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## Cytokine-skewed Tfh cells: functional consequences for B cell help

Adesola C. Olatunde<sup>1</sup>, J. Scott Hale<sup>1</sup>, Tracey J. Lamb<sup>1,\*</sup>

<sup>1</sup>Department of Pathology, University of Utah, 15 North Medical Drive, Salt Lake City, UT 84112, USA

### Abstract

CD4<sup>+</sup> follicular helper T (Tfh) cells play a vital role in providing help for B cells undergoing selection and differentiation into activated antibody-secreting cells in mammalian germinal centers (GCs). Increasing evidence suggests that Tfh cells are a heterogeneous population that generates cytokine-skewed immune responses – a reflection of the microenvironment during differentiation. This has important ramifications for Tfh-mediated B cell help. Because Tfh subsets can have opposing effects on GC B cell responses, we discuss current findings regarding the differentiation and functions of cytokine-skewed Tfh cells in modulating GC B cell differentiation. Antibodies are important weapons against infectious diseases but can also be pathogenic mediators in some autoimmune conditions. Since cytokine-skewed Tfh cells can influence the magnitude and quality of the humoral response, we address the roles of cytokine-skewed Tfh cells in disease.

### Tfh cells are key players in the mammalian humoral immune response

Plasma cells that secrete **class-switched** (see Glossary) high-affinity antibodies and long-lived memory B cells are the main cellular components conferring efficacious and long-lasting humoral responses against invading pathogens [1]. The ability of B cells to undergo selection and differentiation into long-lived plasma cells and memory B cells is dependent on signals from **T follicular helper (Tfh) cells** within GCs [2,3] (Figure 1). Tfh cells are a distinct T cell subset that can be distinguished from other CD4<sup>+</sup> T cell subsets by their surface expression of **C-X-C chemokine receptor 5 (CXCR5)** [4,5], **programmed cell death protein 1 (PD-1)**, and **inducible T cell co-stimulator (ICOS)**, and low expression of chemokine (C-C motif) receptor 7 (CCR7) [4,5] – the latter enabling Tfh cell migration to and retention in the GC [6]. Tfh cells are also characterized by the expression of the transcription factor (TF) **B cell lymphoma 6 protein (Bcl-6)** [7–9] and the production of interleukin (IL)-21 in mice and humans [10].

Tfh-derived IL-21 [3,11] plays several crucial roles, including the formation and maintenance of GCs and the development of plasma cells and memory B cells [1]. Recent

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\*Correspondence: tracey.lamb@path.utah.edu (T.J. Lamb).

Declaration of interests

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studies of responses during infection in mice and humans reveal that Tfh cells can produce a more varied repertoire of cytokines than the canonical IL-21. Indeed, Tfh cells are a heterogeneous population of cells that are capable of coexpressing low, but biologically meaningful, amounts of TFs that are more typical of other T cells subsets, such as **T-box expressed in T cells [T-bet;** type 1 T helper (Th1) cells], **GATA-binding protein 3 (GATA-3;** Th2 cells), **retinoic acid receptor-related orphan receptor  $\gamma$  (ROR- $\gamma$ ;** Th17 cells) and **Forkhead box protein 3 [FoxP3;** T regulatory (Treg) cells] (Figure 2 and Box 1), as well as cytokines associated with these TFs [12–15]. Although the roles of these cytokines are well defined in classical helper T cell responses, the functions of Tfh-derived canonical T helper cell cytokines within the GC are poorly understood, but can have consequences for the resulting humoral response.

The GC response is crucial in the generation of efficacious humoral immunity. An understanding of the conditions that regulate cytokine production by Tfh cells could be harnessed to enhance humoral immunity to pathogens. The limited quantities of canonical T helper lineage cytokines produced by Tfh cells make these subsets difficult to identify through commonly used assays that measure cellular cytokine production following antigen restimulation. To more clearly identify antigen-specific Tfh cells, approaches such as **activation-induced marker assays** were recently developed to examine the upregulation of generic activation markers such as CD25 and OX40 following antigen restimulation, instead of cytokines [16,17]. This has enabled an advance in our understanding of Tfh biology. We discuss here the differentiation and functional consequences of heterogeneous Tfh cells that we refer to as ‘cytokine-skewed’, and discuss the roles of cytokine-skewed Tfh cells during B cell help for different inflammatory environments.

## Development of cytokine-skewed Tfh cells

Tfh cells were first described in human tonsils [18–20], although analyses of human Tfh cells have thus far mostly focused on circulating Tfh (cTfh) cells with the phenotypic profile of PD-1<sup>+</sup>CXCR5<sup>+</sup> surface expression. The use of mouse genetic models has defined crucial TFs that regulate early Tfh cell differentiation and commitment (reviewed in [21]). The dominant TF that is required for and controls the programming of Tfh cells is Bcl-6 [7–9,21].

### Bcl-6 is a key TF in Tfh cell differentiation

Early Tfh cell differentiation is positively influenced by additional TFs including cellular musculoaponeurotic fibrosarcoma oncogene homolog (c-Maf) [22–24], **lymphoid enhancer-binding factor 1 (LEF-1)**, and **T cell factor 1 (TCF-1)** [25,26]. Multipotent in all aspects of the formation and maintenance of the GC reaction, the cytokine IL-21 is crucial for the development of plasma cells (Figure 1) [3,10,11]. Studies in mice [27] and humans [23] have shown that c-Maf is a key inducer of IL-21 production and is therefore a key molecule conferring Tfh function. However, simultaneous expression of Bcl-6 is necessary to repress transcriptional programming that instructs other T helper cell lineage subsets.

Many TFs serve as negative regulators of Tfh cell differentiation. As shown in mice, the repressive activity of Bcl-6 is antagonized by **B lymphocyte-induced maturation protein-1 (Blimp-1)** [7]. Other TFs, including Foxo1 [28,29] and ID2 [30], act to antagonize Bcl-6 or other pro-Tfh programs and promote the differentiation of other T helper lineage cells. These findings suggest that, beyond direct Blimp1-mediated Bcl-6 repression, Bcl-6 indirectly and positively instructs other genes involved in Tfh cell differentiation via a 'repressor of repressor mechanism' [21,31].

### Models of differentiation

Beyond the differentiation of CD4<sup>+</sup> T cells into the Tfh lineage, the expression of cytokines more traditionally associated with T helper cells has been described. The influential role of signature cytokines from T helper cell subsets with respect to humoral responses, combined with the key role of Tfh cells in providing help to B cells for activation, indicates a potentially important role for cytokine-skewed Tfh cells in shaping humoral responses. The developmental pathways leading to cytokine-skewed Tfh cells are incompletely understood. Proposed pathways include a linear route for naïve CD4<sup>+</sup> T cells to become cytokine-skewed Tfh, as well as a pathway that incorporates a transitional hybrid state between Tfh cells and other CD4<sup>+</sup> helper T subsets [12] (Figure 2). Identification of a pathway that involves a transient differentiation state could be important in maintaining flexibility for gene expression profiles of Tfh cells, with ramifications in terms of Tfh cell plasticity, as the immune response progresses. Other studies have shown that classical T helper cells, such as Th2 cells, can further differentiate into Tfh cells [14]. Regardless of the developmental pathways, once induced, Bcl-6 promotes Tfh cell differentiation by repressing other helper T cell lineage-associated signature genes [32]. Bcl-6 accomplishes this repressive function by binding to the promoters of Th1/Th2/Th17 TF genes (e.g., encoding T-bet, GATA-3, and ROR $\gamma$ t), leading to suppression of **interferon  $\gamma$  (IFN- $\gamma$ )** and IL-17 production in human tonsillar cells and mice [8]. The transitional model that incorporates a hybrid cell type can explain the bifurcation of Tfh and classical T helper cells, as well as the identification of cells displaying characteristics of both.

### Differentiation of Tfh1 cells

**Tfh1 cells** are the best-studied subset of Tfh cells that have partial qualities of polarization towards other T helper lineages, and these are generated during type 1 inflammatory responses. A transient increase in ICOS<sup>+</sup>PD-1<sup>+</sup>CXCR5<sup>+</sup>CXCR3<sup>+</sup> circulating Tfh1 (cTfh1) cells was observed in humans following vaccination with influenza virus trivalent inactivated vaccine relative to controls; this increase correlated positively with high-affinity antibody production [33], suggesting that there might be a functional correlation between the number of cTfh1 cells and the generation of high-avidity antibodies. Moreover, the priming of naïve CD4<sup>+</sup> T cells in the presence of the polarizing cytokine IL-12 or infection of mice with *Toxoplasma gondii* (a strong inducer of Th1 cell responses) can increase the expression of *Tbx21*, *Bcl6*, and *Ii21* genes via **signal transducer and activator of transcription 4 (STAT4)**, and transiently generate cells that exhibit features of both Th1 and Tfh cells [12]. This supports the idea that cytokine-skewed Tfh cells may develop through a transitional hybrid state (Figure 2).

*In vitro* polarization of CD4<sup>+</sup> T cells sorted from naïve wild-type (WT) and *Tbx21*<sup>-/-</sup> mice showed that Th1 differentiation proceeds with an increase in T-bet expression which represses Tfh cell differentiation and promotes full differentiation of Th1 cells [12]. However, T-bet can also recruit Bcl-6 to the *Ifng* locus to curtail excessive IFN- $\gamma$  production [34]. Thus, the development of Tfh, Th1, and Tfh1 cells may depend on the ratio of T-bet and Bcl-6 in the cells, thus explaining, at least in part, observations that all three subsets of T cells can coexist in type 1 inflammatory conditions [12,13]. Indeed, this hypothesis is supported by a second study where the expression of both T-bet and Bcl-6 was monitored in CD4<sup>+</sup> T cells cultured under Th1-polarizing conditions, and in the presence of varying concentrations of IL-2 [13]. Although a higher concentration of IL-2 inhibits Bcl-6 expression in Th1 cells, it does not have an impact on T-bet expression, indicating that the T-bet:Bcl-6 ratio, which is modulated in part by IL-2, can dictate the Bcl-6–Blimp1 axis balance, and may be important in maintaining the flexibility between the Th1/Tfh gene profiles [13]. Furthermore, CXCR5<sup>+</sup> Tfh cells circulating in human blood, referred to as cTfh cells, express low amounts of Bcl-6 [35,36]; thus, the lack of Bcl-6-mediated transcriptional repressor activity in cTfh cells in humans might help to explain a higher frequency of cytokine-skewed Tfh cells in which T helper cell-associated transcripts and cytokines are not suppressed once they enter the circulation [37]. Of note, the epigenetic modifications that differentiate cTfh cells from their tissue-resident counterparts in humans are currently unknown, although cTfh cells may functionally resemble GC Tfh cells in lymphoid organs [36–38].

Control of loci by epigenetic modifications that include dynamic DNA methylation, chromatin remodeling through histone modification, and noncoding RNAs seems to play an essential role in the development of cytokine-skewed Tfh cells. For instance, STAT TFs that transduce signals from cytokine receptors can control transcriptional programming by promoting the deposition of repressive or permissive epigenetic marks at specific loci [42,43]. STAT4 transduces signals from the IL-12 receptor and has been found to control permissive H3K36me3 and H3K4me3 modifications that regulate gene loci such as *Bcl6*, *Pdcd1*, and *Ii21* that are important in Th1 and Tfh cell differentiation [12]. Moreover, IL-12-driven differentiation of Tfh1 cells occurs through phosphorylation of STAT1 and STAT4 [44,45] which act by suppressing the histone repressive mark H3K27me3 on the *Tbx21* and *Bcl6* gene loci [45]. This results in IL-12-driven expression of Tfh cell-associated genes such as those encoding ICOS and Bcl-6 in CD4<sup>+</sup> T cells [44]. In addition, STAT3, which transduces signals from the IL-6 receptor, also regulates the commitment of CD4<sup>+</sup> T cells to either a Tfh1 or Th1 phenotype in a *Plasmodium* sp. mouse infection model by regulating T-bet expression to avoid potential immunopathology [46].

### Differentiation of Tfh2 cells

Tfh cells can produce the classical Th2 cytokine IL-4 [14]. This can occur after the production of IL-21 has been initiated following immunization of mice with nitrophenyl-haptenated ovalbumin (NP-OVA) [39], but this is not a strict requirement given that IL-4-producing Th2 cells, when adoptively transferred into naïve mice and challenged with antigen (schistosome egg antigen), can differentiate into IL-4-producing **Tfh2 cells** [14]. IL-4 does not appear to play a role in the formation of mouse GCs until **pre-T follicular**

**helper (pre-Tfh) cells** are in the follicle (Figure 1), thus influencing **centrocyte** formation in the follicular B cell population [39]. Tfh2 cells can be distinguished from other subsets of Tfh cells by the quantity of IL-4 produced. Although all Tfh cells open the *Ii4* locus to facilitate centrocyte formation, not all continue to produce IL-4 [39,40]. Of note, most virus-specific Tfh1 cells do not express IL-4, but the GC Tfh cell subset does in the context of acute lymphocytic choriomeningitis virus (LCMV; Armstrong strain) acute infection in mice [41]. Thus, sustained IL-4 production appears to be the hallmark of Tfh2 cells, and Tfh cells skewed towards other subsets such as Tfh1 cells do not produce IL-4 to any appreciable concentration in mice immunized with unadjuvanted soluble antigen from *T. gondii* [40].

### Differentiation of Tfh17 cells

Tfh cells coproducing IL-17 in addition to IL-21 [24], termed **Tfh17 cells**, have also been described in mice. Although comparatively little is known about these cells, they appear to share many characteristics of Th17 cells in that their differentiation is dependent on the TF ROR $\gamma$ t [47], as well as on receptor ligation via IL-6, IL-21, and transforming growth factor (TGF)- $\beta$  [48–50]. *In vitro* stimulation experiments of mouse naïve T cells suggest that the expansion and maintenance of Tfh17 cells is mediated by IL-23 [24,51], but the interplay of Bcl-6 and ROR $\gamma$ t in the regulation of the coexpression of IL-21 and IL-17 has not been studied in great detail. However, there appears to be a key role for c-Maf, which is important in IL-21 expression, both in the induction of ICOS expression [24] and presumably also in the coinduction of Bcl-6 [22–24], leading to coexpression of IL-17 and IL-21. Thus, these key molecules described in Tfh cells play an important role in the differentiation of Tfh17 cells, but are likely not factors that might directly lead to IL-17 expression in this subset.

### Regulation of GC responses by T follicular regulatory cells

Once formed, GC responses are known to be tempered by **T follicular regulatory (Tfr) cells** in murine studies. Similarly to Tregs, Tfr cells express Treg signature molecules such as the TFs Foxp3, **cytotoxic T lymphocyte-associated protein 4 (CTLA-4)**, and GITR [15,52], but are deficient in the expression of IL-4, IL-21, and CD40L – molecules that are crucial for B cell help [15]. In Tfr-diphtheria toxin receptor (DTR) (*Foxp3<sup>cre</sup>Cxcr5<sup>DTR/WT</sup>*) mice in which Tfr cells were deleted at days 5–9 after immunization with NP-OVA, Tfr cells exerted an early effect on antigen-specific B cells before the formation of GCs, as demonstrated by an increased frequency of GC B cells compared with control mice (*Foxp3<sup>cre</sup>Cxcr5<sup>WT/WT</sup>*) [53]. This finding suggested that Tfr cells might modulate extrafollicular B cell responses. However, FoxP3-expressing Tfr cells also display restrained GC B cell proliferation and promote the generation of high-affinity antibodies; although the vast majority of Tfr cells are generated from infiltrating Tregs (e.g., [54]), they also develop directly in the GC from naïve T cells, but in smaller numbers [55] (Figure 2). In addition, circulating Tfr (cTfr) cells are generated in the peripheral lymphoid tissue before T and B cell interactions [56], and studies in mice in which cTfr cells were sorted from immunized mice, and cocultured with B cells in a suppression assay, suggested that cTfr cells may have a limited capacity to suppress Tfh cells and IgG1 in B cells compared with lymph node-resident Tfr cells [57]. This suppression might be acquired concomitantly with GC infiltration because cTfr cells are able to migrate to secondary lymphoid organs and partake in GC reactions in mice [57]. Once in the GC, Tfr cells can maintain their suppressive

function via the expression of polycomb protein Ezh2 – a chromatin-modifying enzyme that is necessary for the development of T cell homeostasis and cytokine production [42,58,59], and is crucial in maintaining Tfr cell transcriptional programming [60] via direct binding to Foxp3 [59–61].

Although IL-10 was previously thought to play a significant role in the suppression capacity of Tfr cells, B cell-intrinsic IL-10 signaling promoted GC responses and anti-parasite immunity in a mouse model of malaria [62,63]. It is reasonable to speculate that suppression is instead mediated by other molecules on the Tfr surface, such as CTLA-4; indeed, specific deletion of CTLA-4 from Tregs does not restrain the expansion of GC and Tfh cells, despite an increase in the frequency of both Treg and Tfr cells in these mutant mice relative to controls [64]. However, these possibilities remain to be investigated under a more robust condition in which CTLA-4 is specifically knocked out from Tfr cells. Thus, the mechanisms by which Tfr cells function remain speculative and require further investigation.

### Plasticity of cytokine-skewed Tfh cells

Although there is evidence that Tfh cells can maintain Tfh cell commitment [65], it is unclear whether Tfh subsets are plastic with respect to differential skewing of cytokines. However, epigenetic control of specific gene loci in Tfh cells can be altered to facilitate the transition of Tfh-like to conventional T helper cell subsets, as demonstrated in a study that utilized *in vitro* and *ex vivo* repolarized murine Tfh cells [66,67]. Analysis of adoptively transferred CXCR5<sup>+</sup> memory Tfh cells in the acute LCMV infection mouse model suggests that, although the majority of secondary effector cells can recall a Tfh response upon rechallenge with LCMV, those that exhibit some plasticity by downregulating CXCR5 also maintain low expression of Th1 effector molecules, including IFN- $\gamma$  and granzyme B [65]. Of note, DNA methylation at the *Gzmb* promoter is a hallmark of memory Tfh cells compared with memory Th1 cells in mice [65]. Together, these data suggest that, although Tfh cells may exhibit some plasticity, there may still be some epigenetic restrictions on the extent of such plasticity.

Regarding Tfh2 cells in the house dust mite mouse allergy model, those cells that were reactive to the antigen, and which secreted IL-4 on initial sensitization, could also transition to become conventional IL-4 and IL-13 double-producing Th2 cells upon restimulation, suggesting that Tfh2 might be precursors to Th2 cells [66]. However, Th2 cells can also differentiate into Tfh2 cells [14]. These data support the idea that Tfh cells can express permissive histone modifications such as H3K4me at specific gene loci (e.g., *Tbx21*, *Gata3*, and *Rorc*) that are associated with classical Th cell subsets, thereby enabling Tfh cells to express effector molecules and perform effector functions [67]. The plasticity of Tfh cells is vital to the generation of short-term effector Th cells, as well as long-term humoral immunity.

### The role of cytokine-skewed Tfh cells in B cell differentiation

In humans, B cells in the GC interact with Tfh cells through CD40/CD40L, CD28/CD86, and ICOS/ICOS-L interactions [23], constituting the GC reaction in which B cells undergo



multiple rounds of differentiation and proliferation in the GC dark zone. This involves a series of maturation steps, including **somatic hypermutation**, class switching, and **affinity maturation**, eventually resulting in a population of differentiated plasma cells and memory B cells (Figure 1). Tfh cells provide a plethora of signals to differentiating B cells, including IL-21 which is absolutely required for plasma cell formation and somatic hypermutation, as well as producing IL-9, which is essential for the formation of memory B cells in the GC [68] via stimulation of memory precursor cells in both mice and humans. The role of classical T helper cytokines in the GC with respect to the B cell maturation process is incompletely understood but is not inconsequential. Temporal and spatial effects influence the contributions of these cytokines to GC formation and the B cell differentiation process, and some studies demonstrate that cytokine production is asynchronous [69].

The role of pre-Tfh cells compared with GC Tfh cells in class-switch recombination (CSR) is difficult to dissect. However, recent studies have demonstrated that CSR is initiated before GC formation [70]. Therefore, cytokine production by pre-Tfh cells may play a role in class switching before the formation of the GC. There is evidence that IL-17 associated with Tfh17 cells might enhance cognate T–B cell interactions, leading to spontaneous GC development, as shown in autoimmune **BXD2 mice** [71]. This might potentially occur via suppressed mobility of B cells within the B cell follicle, thereby fostering GC reactions, or by stabilizing Tfh cells in the light zone [71]; indeed, interruption of IL-17 signaling abrogates autoreactive B cell responses [72]. However, the mechanism underlying this observation remains unclear. There is stronger evidence for the role of cytokine-skewed Tfh cells in isotype class switching. Specifically, both Tfh1 [44,73] and Tfh2 [74] cells are instrumental in isotype switching: on the one hand, in the absence of Tfh1 cells, there is a lack of isotype switching to IgG2c and IgG2a [73,75] and of enhanced IgG1 [76] isotype switching in response to viral infection in mice (Figure 3), with consequences for the maintenance of long-term neutralizing antibodies [73]. On the other hand, sustained IL-4 production by murine Tfh2 cells, along with IL-13, can drive switching to IgE [74], and in *in vitro* stimulation of murine B cells in the presence of exogenous IL-17 appears to induce switching to IgG2a and IgG3 [77]. Given the proximity of Tfh cells to the GC where B cells undergo phenotypic and functional development, the production of cytokines associated with other T helper cell lineages by cytokine-skewed Tfh cells is paramount for efficacious antibody responses.

Beyond isotype switching, a wider role for Tfh1 cells in the GC reaction has been shown in the induction of T-bet in B cells via IFN- $\gamma$  signaling [78]. This has been linked to retention of GC B cells in the dark zone and affinity maturation during *Plasmodium* sp. infection in mice [79] leading to an enhanced anti-malarial response relative to controls (as evidenced from specific deficiency of T-bet in B cells). However, this finding was not reproduced in another study reporting that T-bet expression in B cells suppressed anti-*Plasmodium* humoral immunity [62]. The discrepancy between these two reports may be due to differences in the models used – the latter study used a model of *Plasmodium* infection that did not require chemotherapeutic intervention to keep the mice alive [79]. Nonetheless, the latter study also determined that intrinsic IL-10 signaling in B cells is necessary to limit the suppressive effects of IFN- $\gamma$  within the GC, presumably supporting

a suppressive IL-10 effect from Tfh1 cells on the generation of humoral immunity [62]. This notion is also supported by studies of LCMV infection in mice where IL-10<sup>+</sup>IL-21<sup>+</sup> double-producing Tfh cells present within the spleen during chronic (clone 13 strain) but not acute (Armstrong) LCMV infection enhanced humoral immunity and viral control [80]. This role of IL-10-producing Tfr cells might also extend to enhancing type 2 helper responses, as suggested by a study in a mouse model of induced peanut allergy in which Tfr-produced IL-10 enhanced GC responses and increased antigen-specific serum IgE in control mice relative to Tfr-deficient (*Bcl6<sup>fl/fl</sup>Foxp3<sup>cre</sup>*) mice [81]. In humans, by contrast, a subset of tonsillar follicular CD25<sup>+</sup> FoxP3<sup>-</sup>IL-10<sup>+</sup> T cells – that otherwise functionally and phenotypically resembled mouse Tfr cells – prevented isotype class switching to IgE without having any suppressive role on plasma cell differentiation [82]; this suggested that suppression was restricted to specific aspects of the humoral response. This difference between species highlights one of the challenges in assessing the role of cytokine-skewed Tfh cells where data from mouse models may not always translate to humans.

## The role of cytokine-skewed Tfh cells in immunity to infection

Cytokine-skewed Tfh cells arise in different infectious disease settings, concomitantly with a diverse suite of effector cytokine-driven immune effector mechanisms that are required for the control of different pathogens, not least of which are those mediated by different antibody isotypes. In line with the role of cytokine-skewed Tfh cells in antibody class switching in GC B cells, there is mounting evidence for their importance in the generation of neutralizing antibodies, particularly in human viral infections such as hepatitis C virus (HCV) [83] and HIV-1 [84,85], in which the frequency of cTfh1 cells correlated with a broadly reactive antiviral humoral response. Similarly, IgE is a key mediator of type 2 inflammation that contributes to anti-helminth immunity in a variety of helminth infections in both mice and humans, and its production is thought to be mediated by IL-4 produced by CD4<sup>+</sup> T cells [86].

Tfh2 cells are now emerging as the most crucial regulatory component of IgE secretion during intestinal helminth infection via the production of IL-4. Depletion of Tfh2 cells, but not of Th2 cells, in the lymph node microenvironment impaired IgE<sup>+</sup> plasma cell differentiation and antibody production during *Heligmosomoides polygyrus* infection in mice [87,88]. Moreover, Tfh2 cells can induce IgG1-producing as well as IgE-producing plasma cells in response to helminth infection, and the production of both isotypes is compromised in the absence of IL-4 signaling [14,88]. These data underscore the importance of Tfh2 cell-derived IL-4 as the crucial cytokine source in directing humoral responses to helminth infections. Although IL-4 might play a role in the formation of centrocytes in the GC, it does not appear to play a role in affinity maturation. IL-4-producing Tfh2 can induce low-affinity IgE or IgG4 responses [89–91], and low-affinity IgE-producing plasma cells develop when there is a direct class switch from IgM to IgE. This is illustrated by the inability of IgG1-deficient mice to produce high-affinity IgE, even after repeated immunization with NP<sub>19</sub>KLH [92]. By contrast, high-affinity antibodies can develop upon sequential class switching from IgM to IgG1 to IgE [92–94], as observed in *Nippostrongylus brasillensis* infection in mice. Noteworthy, low-affinity/directly switched IgE was found in



GC B cells, while high-affinity/sequentially switched IgE was found in plasma cells [92–94].

Although IL-4 remains the key defining cytokine of type 2 immunity, Th2 cells can produce other type 2 cytokines, including IL-5 and IL-13 [95]. A study showed that plasma cells producing higher-affinity IgE during anaphylaxis can be driven by multipotent Tfh2 cells coexpressing IL-4 and IL-13 (termed Tfh13 cells) [74], suggesting that Tfh2 cells might have similar characteristics to Th2 cells in this regard (Figure 3). This observation suggests that cytokines produced by Tfh2/Tfh13 cells might contribute to defining the affinity of the antibodies produced. Although the putative mechanism behind this observation is not fully understood, we posit that the kinetics of antibody isotype class switching driven by different subsets of Tfh2 in the GC might be a key factor in the affinity of the antibody produced.

Cytokine-skewed Tfh cells do not always appear to be beneficial (Box 2). During *Plasmodium* sp. infections, cTfh1 cells isolated from children infected with *Plasmodium falciparum* have been shown to be inferior at providing B cell help compared with conventional cTfh cells, as evidenced by the decrease in their ability to induce isotype class switching in naïve B cells cocultured with superantigen SEB (staphylococcal enterotoxin B) [96]. Specifically, this impaired function of Tfh1 cells has been attributed to IFN- $\gamma$  production by both Th1 and Tfh1 cells during *Plasmodium* infection in both mice and humans; indeed, blockade of IFN- $\gamma$  or deletion of T-bet enhanced Tfh cell differentiation relative to controls [96,97]. This is surprising given the importance of Th1 responses [98,99] and antibodies [100,101] in malaria, as well as the preferential activation of cTfh1 over conventional cTfh cells. However, the observation that IFN- $\gamma$  suppresses GC responses was supported by at least one other study in rodent *Plasmodium* infection, attributing it to the induction of T-bet in GC B cells [62] – which led to the differentiation of atypical memory B cells and antibody skewing towards an IgG3 isotype [78]. Since cTfh1 cells upregulate CXCR3, a chemokine receptor that allows lymphocyte migration, it is also possible that, in this setting, CXCR3 expressed on cTfh1 cells enhances Tfh1 cell trafficking from the GCs towards the T–B cell border [97], leading to expedited and inefficient cognate interaction between Tfh1 cells and B cells, and inferior antibody production. Regardless of the mechanism, it is not clear why these potential explanations might not also apply to the generation of antibodies in viral infection (Figure 3). The function of cTfh1 cells in humoral immunity is thus context-dependent and pathogen-specific because cTfh1 cells can be beneficial in viral infections, but detrimental in some parasitic infections.

## Concluding remarks

The identification of different subsets of cytokine-skewed Tfh cells that develop under different polarizing conditions, mostly investigated in mice, underscores the complexity of the transcriptional programming and inflammatory environment that regulates Tfh cell biology. There is still much to be discovered about the functions of these different Tfh subsets (see Outstanding questions). Furthermore, the varying capacity of different Tfh cell subsets to provide B cell help raises a relevant question about how the functionalities of these cells might be harnessed to modulate the number and type of Tfh cell subsets. Interrogating this aspect of Tfh cells heterogeneity will be important for improving strategies

to design efficacious candidate vaccines against infectious diseases (Box 3) in which key effector mechanisms may develop under polarized immune conditions, and in which skewing humoral immunity might generate an optimal protective response.

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## Glossary

### **Activation-induced marker assay**

utilizes activation markers such as CD25 and OX40 to identify antigen-specific T cells following restimulation with antigen without the need for intracellular cytokine staining

### **Affinity maturation**

a change taking place in B cells that allows improvement of their antigen-binding affinity and the generation of antibodies with higher affinity for a particular antigen

### **B cell lymphoma 6 (Bcl-6)**

a TF that identifies Tfh cells

### **B lymphocyte-induced maturation protein 1 (Blimp-1)**

a TF that represses the expression of *Bcl6*, or of other genes involved in Tfh cell development, thereby serving as a potent inhibitor of Tfh cell differentiation

### **BXD2 mice**

a recombinant inbred mouse strain generated by crossing offspring from C57BL/6J and DBA/2J mice for >20 generations. These mice spontaneously develop autoimmune disease

### **Centrocytes**

nondividing B cells in the light zone of the GC that can undergo selection and terminally differentiate into either memory B cells or antibody-producing plasma cells

### **Class switching**

rearrangement of the heavy chain locus of an immunoglobulin in mature B cells, leading to a different immunoglobulin isotype

### **Cytotoxic T lymphocyte-associated protein 4 (CTLA-4)**

a negative regulator of immune responses

### **C-X-C chemokine receptor 5 (CXCR5)**

a member of the CXC chemokine receptor family that responds to chemokine CXCL13

### **Forkhead box protein 3 (FoxP3)**

a TF that identifies regulatory Tregs

### **GATA-binding protein 3 (GATA-3)**

a TF involved in the development and function of Th2 cells

**Inducible T cell co-stimulator (ICOS)**

a T cell co-stimulatory molecule

**Interferon  $\gamma$  (IFN- $\gamma$ )**

a key cytokine typically found in type 1-skewed immune responses

**Lymphoid enhancer-binding factor 1 (LEF-1)**

a TF that promotes early differentiation of Tfh cells

**Programmed cell death protein 1 (PD1)**

a molecule normally associated with downregulation of T cell effector responses

**Pre-T follicular helper (pre-Tfh) cells**

these are formed immediately after T cell priming, before GC formation. They are important in extrafollicular antibody responses and GC initiation

**Retinoic acid receptor-related orphan receptor  $\gamma$  (ROR- $\gamma$ t)**

a TF mediating the development, maintenance, and function of Th17 cells

**Somatic hypermutation**

point mutations that occur in the variable regions of both the heavy and light chains of immunoglobulins, leading to the generation of high-affinity antibodies

**Signal transducer and activator of transcription (STAT)**

a family of TFs activated by Janus kinases to mediate intracellular signaling

**T-box expressed in T cells (T-bet/TBX21)**

a TF associated with IFN- $\gamma$  transcription

**T cell factor 1 (TCF-1)**

a TF important for T cell development and helper T cell lineage commitment

**Tfh1 cells**

Th1-like Tfh cells that express stereotypical molecules of Tfh cells as well as the canonical Th1 cytokine IFN- $\gamma$

**Tfh2 cells**

Th2-like Tfh cells that express stereotypical molecules of Tfh cells as well as the canonical Th2 cytokine IL-4

**Tfh17 cells**

Th17-like Tfh cells that express stereotypical molecules of Tfh cells as well as the canonical Th17 cytokine IL-17

**T follicular helper (Tfh) cells**

a subset of CD4<sup>+</sup> T helper cells whose key function is to provide help for B cells to become antibody-secreting cells

**T follicular regulatory (Tfr) cells**

express stereotypical molecules of Tfh cells as well as FoxP3, the canonical TF of Tregs

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### Highlights

The inflammatory environment and the cytokine milieu induce T follicular helper (Tfh) cells to differentiate into different subsets that pertain to type 1 inflammatory responses (Tfh1), type 2 inflammatory responses (Tfh2), and T regulatory responses (Tfr), as well as the production of interleukin (IL)-17 (Tfh17) cells with varying capacity to provide help to B cells in mammals.

Cytokine-skewed Tfh cells contribute to class-switch recombination before germinal center (GC) formation and modify B cell maturation within GCs.

The accessibility in conventional Tfh cells of specific gene loci typical of CD4<sup>+</sup> T helper cells through epigenetic programming might facilitate their transition into Tfh1, Tfh2, Tfh17, or Tfr phenotypes.

The heterogeneity of Tfh cells underscores their ability to modulate different humoral immune responses to different pathogens, all with varying consequences. For instance, production of interferon (IFN)- $\gamma$  by Tfh1 cells can impair B cell function during *Plasmodium* sp. infection but enhances B cell function in some viral infections.

Cytokines produced by Tfh2/Tfh13 cells can mediate not only antibody class switching but also the affinity of the IgE produced.

The suppressive activity of Tfr cells depends on their origin, as well as on their location.

### Outstanding questions

Given that Tfh cells do not differentiate in the absence of directional cytokine-skewing, what are the major functions that non-skewed, IL-6-driven, conventional Tfh cells play in the activation of B cells, isotype class switching, and the humoral immune response in different infection models?

*In vitro* studies have clearly demonstrated that cytokine-skewed Tfh cells can differentiate from naïve CD4<sup>+</sup> T cells in the presence of various cytokines. To what extent could these polarizing cytokines drive conventional Tfh cells to become cytokine-skewed?

What are the epigenetic modifications that distinguish conventional Tfh cells from their cytokine-skewed counterparts?

What are the intrinsic mechanisms, such as post-translational regulation or epigenetic modifications, that could regulate the stringency of cytokine production by these cells over and above regulation by Bcl-6?

Does skewing occur before or after T cell follicular commitment?

Is cytokine-skewing reversible?

How heterogeneous are Tfr cells, and what are the mechanisms of GC suppression?

How do different pathogens skew Tfh cell polarization to avoid protective immune responses?

Tfh1 cells can enhance or impair antibody responses depending on the infection model. How could the molecular mechanisms that govern this cell differentiation be targeted in the generation of appropriate immune responses and efficacious antibody responses?

Studies in mice have shown that direct class switching (from IgM to IgE) and sequential class switching (from IgM to IgG1 to IgE) are mediated by cytokines and affect the affinity of IgE in Tfh2 cell responses. How do the kinetics of antibody isotype class switching driven by different subsets of cytokine-skewed Tfh cells dictate the affinity of the antibody produced?

Are cytokine-skewed Tfh cell subsets able to differentiate into a phenotype that can recall the appropriate effector function upon reinfection? If so, what is the longevity of these memory cells?

**Box 1.****Priming of cytokine-skewed Tfh cells****Conventional Tfh cells**

IL-6 and IL-21 are essential for Tfh cell differentiation. Engagement of IL-21R through IL-21 autocrine signaling leads to the activation of STAT3, upregulation of Bcl-6, and CD4<sup>+</sup> T cell differentiation into Tfh cells. Bcl-6 expression enables down-stream expression of E proteins which drive the expression of CXCR5, the follicle homing chemokine receptor that identifies Tfh cells [31]. Differentiated Tfh cells produced copious amounts of IL-2 but remain unresponsive to this cytokine through intrinsic IL-6 signaling [102] that prevents STAT5 from accessing the *Il2rb* gene locus, in turn preventing upregulation of IL-2R $\beta$  and activation of the T cell receptor/IL-2/STAT5 positive feedback loop [102].

**Tfh1 cells**

Adoption of a Th1 phenotype leads to the development of a Th1 and Tfh ‘hybrid’ cell type referred to as Th1-like Tfh (Tfh1) cells. This population is characterized by a phenotypic profile of CXCR3<sup>+</sup>CXCR5<sup>+</sup>CCR6<sup>-</sup> and expression of IFN- $\gamma$  in addition to IL-21 [103]. *In vitro* differentiation of mouse [44] and human [45] naïve CD4<sup>+</sup> T cells into Tfh1 cells appears to require IL-12R signaling, but not IL-6 or IFN- $\gamma$  receptor signaling. Ligation of the IL-12 receptor activates STAT4 and STAT3 in mouse [44] and human [45] CD4<sup>+</sup> T cells, leading to the expression of a Th1 and Tfh transcriptional profile in this hybrid population, driven by T-bet and Bcl-6, respectively [44]. IL-27 signaling also drives a Tfh1 phenotype [46] whereas IL-2 signaling suppresses this differentiation [44].

**Tfh2 cells**

These can be identified based on their production of IL-4 and IL-21 [36,104] and a lack of expression of the two chemokine receptors CXCR3 and CCR6 [28]. The differentiation of Tfh2 cells is dependent on IL-4 but is suppressed by IL-6 via STAT3 signaling [105]. Tfh2 cells express Bcl-6 as well as GATA-3. Given that IL-4 is pivotal to B cell survival and affinity maturation [69,106,107], the importance of Tfh2 cells extends beyond the induction of class switching of B cells to IgE [108,109]. IL-4 secreted by Tfh2 cells supports humoral immunity in situations where IgE is not made, such as in IgG4-mediated diseases [89–91].

**Tfh17 cells**

Characterized by IL-21 and IL-17 production, the differentiation of Tfh17 cells is dependent on IL-23, ICOS, and ROR $\gamma$ t [24]. Tfh17-associated IL-17 may enhance cognate T–B cell interactions, leading to spontaneous GC development [71]. The IL-17 produced from Tfh17 cells also induces switching to IgG2a and IgG3 [77].

**Tfr cells**

Regulation of follicular responses is mediated by T follicular regulatory (Tfr) cells. Tfr cells express Treg signature molecules such as the TF forkhead box (Fox) p3, CTLA-4,



and GITR [15,52], but are deficient in the expression of IL-4, IL-21, and CD40L – molecules that provide help signals to B cells [15]. Tfr cells can differentiate from naïve CD4 T helper cells originating from conventional FoxP3<sup>+</sup> Treg precursor cells as well as after immunization with foreign protein emulsified in incomplete Freund's adjuvant (IFA) in a PD1-ligand dependent manner [15,55,110,111]. Like conventional Tfh cells, IL-6 plays a key role in the differentiation of Tfr cells, and STAT3 deficiency abrogating signaling from IL-6R leads to a decrease in Tfr cells in both the spleen and Peyer's patches following antigen stimulation [112]. IL-21, the major cytokine produced by Tfh cells, suppresses Tfr cell differentiation [113].

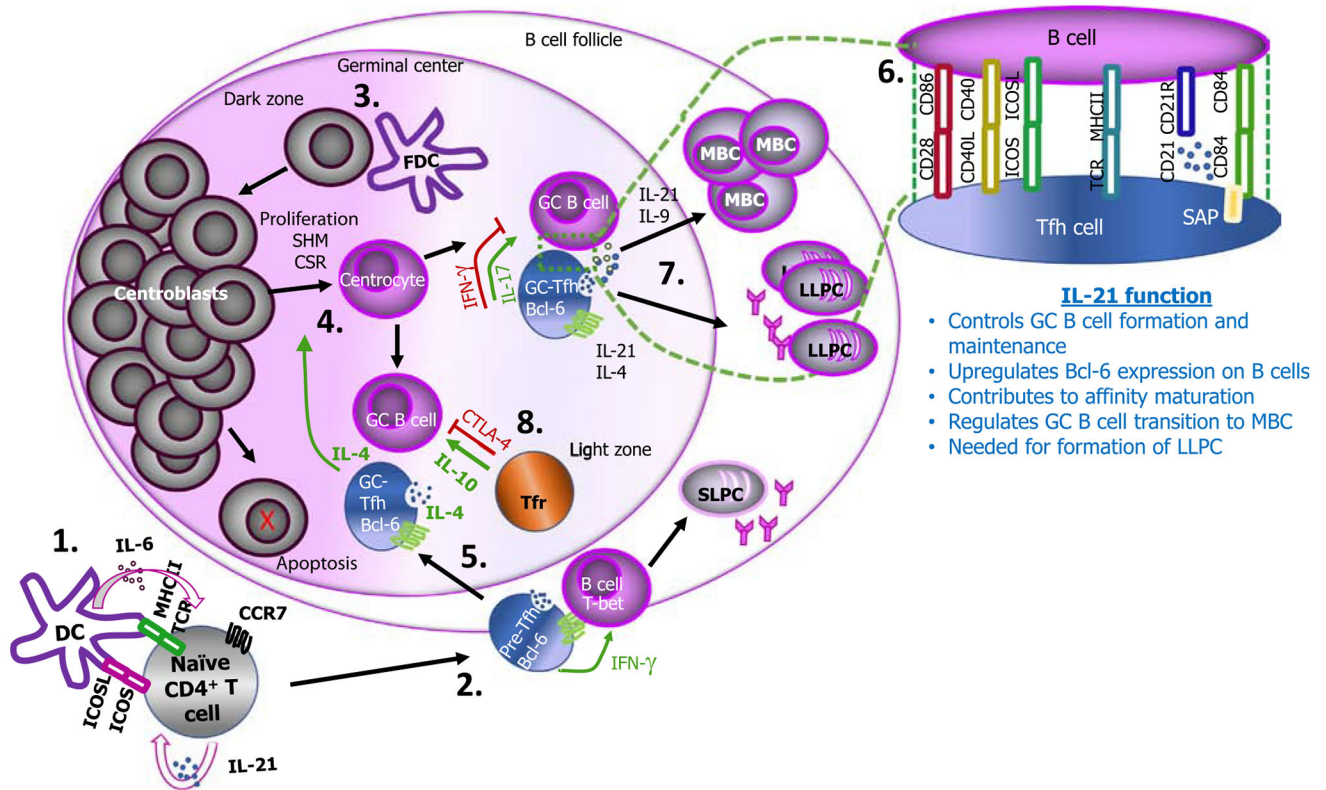
**Box 2.****Contribution of cytokine-skewed Tfh cells to autoimmune and allergic disease**

Many autoimmune diseases are known to be driven in part by autoantibodies. The importance of Tfh cells as driving factors in the development of robust class-switched and high-affinity auto-antibody responses places Tfh cells as a key cell subset in the development of a range of autoimmune and allergic diseases such as systemic lupus erythematosus (SLE) and anaphylaxis. IgE has long been identified as the perpetuator of allergic responses leading to asthma [86] and is the main source of IL-4 that drives class switching to IgE; this can be driven by Tfh2 cells rather than by conventional CD4<sup>+</sup> Th2 cells in mice [74,87]. In peripheral blood, IL-21/IL-4 double-producing Tfh2 cells have been reported to be increased in allergic asthma patients in peripheral blood, and correlate positively with total serum IgE [114,115]. Similarly, a heightened number of Tfh2 cells in the spleen correlated with disease incidence in mice with specific deletion of *Ets1* in CD4<sup>+</sup> T cells – a mouse model of SLE-like disease – concomitant with modulation of IL-4 signaling and increasing IgE titers [116].

Not all autoimmune conditions are characterized by the development of Tfh2 cells alone. Juvenile dermatomyositis (JDM) is a systemic autoimmune disease that alters the frequency of different subsets of cytokine-skewed human cTfh cells, and a mixed population of cTfh2 and cTfh17 cells in the periphery correlates with disease activity [36]. The significance of this finding is unclear, but it may be related to findings in BXD2 mice, a recombinant inbred mouse strain that develops autoimmunity including spontaneous arthritis [71] – when derived from CXCR5<sup>+</sup>ICOS<sup>+</sup>IL-21R<sup>+</sup>IL-17R<sup>+</sup> Tfh (Tfh17) cells in these mice, IL-17 has been implicated in mediating spontaneous GC development [72]. In the absence of a functional IL-17 receptor in BXD2 *Il17ra*<sup>-/-</sup> mice, Tfh cells were defective in their ability to localize to the light zone of the GC, although *in vitro* cocultured B cell responses remained unaffected. These data suggest that IL-17 signaling can influence Tfh cell positioning, perhaps via upregulation of G protein signaling (RGS) and chemokine-mediated positioning within the GC to interact with B cells [72]. This suggests that Tfh17 cells might be relevant targets for therapeutic interventions in some autoimmune diseases, and they therefore merit further investigation.

**Box 3.****Cytokine-skewed Tfh cell development towards a memory phenotype**

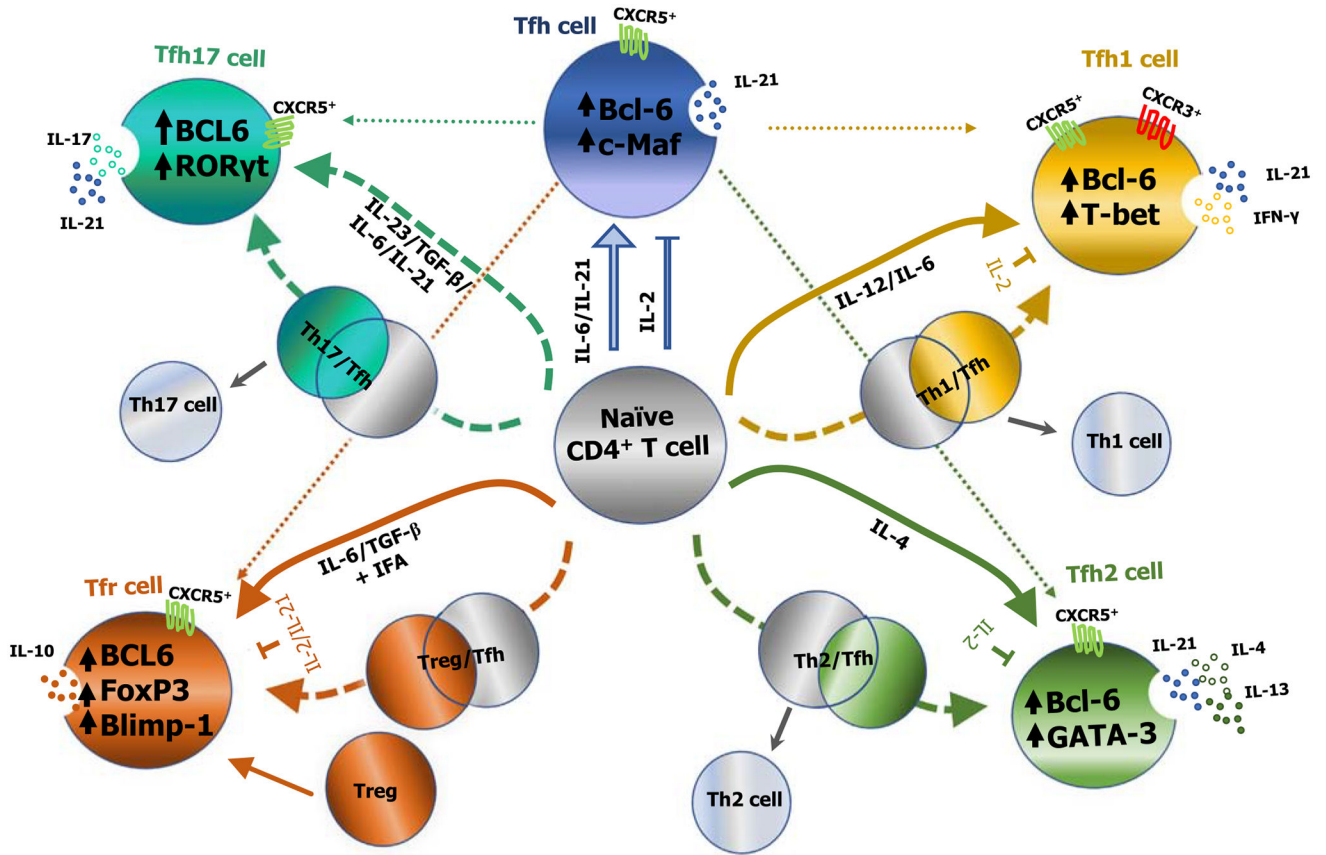
One of the hallmarks of CD4<sup>+</sup> T cells is their central function in immunological memory. Following pathogen clearance, effector CD4<sup>+</sup> T cells undergo a contraction phase, leaving behind only a subset of this cell population with a differentiated memory phenotype [117]. Memory Tfh cells are no exception and have been shown to be present in both mice [65] and humans [37]. They are able to recall Tfh cell–B cell helper function following secondary challenge [65,116,118–120]. Two main subsets of memory Tfh cells have been identified – circulatory Bcl-6<sup>lo</sup>CXCR5<sup>lo</sup>ICOS<sup>+</sup>PD-1<sup>+</sup>CD69<sup>-</sup> memory Tfh cells [37,121] and draining lymph node-resident Bcl-6<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>hi</sup>PD-1<sup>+</sup>CD69<sup>+</sup> antigen-specific memory Tfh cells may [121,122] – both of which are functional in providing B cell help. Many questions remain regarding the plasticity of memory Tfh cell reactivation initiated by antigen-specific memory B cells [123]. However, the epigenetic programming that retains the pattern of gene expression and transcription profiles in Tfh cells may extend to cytokine secretion [65].



**Figure 1. T follicular helper (Tfh) cell roles in mammalian B cell differentiation.**

Dendritic cells (DCs) induce initial priming of naïve CD4<sup>+</sup> T cells in the presence of interleukin (IL)-6 and through inducible T cell co-stimulator (ICOS)/ICOS-L (ligand) interactions. This process is accompanied by antigen recognition through peptide-loaded MHC-II on DCs and differentiation of T cells into pre-Tfh cells, characterized by upregulation of B cell lymphoma 6 protein (Bcl-6) and C-X-C chemokine receptor 5 (CXCR5), while downregulating CCR7 (1). Autocrine signaling of IL-21 on Tfh cells enhances their development and maintenance. Expression of CXCR5 enables Tfh cells to migrate to B cell follicles where they interact with B cells, leading to B cell differentiation into short-lived extrafollicular plasma cells (SLPCs) (2). Follicular dendritic cells (FDCs) interact with follicular B cells, and this allows B cells to migrate to the dark zone where they differentiate into centroblasts (3). Centroblasts undergo somatic hypermutation (SHM), class-switch recombination (CSR), and proliferation, and subsequently migrate to the light zone to form centrocytes (4). In addition, IL-4 produced by Tfh cells may mediate centrocyte differentiation. B cell clones in the dark zone with low affinity or that are autoreactive can undergo additional SHM or die because of apoptosis. The cognate interaction between pre-Tfh cell and B cells allows full differentiation of Tfh cells, and these migrate to the GC (5) where they provide help to B cells activated by FDCs via cytokines that in some cases may include IL-17. CD28/CD86, CD40L/CD40, ICOS/ICOS-L, TCR/MHCII, CD21/CD21R, and SAP-associated receptor (CD84)/CD84, mediate cognate interactions between T and B cells (6). This GC reaction between Tfh and B cells results in memory B cell (MBC) and long-lived plasma cell (LLPC) formation supported by IL21, IL-9, and IL-4 (7). Regulatory T follicular (Tfr) cells can suppress T-B cell

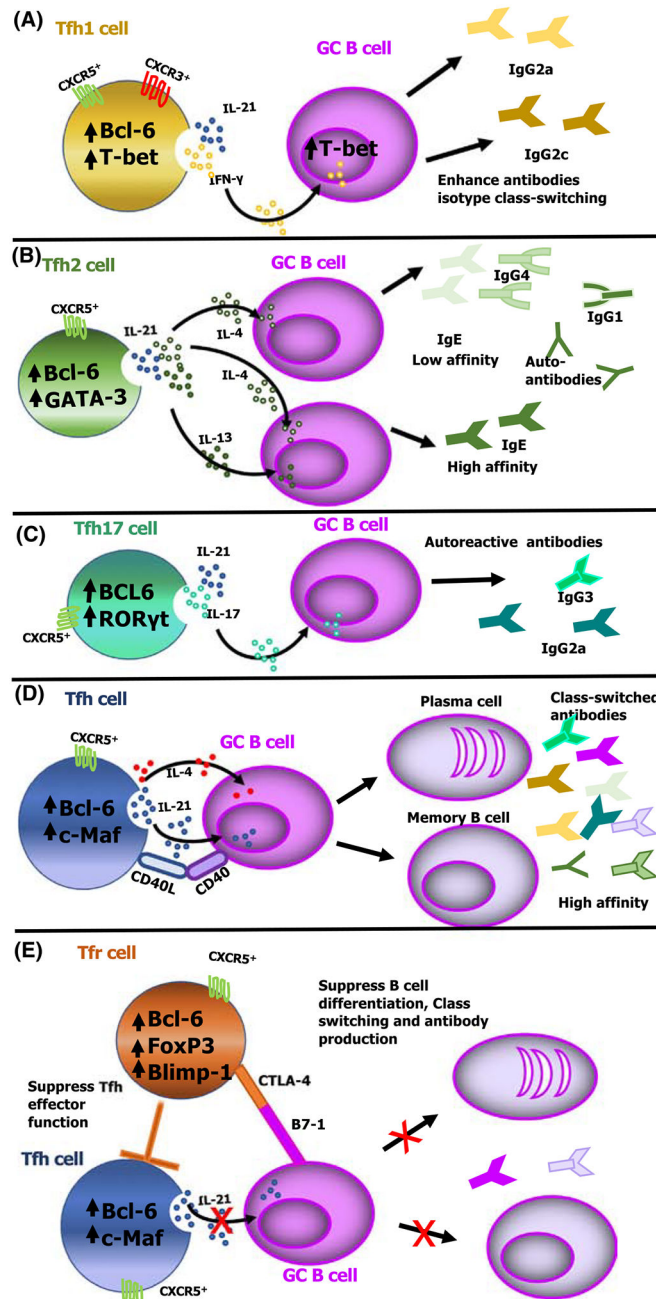
interactions through cytotoxic T lymphocyte-associated protein 4 (CTLA-4) but enhance B cell maturation via IL-10 (8). Arrows in green signify a positive signal for B cell maturation; arrows in red signify a negative signal.



**Figure 2. Proposed models for the differentiation of cytokine-skewed T follicular helper (Tfh) cell subsets in mice and humans.**

A schematic representation of the differentiation of cytokine-skewed Tfh cells from naïve CD4<sup>+</sup> T cells is shown. External stimuli including the cytokine milieu, as well as intrinsic factors such as lineage-specific transcription factors (TFs), induce the differentiation of naïve CD4<sup>+</sup> T cells into subsets of cytokine-skewed Tfh cell subsets. Polarizing cytokines can induce Tfh cells to coexpress transcriptional programming that is typical of other CD4<sup>+</sup> helper T cells, in addition to Bcl-6 that is typically expressed by Tfh cells. It is possible that a partial ‘hybrid’ [T helper (Th)1/Tfh, Th2/Tfh, Th17/Tfh, and T regulatory cell (Treg)/Tfh] state occurs before maturing into different cytokine-skewed germinal center (GC)-Tfh cells. In view of this, Tfh cells can produce limited quantities of canonical cytokines that have been used to identify other T helper subsets, in addition to interleukin (IL)-21, typically associated with classical Tfh cells. The majority of T follicular regulatory (Tfr) cells arise via differentiation from infiltrating Tregs, although some also differentiate from naïve CD4<sup>+</sup> T cells. Broken lines indicate hypothesized differentiation pathways for cytokine-skewed Tfh subsets [12]. Abbreviations: Bcl-6, B cell lymphoma-6; Blimp-1, B lymphocyte-induced maturation protein-1; c-Maf, c-musculoaponeurotic fibrosarcoma oncogene homolog; CXCR5, C-X-C chemokine receptor 5; FoxP3, forkhead box protein 3; GATA-3, GATA-binding protein 3; IFN- $\gamma$ , interferon  $\gamma$ ; ROR $\gamma$ t, RAR-related orphan receptor  $\gamma$ ; T-bet, T-box expressed in T cells; Tfh1, Th1-like Tfh cells; Tfh2, Th2-like Tfh cells; Tfh17, Th17-like Tfh cells.





**Figure 3. Cytokine-skewed T follicular helper (Tfh) cell subsets may play varied roles in modulating antibody isotype class switching and affinity maturation in both mice and humans.** (A) Interferon (IFN)- $\gamma$  produced by Th1-like Tfh (Tfh1) cells induces the expression of T-box expressed in T cells (T-bet) on B cells that promotes IgG2a isotype class switching in response to lymphocytic choriomeningitis virus (LCMV) or Zika viral infection and IgG2c isotype class switching in response to *Plasmodium* parasites, albeit by limiting the overall germinal center (GC) B cell response [73,75,78]. (B) Cytokines secreted from Th2-like Tfh (Tfh2) cells influence the affinity of the antibody produced, where IL-4 from Tfh2 cells promotes low-affinity IgG1 and IgE, whereas coproduction of interleukin (IL)-4 and IL-13 induces high-affinity IgE. (C) IL-17 is produced by Th17-like Tfh (Tfh17)

cells that induce antibody isotype class switching to IgG2a and IgG3, and induction of autoreactive antibodies. (D) Conventional Tfh cells produce effector molecules such as IL-21 and IL-4 that induce B cell differentiation into plasma and memory B cells. (E) T follicular regulatory (Tfr) cells mediate regulatory functions by expressing cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), a coinhibitory receptor that interacts with B7-1/B7-2 on B cells, resulting in direct suppression of IL-21 production by Tfh cells, and disrupting cognate interactions between Tfh and B cells. This suppressive function of Tfr cells on either B or Tfh cells subsequently limits B cell differentiation into memory or plasma cells [57,124]. Abbreviations: Bcl-6, B cell lymphoma 6 protein; c-Maf, cellular musculoaponeurotic fibrosarcoma oncogene homolog; CXCR, C-X-C chemokine receptor; GATA-3, GATA-binding protein 3; ROR $\gamma$ t, RAR-related orphan receptor  $\gamma$ .