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Transcriptional regulation of follicular T-helper (Tfh) cells

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Summary: T-follicular helper (Tfh) cells are a new subset of effector CD4$^+$ T cells that are specialized in helping B cells in the germinal center reaction. Tfh cells are distinct from other established CD4$^+$ T-cell lineages, Th1, Th2, Th17, and T-regulatory cells, in their gene expression profiles. Tfh cell differentiation results from a network of transcriptional regulation by a master transcriptional factor Bcl6 as well as IRF4, c-Maf, Batf, and STAT3/5. During Tfh cell ontogeny, increased CXCR5 expression directs activated T-cell migration to the follicles, and their interaction with B cells leads to Bcl6 upregulation, which helps establish effector and memory Tfh cell program. This review summarizes the recent progress in molecular mechanisms underlying Tfh differentiation and discusses the future perspectives for this important area of research.

Keywords: *dendritic cells, T-cell lineages and subsets, Tfh cells*

Introduction

After activation by antigen-presenting cells (APCs), naive, antigen-specific CD4$^+$ T cells differentiate into effector T cells. More than two decades ago, Coffman and Mosman (1) first discovered the heterogeneity of effector T cells, which were then named as T-helper 1 (Th1) or Th2 cells. Th1 and Th2 cells are differentially induced and regulate immunity against intracellular and extracellular pathogens, respectively, as well as immunopathologies such as autoimmunity and allergy. The Th1/Th2 dichotomy dominated the field of immune regulation until 7 years ago when IL-17-expressing T cells were proposed to be a third lineage of helper T cells (2, 3).

Th cell differentiation is instructed by distinct environmental cytokines, which signals through signal transducer and activator of transcription (STAT) or other inducible but generally ubiquitous transcription factors. These factors then upregulate the expression of lineage-specific transcription factors, which function not only to promote its own lineage differentiation but also to inhibit the alternate differentiation pathways. For example, interferon-γ (IFN-γ)/interleukin-12 (IL-12) through STAT1/STAT4 regulates the transcription of

T-bet in Th1 cells. IL-4 signals through STAT6 to induce the expression of GATA3 in Th2 cells. IL-6 and IL-21 act through STAT3, which functions to upregulate the expression of two Th17-specific orphan nuclear receptors, ROR-γ and ROR-α, that ultimately determine Th17 cell terminal differentiation (4). There are extensive cross-regulations of lineage-determining transcription factors. Moreover, there is growing evidence that Th cell lineage commitment can be plastic in certain circumstances.

T-follicular helper (Tfh) cells, originally characterized by their CXCR5 expression, provide essential help for B-cell affinity maturation, class switch recombination, and plasma and memory B-cell generation within the germinal center (GC) (5–9). Many molecules including programmed death-1, inducible costimulator (ICOS), SLAM adapter protein, B and T-lymphocyte attenuator (BTLA), and IL-21 are highly expressed in Tfh cells (7) and have been used to characterize Tfh cells. Recently, we and other groups identified that Bcl6 plays an obligatory role in programing Tfh differentiation and GC reactions (10–12). Bcl6 deficiency in $CD4^+$ T cells results in a failure in the generation of Tfh cells and GC responses *in vivo*, whereas ectopic expression of Bcl6 drives Tfh cell development (10–12). These studies on Bcl6 support Tfh cells as a unique T-cell lineage.

Since the pioneering work on Bcl6, increased numbers of factors have been found to regulate Tfh generation. In addition, Tfh developmental pathway has been characterized by using Bcl6 antibody staining or reporter mice. Here, we cover the existing literature on the transcriptional mechanism underlying Tfh differentiation, discuss the dynamic process of Tfh generation, and highlight possible future directions.

Transcriptional factors

Differentiation of naive $CD4^+$ T cells into different cell lineages is determined by cytokine signaling and subsequent activation of specific transcription factors. For Tfh cell differentiation, it has been shown that neither Th1- (STAT4, T-bet) nor Th2-determining (STAT6, GATA3) transcription factors are required (13). Despite that both Th17 and Tfh lineages require IL-6/IL-21 and STAT3, Tfh development does not require Th17 transcriptional factors (ROR-α and ROR-γ). Instead, Bcl6 serves as the specific transcriptional regulator for Tfh differentiation (7).

STATs

STAT-mediated cytokine signaling pathways are important regulators of T-helper cell development. It has been shown that IL-21 is induced in T cells by IL-6 in a STAT3-dependent manner and is important for Th17 development (14). In addition, IL-21 has been reported to be important for Tfh differentiation (13). To determine whether STAT3 signaling is required for Tfh development, we analyzed Tfh cell generation in $Stat3^{f/f}$ mice bred with CD4-cre mice (13). Immunization of these mice with keyhole limpet hemocyanin (KLH) in complete Freund's adjuvant (CFA) revealed a great reduction of $CXCR5^+$ Tfh cells in the absence of STAT3. Moreover, STAT3 deficiency in T cells also led to defective GC B-cell generation and reduced production of KLH-specific immunoglobulin G (IgG) and IgM. In addition, Tangye's group(15) reported that STAT3 deficiency compromised the generation of human Tfh cells as well. Mutation in STAT3 abolished IL-6/IL-21/IL-12-induced expression of IL-21 by human T cells, which resulted in diminished Tfh cell generation and abolished B-cell helper activity *in vivo*. Thus, STAT3 functions as a critical factor for mouse and human Tfh cell differentiation.

In contrast to the IL-6/IL-21-STAT3 pathway, IL-2-STAT5 signaling represses Th17 development (16). The role of STAT5 in Tfh development has been addressed this year by us and Crotty's group (17, 18). A constitutive active form of STAT5 efficiently inhibits Tfh differentiation by suppressing the expression of Tfh-associated genes such as CXCR5, c-Maf, Bcl6, Batf, and IL21, whereas STAT5 deficiency greatly enhances Tfh gene expression *in vitro*. Importantly, STAT5 positively regulates the expression of Blimp-1, a Tfh suppressor. Conversely, STAT5 deficiency potentiates Tfh development associated with dampened Blimp-1 expression. Mice with STAT5 deficiency in $CD4^+$ T cells exhibit an increase in Tfh and GC B cells and impairment of B-cell tolerance, suggesting that STAT5 functions to control Tfh development and humoral immunity. This finding highlights an inhibitory crosstalk between IL-2/STAT5/Blimp1 and the IL-6/IL-21/STAT3/Bcl6 pathways in Tfh development and may help us to find ways to treat antibody-mediated autoimmune diseases associated with expansion of Tfh cells.

The physiological activator of STAT5 to restrict Tfh generation is likely IL-2. We showed that inhibition of IL-2 greatly enhanced IL-6-driven Tfh differentiation *in vitro* (18). Moreover, in a recent study using influenza infection model, Ballesteros-Tato *et al.* (19) showed that *in vivo* administration of recombinant IL-2 impaired Tfh cell generation, resulting in a reduction of GCs and long-lived antibody responses, while $Il2ra^{-/-}$ $CD4^+$ T cells are more prone to become Tfh cells.

Bcl6

The B-cell lymphoma 6 (Bcl6) transcription factor is selectively expressed by mouse and human Tfh cells (20, 21). Bcl6 was previously shown to be inhibitory in Th2 responses by blocking STAT6 binding to DNA (22, 23), and Bcl6-deficient mice developed multi-organ inflammatory diseases, enhanced IgE production, and defective GC reaction (24). However, it was not clear whether the GC defect in these mice is caused by lack of proper T and/or B-cell function because GC B cells also express Bcl6 (25).

Expression of Bcl6 at mRNA level can be moderately upregulated by IL-6 or IL-21 *in vitro*, and overexpression of Bcl6 promotes the mRNA expression of several Tfh-associated genes in the absence of exogenous cytokines (10). Interestingly, IL-21 expression was not upregulated by Bcl6 overexpression. Bcl6 expression was not required for the development of Th1, Th2, or Th17 cells, and in fact, overexpression of Bcl6 repressed the production of Th1, Th2, and Th17 cytokines (10). Bcl6 function appears to be dependent on binding to DNA (10). Yu *et al.* (12) found that Bcl6 binds to the promoters for the transcriptional regulators T-bet and ROR-γ, which determine Th1 and Th17 cell development, respectively, resulting in the repression of IFN-γ and IL-17 production. Furthermore, Bcl6 also suppressed the expression of many microRNAs that are thought to control the Tfh generation, including miR-17–92, which repressed CXCR5 expression (12). Thus, Bcl6 regulates Tfh development through repression of microRNAs and Th1, Th2, and Th17 lineage-specific transcription factors. Bcl6 deficiency in T cells resulted in impaired Tfh development *in vitro* and *in vivo*, and Bcl6 expression in both B and T cells is required for germinal canter reactions (10–12). Interestingly, Bcl6 repressor, B-lymphocyte-induced maturation protein-1 (Blimp1), was significantly reduced in Tfh cells compared with non-Tfh $CD4^+$ T cells (11). Overexpression of Blimp1 in $CD4^+$ cells prevented Bcl6 expression and significantly reduced the differentiation of Tfh cells, without affecting Th2, Th17, and Treg differentiation. Moreover, Blimp1-deficient $CD4^+$ T cells showed enhanced Tfh cell differentiation (11). Taken together, these results show that Bcl6 is both necessary and sufficient for Tfh cell development and provide further evidence to support the idea that Tfh cells are a separate lineage of helper T cells.

Batf

Activator protein -1 (AP1) family member Batf (basic leucine zipper transcription factor, ATF-like) contains only a basic region and leucine zipper and is capable of partnering with Jun to control lymphoid development and differentiation, including Th17 (26) and Th2 (27, 28) cell development. Batf also controls GC development including both Tfh and GC B cell generation (27–29). Batf regulates gene expression through direct binding of Batf-Jun complex to target gene locus. Batf was first demonstrated to bind to IL-21 promoter in Th17 cells (26). In Tfh cells, Batf was shown to control Bcl6 and c-Maf expression, while in B cells, Batf regulates class switching recombination events through modulating the expression of activation-induced cytidine deaminase (AID) and germline transcription (29). Very recently, two groups characterized Batf-binding genes at a whole-genome level and observed that Batf-Jun complex could cooperate with IRF4 to bind to AP1-IRF4 composite motif (5′-TGAnTCA/GAAA-3′) in mouse $CD4^+$ T and B cells (30, 31). In addition, Batf-IRF interaction is found to promote dendritic cell (DC) development (32). At this stage, whether Batf-Jun-IRF4 interaction is required for Tfh cells and their interaction with STAT/Bcl6 factors remains to be determined.

IRF4

Mammalian interferon regulatory factor (IRF) family members play multiple roles within and beyond the immune system (33, 34). Many of IRF members are expressed in T cells and play important roles in regulating T-cell differentiation (33). IRF4, a weak DNA binder due to its C-terminal auto-inhibitory domain, is restricted to immune cells in its expression (33, 35). For IRF4 to execute transcriptional regulation function, it needs cofactors for stable binding with chromatin (30). In B cells, the binding of PU.1 or related factor SPIB with DNA facilitates recruitment of IRF4 into ETS-IRF composite element (5′-GGAAnnGAAA-3′) and regulates light chain expression (35, 36). However, in IL-21-treated B cells, IRF4 is frequently found in cooperation with STAT3, regulating IL-21-induced genes including Blimp-1 (37). Conversely, $Irf4^{-/-}$ $CD4^+$ T cells exhibit diminished STAT3 binding and consequentially possess defective Tfh cells (37), due to a T-cell-intrinsic defect (38). Furthermore, as mentioned above, IRF4 can interact with Batf-Jun complex in controlling transcriptional regulation (30, 31). Given the functional importance of Batf-Jun complex in immune system and general impairment caused by IRF4 deficiency in Th1 (39), Th2 (39–42), Th9 (43), Th17 (44, 45), and Tfh (37, 46) differentiation, the mechanism of how Batf-Jun and/or IRF4 controls T-cell function awaits further evaluation.

c-Maf

The cellular homolog of avian viral *V-maf* gene (c-Maf) belongs to the AP-1 family, containing basic region and leucine zipper domains (47). C-Maf was initially found to be selectively expressed in Th2 cells to enhance IL-4 production, but not other Th2-related cytokines (47, 48). In a recent study, $Maf^{-/-}$ chimeric mice exhibited defective Tfh cell differentiation *in vivo* (49). Investigators have indicated that because IL-4 and IL-21 are major products of Tfh cells (7, 50–53), Tfh cell impairment in the absence of c-Maf is due to defective induction or maintenance of IL-4 and IL-21 in an ICOS-dependent manner (49). Also, c-Maf is shown to be able to regulate IL-21 in Th17 and IL-4 in Th2 cells (47–49). IL-21 is an autocrine growth factor for differentiated Th17 cell expression (14, 54). In Th17 cells, c-Maf is able to sustain Th17 cells through maintaining IL-21 expression (49). However, in Tfh cells, c-Maf interacts with NFAT and JunB to regulate IL-4 expression (48), which might provide explanations for Gata3-independent IL-4 induction (55).

Current understanding of Tfh development

As Bcl6 was shown as the master regulator of Tfh cells, considerable studies have been focusing on measurement of Bcl6 expression during Tfh differentiation (56–59). In an acute lymphocytic choriomeningitis virus (LCMV) infection model, Choi *et al.* (58) showed that Bcl6 induction occurs as early as second cell division of antigen-specific $CD4^+$ T cell following DC priming. T cells with enhanced Bcl6 expression exhibited CXCR5 expression, but not CD25 or Blimp1 expression (58), suggesting an early separation of Tfh and non-Tfh lineages.

In a recent work, Kitano and colleagues (57) developed a Bcl6-YFP reporter mouse via fusing Bcl6 with YFP and found that Bcl6-YFP was initially upregulated together with CXCR5 on all T cells as early as day 2 following immunization, peaked at day 3, and then was subsequently downregulated. Through flow cytometry analysis, this pattern of Tfh differentiation was also observed in mice transferred with antigen-specific T cell and B cells (56) as well as in the LCMV-infected mouse model (58). Furthermore, Baumjohann *et al.* (59) specified two waves of Bcl6 expression during Tfh cell differentiation: Bcl6 expression was upregulated in all divided cells as early as first division after $CD4^+$ T-cell activation; a second wave occurred by the fifth cell division.

The above-cited results support that DC priming can induce Bcl6 expression (60). Because Bcl6 is a determining factor in Tfh cell differentiation, it was favored that DC-derived signals determine Tfh differentiation (60). The questions arise as to how DCs orchestrate Tfh versus non-Tfh polarization. A number of studies have shown that DC can promote Bcl6 expression through multiple mechanisms including high-affinity TCR signal (61, 62), ICOS ligand (63, 64), IL-6 (10, 65), and IL-12 (66). However, none of above-mentioned factors is unique for Tfh generation; they may work in concert to promote Tfh development, the precise mechanism of which needs further investigation.

In our recent study, by using a Bcl6-IRES-RFP mouse in which RFP expression can faithfully represent Bcl6 protein expression without compromising Bcl6 function, we remeasured the dynamic expression of Bcl6 and CXCR5 during Tfh differentiation in an immunization system (67). Our results revealed that, from day 2 to day 7, Bcl6 expression was gradually upregulated. Notably, CXCR5 expression does not follow the same kinetics: the expression of CXCR5 was rapidly increased at day 2 or 3 and maintained at high level in activated T cells, suggesting Bcl6 is not involved in initiating CXCR5 expression (67). Indeed, this hypothesis is confirmed by two separate experiments in which Bcl6 deficiency or overexpression did not affect the initiation of CXCR5 expression (67, 68).

At an early stage of Tfh development, when T cells upregulate CXCR5, but not Bcl6, some of the activated T cells upregulate the expression of Tfh genes, while simultaneously expressing Th1, Th2, and Th17 cell-associated genes (67). These precursor Tfh cells can be further matured into $CXCR5^{hi}Bcl6^{hi}$ Tfh cells with the help of cognate B cells (7, 67). The resulting $Bcl6^{hi}$ expression appears to have fully polarized Tfh gene expression profiles and may function to instruct the formation of GC structure. Although several studies reported that Tfh cells in GCs still expressed cytokines including IFN-γ (11, 69, 70), IL-4 (21, 70, 71), and IL-17 (49), the levels of Th1, Th2, or Th17 cytokines and transcription factors in $CXCR5^{hi}Bcl6^{hi}$ Tfh cells are greatly reduced (13, 72). In addition, Th1 and Th2 cells generated *in vitro* can further develop into Tfh cells *in vivo* after adoptive transfer, supporting the plasticity of T-effector cells during early stages of T-cell differentiation.

Therefore, our results favor a somewhat different model (*Fig.* 1), in which Bcl6-independent CXCR5 expression guides activated T cells to migrate to interfollicular regions, the subsequent T-B cell interactions at B-T borders instruct further Tfh cell commitment via upregulating or sustaining the expression of Bcl6. Concomitantly, the gradually increased Bcl6 expression acts together with multiple transcriptional factors to specify and stabilize Tfh cells, resulting in downregulation of Th1, Th2, and Th17 cell-associated genes.

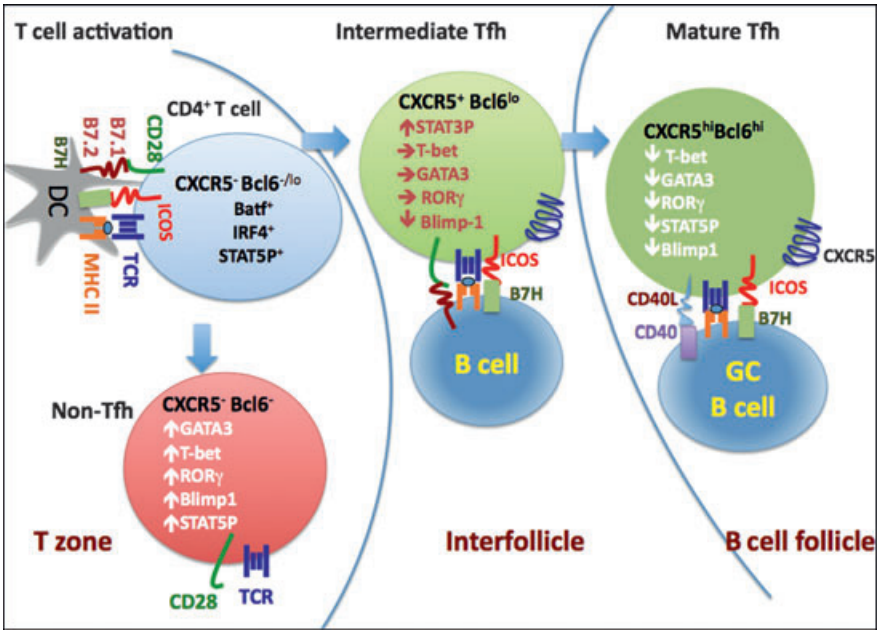

***Fig. 1.* Transcriptional regulation of Tfh development.** $CD4^+$ T-cell priming by DCs leads to the generation of $CXCR5^+Bcl6^{-/lo}$ pre-Tfh cells with increasing activities of transcriptional factors including Batf, IRF4, and STAT3. Bcl6-independent CXCR5 expression drives a portion of T cells to migrate to the interfollicular region, where T-B interaction increases Bcl6 expression. Simultaneously, the suppression of transcriptional factor T-bet, GATA3, and ROR-γ are initiated, whereas IL-6/IL-21-triggered STAT3 signaling is increased. After entry into the follicle, the cognate interaction benefits B and T cells mutually and eventually completes Tfh and GC B-cell commitment. The fully committed Tfh ($CXCR5^{hi}Bcl6^{hi}$) cells downregulate Th1, Th2, or Th17 programs.

Beyond the important role of Bcl6 in programming Tfh cell commitment, it also determines the fate of $CXCR5^{hi}Bcl6^{hi}$ Tfh cells after the effector phase (67). Several recent studies have indicated that $CXCR5^+$ T cells are able to survive the contraction phase in the presence of persistent deports of peptide-major histocompatibility complex class II after the primary immune response and eventually become the long-lived memory compartment with diverse characteristics (62, 73, 74). Actually, the usage of IL-21-YFP and Bcl6-IRES-RFP reporter system provided direct evidence that the transition from effector Tfh to memory Tfh cells could occur in the absence of antigen (50, 67). Although Bcl6 is transiently downregulated in T cells living in an antigen-free environment (75), the recurring antigens drive rapid Bcl6 upregulation, which highlights the effector function of expanded $CXCR5^{hi}Bcl6^{hi}$ memory Tfh cells (either polyclonal or monoclonal) and acceleration of GC responses (67, 75).

Future perspectives

Since the discovery of Tfh cells and the identification of Bcl6 as an obligatory factor in their generation, there has been rapid progress in understanding the signals and transcription factors governing Tfh cell development. A handful factors have been shown to positively or negatively regulate Tfh cell generation. However, many questions remain to be further investigated. (i) How does Bcl6 regulate Tfh program? Bcl6 is traditionally regarded as a transcriptional repressor. The genes bound and regulated by Bcl6 directly need to be identified and their function need to be studied. (ii) Are there other Tfh-specific transcription factors beyond Bcl6? Several factors have been found to be selectively upregulated in Tfh cells. Their function in Tfh cells needs to understood. (iii) How do Tfh transcription factors regulate Tfh cell generation? The hierarchy and interaction of these factors should be addressed. (iv) What is the epigenetic mechanism governing Tfh cell generation? Whether and how Tfh-regulating transcription factors impact epigenetic mechanisms in T cells should be illustrated. (v) What are the molecular mechanisms governing Tfh cell maintenance and memory generation? The extrinsic and intrinsic factors need to be identified. (vi) What are the suitable targets for Tfh-mediated immune diseases? Positive and negative regulatory factors should be explored and targeted by small molecules and biologics, considering the increasing association of Tfh cells with a broad spectrum of diseases. We anticipate that Tfh cells will represent another rapid growing area in immunology. Further understanding of Tfh cell regulation may offer ways to target various immune diseases.

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