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The regulation and role of T follicular helper cells in immunity

Elissa K. Deenick[1,2] and Cindy S. Ma[1,2]

[1]*Immunology Program, Garvan Institute of Medical Research, Darlinghurst, NSW and* [2]*St Vincent's Clinical School, University of NSW, Kensington, NSW, Australia*

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Correspondence: Dr E. K. Deenick, Garvan Institute of Medical Research, 384 Victoria St, Darlinghurst 2010, NSW, Australia.
Email: e.deenick@garvan.org.au
Senior author: Dr. E. K. Deenick

Summary

It is well established that the generation of a high-affinity long-lived antibody response requires the presence of T cells, specifically $CD4^+$ T cells. These $CD4^+$ T cells support the generation of a germinal centre (GC) response where somatic hypermutation and affinity maturation take place leading to the generation of memory B cells and plasma cells, which provide long-lasting protection. Greater insight into the nature of the $CD4^+$ T cells involved in this process was provided by two studies in 2000 that described $CD4^+$ T cells residing in the B cell follicle that expressed CXCR5. As a result these cells were named follicular B helper T cells, now more commonly known as T follicular helper (Tfh) cells. Since then there has been enormous growth in our understanding of these cells, now considered a distinct T helper (Th) cell lineage that can arise from naive $CD4^+$ T cells following activation. This review summarizes some of the most recent work that has characterized Tfh cells and the pathways that lead to their generation.

Keywords: antibody responses; $CD4^+$ T cells; T follicular helper

Phenotype of Tfh cells

Tfh cells express a range of cell surface molecules that not only allow for their identification, but also serve important functions in their interactions with B cells.

The original defining feature of a Tfh cell was the expression of the chemokine receptor CXCR5.[1,2] Expression of this molecule, together with down-regulation of CCR7, facilitates the movement of Tfh cells out of the T cell zone of the lymphoid tissue and into the B cell follicle.[3–5] This movement is essential for positioning the $CD4^+$ T cells in proximity with cognate B cells to which they will provide help.

Typically, Tfh cells are not identified by the expression of CXCR5 alone but by the coexpression of other surface markers, most commonly programmed death-1 (PD-1) and inducible co-stimulator (ICOS). Both these molecules are members of the CD28 family and are up-regulated on T cells following activation. ICOS is a co-stimulatory molecule, while PD-1 provides an inhibitory signal to the T cell.[6,7] Tfh cells, however, also express a range of other molecules including CD40 ligand (CD40L), OX40, CXCR4, CD200, B and T lymphocyte attenuator (BTLA), members of the SLAM family (CD84, NTBA, SLAM), SLAM-associating protein (SAP) and the cytokine interleukin (IL)-21. They also down-regulate expression of molecules such as CD62L and CD127 (IL-7Rα).[8–13]

Like other Th lineages, Tfh cells are associated with expression of a canonical transcription factor. Thus, as the generation of Th1, Th2 and Th17 cells is controlled by T-bet, Gata-3 and Rorγt, respectively,[12,14,15] the generation of Tfh cells is controlled by Bcl-6 expression.[16–18] Not only do Tfh cells possess high levels of this transcription factor,[10,11,19] but several reports have also shown that its expression is both necessary and sufficient to drive Tfh cell development.[16–18]

Problems with identification of Tfh cells

Although a considerable number of studies have sought to phenotype Tfh cells, a significant level of difficulty and controversy still surrounds the identification of these cells. This problem stems from several issues: first, many of the markers used to identify Tfh cells, such as PD-1, ICOS and CXCR5, are also commonly expressed by activated $CD4^+$ T cells.[3,6,7] As a result, Tfh cells are often identified as the cells expressing the highest levels of these molecules; thus, it is easy to see how this can quickly become a problematic definition. Secondly, the term 'Tfh cell' is used by individual researchers to describe different

populations of cells. Hence, while the original reports used the term to describe $CD4^+$ $CXCR5^+$ T cells located in the follicle, in more recent times 'Tfh cell' has come to be used by many to describe only those cells that are found within the germinal centre (GC), while $CD4^+$ $CXCR5^+$ T cells found elsewhere in the follicle are termed 'pre-Tfh cells'. In contrast, others have maintained the usage of 'Tfh cell' to describe all $CD4^+$ $CXCR5^+$ T cells in the follicle and refer instead to those cells located specifically in the GC as 'GC-Tfh cells'. Even given a consensus on the terminology for these cell populations, it remains to be determined whether follicular and GC-Tfh cells can be distinguished phenotypically or whether they can only be identified by imaging which reveals their location. Although some reports have suggested that molecules such as GL7[20] are able to identify specifically cells found in the GC, other reports have suggested that at different times during the response, cells outside the GC can also express these molecules.[21] Once again, this probably reflects the problem that many markers of Tfh cells are also found on activated cells.

The story is complicated further by recent reports that demonstrate that even Bcl-6, considered one of the gold standard markers of Tfh cells, cannot be used on its own to identify Tfh cells. These studies revealed that $CD4^+$ T cells express Bcl-6 very quickly following activation, long before they migrate deep into the follicle, let alone into the GC.[21–23] Moreover, they identified cells with a Tfh-like phenotype (e.g. CXCR5 and PD-1 expression and GC localization) that did not express Bcl-6 as well as cells that expressed Bcl-6, but not other Tfh cell markers such as PD-1.[21,22] This suggests that the role of Bcl-6 in regulating Tfh cell differentiation may be more complex than first anticipated. However, for the purposes of this review we will consider Tfh cells to be $CXCR5^+$ $PD\text{-}1^+$ $Bcl\text{-}6^+$ cells that express IL-21 and are found in the follicle.

Identification of Tfh cells in the clinical setting

A further problem has arisen in studies of human T_{FH} cells, particularly in the investigation of patients suffering from immunodeficient or autoimmune conditions. In these patients it would be helpful to be able to identify Tfh cells to determine whether the generation or function of these cells is dysregulated. However, in most cases it is not feasible to access lymphoid tissue from these patients, and therefore analysis is limited to cells in the blood. Thus, researchers have used enumeration of circulating $CD4^+$ $CXCR5^+$ cells as a measure of Tfh cells even though it was unclear whether these cells represented true Tfh cells. Several studies have reported increased or decreased numbers of $CD4^+$ $CXCR5^+$ cells in the blood of patients with autoimmunity[24,25] or antibody deficiencies,[26] respectively, suggesting that these cells may be a good correlate of Tfh cells. Two recent studies have now addressed the question more closely and demonstrated that circulating $CD4^+$ $CXCR5^+$ cells can secrete IL-21 and CXCL13, express ICOS and Bcl-6, and induce antibody production from naive B cells,[25,27] suggesting that they do indeed represent a Tfh-like population.

Generation of Tfh cells

Given the importance of Tfh cells in the generation of T cell-dependent antibody responses, much interest has focused on the pathways involved in the generation of these cells. Multiple signals appear to be involved in the generation of Tfh cells, including T cell receptor (TCR) signalling, cell surface molecules and cytokines. Furthermore, it is thought that Tfh cell generation is a multi-step process (Fig. 1), with the initial activation signals provided by dendritic cells (DCs) followed by a second stage of signalling that is required for maintenance and/or further differentiation of the cells. This second stage of signalling is thought to be provided largely by B cells.

Cell surface molecules

Numerous molecules, operating at different stages of T cell activation, have been shown to play a role in the generation of Tfh cells (Fig. 1). For example, initial activation of $CD4^+$ T cells by DCs is dependent on CD28 and CD40L. B7.1 (CD80) and B7.2 (CD86) expressed by the DC binding to CD28 is known to provide an important co-stimulatory signal for the activation of $CD4^+$ T cells[28] and CD40L expressed by the T cell is known to activate DCs via CD40, allowing the DCs to support ongoing T cell activation.[29] The importance of these molecules in generating T cell help for B cells is demonstrated by the findings that the absence of CD40 expression on DCs[30] or blocking signalling through CD28[31] inhibited up-regulation of CXCR5 and homing to the follicle. Furthermore, mice deficient in CD28 or CD40L or patients with mutations in *CD40LG* show decreased numbers of Tfh cells.[26,32] OX40–OX40L interactions between $CD4^+$ T cells and DC also seem to be important for the up-regulation of CXCR5 and homing of $CD4^+$ T cells to the follicle,[30,31,33,34] although the requirement for OX40 signalling may also depend upon mouse strain and the immunization protocol.[32]

Following appropriate activation by DCs, $CD4^+$ T cells up-regulate CXCR5 and move towards the follicle, where they encounter B cells and can receive a second round of activation signals. The importance of B cells in generating or maintaining Tfh cells is demonstrated by the lack of Tfh cells when B cells are absent or their interactions with $CD4^+$ T cells are disrupted.[5,9,16,35,36] Once again, a range of cell surface receptors interactions play an important role at this stage. As for DC–T interactions, CD40–CD40L are also important for T–B interactions, as a lack of CD40 expression on B cell prevents activation of B

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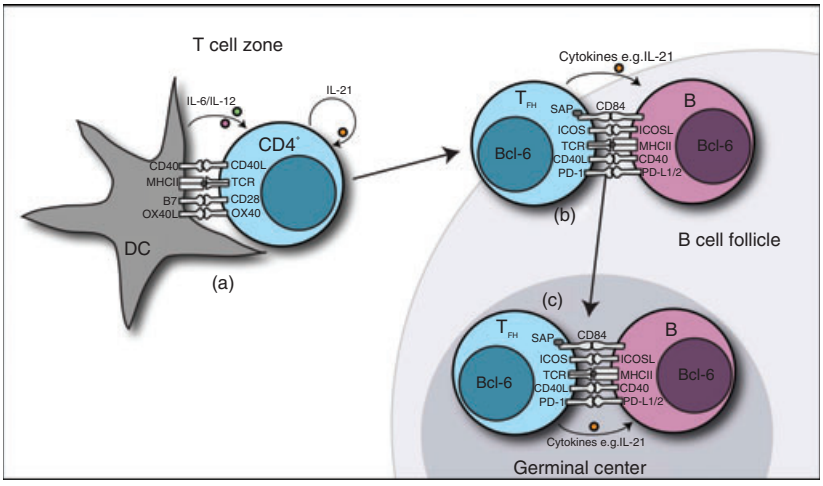

Figure 1. (a) Naive CD4$^+$ T cells are activated when they encounter their antigen presented on dendritic cells (DCs) within the T cell zone. T cell receptor (TCR) signals, together with co-stimulation provided by CD28–B7, OX40–OX40L interactions and cytokines, are able to initiate a programme of differentiation associated with T follicular helper (Tfh) cells including up-regulation of CD40L, CXCR5, PD-1, Bcl-6 and interleukin (IL)-21, as well as the down-regulation of CCR7 and CD127. CD40L stimulation activates DCs, further supporting the activation of the T cells. Activated T cells then move towards the follicle, where they encounter B cells. (b) Cognate B cells will also present antigen and provide co-stimulatory signals allowing ongoing signalling to the T cells which maintains the Tfh cell phenotype. At the same time, Tfh cells provide help to B cells in the form of CD40L stimulation and secreted cytokines such as IL-21. (c) These signals allow the formation of germinal centres (GCs), maintained by the cross-talk between Tfh cells and B cells involving a range of co-stimulatory molecules and cytokines as shown.

cells by T cells which, in turn, results in decreased Tfh cell numbers.[15] In contrast, while CD28 seems to be important at the initial stages of CD4$^+$ T cell activation it does not seem to be as crucial for Tfh cell development at the later stages of T–B interactions.[37,38] A recent study, however, reported that B7.2 expression on B cells was required for GC formation, suggesting the B7–CD28 interactions between T–B cells are important for the function of Tfh cells and the delivery of helper signals to the B cells.[39] For the most part, however, another CD28 family member, namely ICOS, seems to be required at this later stage. Consequently, mice in which ICOS–ICOSL interactions are disrupted, or patients with mutations in *ICOS* (which results in common variable immunodeficiency), have decreased Tfh cells.[26,32,40,41] ICOSL is expressed widely on haematopoietic cells; however, mice that lack ICOSL expression on their B cells show decreased numbers of Tfh cells indicating that, at least in part, this ICOS–ICOSL signal is delivered by B cells.[42] This requirement for ICOS signalling seems to depend on its ability to activate phosphoinositide-3-kinase (PI3K), as mice expressing a mutant ICOS molecule with defective PI3K activation[41] or lacking the p110δ isoform of PI3K in T cells[43] also show decreased Tfh cell generation. Several studies have demonstrated that ICOS signalling, via PI3K, is able to up-regulate Tfh cell-associated genes such as c-maf, IL-4 and IL-21;[40,41,43] however, it remains to be determined whether the primary role of ICOS signalling is to induce the differentiation of Tfh cells or simply to maintain those that have already formed.

It has also become clear that the SLAM family of surface receptors play an important role in Tfh cell generation. The importance of these molecules in T–B interactions first came to light in patients suffering from the immunodeficiency X-linked lymphoproliferative disease (XLP). XLP is caused by mutations in the gene encoding SAP (i.e. *SH2D1A*), which is a cytoplasmic adaptor molecule that signals downstream of the SLAM family of receptors. Patients with XLP, as well as gene-targeted mice that lack SAP expression, display a deficiency in T-dependent B cell responses.[44,45] Furthermore, several groups have demonstrated that loss of SAP can result in decreased numbers of Tfh cells.[9,20,46,47] Members of the SLAM family including SLAM itself, CD84 and NTBA (also known as Ly108 in the mouse) are expressed highly on both activated B cells and activated CD4$^+$ T cells, including Tfh cells.[8,9,11,20,47–50] As these receptors are homotypic receptors, this expression pattern allows for SLAM family interactions between T and B cells. Several recent studies have demonstrated that SAP acting downstream of CD84, and possibly Ly108, is required to regulate adhesion between T and B cells. Thus, in the absence of SAP or CD84, CD4$^+$ T cells are unable to form stable conjugates with B cells and cannot deliver help to B cells.[47,51] In addition, this prevents the CD4$^+$ T cells from receiving signals from B cells that regulate the formation or maintenance of Tfh cells.

While it is thought that Tfh cell development is a multi-step process with initial activation on DCs, followed by secondary signals provided by B cells, several

recent findings have challenged this view. Many reports have demonstrated that Tfh cell numbers are decreased in the absence of B cells or when T–B cell interactions are disrupted.[5,9,16,35,36] However, we recently showed that in the absence of antigen presentation by B cells, Tfh cell development (as indicated by surface phenotype and GC localization) could at least partially be rescued in the presence of abundant antigen, which prolonged presentation by DCs.[9] Consistent with this, a recent study found that Tfh cells also developed in B cell-deficient mice in response to chronic viral infection.[52] This suggests that the requirement for B cells results not from a unique signal that B cells provide, but because Tfh cells need prolonged antigen stimulation and B cells often quickly become the only cells capable of presenting antigen to the T cells.[9] A requirement for prolonged antigen presentation is consistent with data indicating a crucial role of TCR signalling in Tfh cell development. For example, many of the features of Tfh cells, such as up-regulation of CXCR5 and PD-1 and down-regulation of CD127, are observed in T cells following TCR stimulation.[3,6,53,54] Moreover, it has been shown that high-affinity T cells are preferentially selected to become Tfh cells.[55] The restriction of antigen presentation to the B cells presumably occurs ordinarily because, first, the B cell receptor allows for efficient uptake of antigen and secondly, as the T cells move into the B cell follicle and then the GC, these are the antigen-presenting cells (APCs) which the T cells encounter.

Furthermore, several new papers support the idea that early activation on DCs is able to drive differentiation of Tfh cells. They demonstrated that $CD4^+$ T cells with a Tfh cell phenotype – high CXCR5, PD1, IL-21 and Bcl-6 expression – could be identified early on in the response (e.g. day 3)[21–23] in the interfollicular zone or outer follicle.[21,22] This early appearance of Tfh-like cells was independent of B cells;[21,23] however, the continued maintenance of these cells was disrupted in the absence of B cells.[21–23] This suggests that a role of the second round of signalling, usually provided by B cells, may be to maintain a Tfh cell phenotype or the survival of Tfh cells rather than to drive unique differentiation events.

Cytokines

Generation of the different Th lineages is associated with the action of particular cytokines.[12,14,15] Consequently, researchers have sought for cytokines that regulate Tfh cell generation. Several studies in mice have suggested that not only did Tfh cells produce IL-21, but IL-21 could also drive IL-21 production and Tfh cell differentiation.[8,42,56,57] Subsequent studies, however, showed that disruption of IL-21 signals had little or no effect on Tfh cell development.[35,58–62] IL-6 has also been shown to induce IL-21 production and Tfh cell generation.[42,57,63] However, once again, while some studies have shown a decrease in Tfh cell generation in the absence of IL-6,[42] others have failed to see any defect in the absence of IL-6.[35,62,63] These discrepancies probably reflect a level of redundancy in the signals required for generating Tfh cells. Indeed, both IL-6 and IL-21 signal through signal transducer and activator of transcription 3 (STAT3) and a recent paper by Eto *et al.*[62] demonstrated that, although the absence of only one of these cytokines did not affect Tfh cell numbers, the combined absence of IL-6 and IL-21 did result in a significant decrease. This decrease, however, was not complete, and a substantial number of Tfh cells could still be found. In this light it is interesting to note that a recent study demonstrated that another STAT3-activating cytokine, IL-27, can also increase IL-21 production and Tfh cell generation.[64] Thus, the ability of all three of these cytokines to activate STAT3 contributes to a high level of redundancy in their requirement for Tfh cell generation. However, $CD4^+$ $CXCR5^+$ cells can still be identified even in the absence of STAT3 itself, suggesting that it may not be absolutely required for the generation of Tfh cells,[42,63] indicating that an even greater level of redundancy exists. However, in the absence of STAT3 these $CD4^+$ cells were very poor at supporting B cell responses, suggesting that STAT3 may be more important for the functional ability of Tfh cells even if it is not required for the cell surface expression of Tfh-associated molecules.

The role of cytokines in inducing B cell helper function from naive human $CD4^+$ T cells differs from that of mice in that it has been shown to largely involve IL-12, and to a lesser extent IL-6, IL-21 and IL-23.[8,65] IL-12 induced the differentiation of cells expressing IL-21, CXCR5, ICOS and Bcl-6 that facilitated antibody production by B cells *in vitro*.[8,65] Interestingly, several studies have found little effect of IL-12 on the production of IL-21 by murine $CD4^+$ T cells,[42,57,66] although another paper observed a significant proportion of IL-21 secreting cells in response to IL-12.[62] These results suggest that different cytokine pathways may be involved in the generation of human versus murine Tfh cells.

Function of Tfh cells

The key function of Tfh cells is to provide help to B cells to support their activation, expansion and differentiation and the formation of the GC. Several features of Tfh cells enable them to carry out these functions. Possibly the best-characterized B cell helper signal provided by Tfh cells is CD40L, which is highly expressed on these cells. CD40L is a potent activator of B cells and is able to induce proliferation and, in combination with cytokines, isotype switching and differentiation of B cells.[29,67,68] The importance of this molecule for B cell responses is demonstrated by mice lacking CD40 or CD40L, which display

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abortive B cell responses and a failure to generate GCs and long-term memory.[29,69–71] Similarly, in humans, mutations in *CD40LG* or *CD40* result in the primary immunodeficiency hyper-immunoglobulin M syndrome, which is characterized by recurrent bacterial infections, an inability to respond to vaccinations and a lack of serum IgG, IgA and IgE.[72]

Although PD-1 is highly expressed on Tfh cells, little is known about the role of PD-1 in Tfh cell development or function. The ligands for PD-1, namely PD-L1 and PD-L2, are expressed on multiple cells including B cells. Studies in mice deficient in PD-1 or its ligands PD-L1 and PD-L2 suggest that these may regulate GC cells and long-lived plasma cells either positively[73,74] or negatively.[75] It is likely, however, that this is not a direct effect of signalling to the B cell but, rather, reflects a role of B cell expressed PD-L1 and/or PD-L2 in regulating the number and function of the Tfh cells via PD-1, as all three papers reported increased numbers of Tfh cells when PD-1/PD-L1 interactions were ablated.[73–75]

Another important mechanism by which Tfh cells regulate B cell responses is through the secretion of cytokines. Tfh cells are characterized by expression of IL-21, a cytokine capable of modulating B cell differentiation and proliferation.[76–78] Addition of IL-21 to CD40L-stimulated human B cells is able to induce switching to IgG and IgE and the formation of antibody-secreting cells.[76,77] In addition, it has been demonstrated that ablation of IL-21:IL-21R signalling *in vivo* in mice can affect multiple aspects of the B cell response, including formation of GCs, antibody production and the generation and/or function of memory B cells.[59,60,62,78–80] The nature and severity of these effects varied widely, however, depending on the immunization or infectious challenge used. This suggests that, as for the generation of Tfh cells, there may be other signals that can compensate for IL-21 under certain circumstances. None the less, it is clear that IL-21 produced by Tfh cells is able to modulate B cell responses.

While IL-21 is the cytokine associated primarily with Tfh cells, there have been increasing reports of Tfh cells producing other cytokines, including IL-4,[8,20,25,36,81,82] IL-10,[1,8] IL-17[25,40,83,84] and IFN-γ.[16,20,25,40,81] This is consistent with the ability of these cytokines to modulate B cell behaviour such as isotype switching and antibody production.[85–89] This raises questions, however, about the status of Tfh cells as a distinct lineage. Indeed, murine studies have demonstrated that Th2 cells can develop into Tfh cells *in vivo* following infection with the Th2-polarizing pathogens helminths[36,82] and Leishmania.[81] Similarly, murine regulatory T cells (Tregs) transferred into T cell-deficient hosts lost forkhead box P3 (Foxp3) expression acquired Tfh cell characteristics.[90] Furthermore, in the scenario of Th2 cells for example, they maintained IL-4 secretion and *gata3* expression while gaining attributes of Tfh cells (CXCR5, Bcl-6, IL-21 expression). This suggests Tfh cells may not represent a discrete lineage, but a state of differentiation that can be superimposed onto other Th subsets when B cell helper activity is required. This is supported by human studies, wherein the $CD4^+$ $CXCR5^+$ fraction could be subdivided into $CXCR3^+$ Th1-like, $CCR6^+$ Th17-like and $CXCR3^-$ $CCR6^-$ Th2-like Tfh cells.[25] Th2- and Th17-like Tfh cells secreted IL-21 and could subsequently induce antibody production by naive B cells, while Th1-like Tfh cells did not express IL-21, nor could they support antibody production by B cells. Consistently, Th17- and Th2-like, but not Th1-like, Tfh cells were found to be elevated in juvenile dermatomyositis, a chronic multi-systemic autoimmune condition.[25]

The field of Tfh cells has evolved at an extremely rapid pace, which has helped to improve our understanding of this cell type. However, as it stands currently, it appears that multiple varieties of Tfh cells exist. Thus, one of the interesting areas of future endeavour will be to determine whether Tfh cells are a discrete lineage or a state of activation of Th cell lineages when B cell helper function is required. Dysregulation of these cells underpins numerous human disorders, therefore, addressing this question will facilitate our ability to intervene in these diseases by altering the development and/or function of Tfh cells.

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