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T-cell subsets in the germinal center

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Summary: T cells are known to migrate to B-cell-enriched follicles and germinal centers within secondary lymphoid organs to provide help to B cells. Cognate T:B interactions that take place at the T:B border and subsequently within germinal centers are essential for B-cell priming, differentiation into germinal center B cells, and selection of mutated cells into memory B cells or memory plasma cells. In recent years, different stages of maturation within B-cell helper T cells, collectively known as B-follicular helper T (Tfh) cells, as well as heterogeneity amid germinal center T cells are becoming clear. Indeed, germinal centers support not only *bona fide* Tfh cells but also $CD4^+$ and $CD8^+$ follicular regulatory T (Tfr) cells that act to suppress germinal center responses and B-cell helper natural killer T cells. There is a growing need for more precise phenotypic and functional distinction of these specialized T-cell subsets. In this review, we summarize current knowledge on the ontogeny, molecular identity, and functional relevance of the various subsets of germinal center T cells.

Keywords: *T cells, B cells, cell differentiation, lineage commitment, tolerance, cytokines*

Introduction

T cells are critical players in supporting and regulating the efficacy and longevity of humoral immune responses. In the periphery, T-cell help to B cells is first provided to naive B cells that have taken up protein antigen and present the processed peptides complexed with major histocompatibility complex (MHC) class II to cognate effector T cells at the outer T-zone or T:B border in secondary lymphoid organs. Contact-dependent ligation of CD40 on B cells by its inducible T-cell surface CD40 ligand (CD40L) acts as a costimulatory signal to induce B-cell clonal expansion and differentiation (1). In addition, $CD4^+$ T-cell-derived cytokines promote growth, differentiation, and direct immunoglobulin (Ig) class-switching of dividing B cells (2). Activated B cells can then further differentiate either (i) into short-lived extrafollicular plasma cells that secrete low-affinity antibodies important for immediate defense against infection; (ii) into early memory B cells; or (iii) within secondary follicles giving rise to germinal centers.

Germinal centers are dynamic microenvironments that provide a unique niche for B-cell affinity maturation to occur. Germinal center formation in response to protein antigens is thymus dependent, as seen from early studies in athymic *nude* mice that do not form germinal centers after immunization with hapten-protein conjugates (3). Normally, a limited number of clones (1–3) colonize each follicle and grow as B blasts to fill the follicle. They then move to the pole of the germinal center closer to the follicle, where they continue to divide as $CD83^{low}CD86^{low}CXCR4^{high}$ centroblasts and undergo a process of somatic hypermutation (SHM) (4). SHM is unique to B-cell maturation in germinal centers and consists of the acquisition of random single-nucleotide changes in the Ig V-region genes. SHM promotes affinity maturation of the Ig repertoire (5), where clones gaining improved affinity toward the immunizing antigen are positively selected for, and low-affinity, self-reactive or non-functional clones collectively succumb to cell death by apoptosis. The intricate process of selection of mutated B cells remains a highly contentious area of study and has been thoroughly reviewed recently (6, 7). After undergoing SHM, centroblasts exit cell cycle and migrate to the follicular dendritic cell (FDC)-rich zone called the light zone as centrocytes that can be distinguished phenotypically on the basis of high CD83 and CD86 surface expression with low CXCR4 expression (4). Centrocytes take up antigen from FDCs from which they also appear to receive survival signals (8). It is thought that those centrocytes expressing higher affinity receptors can take up and present more antigens to limiting germinal center T cells, thus having a competitive advantage for selection (4). Centrocytes that receive T-cell-derived survival signals then proceed to become either long-lived plasma cells, form memory B cells, or re-enter the dark zone for further rounds of SHM and continued selection. T-cell help is thus central to germinal center maturation and centrocyte selection in the light zone and therefore critical for the genesis of a high affinity and long-lasting antibody response, and for the formation of quality memory B cells (9, 10).

Here, we examine the heterogeneity and phenotypic and functional complexity of the effector T cells that are important for promoting various stages of germinal center B cell development and selection. We also discuss the functional diversity of 'non-helping' follicular/germinal center-homing T cells that directly negatively regulate germinal center responses.

Becoming a follicular homing T cell

Maturation of T cells into cells specialized in provision of help to B cells commences with migration of primed T cells to the border between the T-zones and the follicles (T:B border) in secondary lymphoid tissues (11). Follicular homing by effector T cells is reliant upon a two-step chemotactic process, beginning with the downregulation of CCR7 expression, the receptor for the chemokines CCL19 and CCL12 produced in the T-zone of secondary lymphoid organs (12, 13). Escape from the T zone occurs concurrently with the induction of CXCR5 surface expression, important for follicular homing by binding CXCL13 abundant in B-cell zones (14). In early studies of human tonsils (15–17), CXCR5 was used to mark a subpopulation of effector T cells that could support antibody responses. T cells that express CXCR5, named 'follicular B-helper T cells or 'T-follicular helper' (Tfh) cells, are almost all $CD4^+$ (>95%), phenotypically distinct from T-helper 1 (Th1) and Th2 cells, and localize to primary follicles and within active germinal centers.

Downregulation of CCR7 and upregulation of CXCR5 is coordinated by B-cell lymphoma 6 protein (BCL6), the transcription factor that directs Tfh differentiation (18–20). BCL6 expression identifies Tfh cells from other effector $CD4^+$ T cells, namely Th1, Th2, Th17, and T-regulatory (Treg) cells, which are instead genetically programmed by the transcription factors Tbet, GATA3, retinoid orphan receptor γt (RORγt), and forkhead box protein 3 (FoxP3), respectively (21). Initial studies on the kinetics of BCL6 expression in developing Tfh cells noted that induction of BCL6 protein expression in activated $CD4^+$ T cells occurs during priming by antigen-presenting dendritic cells and independent of cognate B-cell signals (22, 23). BCL6 levels in primed T cells then gradually diminish over 1–3 rounds of cell division before a second wave of BCL6 upregulation is triggered. Secondary signals (discussed later) that stabilize and potentially enhance BCL6 expression in activated $CD4^+$ T cells are required for follicular entry and Tfh cell lineage dedication (22–24). Although BCL6 expression in T cells coincides with follicular migratory behavior (11), the exact mechanisms for BCL6-regulated Tfh cell differentiation are yet to be fully elucidated. It is likely that BCL6 also programs functional properties in Tfh cells important for B-cell help. BCL6 is known to transcriptionally repress *Prdm1*, encoding for BLIMP1, in activated $CD4^+$ T cells, which otherwise inhibits Tfh cell differentiation (18), at least in part via transcriptional silencing of CXCR5 (25).

Pre-Tfh cells

The possibility to visualize BCL6 expression in T cells and to perform intravital imaging of developing germinal centers has provided important insights into the early stages of Tfh cell differentiation. Several recent studies have shown that

antigen-specific $CXCR5^+$ $BCL6^+$ T cells are detectable at the T:B border soon after T-cell priming, before germinal centers form in immunized mice (26–28). The minimal cues that are important for the formation of these nascent pre-germinal center Tfh or 'pre-Tfh' cells that establish cognate interactions with antigen-activated B cells are not known. CD28 T-cell costimulation during initial priming may play a major part in pre-Tfh cell induction owing to the requirement of CD28 in germinal center formation (29) and its dispensability within fully established GC T cells (30). Inducible costimulatory (ICOS) costimulation at the time of T-cell priming by DCs has also been proposed to play an important role in this initial stage of Tfh cell differentiation (23). Pre-Tfh cells are essential for the initiation of both germinal center and extrafollicular antibody responses (28) as demonstrated by the comparably severe impairment in the induction of both germinal center and extrafollicular antibody responses when T cells lack *Bcl6* (28). This observation is also consistent with T-cell CXCR5 deficiency in mice showing defective germinal center formation (14) and to a lesser extent compromised extrafollicular antibody responses (31, 32). In response to immunization with sheep red blood cells and to *Salmonella* infection, pre-Tfh cells located at the T:B borders exhibited lower surface expression of PD-1 than that found on Tfh cells located within mature germinal centers (28).

Commitment of pre-Tfh cells to enter germinal centers requires secondary signals that stabilize BCL6 expression (22). These secondary signals are largely dependent on B-cell contact and the formation of stable conjugates between pre-Tfh cells and B cells *in vivo* (lasting >15 min) (26, 27). The homophilic signaling lymphocytic activating molecule (SLAM) family members including CD84 and Ly108 have emerged as essential adhesion molecules necessary for prolonged T:B cell engagement (33). These molecules self-associate with high affinity at the surface interface between T and B lymphocytes. Like other SLAM family members, CD84 and Ly108 provide stimuli to intracellular SLAM-associated protein (SAP). Although the nature of effector signals downstream of SAP in Tfh cells remains unknown, in the absence of SAP signaling, T:B cell interactions are unstable, resulting in a failure to form germinal center Tfh cells and thus germinal centers themselves (34).

ICOSL is also expressed by activated B cells and binds ICOS receptor on primed T cells, contributing to enhancing and/or stabilizing *Bcl6* expression, Tfh differentiation and Tfh-driven germinal center responses (23, 35–37). ICOS transduces intracellular signals to phosphoinositide 3-kinase (PI3K) which is a potent inducer of GC Tfh cell effector cytokines, namely IL-4 and IL-21 (38, 39). IL-21:IL-21R signaling seems to be important during early T:B cell responses in immunized mice (28) and therefore its production may represent a critical factor in pre-Tfh cell responses. It has been shown to exert autocrine effects on T cells to enhance Tfh cell formation under certain – albeit not all – immunization conditions (40–43), and exert paracrine effects on B cells promoting their growth and differentiation.

It is likely that $CXCR5^+$ $BCL6^+$ pre-Tfh cells at the T:B border represent a transitional effector $CD4^+$ T-cell phase preceding germinal center Tfh cell formation (*Fig. 1*). Maintained *Bcl6* expression as a consequence of stable cognate T:B interactions is required for entry of pre-Tfh cells into germinal centers (26, 27) and thus maturation to differentiated germinal center Tfh cells.

Germinal center Tfh cells

After initial cognate interaction of pre-Tfh and B cells at the T:B border, some pre-Tfh cells gain access to and are retained in follicles that harbor nascent germinal centers. Migration of $CXCR5^+$ $BCL6^+$ pre-Tfh cells into seeded germinal centers does not occur during conjugation with a B cell, but rather as unpaired T cells after significant differentiation is achieved due to T:B interactions and maintained *Bcl6* expression (26). In addition to CXCR5 and BCL6, $CD4^+$ Tfh cells located within mature germinal centers also highly express PD-1 (14), IL-21, CD84, and ICOS (44).

In human tonsils, further heterogeneity is observed among $CXCR5^+$ GC Tfh cells, with those localized more internally within the light zones of germinal centers showing high surface expression for CD57 in contrast to outer zone germinal center Tfh cells that appear to be $CD57^-$ (16). To date, the physiological and functional divergence between human $CD57^+$ versus $CD57^-$ Tfh cells in germinal center responses remains uncertain. Although the genetic landscape between $CD57^+$ versus $CD57^-$ human Tfh cells appears to be largely similar, some molecules with little known function in T cells are specifically upregulated in $CD57^+$ germinal center T cells, for instance V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (*MAFB*) (44). Strikingly, $CD57^-$ but not $CD57^+$ Tfh cells appear to contain pre formed CD40L, which may have important implications in the provision of differentiation signals to B cells (45). It has been suggested that CD40L signals differentially promote long-lived memory B cells and plasma cells, favoring the former (46). Indeed, experiments using tonsil grafts into immunodeficient mice have also shown that CD40:CD40L signals, but not T cells are dispensable for

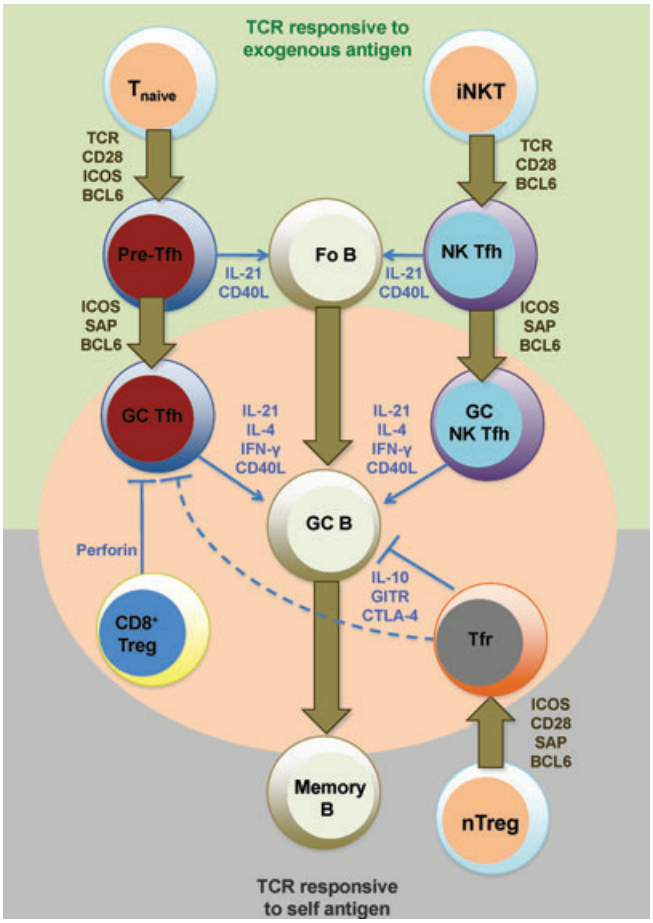

***Fig. 1.* Heterogeneity and differentiation of germinal center T cells.** The commitment of T cells in promoting germinal center B-cell responses against immunizing antigens is largely dependent at least initially on TCR engagement and CD28 costimulation by antigen-presenting dendritic cells. Upon transient BCL6 induction after activation, $CD4^+$ naive T cells (T_{naive}) give rise to pre-germinal center follicular helper T (pre-Tfh) cells that subsequently require contact with cognate B cells to receive re-enforcement signals via ICOS, PD1, and SAP signaling for BCL6 stabilization and commitment to the germinal center (GC) Tfh cell differentiation pathway. B-cell conjugation and SAP signaling is also important for NK Tfh cell formation from invariant NKT (iNKT) cells and their response toward glycolipid-containing antigens. T-cell-mediated suppression of autoreactive and/or or non-antigen-specific germinal center B cells is mediated by Qa-1-restricted $CD8^+$ regulatory T ($CD8^+$ Treg) cells (that directly inhibit Tfh cells) and follicular regulatory (Tfr) cells. Tfr cells develop from BCL6-dependent natural $CD4^+$ regulatory T cells (nTregs) that receive TCR and CD28 signals and also and SAP-mediated signals during cognate B-cell interactions. Fo B, follicular B cells; GC B, germinal center B cell; memory B, memory B cell.

survival of plasma cells in these human secondary lymphoid tissues (47). Together these observations suggest a physiological B-cell dependence on T-cell-derived CD40L signals for memory B-cell survival, and these signals may be delivered by outer zone $CD57^-$ Tfh cells, as cells selected in the light zone by $CD57^+$ Tfh cells prepare to leave the germinal center. Considering BLIMP1-mediated transcriptional repression of *MAFB* during osteoclast differentiation (48) and the ubiquitous mutual antagonistic relationship between nuclear factors BCL6 and BLIMP1 in mammalian cells (49, 50), high *MAFB* gene expression in $CD57^+$ Tfh cells may reflect increased BCL6 nuclear activity or stability. Indeed another MAF oncogene family member, c-MAF, has recently been reported to act cooperatively with BCL6 in promoting Tfh cell differentiation (51). Interestingly, *in vitro* studies have shown $CD57^+$ Tfh cells produce high levels of the immunosuppressive cytokine IL-10 (16). The presence of low IL-10 expressing $CD57^-$ Tfh cells at the periphery of germinal centers aligns well with two independent but related observations: (i) the inhibitory action of IL-10 on post germinal center memory B-cell expansion (52), and (ii) the observation that memory B cells localize adjacent to germinal centers, thus close to the location of $CD57^-$ Tfh cells (52, 53). Whether or not $CD57^-$ Tfh cells are required for optimal germinal center-derived memory B-cell proliferation warrants future study. Furthermore, IL-10-producing $CD57^+$ cells may include cells with regulatory potential. Follicular regulatory T (Tfr) cells are strong producers of IL-10 (54), and at least in mice, most regulatory T-cell subsets including activated conventional $FoxP3^+$ Tregs (55) and Tr1 cells produce high amounts of IL-10 (56).

An important role for germinal center Tfh cells is to provide selection and survival signals to centrocytes that have mutated their Ig V-region genes (*Fig.* 1). What are the important intercellular signals exchanged within germinal center T-B cell conjugates? This question has been the focus of much research in the past decade from which we now know that cross talk during germinal center T:B cell contact is importantly bidirectional, highlighting a co-dependence between germinal center T and B cells. As seen in early T-cell induction of B cells at the T:B border, activation of surface CD40 on B cells by Tfh cell expressing CD40L represents an important factor for promoting germinal center survival as demonstrated by Han *et al.* (57) via the injection of neutralizing anti-CD40L antibodies that caused the dissolution of established germinal centers. In return, germinal center B cells also present costimulatory ligands to Tfh cells. Recently, it was shown in mice that T-cell costimulation by CD80 on B cells provides an important survival and maturation signal to Tfh cells, promoting the expression of essential Tfh cell molecules including ICOS, programmed death-1 (PD-1), and IL-21 (58). PD-1 is an inhibitory surface receptor on mouse and human $CD4^+$ and $CD8^+$ T cells (59). Although PD-1 signaling does appear to moderately inhibit

Tfh cell expansion, it has been shown by Good-Jacobson *et al.* (60) to be required *in vivo* for optimal germinal center reactions and long-lived memory plasma cells by promoting IL-21 synthesis. However, a separate report published shortly afterwards described an inhibitory effect of PD-1 on antigen-specific antibody responses (61). The major discrepancies between these reports may stem from the different immunization conditions.

Cytokine production is an important mode of B-cell regulation by germinal center Tfh cells (*Fig. 1*). Germinal center Tfh cells express high levels of IL-21 and variable levels of IL-4, interferon-γ (IFN-γ), and IL-2. IL-21 is important for germinal center Tfh cell differentiation and function (40–42). Using 50:50 mixed wildtype:IL-21R knockout bone marrow chimeras, IL-21 was shown to signal predominantly via IL-21R on germinal center B cells to enhance *Bcl6* expression and therefore maintain germinal center B cells and sustain the germinal center Tfh cell niche (40, 43). In some, but not all of these studies, IL-21 was also shown to act cell-autonomously in Tfh cells themselves (40–42). Recently, genetic regulation for IL-4 expression in germinal center Tfh cells was shown to be distinct from Th2 cells; IL-4 was shown to be important for augmenting germinal center reactions (62, 63). The need for IL-4 signaling is likely to be more critical in germinal center Tfh cells than pre-Tfh cells, since it has previously been reported to be dispensable in the initial stages of Tfh cell formation (64, 65) and T:B cell priming (66). IFNγ is another cytokine that is emerging to be important for germinal center Tfh cell-mediated modulation of germinal centers. Excessive IFN-γ in germinal centers can enhance Tfh cell formation and lead to pathogenic Tfh cell accumulation and autoantibody responses, as seen in various strains of lupus-prone mice (67). Indeed, it has previously been documented that IFN-γ expression is also transcriptionally repressed by BCL6 in developing Tfh cells *in vitro* (20). IFN-γ is lowly expressed in CXCR5^{hi} GC Tfh cells of human tonsils (20, 68) and mice immunized with sheep red blood cells (18, 69), compared with expression by CXCR5^{int} CD4^{+} T cells likely to include pre-Tfh cells. IFN-γ production by GC Tfh cells can increase substantially in the context of viral infection, although on a per cell basis, the amount produced still appears to be lower than on non-GC CD4^{+} T cells (70). *In vitro* studies suggest that this cytokine may also participate in a negative feedback autocrine loop to dampen germinal center Tfh cell differentiation (71). In contrast with the cytokines described above, there seems to be very little production of IL-17 by human and mouse GC Tfh cells in physiological circumstances (20, 60, 68). In fact, elevated production of IL-17 by GC Tfh cells or their precursors, i.e. T cells shown to induce germinal centers, have been associated with autoimmune and/or ectopic germinal center reactions associated with autoimmune arthritis (72), lupus (73), and multiple sclerosis (74).

Through the use of experimental mouse models, it is slowly becoming clear that the deregulation of Tfh cell differentiation, Tfh cell accumulation, and Tfh cell-mediated germinal center responses are key to the emergence of many antibody-dependent immune disorders in mice and humans (75, 76). We and others have shown that unrestrained Tfh cell responses can lead to excessive germinal center formation, which through SHM and a lowered threshold of selection by T cells may result in the production of autoantibodies (29, 73, 77, 79). One genetic pathway found important for the maintenance of tolerogenic Tfh cells was elucidated in lupus-prone *sanroque* mutant mice, harboring a single M199R substitution in the RING and C3H zinc finger domain-containing protein ROQUIN and exhibiting a phenotype reminiscent of human systemic lupus erythematosus (78). The mice presented with early onset of circulating antibodies against nuclear antigens and double-stranded (ds) DNA, nephritis, splenomegaly, lymphadenopathy, and hypergammaglobulinemia. Many Tfh cell-specific genes were upregulated in *sanroque* T cells, including *Il21*, *Cxcr5*, *Pdcd1*, *Cd84*, and *Bcl6*, and large numbers of CD4^{+} PD1^{hi} T cells accumulated in germinal centers (29, 78). ROQUIN was shown to act cell-autonomously in T cells to repress Tfh cell formation. ROQUIN normally acts to post-transcriptionally destabilize messenger RNA substrates like *Icos* (82–84) and *Ifng* (67). Inactivation of this RNA-regulatory function of ROQUIN in *sanroque* mice led to excessive Tfh cell formation and autoreactive germinal centers that drive the systemic autoimmune phenotype. More recently, *sanroque* Tfh cells were shown to play a major role in the onset of tissue-specific autoimmunity in a diabetes-prone mouse model through their ability to induce anti-islet autoantibodies (83). It is likely that ROQUIN can target other Tfh-relevant RNA species. Whether or not the enzyme acts as a *bone fide* E3 ubiquitin ligase enzyme via its RING domain in Tfh cells remains unknown. In nematode worms, ROQUIN's ancestor, RLE-1 has been shown to mediate the polyubiquitination of DAF-16, homolog to the FOXO transcription factors in mammals (84). Whether this putative E3 ligase activity is related or in any way regulates the RNA-repressing function of ROQUIN is also unknown.

Although Tfh cell overactivity causes autoimmunity, absence of Tfh cell help to B cells can also cause severe immunodeficiencies and defects in protective immunity (85).

An example is common variable immunodeficiency (CVID) seen in adult patients with genetic lesions in *ICOS* (86). In individuals with CVID, normal T-cell responses appear widely intact, but naive, switched, and memory B cells are significantly diminished, suggestive of selective defects in Tfh formation and function. Another pathology arising from deregulated Tfh cells is angioimmunoblastic T-cell lymphoma, a peripheral T-cell lymphoma in which the normal counterpart of the neoplastic T cells appears to be Tfh cells (87).

NK Tfh cells

Natural killer T (NKT) cells express invariant $\alpha\beta$ TCRs that respond to glycolipid antigens that can be found on various bacteria, presented on CD1d (88). A role for NKT cells in promoting antibody responses has been known for some time (89). NKT cells were shown to be capable of establishing cognate interactions with B cells that have bound lipid antigens and provide help for extrafollicular antibody responses (90, 91). More recently, NKT cells have also been described to be involved in germinal center reactions (92, 94). Furthermore, NKT cells can co-opt the same differentiation pathway described for $CD4^+$ Tfh cells to become germinal center-bound NKT cells, so called NK Tfh cells (95). It was shown that in response to CD1d-α-Galceramide gycolipid immunization, NK Tfh cells expressed the canonical markers that identify germinal center Tfh cells, including high levels of surface CXCR5 and PD-1. NK Tfh cell formation was dependent on *Bcl6* and *Cd28* expression and on cellular interactions with B cells (95). NK Tfh cells formed stable motile conjugates with B cells (lasting >20 min) and localized in the germinal center. More recently, NKT-dependent cognate help to B cells has been shown to be dependent on SAP, again pointing to an important role for stable NKT:B interactions to promote NK Tfh formation and adequate terminal B-cell differentiation (95). NK Tfh cell help to B cells is at least in part dependent on the production of IL-21 cytokine (93, 94), as selective deficiency of IL-21 diminished both follicular and extrafollicular antibody responses.

A major difference between NK Tfh cells and their germinal center Tfh cell counterpart is the kinetics of the antibody response: NK Tfh cells accelerate antibody responses leading to early appearance of extrafollicular plasma cell foci and germinal centers. Although the extrafollicular response is robust, germinal centers fail to grow and rapidly involute. Furthermore, unlike other thymus-dependent responses, NK Tfh cell-driven germinal center responses to glycolipid antigens did not yield memory B cells nor bone marrow plasma cells and led to limited (94) affinity maturation of germinal center B cells (93). This has led to denominating NK Tfh-dependent responses as thymus-dependent type 2 (TD-2), to distinguish them from the conventional TD responses to protein antigen capable of inducing long-lived high affinity antibody responses. Conventional $CD4^+$ T cells, however, appear capable of enhancing NK Tfh-derived help for germinal center responses (92).

Tfr cells

Conventional $CD4^+$ $FoxP3^+$ Treg cells are central to limiting effector T-cell responses and maintaining peripheral T-cell tolerance. Treg cells are classified into two major subsets: (i) natural Tregs (nTregs) directly derived from the thymus and reactive to self-antigens, and (ii) inducible Tregs (iTregs) generated from naive $CD4^+$ T cells stimulated by exogenous antigen in the context of MHC class II and in the presence of IL-2 and TGFβ (96).

There is early evidence from mouse studies suggesting that $CD4^+$ Treg cells can negatively regulate humoral responses (97). $CD4^+CD25^+$ T cells had also been visualized in human germinal centers, but their ontogeny, relationship with Treg cells, and *in vivo* function was uncertain (98, 99). We and others have recently added insight into the identity of these B-zone-homing regulatory cells with the description of $CXCR5^{hi}$ $PD1^{hi}$ $FoxP3^+$ Treg cells in germinal centers, designated follicular regulatory T (Tfr) cells (54, 100, 101). As described for Tfh cells, BCL6 is essential for Tfr cell formation. Furthermore, up to 75% of these Tfr cells express high levels of BLIMP1 while still co-expressing BCL6. These cells have an activated Treg phenotype, with high expression of GITR, CTLA-4, and IL-10. Importantly, they do not express B-cell-helping cytokines or CD40L. Like their $FOXP3^-$ germinal center Tfh cell counterpart, Tfr cell formation requires the presence of B cells and intact SAP signaling suggesting a requirement for SLAM-mediated intercellular interaction between Tregs and B cells for entry into germinal centers. Although Tfr cells express high levels of T-cell immunosuppressive genes, the exact molecular mechanism for Tfr cell-driven germinal center repression is yet to be fully elucidated. Germinal center Tfr cells that emerge after T-dependent immunization were shown to originate predominantly from nTregs and not from extrathymic $CD4^+$ T-cell populations, including naive T or $Foxp3^-$ Tfh cells (100, 101), but the possibility that Tfr cells can differentiate from TGFβ-generated iTreg cells in the periphery to suppress antigen-specific B-cell responses has not been formally excluded. It has recently been demonstrated

that *in vivo* blockade of TGFβ results in abnormal germinal center growth in response to sheep red blood cell immunization (102). In addition, expanded germinal centers within Peyer's patches and hyperreactivity of serum to mucosal-associated antigens in mice with a highly selective blockade in iTreg cell differentiation (103) suggests that inducible Tfr cells derived from iTregs might develop under particular experimental and physiological conditions. Alternatively, iTregs may suppress the initiation of germinal center T-cell responses against exogenous T-dependent antigens simply by virtue of their ability to access the outer follicles and T:B border. At these sites, it is possible that iTreg cells act early on in competing against or antagonizing cognate pre-Tfh cell responses.

Through the use of either adoptive transfers or mixed bone marrow chimeras, Tfr cells were shown to inhibit both germinal center Tfh cell and B-cell compartments (56, 102, 103), although at this point it is still not clear if both of these effects are direct, or one could be the consequence of the other, given the mutual dependence of Tfh and GC B cells for their growth and survival. Tfr cells were shown to dampen antigen-specific antibody production. Interestingly, our group observed an outgrowth of non-antigen-specific B cells in mice lacking functional Tfr cells (56). This suggests that Tfr cells may be specialized in repressing self-reactive germinal center B cells that have either received linked-help from non-self-reactive T cells to enter germinal centers or have arisen as a consequence of somatic hypermutation. Tfr cells emerge as a much-needed cellular candidate to mediate negative selection of self-reactive B cells in germinal centers, a process which to date has remained a conundrum. Indeed, there is abundant evidence that self-reactive B cells can both enter and arise in germinal centers. To date, plausible cellular candidates and mechanisms to explain how self-reactive B cells, some of which may still bear high affinity for exogenous antigens, are deleted have remained elusive. Further experiments are needed to experimentally test this hypothesis.

Another plausible role for Tfr cells is curtailing germinal center reactions; this is suggested by their kinetics as they peak later than Tfh cells, coinciding with a decline in Tfh cell numbers (101) (*Fig.* 2). It is possible that a bias in Tfr cell accumulation over FoxP3$^-$ germinal center Tfh cells acts as a regulatory brake once sufficient antigen has been cleared within a mature germinal center. Regardless of the underlying mechanism, the Tfh:Tfr ratio may be a useful biomarker to determine germinal center maturation and longevity. It emerges that identification of the biochemical and genetic mechanisms that control the balance between Tfr and Tfh cells in germinal centers may be important in the design of vaccines and the control of Tfh overactivity associated with autoimmunity. Given the thymic origin of the Tfr cells described to date and the anticipated ability of their TCRs to recognize self-antigens, elucidation of the regulatory signals that dictate the Tfh:Tfr ratio is likely to have important therapeutic potential in autoantibody-driven diseases.

CD8$^+$ Treg cells

Kim *et al.* (104) reported a CD8$^+$ Treg-like cell in mice, capable of directly suppressing germinal center Tfh cells expressing the non-canonical major histocompatibility complex class 1 molecule Qa-1. Analogous to classical CD8$^+$ T-cell cytotoxicity, CD8$^+$ Tregs were dependent on IL-15 and perforin expression for their repressive effects. Unlike conventional CD8$^+$ cytolytic T cells, CD8$^+$ Tregs were identifiable by high expression of CD44, ICOSL, and CXCR5, and were thus capable of homing to Qa-1-peptide-presenting B cells in germinal centers. Hence, through their specific mode

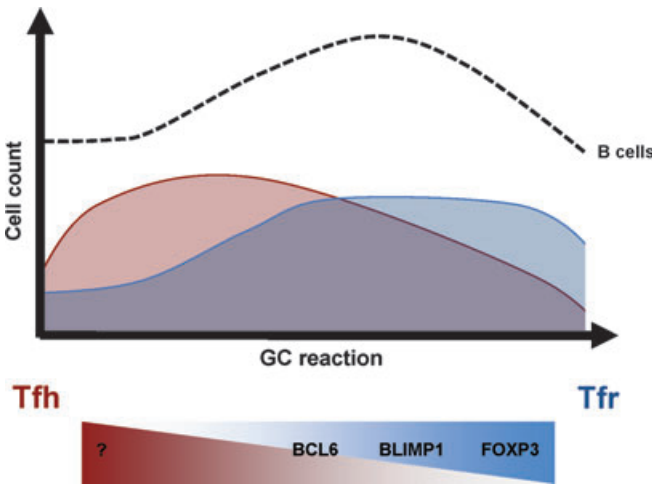

***Fig.* 2. Differential kinetics and development of follicular helper and follicular regulatory T cells during a germinal center response to immunization.** CD4$^+$ follicular helper T (Tfh) cells are induced early on in T-dependent follicular B-cell responses. After receiving contact-dependent 'help' signals from Tfh cells, activated cognate B cells proliferate and initiate germinal center (GC) reactions. As Tfh cells decline, GC B-cell responses are dampened and follicular regulatory T (Tfr) cell responses peak. This later peak of Tfr cells may help coordinate GC B-cell clearance and prevent the survival of mutated autoreactive GC B-cell clones. The emergence of Tfr cells is tightly regulated genetically and dependent on co-expression of BCL6 and FOXP3. Most, albeit not all, Tfr cells also express high levels of the nuclear factor BLIMP1 that opposes Tfh cell formation and promotes Treg activity, including IL-10 production. Genetic or other cues that preferentially drive Tfh cell differentiation against Tfr cell responses are yet to be elucidated.

of cellular targeting and killing, which is highly dependent on TCR-based recognition of surface-bound Qa-1-peptide (105, 106), $CD8^+$ Treg cells are distinct from $CD4^+$ Tfr cells.

In immunized mice, Qa-1-restricted immunosuppression within germinal centers was shown to play an important role in blocking affinity maturation of antigen-specific antibodies (104). In addition, disruption of Qa-1 activity in mice sufficient in conventional $FOXP3^+$ $CD4^+$ Tregs was critical in preventing glomerulonephritis and the production of autoreactive antibodies against dsDNA. The emergence of $CD44^+CXCR5^+ICOSL^+CD8^+$ Tregs in the germinal center therefore highlights a new mechanism for intercellular cross talk between T cells of the germinal center that is fundamentally critical for shaping protective and pathological Ig production.

Perspectives

Much has been uncovered in recent years regarding the functional requirements of a T-cell pool in B-cell zones for direct modulation of germinal center humoral immunity. It is therefore critical that future work focuses on refining the identity, regulation, and functions of these germinal center T cells. How do the various germinal center T cells differentially emerge, and what unique development cues are they dependent on? One possibility is that differentiation of one subset may be a product of reciprocal changes in other subsets since all cells compete for a common germinal center niche. It appears that BCL6 and CXCR5 expression is essential for formation and possibly maintenance of all germinal center T cells, although this idea has not been formally tested in Qa-1-restricted $CD8^+$ Tregs.

Are there yet to be identified unique germinal center T cells within B-cell zones from different lymphoid tissues and are those belonging to the same subset identical across the different tissues? Assessment of germinal center T-cell phenotypes is often performed in cells derived from lymph node or spleens. *Bcl6*-expressing T cells also exist in Peyer's patches in the gut, where these cells can differentiate from $CD4^+$ T cells that express FoxP3 (107), although they may not be stable Treg cells. In the central nervous system of EAE-induced mice, Tfh-like cells aberrantly co-express Th17-relevant genes (74), and Tfh-like cells have also been found in bone marrow samples from human patients of angioimmunoblastic T-cell lymphoma (108).

What is the extent of phenotypic and functional translatability between mouse and human studies of all germinal center T-cell subsets? For future studies, it will be important to compare germinal center T-cell subsets identified in mice with those from humans. This will be critical to identify reliable biomarkers, factors regulating germinal center responses in humans, novel insights for vaccine design to boost the quality of B-cell memory, and ultimately, more specific and effective drugs against human autoimmune syndromes and immunodeficiencies.

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