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Bcl-6 controlled T_{FH} polarization and memory: the known unknowns

Hai Qi, Dan Liu, Weiwei Ma, Yifeng Wang and Hu Yan

Upon antigenic activation *in vivo*, naïve CD4 T cells can differentiate into one of several helper (Th) subsets under the control of lineage-specifying transcription factors to tailor immune responses against different types of pathogens. Follicular T-helper (T_{FH}) cells are a recently defined subset that is controlled by Bcl-6 and specializes in promoting B cell-mediated humoral immunity. T_{FH} cells exhibit unique spatiotemporal and functional features, but it is not settled as to how Bcl-6 promotes the T_{FH} development, how T_{FH} cells relate to other Th subsets, and how T_{FH} cells relate to memory. Here we review recent advances and crucial gaps in our understanding of Bcl-6-controlled T_{FH} development and function.

Addresses
Tsinghua-Peking Center for Life Sciences, Laboratory of Dynamic Immunobiology, School of Medicine, School of Life Sciences, Tsinghua University, Beijing 100084, PR China

Corresponding author: Qi, Hai (qihai@biomed.tsinghua.edu.cn)

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Introduction

The most distinct feature of T_{FH} cells is the follicular site of helper actions [1,2]. As neutralizing antibodies re-emerge as the hope for anti-viral vaccines [3] and dysregulated T_{FH} biology is increasingly implicated in diseases [4], how T_{FH} cells develop, acquire localization features, and function to regulate the quality and magnitude of humoral immunity has become not only a fascinating basic question but also a key area of potential translational research [5•,6]. As such, the T_{FH} subset has been the main topic of more than 30 review articles since 2011. Not to be excessively repetitive, we synthesize recent results on mapping T_{FH} development and its relationship with memory formation. We highlight conceptual roadblocks on our way forward to better understand the essence of a Bcl-6-controlled T_{FH} program.

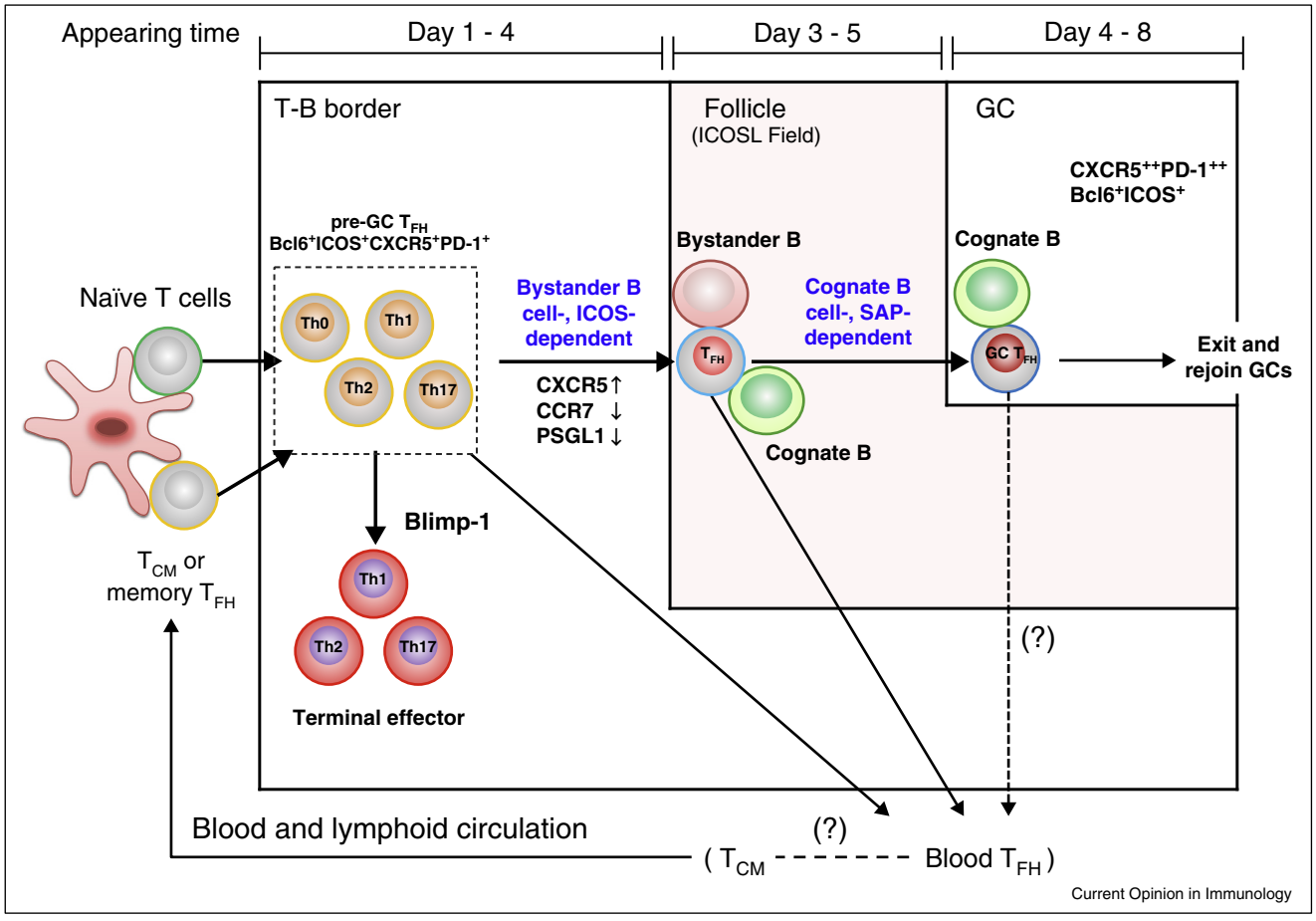
GC T_{FH} cells and T_{FH}-associated molecules

Whereas histologically defined, GC-associated Th cells are *bona fide* GC T_{FH} cells, $CXCR5^+$ Th cells at the T–B border can engage in cognate interactions with antigen-specific B cells [7••,8••], promote B cell proliferation and differentiation [9,10•], and are considered pre-GC T_{FH} cells [1]. These cells contain precursors that subsequently become GC T_{FH} cells (Figure 1). GC accumulation of T_{FH} cells, as identified by the $CXCR5^+PD\text{-}1^+$ phenotype [11] or by dynamic visualization in the GC structure [9], requires cognate interactions with antigen-specific B cells [12,13••], in part reflecting their dependence on continuous antigen presentation inside the follicle and GCs [14,15•]. $CXCR5^+PD\text{-}1^+$ T cells fail to develop without T cell-intrinsic Bcl-6 expression, and Bcl-6 overexpression promotes the T_{FH} phenotype [16•,17•,18•]. By histology, Yu *et al.* showed a complete absence of *Bcl-6*$^{-/-}$ Th cells from GCs formed in *Bcl-6*$^{+/+}$:*Bcl-6*$^{-/-}$ mixed bone-marrow chimera [18•]. These data establish that the absence of *Bcl-6*$^{-/-}$ GC T_{FH} cells is a primary T-cell defect and not secondary to a lack of GC B cells or the organized GC structure. Given the complexity of reciprocal regulation between T and B cells [19], such tests in mixed chimera are crucial for precisely assigning gene functions involved in T_{FH} and GC development, a point illustrated again by the recent discovery that distinct biochemical mechanisms are responsible for Bcl-6-controlled T_{FH} development and GC B cell differentiation [20•]. Based on known functions, major T_{FH}-associated molecules can be categorized into three functional modules (Figure 2), with the positioning and interaction modules together specifying where and how helper signals are delivered to B cells. In addition to Bcl-6, c-Maf [21•], IRF-4 [22] and BATF [23] may also contribute to T_{FH} programming. However, these other transcriptional factors have not been tested in systems where GCs can form with 'help' provided by wildtype T_{FH} cells.

Bcl-6 induction and pre-GC T_{FH} cells at the T–B border

Bcl-6 is upregulated in T cells within 30 minutes upon activation *in vitro* [24]. T cells substantially increase Bcl-6 protein expression from 2 to 4 days following activation *in vivo* [7••,8••,13••,25•], and essentially all divided T cells at day 2 highly express Bcl-6 [25•]. Cells with a $CXCR5^+PD\text{-}1^+$ phenotype also rapidly appear, exhibiting kinetics identical to or slightly slower than that of Bcl-6 [7••,8••,25•]. Early-arising $Bcl\text{-}6^+CXCR5^+$ cells are located in the T–B border and interfollicular regions, poised to migrate into the follicle [7••,8••]. The appearance of these pre-GC T_{FH} cells requires DC- but not B cell-mediated antigen presentation [8••,13••,26•], and Bcl-6 upregulation is promoted by IL-6 [27–29] and IL-12 [30–32]. Because ICOS signaling is required for T_{FH} development and GC formation [33]

Figure 1

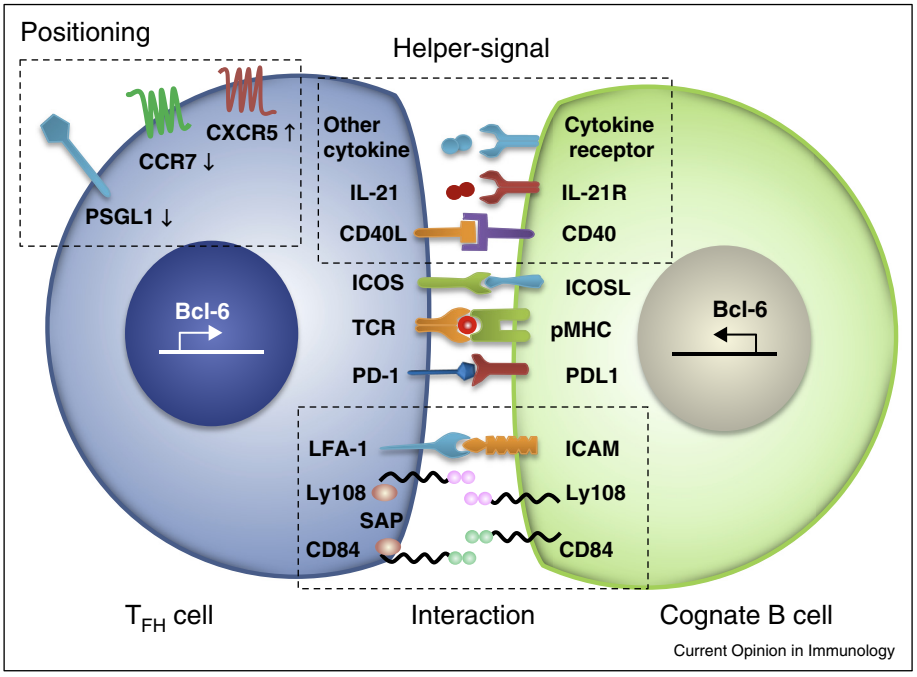


Spatiotemporal development of the T_{FH} subset and its relationship to other lineages and memory. Upon activation, naïve T cells rapidly upregulate Bcl-6 and CXCR5 and become pre-GC T_{FH} cells at the T–B border. Varied biases toward Th1/Th2/Th17 potentials likely exist among individual cells at this stage, and such T cells can productively interact with cognate B cells here (not depicted). CXCR5 and ICOS upregulation combined with CCR7 and PSGL-1 downregulation facilitates follicular re-localization, for which the ICOSL molecule expressed by bystander B cells is crucial (red shade in the follicle). Some pre-GC T_{FH} cells at the T–B border may not enter the follicle but encounter milieu that favors Blimp-1 induction and drives the terminal effector differentiation. The entry of T_{FH} cells into the GC requires their SAP expression and productive interactions with cognate B cells. Besides location, functional polarization in other aspects of GC T_{FH} cells is not well characterized. T cells may exit the secondary lymphoid organ from both T–B border and the follicle, eventually joining the general circulation to become blood T_{FH} cells. GC T_{FH} cells can exit from GCs and join other GCs. They might also enter general circulation and constitute a numerical minority of the blood T_{FH} compartment. Blood T_{FH} cells are more quiescent and memory-competent, and their relationship with T_{CM} cells that survey lymphoid organs requires further investigation. A provocative possibility is that T_{CM} cells are essentially derived from cells along the T_{FH} developmental pathway. Upon re-challenge, such memory cells can give rise to not only non-polarized or Th1-/Th2-/Th17-biased T_{FH} cells but also terminally differentiated and Th1/Th2/Th17-polarized effectors. Alternatively, blood T_{FH} cells may be memory cells committed to the T_{FH} pathway and cannot generate terminal Th1/Th2/Th17 effectors in a recall response. Dotted lines and question marks indicate uncertainties. Time axis on the top indicates the approximate time frame in which T_{FH} cells of a given stage appear before the next stage in the lymphoid organ, thus giving the overlap. The time axis does not imply a schedule for T_{FH} cells to exit from a give location into the blood. The progressive localization from the T–B border into the GC is likely a feature shared by follicular NKT cells and follicular T regulatory cells (not depicted).

and ICOS-deficient T cells fail to generate Bcl-6$^+$CXCR5$^+$ cells [13••], ICOS co-stimulation is thought to specifically induce Bcl-6 [1], although evidence for a direct signaling connection is lacking.

How Bcl-6 promotes pre-GC T_{FH} development is not clear. A very high level of Bcl-6 overexpression in human T cells led to upregulation of CXCR5, PD-1, SAP, ICOS, and CD40L in five days [21•], implying that Bcl-6 programs all functional modules characteristic of T_{FH} cells. However, this kinetics is slower than the appearance of pre-GC T_{FH} cells *in vivo*. Bcl-6 was shown to promote CXCR5 transcription [17•], possibly through suppression of microRNA species including the mir-17∼92 family that might target CXCR5 [18•]. Following *Listeria* infection, antigen-specific *Bcl-6*$^{-/-}$ T cells failed

Figure 2



Modular characteristics of T_{FH} cells. The 'positioning' module facilitates T cell migration into the follicle and promotes T cell co-localization with cognate B cells. ICOS plays a key role in this module, and the ligand it requires in regulating follicular recruitment of T cells is on bystander B cells (see Figure 1). The 'interaction' module controls the strength of physical interactions with cognate B cells, which likely impinge on the efficiency with which not only surface-bound but also soluble 'helper-signal' molecules are delivered to cognate B cells across the synapse. Bcl-6 is required for activated T cells to develop into T_{FH} cells and for cognate B cells to differentiate into the GC state. Precise functions of ICOS and PD-1 in the context of antigen-specific interactions between T_{FH} and GC B cells are not yet clarified.

to express CXCR5 [34••], but *Bcl-6*$^{-/-}$ OT-II T cells normally expressed CXCR5 3 days after OVA immunization in CFA [35••], suggesting requirement of Bcl-6 for CXCR5 expression is context-dependent. New evidence indicates that mir-17∼92 does not inhibit but actually promote T_{FH} development and GC formation [36••,37••], making this microRNA cluster an unlikely link between Bcl-6 and CXCR5. Very recently, Achaete-Scute homologue 2 (Ascl-2), a new basic helix–loop–helix transcription factor, was found to bind directly to the *Cxcr5* locus, trigger CXCR5 upregulation, and initiate the T_{FH} development [38••].

Transition from pre-GC T_{FH} cells to GC T_{FH} cells

All recently primed T cells move to the T–B border but do not necessarily move into the follicle (Figure 1). When activated T cells were blocked from exiting the lymphoid organ, it became evident that the GC T_{FH} cell compartment constitutes a numerical minority of all activated T cells of the same specificity [39]. It is not yet clear how the GC T_{FH} cell compartment is selectively established from the pool of all activated T cells that have ever arrived at the T–B border [19,40], although the strength of TCR signals appears to be an important factor [39,41].

Does Bcl-6 commit T cells to the unique follicular location and/or functions?

Although Bcl-6 does not seem to regulate CXCR5 directly, it could control other factors that promote follicular localization, a possibility that remains to be vigorously tested. It is also possible that, between individual pre-GC T_{FH} cells at the T–B border and their clonal progenies eventually in the GC T_{FH} compartment at a later time, Bcl-6 instructionally polarizes biological features of T_{FH} cells [1] so as to fulfill a particular set of helper functions inside the follicle or GCs. Somewhat contrary to this notion, Bcl-6 protein levels on individual CXCR5^{+}PD-1^{+} T cells were found to peak around day 3 after immunization at the T–B border and decrease thereafter, leading to some GC T_{FH} cells with undetectable Bcl-6 expression [7••]. Studies with an IRES-driven Bcl-6 reporter line revealed the same peak time but not the subsequent declining phase [35••]. This difference may result from the complex transcriptional and post-translational regulation of Bcl-6 protein expression, extensively characterized in B but not yet in T cells [42].

Importantly, we cannot yet define T_{FH} function properties unique to the follicular or GC phase, as the entire helper signal module does not distinguish GC from pre-GC T_{FH}

cells (Figure 2). PD-1 is involved in regulating plasma cell differentiation [43,44], but cellular mechanisms remain to be clarified. IL-21 is arguably the most specific T_{FH}-associated helper signal molecule [45,46], although it is not controlled by Bcl-6 but c-Maf [21•,47]. Experiments with an IL-21 reporter mouse strain showed no sign of IL-21 polarization, as a comparable fraction of $CXCR5^+PD\text{-}1^+$ T_{FH} cells expressed this cytokine from the pre-GC to the GC stage [48••]. In fact, the magnitude of GC formation remains the most frequently used readout to evaluate T_{FH} functions in general, even though it depends more on 'help' delivered before GC formation. Until we come to grasp how T_{FH} cells function to help B cells inside the follicle and GCs, possibly in a manner that is distinct from that at the T–B border, we may not be able to define T_{FH} functional polarization in substance, that is, other than location.

ICOS-controlled follicular localization of T_{FH} cells and bystander B cells

CXCR5 expression is necessary but not sufficient for activated T cells to localize into follicle, even when CCR7 is downregulated [49]. Another key factor is ICOS. Our recent work demonstrates that ICOS exerts direct motility control on T cells [50••]. This effect does not involve classical co-stimulation but hinges on ICOS engagement by ICOSL on the follicular bystander B cells. The resultant p110δ-mediated signaling promotes pseudopod formation and persistent T cell migration, which is necessary for $CXCR5^+$ T cells to move efficiently from the T–B border into the follicle [50••]. Interestingly, the T_{FH}- and GC-promoting effect of mir-17∼92 is in part due to its suppression of Phlpp2, a phosphatase that antagonizes ICOS-triggered PI3K signaling and inhibits follicular localization of T cells [36••], further highlighting the importance of direct ICOS-mediated motility control. Given its crucial role, ICOS transcription is under redundant control by RNA-binding protein Roquin-1 and Roquin-2 [51,52]. Because enforced Bcl-6 overexpression cannot correct the deficient T_{FH} recruitment and GC formation in the absence of ICOS [50••], Bcl-6 alone is insufficient to instruct a pre-GC to GC T_{FH} transition, at least in terms of follicular localization (Figure 1). Given the role of bystander B cells in regulating T_{FH} development, studies involving gene ablation in the entire B or T cell compartment should not always be interpreted solely in the context of antigen presentation and cognate interactions.

Bcl-6 and other Th fates

Bcl-6 may antagonize Th1, Th2, and Th17 development by suppressing T-bet, GATA-3, and ROR-γ expression and activities [17•,18•,53], implying a zero-sum competition between the T_{FH} fate and the other lineages. However, T_{FH} cells sorted from immunized animals still exhibit positive histone marks at T-bet, GATA-3 and ROR-γ loci [54••]. Th1 and Th2 cytokines are needed for regulating isotype-switching inside GCs and are indeed expressed by T_{FH} cells [48••,55]. IFN-γ may even promote T_{FH} development under certain conditions [56]. In Peyer's Patches, IL-17-producing cells are T_{FH}-like and induce IgA-producing GC B cells [57]. T cell-specific Bcl-6 ablation does not cause exaggerated Th1, Th2, or Th17 development [58,59]. In the strictest sense, therefore, the T_{FH} fate is not a parallel alternative to the other effector lineages (Figure 1).

Bcl-6, Blimp-1 and memory competence of T_{FH} cells

From either the T–B border or the follicle, recently activated T cells can exit the lymphoid tissue to eventually join the circulation (Figure 1). Cells with a T_{FH}-like phenotype are found in the blood, and their numbers and helper qualities correlate with ongoing immune responses [60•,61•,62•,63•]. These cells are of a more quiescent phenotype (e.g. low in PD-1 expression) compared to T_{FH} cells in the lymphoid organ, suggestive of memory formation [62•,63•].

In B cells, Bcl-6 is required to maintain the highly proliferative GC state, which arguably represents a differentiation stage in transition to humoral memory [5•]. In T cells, Bcl-6 is essential for the memory competence, in part because it helps maintain T cell proliferative capacity and suppresses apoptosis [59,64,65]. Interestingly, central memory T (T_{CM}) cells identified during a *Listeria* infection express CXCR5, and their development requires Bcl-6, intact B cell compartment, and ICOS signaling triggered by B cell-derived ICOSL [34••]. These are the same set of requirements for generating GC T_{FH} cells. $CXCR5^+$ T_{CM}-like memory CD4 T cells have been identified in other systems [66•,67,68]. An intriguing possibility is that T_{FH} cells at the T–B border and inside the follicle are a shared precursor pool for GC T_{FH} cells and T_{CM} cells (Figure 1). Consistent with this scenario, IL-2-driven upregulation of Blimp-1, which antagonizes Bcl-6 [69], promotes terminal effector T cell differentiation, suppresses T_{CM} development, and inhibits T_{FH} formation [16•,70,71•,72–74]. Chemokine receptors other than CXCR5 may guide recently activated T cells to milieu particularly suited for terminal effector differentiation, as in the case of CXCR3 for Th1 differentiation [75].

T_{FH}-polarized memory cells or T_{CM} cells capable of T_{FH} development

Given the relatedness of T_{FH} and T_{CM} cells, it is challenging to vigorously establish whether an immune response produces memory T cells with T_{FH} functional polarization, at least until the latter concept can be better defined as discussed above. Compared to terminally differentiated effector cells, which are enriched in the non-T_{FH} compartment and lack memory or T_{FH} potential owing to high levels of Blimp-1, T_{CM} cells undergo secondary expansion of much higher magnitudes. Thus, even if secondary T_{FH} development and GC formation is only coincidental and proportional to the recall response, a T_{FH} 'bias' can be

detected in the T_{CM}- as compared to the effector-mediated recall. Indeed, when T_{FH} and non-T_{FH} cells isolated late during influenza infection were recalled in naïve hosts after a period of 'rest' [48••], a huge difference in proliferative capacity was observed, reflecting inherent differences in the memory competence between the seeding populations. Such difference in the proliferative capacity between T_{FH} and non-T_{FH} cells after transfer was reported in two studies of LCMV infection [66•,71•] and in one [35••] but not another [76] model of protein immunization. Optical-highlighting experiments demonstrate that GC T_{FH} cells could exit GCs [77•], and some GC T_{FH} cells downmodulate Bcl-6 [7••] and increase IL-7Rα expression [7••,71•], making it possible that even GC T_{FH} cells are not terminal but maintain memory competence. Future studies have to determine whether the same or different biochemical mechanisms underlie the Bcl-6 requirement for T_{FH} and T_{CM} formation and to test how B cells, bystander or cognate, may help maintain joint or separate GC T_{FH} and T_{CM} competence.

Conclusions

Moving forward, we must clarify two key concepts for T_{FH} cells, functional polarization and memory potential. To help B cells to form GCs or differentiate into memory or plasma cells, do T_{FH} cells work in different manners or by different mechanisms inside follicle or GCs as compared to any Th cells at the T–B border? Regarding memory, it seems 'T_{FH}-ness' could be important for T_{CM} formation. Alternatively, is the T_{CM} competence somehow important for B helper functions of T_{FH} cells? Or, are some aspects (e.g. cell cycling, quiescence, metabolism) of T cell biology potentially controlled by Bcl-6 important for both? It is provocatively possible that a single cell and its progenies may traverse the pre-GC and GC T_{FH} compartments before becoming T_{CM} cells in a primary response, and can still give rise to Th1/Th2/Th17/T_{FH} cells in a recall response (Figure 1). The crux of these issues remains to understand what Bcl-6 does. Solving the puzzle should also help to identify the most inclusive aspect of T_{FH} biology that may unify follicular NKT cells and regulatory T cells with the conventional helper [78]. Is it reliance on B cells, follicular passage, functional polarization, memory competence or still just that, Bcl-6?

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