ROR γ t expression alone is insufficient to drive Th17 differentiation, and STAT3 activation is critical for the normal expression of IL-17 and IL-21 (50, 51). Additional transcription factors, including BATF, IRF4, I κ B ζ , and E2A, act as positive regulators of the expression of ROR γ t and IL-17 (52–

55). BATF, which is discussed below in greater detail, is a FOS-like AP-1 family transcription factor that is required for the differentiation of Th17 cells, with *Batf*-deficient mice exhibiting defective Th17 differentiation while maintaining normal Th1 and Th2 differentiation (52). The inducible costimulator (ICOS) induces c-MAF, which induces IL-21 production, and in turn IL-21 drives the expansion of Th17 cells (56). Transcription factor E-proteins E2A and HEB act as transcription factors that positively regulate ROR γ t and IL-17 expression (55). ChIP-Seq studies (57, 58) of a range of transcription factors in Th17 cells indicate the co-localization at the *Il17* and *Il21* promoters of STAT3, ROR γ t, BATF, IRF4, c-MAF, JUN/JUNB/JUND, FOSL2 (a FOS family member), as well as other additional factors, suggesting the formation of large protein complexes in which transcription factors either cooperatively or competitively bind to DNA to regulate gene expression (Fig. 2).

In contrast to these inducers of Th17 differentiation, a number of transcription factors, including TCF-1, GFI-1, IRF8 (interferon regulatory factor 8), and T-bet can inhibit Th17 cell differentiation. For example, TCF-1 directly binds to and represses the *Il17* gene and can also repress the expression of IL-7R on Th17 cells, thereby constraining the survival of inflammatory Th17 cells (59). Downregulation of GFI-1, which is driven by TGF- β , is important for Th17 differentiation (32, 60). IRF8 also plays a critical role in silencing Th17 cell differentiation (61), acting at least in part through its physical interaction with ROR γ t. Interestingly, T-bet can negatively regulate Th17 lineage commitment by suppressing IRF4 (62), which is required for optimal Th17 development (53, 63). As noted above, T-bet can also interact with RUNX1, preventing its association with ROR γ t (14); thus, there appear to be at least two molecular mechanisms by which T-bet can inhibit Th17 differentiation.

Whereas IL-2 promotes Th1, Th2, and Th9 differentiation, it potently inhibits Th17 differentiation (64), although IL-2 can amplify the response of committed Th17 cells (65). A number of mechanisms have been suggested for the inhibitory effect of IL-2 on Th17 differentiation. First, a possible competition of binding at the *Il17a* gene between IL-2-induced STAT5 and IL-12 induced STAT3 was suggested (64). Second, IL-2 represses expression of *Il6ra* and *Il6st*, the genes encoding IL-6R α and gp130 (16), which are the components of the functional IL-6 receptor. Third, IL-2 induces T-bet, which as indicated above, has two mechanisms by which it represses Th17 differentiation (14, 16)

Treg differentiation

Treg cells serve to limit immune responses and thereby prevent autoimmune and other deleterious immune reactions (45, 66, 67). In naive T cells, TGF- β induces FOXP3 (forkhead/winged helix transcription factor 3), which is a master regulator for the development and function of iTreg and nTreg cells (68–70). IL-2-activated STAT5 plays a critical role in maintaining Treg differentiation by directly binding to and inducing expression of the *Foxp3* gene, with FOXP3 in turn regulating the expression of Treg-specific genes (71). TGF- β decreases methylation of a key CpG island in the *Foxp3* gene, resulting in increased FOXP3 expression (72). FOXP3 can bind to and block the actions of ROR γ t, leading to diminished Th17 cell development (73). RUNX1 promotes Treg induction via its association with FOXP3, with a RUNX1/FOXP3 complex maintaining

Foxp3 expression (74–77). In the absence of FOXP3, the interaction of RUNX1 with ROR γ t is favored, with enhanced Th17 differentiation and IL-17 production (75).

Transcription factors downstream of PI3-kinase/AKT activation, particularly FOXO family members FOXO1 and FOXO3, are indispensable for Treg cell function (78). NF- κ B family member c-Rel binds to a conserved non-coding sequence, CNS3, in the *Foxp3* promoter, thereby augmenting expression of the gene (79, 80). NFAT and SMAD3 are key transcription factors that induce *Foxp3* gene expression and control the differentiation of CD4⁺CD25⁺ Treg cells, and both factors are essential for histone acetylation in the *Foxp3* enhancer region and the induction of *Foxp3* expression (81). Transcription factor BACH2 acts as a broad regulator of immune activation, helping to maintain Treg cells while repressing genes associated with the differentiation of other effector populations of CD4⁺ T cells (82). In the absence of BACH2, Treg cells had diminished expression of FOXP3 and were depleted in number (83). Nr4a nuclear receptors, which are encoded by immediate-early genes induced by TCR stimulation in thymocytes, also have been shown to be essential for the development of Treg cells, with early death in *Nr4a*-deficient mice due to severe autoimmunity. Interestingly, Nr4a nuclear receptors activate the *Foxp3* promoter (84). A range of studies have indicated that a range of other transcription factors, including GATA-3, ETS-1, EOS, Helios and SATB1 are also important in Treg cells. GATA-3 controls *Foxp3* expression by directly binding to and promoting the activity of *cis*-regulatory elements of the *Foxp3* gene (85). Transcription factor ETS-1 can interact with the FOXP3 intronic enhancer and is required for the development of natural Treg cells (86). EOS, an Ikaros family zinc-finger transcription factor, directly interacts with FOXP3 and mediates FOXP3-dependent gene silencing in Tregs (87). Another recent study shows downregulation of EOS, rather than loss of FOXP3, underlies reprogramming of Treg cells in response to inflammation (88). Helios, encoded by *Ikzf2*, is expressed in a subset of Foxp3⁺ Treg cells and expression of Helios is restricted to the thymic-derived Treg cell population (89). Interestingly, repression of SATB1, which functions as genome organizer to regulate chromatin structure, is crucial for Treg development and function (90).

Tfh Differentiation

T-follicular helper (Tfh) cells provide help to B cells, promoting affinity maturation, germinal center induction, and the generation of plasma cells and memory B cells (91). BCL6 serves as master regulator for the differentiation of Tfh cells. Mice in which *Bcl6* is conditionally deleted in T cells have defective Tfh development and lesser effects on other effector T cell lineages (92, 93). IL-6 and IL-21 activate STAT3 and drive *Bcl6* expression and Tfh cell differentiation via STAT3 binding to the *Bcl6* promoter (94). BLIMP1 is a transcriptional repressor encoded by the *Prdm1* gene. BLIMP1 can inhibit *Bcl6* expression and thus Tfh differentiation, thereby diminishing germinal center B cell responses (95). Several studies have indicated that STAT1, STAT4, and STAT5 are also involved in Tfh differentiation. IL-6-mediated STAT1 was reported to be required for *Bcl6* induction and early Tfh differentiation *in vivo* (96) and IFN- γ -induced STAT1 directly binds to and regulates the *Bcl6* gene (97). STAT5 acts as a negative regulator of Tfh differentiation by suppressing the expression of Tfh-related genes including *Bcl6*, *Batf*, *Maf* and *Il21* (98, 99). BATF is an AP-1 family member that directly regulates expression of two transcription

factors, BCL6 and c-MAF, which are required for the development of Tfh cells (100). Notably, both BCL6 and c-MAF are required for restoring Tfh cell activity to *Batf*^{-/-} T cells (101). Recently, achaete-scute homologue 2 (ASCL2), a basic helix–loop–helix transcription factor, was shown to initiate Tfh cell development and promote T-cell migration to the follicles by inducing C-X-C chemokine receptor type 5 (CXCR5), while suppressing the expression of signature genes related to Th1, Th2, and Th17 differentiation (102).

Cross-regulation, diversity, and plasticity in T-cell differentiation

Although a number of transcription factors have been considered to be master regulators of T-helper differentiation, it has become clear that the determination of distinct T-cell subsets is not governed solely by these factors and that additional co-expressed factors are involved as well. These additional factors can sometimes act to suppress other T-cell subsets and balance the commitment towards the ‘favored’ subsets. The CD4⁺ T-cell phenotype displayed by cells expressing one of these master regulators can be influenced by the cellular context of either cytokine signals or interacting cell signals that influence or refine the activity of the master regulators (103, 104). For example, T-bet is critical for promoting Th1 differentiation, but it also plays a critical role in the inhibition of Th2 differentiation by suppressing GATA-3 expression (105). T-bet induces expression of RUNX3, which can then interact with and attenuate GATA-3, thereby augmenting Th1 differentiation while inhibiting Th2 differentiation (106). As noted above, T-bet also represses Th17 differentiation by interacting with RUNX1 and by decreasing expression of IRF4. GATA-3 promotes Th2 differentiation but also suppresses STAT4, which is critical for Th1 differentiation (107). GATA-3 can also interact with RUNX3 and thereby inhibit RUNX3-dependent IFN- γ production (108). GFI-1 also promotes Th2 expansion (31, 33) but conversely suppresses Th1, Th17 and Treg differentiation (32, 33). Thus, master regulators can induce other factors that serve to re-enforce their own program and/or inhibit other differentiation programs.

Master regulators can be co-expressed. For example, cells co-expressing IL-17 and IFN- γ can be detected *in vivo*, and remarkably these cells co-express ROR γ t and Tbet at a single cell level (109). Although BCL6 promotes expression of the classical Tfh program, subsets of Tfh cells within germinal centers can also express IL-4 or IFN- γ , suggesting that there is flexibility in the extent to which other populations of cells are eliminated. Another example of co-expression of master regulators is provided by the ‘Th2+1’ phenotype, in which infection with Th1 cell-promoting lymphocytic choriomeningitis virus (LCMV) can reprogram fully differentiated virus-specific Th2 cells into GATA-3⁺Tbet⁺ cells that can produce both IL-4 and IFN- γ (110, 111). Furthermore, IL-12-mediated STAT4 activation can induce expression of *Il21* and *Bcl6* to generate cells with features of both Tfh and Th1 cells (112), indicating another complexity and underscoring the idea that these populations of Th cells are perhaps best not viewed as rigid lineages.

FOXP3, which is the master regulator of Treg cells (45, 66), can co-express with a range of transcription factors including T-bet, GATA-3, STAT3, and BCL6 to suppress Th1, Th2, Th17, and Tfh cell responses, respectively (85, 104, 113–116). When the composition of FOXP3 complexes in Treg cells was analyzed by mass spectrometry, approximately 100

transcription-related proteins, including lineage-specific transcription factors, were identified (117). The combined activities of co-expressed transcription factors in Treg differentiation thus may result in a range of actions that are influenced or determined by interactions between Th lineage-specific factors and FOXP3. As we discussed above, the classical paradigm of the master regulator in the differentiation of specialized CD4⁺ T-cell lineages may not truly determine a stable developmental cell fate. Instead, CD4⁺ T-cell differentiation is flexible, with the potential to respond to the changing environmental conditions and fine-tune the immune functions by altering the expression of critical specific transcription factors.

A BATF/JUN/IRF4 complex in T-cell differentiation

Over the past several years, a distinctive cooperative relationship between IRF4 and other transcription factors in T cells has emerged. The first such functional relationship to be recognized was with STAT3 (118), and then cooperation with AP-1 complexes including BATF and JUN family proteins was subsequently recognized as critical in transcriptional regulation in immune cells, especially in T cells (57, 58, 119, 120). The first indication of global association between IRF4 and STAT3 in T cells emerged from studies of the *Prdm1* gene in which a bipartite IL-21 response element that required STAT3 and IRF4 was identified (118). The *Prdm1* gene encodes BLIMP1, a zinc finger protein that represses the expression of PAX5 and BCL6 and acts as a master regulator to drive terminal B cell differentiation into plasma cells. Consistent with its regulation of BLIMP1 expression, IRF4 promotes plasma cell maturation and immunoglobulin class switch, but it also has critical actions for T cells (see below). IRF4 and STAT3 are highly expressed in immune cells and play critical roles in numerous cellular processes and regulate the immune response and lymphoid development.

Interferon regulatory factor 4

Interferon regulatory factors (IRFs) were first characterized and identified as a group of transcription factors induced by type I interferons (IFN- α/β) (121, 122). There are a total of nine mammalian IRF family members, denoted IRF1 to IRF9 (121, 122), which collectively play pivotal roles in the regulation of many biological processes in the immune system (123). Each IRF contains a well-conserved N-terminal DNA binding domain that forms a helix-turn-helix domain and recognizes DNA sequence corresponding to the IFN-stimulated response element (ISRE; 5'-GAAAnnGAAA-3') (121, 124–126). The crystal structure analysis of the DNA binding domain revealed that 5'-GAAA-3' is the core motif recognized by IRF family proteins (125).

Among IRF family proteins, the expression of IRF4 (historically also known as Pip/MUM1) and its closely related family member IRF8 (also known as ICSBP1) are restricted to the immune system and are induced in T cells by TCR stimulation. IRF4 is also expressed in other cell populations, including B cells and dendritic cells. IRF4 has a transactivation domain (127)(Fig. 3), and functions as a positive regulator, playing critical roles in mediating development of lymphoid and dendritic cells (128–131). Alone, IRF4 only weakly binds DNA due to the presence of a carboxy-terminal auto-inhibitory domain.

However, cooperative binding with the ETS family factor, PU.1, or the closely related ETS family protein, SPIB, can relieve this auto-inhibition and increase binding affinity, allowing IRF4 to recognize composite ETS–IRF consensus motif elements, or EICEs, 5'-GGAAnnGAAA-3' (125, 127), and regulate a range of genes, including those encoding the κ and λ immunoglobulin light chain genes. Similarly, IRF8 can also interact with PU.1/SPIB because of its structural similarity with IRF4 (125, 132).

IRF4 plays pivotal roles in both T and B cell biology (133, 134). IRF4 is known to be important for the maturation of B cells (131), plasma cells (135) and T cells (133). We previously demonstrated that IRF4 regulates IL-21-induced *Prdm1* expression in B and T cells and that IRF4 globally cooperates with STAT3 as a complex to regulate IL-21-mediated gene expression (118). Notably, STAT3 binding in CD4⁺ T cells is diminished in *Irf4*^{-/-} mice, suggesting that the expression of IRF4 is required for STAT3 binding *in vivo* in CD4⁺ T cells. IRF4 contributes to the development of multiple T-helper cell subsets (123), with defective Th1 (136), Th2 (30, 137, 138), Th9 (39, 44), Th17 (53, 58, 63), Treg (79), and Tfh (118) differentiation in its absence. IRF4 appears to be dependent on TCR signaling strength and can function to regulate the maintenance of CD8⁺ T-cell effector differentiation (139, 140). Among dendritic cells (DCs), IRF4 is essential for the development of CD4⁺CD8 α ⁻CD11b^{high} DCs (141, 142), and recent studies indicate that it is the migratory function rather than overall development that is dependent on IRF4 (143–145). Notably, *Irf4*^{-/-} mice did not show apparent phenotypes outside of lymphoid and myeloid lineages (146), underscoring the restriction of important non-redundant actions of IRF4 to the immune system.

AP-1 family members, BATF and JUN

BATF family proteins BATF, BATF2, and BATF3 (147, 148) are AP-1 family transcription factors, along with JUN, FOS, MAF (musculoaponeurotic fibrosarcoma) and ATF (activating transcription factor) family proteins (149). Each of these proteins is a basic leucine-zipper (bZIP) protein (Fig. 3), with the leucine-zipper motif mediating dimerization and the basic domain mediating DNA binding activity (150). In T cells, BATF is highly expressed in pre-activated CD4⁺ T cells, as well as in Th1, Th2, Th9, Th17, and Treg cells (57, 119). BATF is essential for normal class-switch recombination in B cells and controls Th17 cell differentiation by regulating expression of IL17 and other Th17-specific target genes, including *Il22*, *Rorc*, *Rora* and *Ahr*. *Batf*^{-/-} mice are resistant to the induction of EAE, and T cells from these animals have defective expression of the master transcriptional regulator ROR γ t and of IL-21 (52, 58, 101, 151), both of which are crucial for Th17 differentiation (46–48, 152). The role for BATF in Th2 differentiation remains somewhat unclear, as in one study, it was reported to be required for normal Th2 development, although some Th2 differentiation occurred in its absence (153), whereas in another study, expression of IL-4, IL-10, and CTLA4 were not significantly decreased in *Batf*^{-/-} mice, whereas, Th2 differentiation was reduced dramatically in *Batf*^{-/-}*Batf3*^{-/-} double knockout mice. BATF is essential for the development of Tfh cells (101). In Th1 cells, *Ifng* expression is not altered in either *Batf*^{-/-} or *Batf3*^{-/-} single or *Batf*^{-/-}*Batf3*^{-/-} double knockout mice, indicating that IFN- γ and T-bet are not regulated by these BATF family proteins (119). In B

cells, BATF can directly regulate the expression of AID (activation-induced cytidine deaminase) and germline transcripts (101, 153).

Like FOS family proteins, BATF, BATF2, and BATF3 can heterodimerize with JUN family proteins. Initially, BATF family proteins were considered as inhibitors of AP-1-dependent transcription, as they lack transactivation domains (Fig. 3) and presumably can function as dominant-negative regulators to block AP-1-mediated gene expression and transcription activity, at least in some cells (154–157). However, BATF and BATF3, respectively, can have positive transcriptional effects on Th cell differentiation and CD8 α^+ lymphoid-resident dendritic cells (52, 158). BATF and JUN family proteins can form heterodimers and exhibit similar DNA binding profiles as FOS/JUN dimers, preferentially recognizing palindromic AP-1 or TRE (12-O-tetradecanophorbol-13-acetate-response element) motifs, with consensus TGA[C/G]TCA DNA binding motifs being preferred to CRE (cyclic AMP-response element) consensus TGACGTCA motifs (148, 155). In addition to JUN, BATF can also heterodimerize with the other two JUN family proteins, JUNB and JUND. Each of these complexes can interact with AP-1 consensus DNA sites, but it remains unclear how the functions of different combinations of BATF-JUN family heterodimers may differ. BATF cannot form homodimers nor dimerize with FOS (155).

Transcriptional regulation via BATF/JUN/IRF4 complexes

As mentioned above, in B cells, IRF4 can form ternary complex with ETS family factors PU.1 or SPIB (Fig. 4A), relieving auto-inhibition and augmenting IRF4 binding affinity to EICEs (146, 159). In B cells, where PU.1 and SPIB are highly expressed, ChIP-Seq analysis of IRF4 binding from previously published B-cell libraries (118) confirmed that EICEs are the dominant IRF4/PU.1 DNA binding motifs (57). In contrast, in T cells where PU.1 and SPIB are poorly expressed, it was unclear how the C-terminal autoinhibitory effect could be relieved to allow IRF4 binding. Indeed, ChIP-Seq analysis from TCR pre-activated T cells (either unstimulated or stimulated with IL-21), Th2 and Th17 cells (57, 58, 119, 120) revealed that EICEs were not enriched at IRF4 binding sites. Instead, *de novo* motif discovery from IRF4 binding sites in T cells strikingly revealed that 5'-TGAnTCA-3' AP-1 motifs were significantly enriched (57, 58, 120). Interestingly, AP-1 like motifs were found at IRF4 ChIP-Seq peaks in B cells as well, although to a much lower extent than EICEs (57). IRF8, which is structurally similar to IRF4 and also can interact with PU.1 (159), instead preferentially bound to tandem canonical IRF core motifs separated by two base pairs (5'-GAAAnnGAAA-3') in both un-stimulated and IL-21 stimulated cells rather than binding to AICEs (57). Further analysis of how IRF4 interacts with AP-1-like motifs revealed the presence of IRF4 core motifs (5'-GAAA-3') either adjacent to or separated by four intervening nucleotides (Fig. 4B) from AP-1 motifs (57), indicating binding cooperativity for AP-1 and IRF4. These AP-1-IRF4 composite elements were denoted as AICEs (AP-1-IRF4 composite elements) to correspond to the EICE nomenclature used for classical IRF4 elements binding PU.1/IRF4 complexes in B cells (57, 120). Thus, there are two distinctive IRF4 binding complexes, with PU.1 in B cells and with BATF-JUN in T cells (see Fig. 4).

A motif analysis of BATF-binding sites in T cells, like the analysis for IRF4 binding sites, identified AICEs as well, and BATF binding sites co-localized with those identified for

IRF4 and JUN family proteins. BATF binding to a AP-1 consensus element lacking the accessory IRF4 motif does not require interaction with IRF4 (57, 58, 120); however, IRF4 binding to AICEs is substantially dependent on BATF, analogous to the requirement of PU.1/SPIB for the binding of IRF4 to EICEs in B cells. Importantly, BATF binding was markedly diminished in *Irf4*^{-/-} T cells and conversely IRF4 binding was markedly diminished in *Batf*^{-/-} T cells, underscoring a functional cooperation between these two factors (57).

Many immunologically important genes that are potently regulated by IL-21 are dependent on AP-1-IRF4 complexes, including *Prdm1*, *Il17a*, *Il21*, *Il10*, *Irf2* and *Ctla4*, and electrophoretic mobility shift assays (EMSAs) revealed direct cooperative binding interactions of AP-1-IRF4. For example, when Th17 nuclear extracts were used with a probe corresponding to a conserved noncoding sequence, CNS9, derived from the *Il10* gene, both IRF4 and AP-1 motifs were required for normal binding activity (57). Although BATF2 and BATF3 also interact with JUN family proteins, it is not yet clear whether BATF2-JUN or BATF3-JUN heterodimers are functional binding partners of IRF4 or IRF8. It will be interesting to study how the different BATF family proteins differ in the complexes they form and the degree of overlap in genes that they regulate.

In Th17 cells, BATF and IRF4 appear to function as ‘pioneer’ factors that govern the initial chromatin accessibility for subsequent binding of other transcription factors that are activated following cytokine stimulation (58). For example, together with STAT3, BATF and IRF4 then recruit lineage-specific transcription factor ROR γ t to regulate a set of Th17-relevant genes. The BATF-IRF4 complex also plays a central regulatory role in IL-6-mediated signaling, particularly in the absence of T-bet (160).

The binding of IRF4 can be complex. For example, in activated B cells, IRF4 can bind to DNA by three different mechanisms—in the context of complexes binding to EICEs and AICEs as noted above, as well via as classical ISREs. Although the binding affinity of these modes varies, these complexes might play distinctive roles in B cell differentiation and development (161). In CD8⁺ T cells, it has been shown that IRF4 and BATF function as major regulators of antiviral cytotoxic T-cell immunity and are required for sustained CD8⁺ T-cell effector following infection with lymphocytic choriomeningitis virus (LCMV) (162). The BATF-JUN-IRF4 complex also can function as a “checkpoint” in the differentiation of effector CD8⁺ T cells via direct binding to and activation of the expression of lineage-specific genes including *Tbx21*, *Prdm1* and *Runx3*, while simultaneously repressing genes that encode effector molecules such as IFN- γ and granzyme B (163).

As noted above, we previously used genome-wide ChIP-Seq analysis to show that IL-21-mediated activation of STAT3 resulted in the binding of this factor to thousands of loci in pre-activated CD4⁺ T cells and that the majority (over ~70%) of STAT3 binding regions co-localized with IRF4, indicating broad cooperative gene regulation by these two factors (118). Importantly, although no difference in STAT3 phosphorylation was observed in *Irf4*^{+/+} and *Irf4*^{-/-} cells, STAT3 binding was markedly diminished to absent in *Irf4*^{-/-} T cells, suggesting that IRF4 is essential for normal STAT3 binding activity (118). No direct co-precipitation of IRF4 and STAT3 was reproducibly observed, but it is conceivable that

there is a direct or indirect physical association of these two factors, or alternatively it is possible that IRF4 affects the chromatin structure in a fashion that influences STAT3 binding. More recently, it was recognized that in IL-21-stimulated T cells or in Th17 cells, there is co-localization not only of IRF4 and STAT3 but also of BATF and JUN family proteins, generally clustered in very close proximity (within 100 bp) (57, 58, 120), including at the *Prdm1* IL-21 response element previously studied (118), as well as in the *Il21* promoter and *Il17a/Il17f* region (57). Notably, STAT3 binds to GAS (γ -interferon activated site) or GAS-like motifs instead of AP-1 motifs, but its co-localization with BATF and IRF4 can potentially be explained by the known physical interaction between the coiled-coil domain of STAT3 and a large COOH-terminal segment of JUN (164–167). However, the mechanism by which STAT3 is recruited to AP-1/IRF4 complex to regulate gene expression is not yet established.

Noncoding RNAs in T-cell differentiation

As noted above, the differentiation of distinctive CD4⁺ T-cell populations are complex processes involving various cytokines, receptors and transcription factors to determine the ultimate outcome. In addition, a tremendous amount of information has been emerging regarding the functional and dynamic roles of non-coding RNAs, including microRNAs (miRNA) and long non-coding RNAs (lncRNAs) that contribute to the regulation of CD4⁺ T cells differentiation programs. These RNAs represent additional layers of complexity. Although 20,000 protein-coding genes have been annotated by GENCODE reference database (168, 169), these cover only ~3% of the genome (170) and the remainder largely consists of non-protein coding sequences (171), some of which correspond to microRNAs and lncRNAs.

MicroRNAs

MicroRNAs are endogenous small (~18–23 nucleotide long) non-coding RNAs that function in the transcriptional and post-transcriptional regulation of protein-coding genes (172). Each microRNA can target hundreds of genes or pseudogenes by binding to microRNA response elements, which are generally 6–8 nucleotides long regions within the 3'-UTR (untranslated region) of target mRNAs. MicroRNAs have been shown to influence many important processes, including development, survival, proliferation, differentiation, and the function of immune cells. Growing evidence suggests that microRNAs can critically modulate the development, maintenance, and differentiation of T-helper cells (10, 173, 174). The expression of various cytokines, growth factors, and transcription factors reportedly can be regulated by microRNAs (175).

For CD4⁺ T cells, microRNAs can control lineage-specific functions at different stages of differentiation (176) (Table 1). MiR-29 is a potent inhibitor of Th1 differentiation, suppressing IFN- γ production to control innate and adaptive immune responses to intracellular bacterial infection by directly targeting 3' UTR of IFN- γ (177). Moreover, miR-29 also can directly repress expression of T-bet and Eomes, two transcription factors known to induce IFN- γ production that promote Th1 differentiation (178). Several other microRNAs also critically regulate Th1 differentiation. MiR-146a can inhibit Th1 differentiation by directly targeting and inhibiting expression of *Stat1*, *Traf6* and *Irak1* (179,

180). MiR-155 enhances Th1 cell-dependent tissue inflammation (181) and inhibits expression of *Socs1* and *Ship1*, which are negative regulators of cytokine signaling (182, 183). Meanwhile, miR155 is upregulated by FOXP3 in Treg cells and it is required to maintain Treg cell proliferative activity by targeting SOCS1 (183). The miR-17~92 cluster (184, 185) can promote Th1 responses while preventing inducible Treg differentiation. MiR-21 (186) plays inhibitory roles in Th1 cells, and loss of miR-21 significantly enhanced the Th1-associated delayed type hypersensitivity cutaneous responses.

Th2 differentiation is also influenced by multiple microRNAs. MiR-155 contributes to the regulation of allergic airway inflammation by promoting Th2 responses through its inhibition of PU.1 expression (187). MiR-21, which is repressed by BCL6 but activated by STAT3, can promote Th2 cell differentiation by its induction of GATA-3 and inhibition of Sprouty1 (188). MiR-126 and miR-145 are also shown to be important for Th2 differentiation and effector functions (189, 190). Blocking miR-126 resulted in increased PU.1, diminished GATA-3, and thus diminished Th2 differentiation (189). MiR-145 is also a positive regulator of Th2 differentiation. Inhibition of miR-145 resulted in suppressed Th2 cytokine production and airway hyperresponsiveness (190). In Th17 cells, TGF- β -induced miR-10a can target BCL-6 and functionally attenuate the differentiation of Th17 cells, thus to fine-tune the plasticity and fate of helper T cells (191). MiR-326 promotes Th17 differentiation by directly targeting ETS-1, a negative regulator of Th17 differentiation, and plays a critical role for the pathogenesis of multiple sclerosis and mice with experimental autoimmune encephalomyelitis (EAE) (192). MiR-155 promotes the development of Th17 cells, promoting production by dendritic cells of key cytokines such as IL-6, IL-12 and IL-23 that promote Th17 cell formation (181). In Tfh cells, the miR-17~92 cluster functions to critically regulate Tfh cells differentiation and function (193) and control the migration of CD4⁺ T cells into B cell follicles, by suppressing expression of the phosphatase PHLPP2 (194). MiR-10a (which can limit Th17 differentiation, as discussed above), can also limit the conversion of iTreg cells into Tfh cells by inhibiting expression of *Bcl6* and its co-repressor *Ncor2* (191).

Long non-coding RNAs

Long non-coding RNAs (lncRNAs), including long intergenic non-coding RNAs (lincRNAs), are defined as non-protein coding transcripts with lengths ranging from 200 to thousands of nucleotides (195). lncRNAs typically overlap with protein-coding genes or other non-coding RNAs, are often polyadenylated, and lack open reading frames (ORFs) (196, 197). High-throughput transcriptome profiling analysis such as RNA-Seq indicates that there are tens of thousands of mammalian lncRNAs. It has been suggested that the majority of lncRNAs may not have functions (198), and only a very small proportion have proven biological roles according to lncRNAs database (199).

Nevertheless, some lncRNAs are known to regulate cellular processes, including the differentiation and activation of lymphocytes. For example, lincRNA *TMEVPG1*, which is located near the *Ifng* gene, is dependent on STAT4 and T-bet. *TMEVPG1* expression contributes, although by itself it is not sufficient, to drive the Th1 program and *Ifng* expression (200). Another lincRNA called LincR-*Ccr2*-5'AS, along with GATA-3, plays

essential roles for the migration of Th2 cells (201). Knockdown of this lincRNA showed decreased expression of neighboring chemokine receptor–encoding genes such as *Ccr2* and *Ccr3*, and compromised the migration of TH2 cells to the lung tissues, indicating a critical function for this lincRNA in Th2 cell differentiation.

Concluding remarks

CD4⁺ T-cell differentiation into multiple specialized populations of cells comprises multiple layers of regulation, including the induction of a range of cytokines, expression of cytokine receptors, the expression of master regulators and cell-type specific transcription factors, as well as epigenetic control, and the contribution of non-coding RNAs. Here, we discussed T-helper differentiation networks in Th1, Th2, Th9, Th17, Treg, and Tfh cells and also discussed the roles of the BATF/JUN/IRF4 protein complex in the development and differentiation of helper T cells. STAT3 co-localizes with these other factors in a genome-wide fashion and has previously been shown to be capable of interacting with JUN. It remains to be determined whether other cell-type specifying STAT proteins can also interact with this complex. In addition to transcription factors, non-coding RNAs such as shorter ncRNA (miRNA) or longer ncRNAs (lncRNAs, lincRNAs) may affect the expression of neighboring protein-encoding genes to reinforce or attenuate gene regulation by transcription factors, thereby adding another layer of complexity to the regulatory network of transcription factors underlying T-cell development and differentiation.

With the help with next generation sequencing, it has become possible to globally examine the genome and the transcriptome and to integrate these multiple datasets together. Combining multiple complex data sets will help to more completely define the molecular mechanisms underlying CD4⁺ T-cell differentiation.

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Fig. 1. Transcription factors involved in the differentiation of CD4⁺ T cells

In addition to ‘master regulators’ in the T-helper cells lineages (T-bet in Th1 cells, GATA-3 in Th2 cells, ROR γ t in Th17, FOXP3 in Treg cells, and BCL6 in Tfh cells), shown are the key STAT proteins that are required for differentiation of these populations of cells as well as other transcription factors known to contribute. For example, BATF-JUN and IRF4 bind to AP-1-IRF4 composite element motifs and form ternary DNA binding complex to regulate IL-21 responsive genes in a range of CD4⁺ T cells including Th17 cells (discussed later, related to Fig. 4). The PU.1/IRF4 complex is important in B cells and DC and these factors are each known to contribute to the development of Th9 cells.

Fig. 2. Genome browser graph of *IL17A/IL17F* locus in Th17 cells, showing a high degree of co-localization among a number of transcription factors including BATF, IRF4, JUN family protein, RORγt, STAT3, c-Maf (MAF), insulator CTCF, co-activator p300 and histone mark H3K4me3

These data are assembled from Li *et al.* and Ciofani *et al.* (57, 58).

Fig. 3. Domain structure of transcription factors that form the BATF-JUN-IRF4 complex
 IRF4 is composed of a DNA-binding domain (DBD; blue), an IRF-association domain (IAD; green), a repression domain (yellow) and a nuclear-localization signal (red). JUN family proteins contain transcription-activating domain (TAD), DNA binding domains and leucine-zipper domain (LZD). The structure of BATF is similar to JUN, JUNB, and JUND, but it lacks a transactivation domain. PU.1 has a PEST domain in addition to transactivation and DNA binding domains.

Fig. 4. Schematic cartoons of the interaction between IRF4 and PU.1/SPIB, and between IRF4 and AP-1 (BATF-JUN) factors

(A) In B cells, ETS transcription factors PU.1 or SPIB interact with IRF4 to form ternary complexes with ETS-IRF consensus elements (EICEs, 5'-GGAAnnGAAA-3') on DNA. (B) In contrast, in T cells, where PU.1 is less expressed, AP-1 family heterodimers (mainly BATF-JUN) form complex with IRF4 and recognize AP-1-IRF4 composite elements (AICEs; 5'-TGAnTCAnnnnGAAA-3', or 5'-GAAATGAnTCA-3'). These AICE complexes either can form with 4 nucleotides or 0 nucleotides between the AP-1 and ETS motifs. These AICEs can also form in B cells.

Table 1

MicroRNAs that influence the differentiation of T helper cells

miRNAs	Targets	Functions	Cells	References
miR-29	Tbet and Eomes 3'UTR of IFN γ	Suppresses IFN γ and inhibits Th1 differentiation	Th1	177, 178
miR-146a	Stat1, Traf6 and Irak1	Directly targets Stat1 and inhibits Th1 differentiation	Th1	179, 180
miR-155	SOCS1, SHIP1 and PU.1	Enhances Th1 tissue inflammation; promotes development of Th2 and Th17; maintains Treg cell activity	Th1, Th2, Th17, Treg	181–183, 187
miR-17-92	IL-12p35	Promotes Th1 responses; promotes Tfh differentiation; prevents iTreg differentiation	Th1, iTreg, Tfh	184, 185, 193
miR-21	IL-12, GATA3	Inhibits IL-12 and limits Th1 differentiation; promotes Th2 differentiation	Th1, Th2	186, 188
miR-126	PU.1	Indirectly inhibits PU.1, which then increases GATA3 and promotes Th2 differentiation	Th2	189
miR-145	?	Promotes IL-5 and IL-13 production by Th2 cells	Th2	190
miR-10a	BCL6, Ncor2	Targets BCL-6 and limits the Th17 differentiation	Th17	191
miR-326	ETS-1	Directly targets ETS-1 and promotes Th17 differentiation	Th17	192