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REVIEW

Control of TFH cell numbers: why and how?

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T follicular helper (TFH) cells are essential for formation of germinal centres (GCs) and selection of mutated GC B cells. It has become clear over the last decade that precise control of TFH cell numbers is important to produce optimally affinity-matured antibody responses that are devoid of self-reactivity. Indeed, limiting the number of TFH cells appears important to impose competition amongst B-cell clones and set a selection threshold that will favour survival of high affinity clones. In contrast, excessive number of TFH cells appears to lower the selection threshold and allow survival of low affinity or self-reactive clones. Here, we review the cell-intrinsic and cell-extrinsic mechanisms that influence TFH cell homeostasis, including recent insights into novel modes of regulation by T-cell costimulators, toxic metabolites, microRNAs, RNA-binding proteins and regulatory cells.
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$CD4^+$ T cells have a crucial role in helping B cells produce antibodies in response to microbes. The interaction between these cell types typically occurs in germinal centres (GCs), which are histologically distinct structures that develop within B-cell follicles of secondary lymphoid tissues. In recent years, T follicular helper (TFH) cells have emerged as the key cell type required for the formation of GCs and the generation of long-lasting antibody responses.[1,2] TFH cells are able to localise to the B-cell follicle and to the GC[3–5] to provide essential help for B-cell affinity maturation, class switch recombination and memory B cell and plasma cell generation within the GC.[1,3–5] These features make the GC essential for long-lived protective immunity against many pathogens.

TFH cells upregulate chemokine (C-X-C motif) receptor 5 (CXCR5) and downregulate chemokine (C-C motif) receptor 7 (CCR7), which facilitate migration towards B-cell follicles.[5–7] Many molecules including programmed death-1 (PD-1), inducible costimulator (ICOS), signalling lymphocyte activation molecule (SLAM) family of receptors and IL-21 are also highly expressed by TFH cells[1] and have been used for the phenotypic identification of TFH cells. Five years ago, we and others identified BCL-6 as the key transcriptional regulator signalling in T cells to programme TFH cell differentiation and initiate GC reactions.[8–10] BCL-6 deficiency in $CD4^+$ T cells causes failure in the generation of TFH cells and GC responses *in vivo*, whereas ectopic expression of BCL-6 drives TFH cell development.[8–10] These studies supported the notion that TFH cells are a unique effector T-cell subset. $CXCR5^{hi}$ $PD\text{-}1^{hi}$ $BCL\text{-}6^+$ TFH cells have been shown to localise to the GCs and are thus the most terminally differentiated TFH cells.[1,11] The precursors of GC TFH cells—or pre-TFH cells—express lower amounts of these proteins and are typically located at the T:B border where they prime B cells and trigger their proliferation and differentiation along the follicular or extrafollicular pathway.[12] These GC TFH cell precursors can also recirculate to colonise other GCs.[13]

The GC can be divided into two anatomically distinct areas called the dark zone and the light zone. The dark zone is densely packed with proliferating B cells (or centroblasts; defined as $CXCR4^+$ $CD83^-$ $CD86^-$) in which somatic hypermutation is targeted at their *immunoglobulin V* region genes, whereas in the light zone, centrocytes (defined as $CXCR4^-$ $CD83^+$ $CD86^+$), which have exited cell cycle, interact with follicular dendritic cells (DCs) and TFH cells. Cognate interactions between antigen-specific TFH cells and antigen-specific GC B cells are critical to provide B cells with signals for survival and differentiation. This is in part mediated by T-cell receptor–major histocompatibility complex class II engagement and TFH cell-expressed molecules including CD40 ligand (CD40L) and IL-21.[1,14] Selected B-cell clones differentiate into long-lived plasma cells and memory B cells, but a proportion migrate back to the dark zone to undergo further rounds of proliferation and somatic hypermutation.[15,16]

Other $CD4^+$ T-cell subsets with TFH cell-like characteristics have also been recently discovered. Extrafollicular T helper cells, which share developmental mechanisms, phenotypes and functional properties with TFH cells[12,17–19] have been described in humans and lupus-prone MRL/Fas^{lpr} mice and typically express PSGL-1.[18] These cells appear to provide help to plasmablasts at extrafollicular sites.[12] Circulating $CD4^+$ $CXCR5^+$ TFH-like cells are also found in the blood and amongst these cells, those expressing low levels of CCR7 and higher PD-1 represent the best surrogate of GC TFH cell numbers and GC function in humoral immunity-related diseases.[13,20] In addition, we and others have shown invariant natural killer T (iNKT) cells can upregulate BCL-6 and turn on the follicular

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differentiation programme to become iNKTFH cells and migrate into the GC. These iNKTFH cells support a distinct type of thymus-dependent antibody response (designated TD-2) to lipid antigens.[21,22] Also, after protein immunisation or infection, regulatory T cells can co-opt the TFH differentiation pathway and become T follicular regulatory (TFR) cells. TFR cells migrate into the GC where they exert a suppressive function over TFH and GC B cells and limit the response.[23–25]

Understanding TFH cell differentiation and function is of central importance for rational vaccine design, as most vaccines rely on long-lived high affinity T-cell-dependent antibody responses. It is becoming clear that improving vaccine efficiency will not be exclusively a matter of maximising the number of TFH cells, in fact, too many TFH cells may dampen, or even corrupt affinity maturation. Restricting TFH cell numbers in GCs is crucial for optimal selection. Rather, it is likely that an appropriate balance between TFH and TFR cells at the different stages of the GC response will prove critical for optimal induction, function and termination of GCs for effective protection against infection whilst preventing autoimmunity. In this review, we delve into the importance of controlling TFH cell numbers for optimal GC reactions and provide an overview of the T cell-intrinsic and T cell-extrinsic molecular mechanisms regulating the dynamic process of TFH cell homeostasis.

WHY TFH CELL NUMBERS MATTER

Since the early 90s, mathematical models incorporating observations made in mouse and human regarding the GC B-cell cycling rates, compartmentalisation into dark zones and light zones, rates of somatic mutation and clonal diversification, and ongoing B-cell selection and exit from GCs proposed that GC T cells had to be the limiting factor for optimal affinity maturation.[15,26] These models predicted that GC T cells would not only provide signals for B-cell differentiation, but would also induce some B cells found in the light zone to re-enter cell cycle and migrate back to the dark zone to undergo further rounds of somatic mutation and proliferation. Experimental evidence described below (summarised in Figure 1) has shown these predictions to be largely true.

Lack of TFH cells: abortive GCs and immunodeficiency

In the context of protein antigens, T cells—and TFH cells specifically—are essential for the induction of GCs.[8–10,15] In exceptional circumstances, GCs can also form in the absence of T-cell help: in response to a T-independent antigen, 4-hydroxy-3-nitrophenylacetyl-Ficoll, large GCs can form in the absence of T cells, or B-cell-helping molecules CD40L and CD28.[27] However, these GCs synchronously abort at a point when the first wave of centrocytes is predicted to require selection signals from T cells. This suggested that T-cell signals are not only required for centrocyte selection[27] but also for the recycling of a defined number of centrocytes to renew the pool of dividing cells (Figure 1) and, therefore, maintain the GC reaction.

Not surprisingly, genetic mutations causing reduced TFH cell numbers lead to primary immunodeficiency syndrome. Given the difficulty of accessing and analysing secondary lymphoid organs in human patients, the frequency of circulating $CD4^+CXCR5^+$ T cells and antibody titres in the serum are increasingly used in human studies as biomarkers of GC activity and disease severity. Patients with CD40L-deficient hyper-IgM syndrome,[28] ICOS-deficient CVID,[29] STAT3-deficient hyper-IgE syndrome[30] or IL-12Rβ1 loss-of-function mutation[31] display obvious defects in humoral immune responses, including reductions in the numbers of circulating $CD4^+CXCR5^+$ T cells, memory B cells and serum antibody levels. Also, X-linked lymphoproliferative syndrome patients due to mutations in the gene encoding SLAM-associated protein (SAP), *SH2D1A*, which is important for sustained T:B interactions and GC TFH cell formation,[32] lack TFH cells and GCs.[1,2] Taken together, these analyses of monogenic immunodeficiencies demonstrate that mutations that severely reduce TFH cell numbers substantially dampen GC reactions.

Limiting TFH cell numbers are essential for optimal GC reaction

Recent studies have provided experimental evidence backing up the theories that T cells are present in limiting amount in the GCs and this is essential for optimal affinity maturation. Increasing the number of antigen-specific T cells in an adoptive transfer system resulted in an increased proliferation and activation of antigen-specific B cells at the early stage of the GC reaction, suggesting that T-cell help is indeed a limiting factor.[33] Also, B cells expressing the highest affinity receptors after somatic hypermutation can capture more antigens and, therefore, present increased peptide concentrations to cognate T cells. This will provide high affinity B cells a competitive advantage in establishing sustained interactions and eliciting survival signals (Figure 1b). This was clearly demonstrated in the recent work of Nussenzweig and colleagues using targeted antigen delivery through DEC205, which is a surface lectin that efficiently delivers antigen to the MHC II processing compartment. The subset of DEC205-expressing antigen-specific B cells that took up the antigen engaged in longer-lasting motile T:B cell interactions indicative of increased T-cell help, and as a consequence became more activated and proliferative.[16,33]

Excessive TFH cell numbers are associated with autoimmunity

Owing to the random nature of the somatic hypermutation of B-cell receptors in GCs, B cells can acquire self-reactivity in the GCs. The suggestion that excessive TFH cell accumulation or overactivity may corrupt positive selection, allowing survival of self-reactive B cells (depicted in Figure 1), first came from the analysis of lupus-prone Roquin mutant (*sanroque*) mice.[34] The 'san' mutation in the RNA-binding protein, Roquin, leads to impaired mRNA decay and cell-intrinsic accumulation of molecules including ICOS, IFN-γ, OX40 and TNF.[34–37] Overexpression of these proteins results in excessive TFH cell accumulation, spontaneous B-cell activation and GC formation, autoantibody production and immune-complex-mediated glomerulonephritis.[34] These mice also develop early onset and/or more severe autoimmune diabetes and arthritis in genetic-susceptible backgrounds.[36,38] Overactive TFH cells were shown to contribute to the systemic lupus erythematosus-like phenotype in *sanroque* mice.[39] Similar to *sanroque* mice, other autoimmune mouse models, BXSB-Yaa and MRL/Fas^{lpr} mice, show excessive TFH and/or extrafollicular helper T-cell formation contributing to autoantibody-mediated diseases.[18,40] Taken together, these studies show that TFH cells are crucial to the pathogenesis of lupus in mice.

Several studies have reported increases in circulating $CD4^+$ $CXCR5^+$ T cells that correlate with autoantibody titres in patients with systemic lupus erythematosus,[13,41] Sjögren's syndrome,[42] myasthenia gravis,[43] rheumatoid arthritis,[13,44] juvenile dermatomyositis[20] and autoimmune thyroid diseases.[45] In multiple sclerosis and experimental autoimmune encephalomyelitis, self-reactive T cells—predominantly TH17 cells—have been shown to acquire a GC TFH phenotype and give rise to ectopic lymphoid tissues containing GCs.[46] These studies suggest that TFH cell overactivity contributes to the pathogenesis of autoantibody-

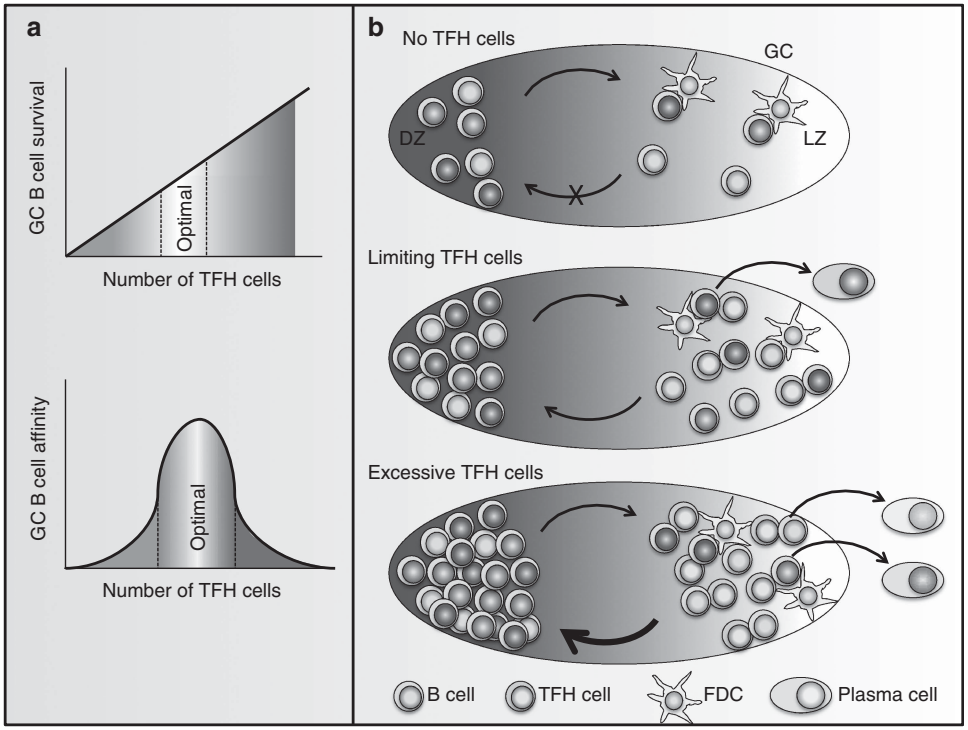

Figure 1 Limiting TFH cell number is important for optimal germinal centre (GC) reactions. (**a**) Schematic diagrams illustrating the predicted effect of TFH cell numbers on GC B-cell survival (top) and affinity maturation (bottom). The lack or low amount of TFH cells diminishes GC B cell recycling, GC lifespan and also impairs their affinity maturation. This may lead to primary immunodeficiency syndromes. On the other hand, excessive TFH cells drives aberrant GC B-cell survival possibly due to lowered threshold for GC B-cell selection. This may lead to survival of low affinity or even self-reactive B cells and eventually autoimmunity. Limiting TFH cell number promotes competition amongst developing GC B cells on the basis of their affinity for antigen, thus leading to emergence of high-affinity antibody-secreting cells that are important for fighting infection. (**b**) Schematic diagrams illustrating GC reactions when TFH cells are absent (top), present in limiting number (middle) or present in excess (bottom). TFH cell-derived signals are required for both B-cell selection and recycling of centrocytes to maintain the GC reaction. When TFH cells are lacking, GCs can still form in response to a T-cell-independent antigens (for example, NP-Ficoll), but rapidly abort at the time when T-cell signals are required for centrocyte recycling to renew the pool of dividing cells in the dark zone. In the presence of limiting TFH cells, GC B cells with high affinity receptors are able to capture more antigen from follicular dendritic cells (FDCs) and present them at increased concentrations to TFH cells, thus establishing sustained interactions and eliciting survival signals. This gives rise to high-affinity plasma cells. However, when excessive TFH cells are present, there is less competition amongst developing B cells for T cell help and hence those with low affinity or even self-reactive receptors are able to survive and eventually differentiate to plasma cells. Increasing colour intensity of B cells and plasma cells represent increasing B-cell receptors' affinity. TFH, T follicular helper; GC, germinal centre; FDC, follicular dendritic cell; DZ, dark zone; LZ, light zone. A full colour version of this figure is available at the *Immunology and Cell Biology* journal online.

mediated autoimmune diseases in at least a subset of patients, potentially by allowing survival of self-reactive B cells.

Paradoxical expansion of TFH cells in patients with chronic HIV infection has also been suggested to be responsible for the dysgammaglobulinemia, defective T-dependent responses[47,48] and even potentially the autoimmune phenomena found in these patients.[49] Despite the ability of HIV to induce the death of virus-specific $CD4^+$ T cells, chronic HIV infection appears to promote expansion of the TFH compartment (about 10-fold increase in TFH cell frequency), particularly in HIV-specific TFH cells.[47] Similarly, during chronic simian immunodeficiency virus infection, there is an eightfold increase in TFH cell frequency[48] and approximately a fourfold increase in TFH cell number.[50] In these studies, the increases in TFH cells were accompanied by parallel increases in GC B cells, plasma cells and serum IgG concentrations, but reduced memory B cells. Given the numerous studies reporting impaired responses of HIV-infected individuals to vaccination with protein antigens, it is likely that the lowered threshold for GC B-cell selection imposed by excessive TFH cells no longer favours selection of antigen-specific affinity-matured B cells.

MULTISTAGE PROCESS OF TFH CELL DIFFERENTIATION

The control of TFH cell numbers is achieved by key checkpoints during their formation, migration, expansion and survival. There has recently been an outburst of studies reporting multiple molecules and pathways important for the control of TFH cell formation or homeostasis (summarised in Figure 2). TFH cell development has been described elsewhere.[1,2] In brief, TFH cell differentiation requires multiple signals including TCR signalling, costimulation, motility/guidance signals and cytokines (Figure 2). These signals collectively induce the expression of transcription factors, microRNAs (miRNAs) and other regulatory proteins that activate a TFH lineage-specific differentiation programme. DCs and their cytokine products (IL-12 and IL-6) are crucial for $CD4^+$ T-cell priming and initial acquisition of TFH cell characteristics, including the induction of BCL-6 expression.[51] Antigen-primed $CD4^+$ T cells, in the T-cell zone, reduce CCR7 and upregulate CXCR5 expression, which direct them to the T:B border.[6,7] Antigen-specific $CD4^+$ T cells and cognate B cells can then stably interact, forming mobile conjugate pairs. Interaction with antigen-presenting B cells in a SAP-dependent manner stimulates a second wave of BCL-6 induction, which leads

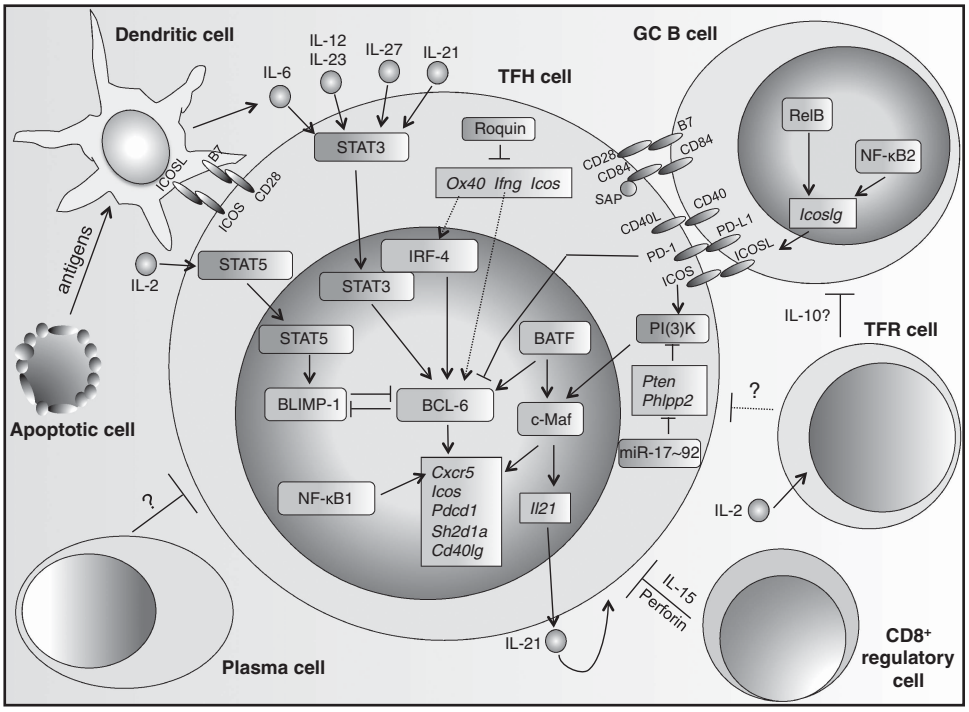

Figure 2 Molecular mechanisms regulating TFH cell differentiation. Cell-intrinsic mechanisms include a set of transcription factors (highlighted in brown), signalling intermediates,[80] and RNA-binding proteins and microRNAs (green), whereas cell-extrinsic mechanisms include cytokines (red), cell−cell signalling molecules (grey) and regulatory cells. Dendritic cells (DCs) are crucial for CD4$^+$ T-cell priming and initial acquisition of TFH cell characteristics, including the induction of BCL-6 expression, which downregulate chemokine (C-C motif) receptor 7 (CCR7) and upregulate CXCR5 expression, preparing for recruitment to the B-cell follicle. Antigen-specific CD4$^+$ T cells and cognate B cells can then stably interact, forming mobile conjugate pairs leading to T-cell migration towards the follicle. DCs also secrete cytokines including IL-6, IL-12 and IL-27, which induce phosphorylation and activation of STAT3. STAT3 can then migrate to the nucleus and contribute to the upregulation of BCL-6, the transcriptional driver of TFH cells. BCL-6 expression induces a module of mRNA expression critical for T:B cell interaction including *Cxcr5*, *Icos*, *Pdcd1*, *Sh2d1a* and *Cd40lg*. Other transcription factors IRF4, BATF and c-Maf also cooperate with BCL-6 to upregulate the expression of these TFH cell signature genes as well as *Il21* expression. Autocrine signalling by IL-21 has been shown to induce STAT3 and drive TFH cell development. In addition, members of the NF-κB family of transcription factors have also been shown to upregulate *Cxcr5* expression. In contrast, IL-2, which signals through STAT5, represses TFH cell differentiation by upregulating BLIMP-1, a transcription factor that is mutually antagonistic to BCL-6. IL-2 is also important for the development of TFR cell, which inhibits TFH cell differentiation. Post-transcriptional regulation by RNA-binding protein Roquin represses a number of mRNAs, such as *Icos*, *Ox40,* and *Ifng*, to limit TFH cell formation. Further, the microRNA-17−92 cluster has been recently shown to be required for TFH cell development by post-transcriptional repression of *Pten*, *Phlpp2* and *Rora* (not depicted) mRNAs. Both *Pten* and *Phlpp2* are phosphatases that inhibit the PI(3)K signalling pathway downstream of ICOS. Cell–cell interactions of developing TFH cells with antigen-presenting cells (DCs and B cells) also promote TFH cell formation. ICOS−ICOSL, CD40L–CD40 and CD28–B7 interactions are central to this process. Notably, ICOSL expression on B cells is partly controlled by NF-κB transcription factors. Members of the SLAM family of receptors, particularly CD84, are also important for stable T:B cell interactions, which also contribute to TFH cell differentiation through SAP-mediated signalling. Other cells in the GC have also been recently shown to influence TFH cell differentiation. Regulatory cells, such as TFR, CD8$^+$ regulatory and plasma cells limit TFH cell formation, whereas impaired clearance of apoptotic cells in the GC promotes TFH cell formation, presumably by providing additional amount of antigens to the DCs, which are then presented to the developing TFH cells. TFR, T follicular regulatory; IL, interleukin; STAT, signal transducer and activator of transcription; IRF, interferon regulatory factor; PI(3)K, phosphoinositide-3 kinase; ICOSL, inducible costimulator ligand; PD-L1, programmed cell death 1 ligand 1; CD40L, CD40 ligand. A full color version of this figure is available at the *Immunology and Cell Biology* journal online.

to a stable CXCR5 expression,[51] migration inside the follicle and differentiation into GC TFH cells. Non-cognate B-cell interactions providing ICOS signals stimulate TFH cell persistent motility that aids follicular migration.[52] Thus, GC B cells and TFH cells are reciprocally dependent on each other for survival, proliferation and differentiation.

CELL-INTRINSIC MECHANISMS CONTROLLING TFH CELL NUMBERS

Transcriptional regulation

BCL-6. This transcriptional repressor is recognised as a key transcriptional driver of TFH cell differentiation.[1] As mentioned above, BCL-6 expression is induced in two waves:[51] after priming by DCs and during interaction with cognate B cells. In humans, DC-derived IL-12 is a potent inducer of BCL-6.[53,54] Expression of *Bcl6* transcript can be upregulated by IL-6 or by IL-21 in an autocrine manner.[9] T cell-intrinsic activity of BCL-6 is required for TFH cell development and T-cell-dependent GC responses[8–10] as well as for extrafollicular antibody responses.[12,19] The introduction of BCL-6 through lentiviral overexpression in CD4$^+$ T cells resulted in CXCR5 upregulation as well as CCR7 and EBI2 downregulation,[55] all required to promote T-cell migration to B-cell follicles. BCL-6 expression also induced a module of protein expression critical for T:B interactions, including SAP, CD40L, PD-1, ICOS and CXCL13.[55] These findings firmly established a critical role for BCL-6 in coordinating TFH cell formation.

BLIMP-1. This transcription factor limits TFH cell formation by directly suppressing BCL-6.[8,56] Deletion of BLIMP-1 from CD4$^+$ T cells enhances TFH cell formation in response to viral infection. In contrast, BLIMP-1 overexpression severely blunts TFH cell development and attenuates GC reactions and antibody responses.[8] BLIMP-1 is induced by IL-2 in CD4$^+$ T cells and hence, repeated administration of IL-2 reduced TFH cell formation and antibody production in an influenza virus infection model.[57] IL-2 signals through STAT5 to limit TFH cell differentiation.[58,59] Thus, the IL-2-STAT5-BLIMP-1 axis is likely to limit TFH cell generation by suppressing BCL-6.

c-Maf. Induction of the transcription factor c-Maf is required for the formation of optimal numbers of TFH cells after immunisation.[60] In a recent study, mice lacking c-Maf specifically in immune cells exhibited defective TFH cell differentiation *in vivo*.[60] The c-Maf expression can induce ICOS-dependent IL-21 production, which is important for TFH cell formation, *in vivo*[60] and *in vitro*,[55] and this is through direct binding to the *Il21* promoter.[61] Furthermore, c-Maf overexpression upregulates other BCL-6-regulated TFH cell-inducing molecules, such as CXCR5, ICOS and PD-1.[55] The c-Maf expression itself is induced by IL-6[61] or IL-27.[62]

STATs. Members of this transcription factor family coordinate responses from certain cytokines to either promote (STAT1, STAT3 and STAT4) or dampen (STAT5) TFH cell development. STAT3 deficiency in mice and humans results in a great reduction of TFH and circulating TFH-like cells, respectively.[30,63] Mechanistically, STAT3 is required for IL-21 production by murine and human CD4$^+$ T cells following stimulation with IL-6, IL-21, IL-23 and IL-27.[63–65] Furthermore, STAT3 was shown to cooperate with STAT1 in promoting TFH differentiation, as the reduction of TFH cells in IL-6-deficient mice was recapitulated only when both STAT1 and STAT3 were deleted from CD4$^+$ T cells.[66] STAT4 also appears to contribute to TFH cell development: TFH, GC B cells and IgG2b titres were significantly reduced following immunisation of *Stat4*$^{-/-}$ mice.[67] Taken together, these findings show that STAT1, STAT3 and STAT4 positively regulate TFH cell differentiation. In contrast, STAT5 deficiency greatly enhances the TFH gene expression *in vitro*[58,68] and TFH and GC formation *in vivo*, also compromising B-cell tolerance.[58] In addition, STAT5 positively promotes the expression of BLIMP-1,[69] which antagonises BCL-6 expression and in turn inhibits the expression of TFH-associated genes, such as *Cxcr5*, *Maf*, *Batf* and *Il21*.[68] These studies indicate that STAT5 functions to limit TFH cell development and humoral immunity.

IRF4. This transcription factor is required for TFH cell differentiation, as *Irf4*$^{-/-}$ mice failed to generate TFH cells due to T cell-intrinsic defect in upregulating BCL-6, CXCR5 and ICOS.[70] In CD4$^+$ T cells, IRF4 may cooperate with STAT3[71] to bind to the *Il21* promoter. Given that the phenotype of *Irf4*$^{-/-}$ mice is much more dramatic than that of *Il21*$^{-/-}$ mice, IRF4 may also regulate the expression of other transcription factors required for TFH cell development.

BATF. Deficiency in BATF, a transcription factor first shown to be important for TH17 cell differentiation, also causes a severe defect in TFH cells, GC development and B-cell somatic hypermutation due to a cell-intrinsic requirement for BATF in both T and B cells.[72,73] BATF has been shown to promote the expression of BCL-6 and c-Maf.[72,73] A recent study has reported that BATF–Jun complex cooperates with IRF4 in mouse CD4$^+$ T and B cells;[74] it remains to be determined whether this BATF–Jun–IRF4 interaction contributes to TFH cell differentiation or function.

NF-κB. The NF-κB family of transcription factors consists of five subunits: RelA (p65), RelB, c-Rel, NF-κB1 (p105) and NF-κB2 (p100). In NF-κB1-deficient mice, TFH cell development was defective upon immunisation with protein antigens.[75] NF-κB1 deficiency particularly impaired CXCR5 expression.[75] Also, IL-21 production was reduced in c-Rel-deficient mice, and the development of TFH and GC B cells was consequently inhibited in those mice.[76] Furthermore, c-Rel was shown to directly bind to the *Il21* promoter to regulate its expression.[76]

Post-transcriptional regulation

Roquin family. This family consists of Roquin (*Rc3h1*) and Roquin-2 (*Rc3h2*). Both genes exhibit high sequence homology particularly in their N-termini including the RING, ROQ and CCCH-type zinc finger domains.[36] As described above, s*anroque* mice homozygous for a loss-of-RNA-regulating function mutation in Roquin exhibit TFH cell accumulation, spontaneous formation of GCs and systemic autoimmunity.[34] Naive and activated T cells from *Rc3h1*$^{san/san}$ mice have increased ICOS expression compared with the wild type cells. Mechanistically, Roquin binds to *Icos* 3′UTR via its ROQ domain and represses *Icos* mRNA through post-transcriptional gene regulation.[77] Induction of mRNA decay occurs through removal of the mRNA 5′ cap.[77,78] mRNA degradation through deadenylation has also been recently proposed as an additional mechanism utilised by Roquin to limit mRNA expression, and this is dependent on recognition of a distinct secondary structure, called the constitutive decay element, in the target mRNA 3′UTR.[79]

TFH and GC B cell spontaneous accumulation and ICOS and OX40 overexpression comparable with those of *sanroque* mice were also observed in mice with compound (but not single) deletion of Roquin and Roquin-2 proteins in T cells, suggesting that Roquin-2 can compensate for the RNA-regulating function of Roquin in its absence.[37] Intriguingly, Roquin-2 cannot compensate for the loss of RNA-repressing function of Roquin^{san}, pointing to this mutant acting as a niche-filling allele.[36] The Roquin target OX40 is a potent inducer of NF-κB signalling[37] that also promotes *Irf4*, and it was significantly increased in Roquin-1/2-deficient T cells.[37] In addition, analysis of mice with combined (but not single) deletion of the RING domain in both Roquin and Roquin-2 revealed a modest increase in the frequencies of TFH and GC B cells as well as increased ICOS and IFN-γ expression on CD4$^+$ T cells.[36] Excessive IFN-γ signalling is involved in the pathogenic accumulation of TFH cells and spontaneous GC formation in *Rc3h1*$^{san/san}$ mice.[35]

MicroRNAs. There is accumulating evidence that miRNAs regulate the TFH cell gene expression programme. BCL-6 itself was shown to repress numerous miRNAs, suggesting that this may be important for some aspects of TFH cell function.[10] Deficiency in the miRNA biogenesis factor DGCR8, leading to deficiency in miRNAs, reduced TFH cell differentiation following immunisation with protein antigens.[80] The miRNA cluster 17–92 encoding six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1) appears to be particularly important for TFH cell development.[80,81] Mice lacking miR-17–92 in T cells had a selective reduction in TFH cell formation and defective long-lived antibody responses to viral infection and protein immunisation.[80,81] miR-17–92 act cell-intrinsically to indirectly upregulate essential TFH lineage-inducing molecules: BCL-6, CXCR5 and IL-21.[80,81] Overexpression of miR-17–92 in

T cells caused lupus-like autoimmune symptoms, with spontaneous accumulation of TFH and GC B cells and production of autoantibodies.[80,81] miR-17–92 directly repress *Pten*, *Phlpp2* and *Rora* mRNAs.[80,81] PTEN and PHLPP2 are phosphatases that antagonise PI(3)K signalling pathway, which is the only known pathway downstream of ICOS required for the development of TFH cells and GCs.[82,83] RORα, on the other hand, is a transcription factor for the gene encoding CCR6 and other genes associated with TH17 or TH22 cells.[80] Hence, miR-17−92 cluster promotes TFH cell differentiation directly by activating the PI(3)K pathway and indirectly by repressing non-TFH cell gene-expression programs.

Post-translational regulation

Ubiquitin ligases. A recent study showed that the E3 ligase Cullin-3 could bind to the BTB-ZF domain containing transcription factor BCL-6.[84] T cell-intrinsic TFH cell accumulation and spontaneous GC B cells occurred in aged Cullin-3-deficient mice.[84] As observed with BCL-6 deficiency, Cullin-3 appears to act selectively in TFH cells, as other $CD4^+$ effector subsets appeared unperturbed in Cullin-3-deficient mice.[84] Although ubiquitylation of BCL-6 by Cullin-3 was not formally demonstrated,[84] it is likely that binding of Cullin-3 to BCL-6 in TFH cells results in the repression of BCL-6 or BCL-6 cofactors, and as a consequence, repression of TFH-associated genes.

Toxic metabolites. Mutations in isocitrate dehydrogenase 2 occur in a number of human cancers. The mutations occur at specific arginine residues and result in the acquisition of a novel enzymatic activity that converts 2-oxoglutarate to the oncometabolite D-2-hydroxyglutarate. Interestingly, isocitrate dehydrogenase 2 mutations were identified in ~20–45% of angioimmunoblastic T-cell lymphomas,[85] which is thought to arise from TFH cells;[86] these mutations were not present in other peripheral T-cell lymphomas.[85]

CELL-EXTRINSIC MECHANISMS CONTROLLING TFH CELL NUMBERS

Regulation of TFH cell numbers by cytokines

Multiple cytokines are important for TFH cell generation *in vivo* and *in vitro*. Numerous cytokines appear capable of inducing, enhancing or sustaining TFH cell-like phenotypes including IL-6, IL-21, IL-12, IL-23 and IL-27 (Figure 2). These cytokines act through phosphorylation of STAT1, STAT3 or STAT4 to regulate the TFH cell programme of gene expression.[53,87,88] In contrast, cytokines like IL-2 acting through STAT5 and IL-10 can suppress TFH cell development at least in mice.[57,89] The specific actions of these cytokines in TFH cell and GC biology have been reviewed in detail elsewhere.[90,91]

Regulation of TFH cell numbers by neighbouring cells in the GCs

Antigen-presenting cells (DCs and B cells)

Cognate interactions via multiple cell surface molecules between developing TFH cells and GC B cells or DCs are vital for TFH cell development. Initial priming by DCs (through T-cell receptor–major histocompatibility complex class II interaction and costimulatory molecules) is crucial for upregulation of CXCR5.[6] In addition, many reports have demonstrated that mice lacking B cells or those with B cells deficient in functional molecules (CD19, CD40, MHC II and ICOSL) exhibited decreased numbers of TFH cells after immunisation or infection.[7,8,19] Here we will review the contribution of the CD28, CD40L and SLAM family in controlling TFH cell numbers.

CD28-B7. B7.1 (CD80) and B7.2 (CD86) expressed on DCs bind to CD28 on T cells to provide an important costimulatory signal for T-cell activation and subsequent differentiation into all effector subsets.[11,92] Mice deficient in CD28 show absence of TFH cells and blocking of CD28 inhibited upregulation of CXCR5 and thus migration to the follicle.[1,11] Although CD28 seems to be important at the initial stages of $CD4^+$ T-cell activation, a CD28-homologue CTLA-4 has additional roles in later stages of GC reaction to limit the number of TFH cells.[92]

ICOS-ICOSL. Costimulatory signals through ICOS are required for optimal TFH formation but are not essential for the development of other T-cell subsets.[60] Mice in which ICOS–ICOSL interactions are disrupted, or patients with mutations in ICOS, have decreased TFH cells.[60,63,82,93–95] ICOSL on DCs and B cells have been shown to be important for the early (T-cell priming) and late (T:B interaction at the T:B border) BCL-6 upregulation and TFH cell differentiation, respectively.[56,63] The expression of ICOSL is regulated by members of the NF-κB and B-cell-activating factor receptor (BAFFR) protein families. Expression of BAFFR and NF-κB-inducing kinase (NIK) on B cells is required for the constitutive expression of ICOSL. NIK deficiency on B cells compromises TFH cell induction.[96] The non-canonical NF-κB members, RelB and NF-κB2, have also been shown to bind *Icoslg* promoter and drive its expression.[96]

Several studies have demonstrated that ICOS signals via recruitment of PI(3)K to the tyrosine within its cytoplasmic YFMF motif.[60,82,83] Mice expressing ICOS with mutated PI(3)K-binding residue in its cytoplasmic tail[82] or lacking the p110δ isoform of PI(3)K in T cells[83] show decreased TFH cell numbers. In addition, p110δ is required for ICOS-mediated upregulation of *Il21*, *Il4* and *Maf* mRNAs.[83] These observations are in accordance with ICOS ligation regulating the c-Maf expression, which in turn drives IL-21[60] and IL-4.[97]

Very recently, another study described a novel role for ICOS as a regulator of persistent directional motility and follicular recruitment of activated $CD4^+$ T cells.[52] This role does not involve BCL-6 as an intermediate nor ICOSL-mediated ligation with cognate B cells.[52] Instead, developing TFH cells require ICOSL signals from bystander follicular B cells, which do not present antigen to these T cells but collectively form an ICOS-engaging field that facilitates directional persistent movement.[52] This effect is also likely to be mediated by PI(3)K signalling.

PD-1-PD-L1. PD-1 was initially shown to exert an inhibitory role in TFH cell differentiation, as mice with impaired PD-1 signalling showed increased TFH cell numbers[98–100] due to increased proliferation and reduced apoptosis.[99] These PD-1-deficient TFH cells expressed higher BCL-6[100] but produced lower amounts of B-cell-helping cytokines IL-4 and IL-21.[98] As a result, there was reduced antibody-producing plasma cells in mice lacking PD-1 or its ligands.[98] The specific increase in TFR cells—which lack IL-21 production—in PD-1-deficient mice[101] may explain the previous observations of reduced IL-21 by total $CXCR5^{hi}$ $PD\text{-}1^{hi}$ or $CCR7^{lo}$ $ICOS^{hi}$ cells (that included both TFH and TFR cells).[98,100] Interestingly, the expansion of TFH cells in PD-1-deficient mice also caused alterations of the microbial composition in the gut leading to dysbiosis.[100]

CD40–CD40L. The absence of CD40 expression on DCs inhibited upregulation of CXCR5 and homing of TFH cells to the follicle.[102] CD40−CD40L interactions are also crucial for the B-cell-helping capacity of TFH cells. As TFH cell and GC B cell are mutually

dependent on each other for their survival, the lack of CD40 expression on B cell prevents activation of B cells and GC formation,[102] which in turn, results in decreased TFH cell numbers. Consistent with the requirement of these interactions for TFH homeostasis, circulating $CD4^+$ $CXCR5^+$ T cells are absent in patients with mutations in *CD40LG*,[93] and mice with CD40L deficiency fail to form GCs following immunisation.[103]

SLAM family. This family of receptors includes SLAM (also known as SLAMF1), CD84, natural killer cell receptor 2B4, T lymphocyte surface antigen Ly9 and Ly108. They are homotypic receptors and facilitate prolonged interactions between activated antigen-specific T and B cells via the recruitment of SAP to their cytoplasmic tails for downstream signalling.[32] The importance of SAP on TFH cell development has been established from analysis of SAP-deficient mice and patients suffering from X-linked lymphoproliferative syndrome (discussed above).[32,39,104] Although SAP-deficient mice were capable of generating $CXCR5^+$ $PD\text{-}1^+$ TFH cells at day 4 post-immunisation, these could not be retained in GCs[104] and did not differentiate into GC TFH cells. Analysis of gene-targeted mice has failed to show a requirement for individual SLAM family members, except for CD84, in TFH cell formation.[32] Moreover, the TFH cell deficiency in $Cd84^{-/-}$ mice was less severe than in SAP-deficient mice.[104] *In vitro* experiments have suggested that CD84 and Ly108 additively contribute to T:B cell interaction and the resulting induction of a TFH phenotype.[104] This indicates that the severe effect of SAP deficiency in TFH cells reflects the requirement of signals from multiple SLAM receptors during TFH cell differentiation.

Apoptotic cells

Large numbers of low-affinity and autoreactive B cells are thought to either be actively negatively selected or die by neglect in the GCs. The clearance of apoptotic cells is necessary to maintain immune tolerance to self-antigen.[105] A specialised subset of macrophages in GCs, called tingible body macrophages, express Mer (a surface receptor that facilitates apoptotic cell clearance) and is associated with the clearance of apoptotic cells within GCs.[106] Mer-deficient mice were found to develop a lupus-like syndrome, and this was due to significantly increased activation and proliferation of GC B cells and $CD4^+$ effector T cells, including TFH cells.[106] In addition, there was a significant increase in IFN-γ production by $CD4^+$ T cells obtained from $Mer^{-/-}$ mice;[106] this can contribute to aberrant TFH cell formation by inducing overexpression of BCL-6.[35]

Regulatory cells

TFR cells. We and others have recently shown that a subset of $Foxp3^+$ cells migrates into the follicles to restrain TFH cell differentiation; these cells have been designated as TFR cells.[23–25] TFR cells colocalise within B-cell follicles and they show characteristics of both TFH and regulatory T cells (for example, BCL-6, Foxp3, CXCR5, PD-1, ICOS and CTLA-4), but lack expression of the B-cell-helper molecules CD40L, IL-4 and IL-21.[23] Deletion of TFR cells or impairing their follicular localisation led to increased numbers of TFH and GC B cells.[23–25] The mechanisms by which TFR cells limit TFH cell differentiation remain to be determined. They may repress TFH cells directly or attenuate GC B-cell development, which in turn will dampen TFH cell numbers.

$CD8^+$ regulatory cells. A subset of Qa1-restricted $CD8^+$ T cells has been found to dampen TFH cell development.[107] These $CD8^+$ T cells express CXCR5 and hence are able to migrate to B-cell follicles.[107] Furthermore, these regulatory $CD8^+$ T cells do not express Foxp3 nor CTLA-4 and they are dependent on IL-15 and perforin to execute their suppressive role.[107] Disrupting the interaction between TFH cells and regulatory $CD8^+$ T cells resulted in a dramatic expansion of TFH cells and the development of a lethal lupus-like disease.[107]

Plasma cells. Antigen-specific plasma cells appear to retain their capacity to present antigens to naive $CD4^+$ T cells but are unable to induce expression of *Bcl6* and *Il21*,[108] suggesting that plasma cells can prime T cells to differentiate into effector cells other than TFH cells. Moreover, co-cultures of plasma cells with differentiated TFH cells resulted in the reduction of *Bcl6* and *Il21* expression on TFH cells.[108] Plasma cell-mediated inhibition of TFH cell differentiation may constitute a negative feedback mechanism to limit the GC reaction after sufficient end products of this process (antigen-specific plasma cells) have been generated. The mechanism by which plasma cells repress TFH cells remains elusive.

Regulation of TFH cell numbers by antigen availability

A recent study showed that the percentage of TFH cells was significantly higher with increased amounts of available antigen.[109] Also, chronic antigenic stimulation through multiple immunisations led to a larger increase in the frequencies of TFH and GC B cells as compared with a single injection.[109] These findings may contribute to explain aberrant TFH cell formation in chronic infection settings, such as chronic HIV infection, which leads to selection of low-affinity B cells and failure to clear the virus.

PERSPECTIVES

The TFH cells constitute a specialised $CD4^+$ T-cell subset that provides essential help for B-cell proliferation and differentiation within the GCs. TFH cell numbers have to be tightly regulated for optimal production of high-affinity antibody responses. Studies in the past decade have collectively characterised the major molecular mechanisms required for TFH cell differentiation, although this is likely to be just the tip of the iceberg, as important roles for recently characterised regulatory elements such as miRNAs and long non-coding RNAs, as well as the effect of diet and gut microbiota composition on TFH cell differentiation/function are just being discovered. This surprisingly large amount of 'molecular rheostats' controlling TFH cell homeostasis may have evolved to enable rapid and effective control of this population that is crucial for mounting protection against potentially lethal toxins and microbes, but can also trigger autoimmunity when overactive. Because TFH cell selection gives rise to memory B cells and plasma cells that can survive for years or decades in an individual, it appears favourable that multiple layers—transcriptional, post-transcriptional, and post-translational—operate to finely tune and curtail the TFH response. Although little is known about epigenetic regulation of TFH cells, it is likely that this level of control will also be important. It is also becoming clear that the proportion of TFH cells relative to the neighbouring regulatory cells (especially TFR cells) will be an important biomarker to determine the magnitude of the B-cell helper response and possibly also the quality of B-cell selection. In order to be able to use these new biomarkers in TFH cell-mediated disease, it will be important to improve our understanding of human TFH and TFR cell development and regulation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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