Review

Emerging cellular networks for regulation of T follicular helper cells

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The cellular networks that regulate humoral immune responses have been a focus of research over the past three decades. Studies have shown that inhibition of immune responses can be attributed to both suppressor T cells and B cells. More recently, T follicular helper (Tfh) cells have been identified as a target of immune regulation. Tfh cells are a subset of highly activated T helper cells specialized for providing cognate help to B cells during germinal center reactions. In this review, we describe emerging evidence for cellular networks that alter Tfh cell phenotype and function and regulate antibody production during the germinal center reaction. We discuss how these new findings influence our understanding of Tfh cells.

T follicular helper cells and the humoral immune response

During the humoral immune response, antigen is transported into the T cell zone and B cell follicles, which initiates the activation and interaction of T and B cells resulting in the germinal center (GC) reaction. GC are discrete structures within the B cell follicles of secondary lymphoid organs in which the processes of somatic hypermutation (SHM), class switch recombination and affinity maturation of activated B cells occur accompanied by production of memory B cells and antibody secreting plasma cells (PC) [1–3]. *In vivo*, PC can arise from two developmental routes. Short-lived PC are generated in the extra-follicular pathway. Antibody from these PC are critical for the immune response to acute and cytolytic infections and constitute the majority of antibody detected soon after primary infection or immunization. By contrast, PC generated in the GC reaction are selected after the time-consuming processes of SHM and affinity maturation; these PC secrete high-affinity class switched antibody and persist in survival niches such as the bone marrow [4,5]. PC and memory B cells generated from the GC are poised to respond rapidly to re-encounter with antigen.

For GC to develop, B cells must receive cognate help from $CD4^+$ T helper cells [6,7]. The absence of T cell help during B cell priming leads to apoptosis, thereby preventing differentiation of B cells into memory B cells or plasma cells [8]. T follicular helper (Tfh) cells are the specialized subset of T helper cells that provide help within GC. The migration of Tfh cells into the B cell follicle and GC is dependent upon the coordinated downregulation of CC chemokine receptor 7 (CCR7), reducing attraction to CC chemokine ligand (CCL)19 and CCL21 in the T cell zone, and upregulation of C-X-C chemokine receptor type 5 (CXCR5), increasing attraction to CXCL13-rich follicular dendritic cell (FDC) networks in the GC [9–13]. Tfh cells are a highly activated subset of T helper cells, but whether Tfh cells comprise a distinct lineage remains controversial.

T cell help in the GC is a crucial component of the generation of an affinity-matured antibody response. However, the important functional role of Tfh cells in driving the affinity maturation of PC has been an underappreciated feature in recent studies describing Tfh-phenotype cells. This is partly because of the focus on the early primary response to infection or immunization when the contribution of affinity-matured antibody remains unclear; and the fact that many recently activated $CD4^+$ T cells in the T cell zone and at the T cell–B cell border share phenotypic features with Tfh cells, including expression of CXCR5, GL7, inducible T-cell co-stimulator (ICOS), programmed death 1 (PD-1), GL7 and the transcriptional repressor B cell lymphoma 6 protein (Bcl6). Therefore, many aspects of Tfh cell biology remain unresolved. An improved appreciation of the similarities and distinctions between T helper cells that provide help to B cells at extrafollicular sites, the T cell–B cell border of lymphoid tissues, and Tfh cells that reside in the GC will be an important step forward.

The cellular and humoral arms of the immune system share many features both in terms of their activation kinetics and migration through lymphoid tissues as well as the growth factors that generate and sustain immune responses following infection or immunization. This offers the potential for a variety of regulatory mechanisms to control the expansion of lymphocyte clones with specificity for self-antigens as well as imposing limits upon the magnitude and duration of immune responses to foreign antigen. These control mechanism are those mediated by T cells and B cells, which operate through antigen-specific means as well as non-specific mechanisms, such as through the release of inhibitory cytokines. Recent studies have identified Tfh cells as a target of immune regulation. In this review, we discuss recent findings of Tfh cell inhibition by $CD8^+$ suppressor T cells, $FoxP3^+$ $CD4^+$ T regulatory cells

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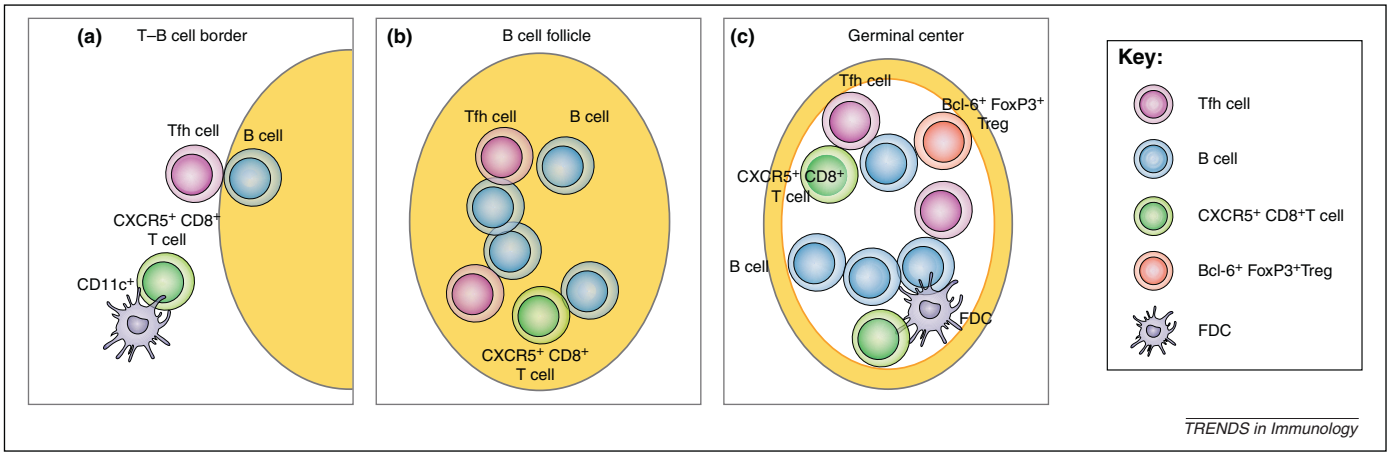

Figure 1. Cellular interactions that regulate Tfh cells in lymphoid tissues. (a) Naive $CD4^+$ T cells interact with $CD11c^+$ dendritic cells in the T cell zone. Activated $CD4^+$ T cells that have upregulated CXCR5 and downregulated CCR7 migrate towards the B cell follicle. At the border of the T cell zone and B cell follicle, Tfh cells interact with B cells. (b) $CXCR5^+$ $CD8^+$ T cells interact with B cells and Tfh cells in the B cell follicle and within the germinal center. Whether this interaction occurs directly or indirectly remains incompletely understood. (c) $CXCR5^+Bcl\text{-}6^+FoxP3^+$ Treg cells regulate T follicular helper cells and reduce the magnitude of the germinal center reaction. (b and c) Plasma (B) cells can also regulate Tfh cells by imposing a negative feedback loop.

and B cells (Figure 1) in the context of our current knowledge of immune regulation of antibody responses.

A brief history of $CD8^+$ suppressor T cells

T cell-mediated inhibition of antibody responses was initially focused on the role of a subset of $CD8^+$ T cells, named $CD8^+$ suppressor T cells. The molecular mechanisms that explain $CD8^+$ T cell suppression of humoral immune responses have been controversial for longer than 3 decades. In the 1970s, a subset of $CD8^+$ T cells was described that caused a progressive decrease in the proliferation of $CD4^+$ T cells in response to the antigen-presenting cell (APC) used for priming during multiple mixed-lymphocyte reactions (MLR) [14,15]. These and subsequent studies resulted in a substantial body of literature demonstrating that $CD8^+$ T cells could broadly inhibit immune responses. $CD8^+$ suppressor T cells were found to be antigen specific, and were $abTCR^+$ and major histocompatibility complex (MHC) class I or HLA restricted [16,17]. The suppressive effects of $CD8^+$ T cells applied to IgE as well as IgA and IgG production and occurred in both primary and secondary responses to both virus infection and non-replicating antigens [18,19]. Infection with LCMV provided a sound model of $CD8^+$ T cell-mediated suppression, establishing that virus-triggered, T cell-mediated immunopathology caused the antigen-specific suppression of B cells and antibody responses and led to the suggestion that such a mechanism may permit certain viruses to establish persistent infections [19–22].

However, despite the wealth of evidence for $CD8^+$ T cell-mediated suppression of immune responses, interest in the field began to wane when these cells were reported to be phenotypically and functionally distinct from other effector and memory-phenotype $CD8^+$ T cells, and especially in being able to secrete soluble suppressive factors mapped to the I-J region within the MHC. The nail in the coffin came when DNA sequencing of the MHC failed to identify a coding region for I-J [23,24]. The idea that T cells could have suppressive function then collapsed abruptly, but was revived a number of years later following the identification of $CD4^+$ $CD25^{hi}$ FoxP3 regulatory T cells and then $CD8^+$ $FoxP3^-$ T regulatory cells. These two subsets were shown to play a crucial role in the maintenance of self-tolerance.

Mechanisms of CD8 T cell suppression

The mechanisms reported to account for $CD8^+$ T cell-mediated suppression of immune responses include the same effector mechanisms employed by $CD8^+$ T cells to defend against infection. They can be broadly divided into lytic and non-lytic mediated mechanisms. $CD8^+$ T cells activated in response to peptide ligand–MHC class I complexes can lyse target cells; for example, via the perforin-mediated granule exocytosis pathway and by inducing Fas-mediated death via upregulation of FasL (CD95L). In addition, $CD8^+$ T cells may exhibit non-lytic mechanisms of suppression that remain poorly understood, but include competition for growth factors or release of soluble factors such IL-10 and TGF-β [25,26] and production of IFN-γ, which has been shown to skew switching patterns in responding B cells from IgG1 to IgG2a and IgG2b [27].

$CD8^+$ T cells in B cell follicles

In considering the ability of $CD8^+$ T cells to regulate Tfh cells and antibody responses, it is notable that $CD8^+$ T cells enter B cell follicles during the GC reaction (Figure 1). Several studies indicate that $CD8^+$ T cells are activated close to B cells during immune responses and this proximity is likely to be important whether $CD8^+$ T cells act on Tfh cells directly or indirectly, which is still unclear.

$CD8^+$ T cell division has been observed inside murine B cell follicles after T-dependent immunization; also, in HIV infection, mitotic $CD8^+$ cells containing TIA-1, a granule-associated protein, are observed inside follicles [28]. More recently, a subset of human $CD8^+$ T cells was described in human tonsil tissue that expressed the chemokine receptor CXCR5 [29]. $CXCR5^+$ $CCR7^-$ $CD8^+$ T cells were found scattered in the follicular mantle and dark zone of B cell follicles [29]. Antigen-specific $CD8^+$ T cells identified with MHC class I-ovalbumin (OVA) peptide tetramers (Tet) have been defined in histological sections of spleen following

infection with listeria M-expressing OVA [30]. Most Tet$^+$ CD8$^+$ T cells were observed within the T cell zones and at the T cell–B cell border but also in the red pulp, marginal zone and B cell areas, where they were found in discrete clusters associated with CD11c$^+$ cells. Tet$^+$ CD8$^+$ T cells migrated out of the spleen via the bridging channels at about day 5 post-infection, a time that coincides with the presence of plasmablasts at this site. It was notable that over 60% of tet$^+$ memory CD8$^+$ cells were found in the B cell follicles 30 days after infection [30]. Taken together, these findings indicate that responding CD8$^+$ T cells can be found in secondary lymphoid tissues at the T cell–B cell border and within B cell follicles during an immune response where they are poised to influence Tfh cells.

B cell antigen presentation to CD8$^+$ T cells

The inhibition of Tfh cells by CD8$^+$ T cells occurs either directly or through an intermediate cell type. Antigen-presenting cells, which are necessary for Tfh cell generation and survival, can become targets of CD8$^+$ cytotoxic T cells by presentation of peptide antigen on their surfaces in the context of MHC class I. Priming of CD8$^+$ T cells typically occurs in response to antigens that gain access to the cytosol of APC (such as viruses, intracytoplasmic bacteria and protozoa) for processing by the endogenous MHC class I presentation pathway [31]. However, CD8$^+$ T cells also respond to antigens from non-cytosolic sources, including apoptotic bodies, whole or degraded cell-associated and soluble antigens, processed by exogenous MHC class I 'cross' presentation pathways that may be limited to professional APC [32–34]. B cells efficiently acquire class I peptides from other APC and could thus become direct targets of CD8$^+$ T cells. However, whether B cells present non-cytosolic antigen in the context of MHC-class I remains controversial [35].

T cell inhibitory pathways

The expression of co-inhibitory receptors attenuates signals through the T cell antigen receptor. In the absence of these inhibitory signals, activated T cells can induce immune mediated pathology, which includes the suppression or elimination of other immune cells and damage to non-hematopoietic tissues. In considering how CD8$^+$ cells act as T regulatory cells, there is increasing evidence that inhibitory pathways, including NKG2A:Qa-1^b, may play a role.

The Qa-1 protein is a member of the class I^b MHC family and its human homolog is HLA-E. Most class I^b MHC gene products interact with CD8$^+$ on the surface of T cells and are expressed with β2 microglobulin (β2m) molecules. Under homeostatic conditions, Qa-1 predominantly binds a single species of peptide termed Qdm (for Qa-1 determinant modifier), derived from amino acids 3–11 of the leader sequence of the class I MHC D region-encoded molecules. The murine CD94/NKG2A receptor, expressed on both NK cells and activated/memory phenotype CD8$^+$ T cells, is inducible [36], recognizes Qa-1^b and, like its human counterpart, contains a consensus immunoreceptor tyrosine-based inhibition motif (ITIM) and a cytoplasmic domain ITIM-like sequence. The functional consequence of Qdm–NKG2A interactions is inhibition of NK or CD8$^+$ CTL activity [37].

A second class of Qa-1 ligands contains a more extensive range of peptides, including a wide array of peptides presented by APC with antigen-processing defects [38]. Interaction between this class of Qa-1 ligands and the TCR on CD8$^+$ T cells can promote activation, expansion and expression of effector cell activity [39]. Studies linking Qa-1 to CD8$^+$ T cell suppression have shown that a subset of activated Qa-1-expressing CD4$^+$ T cells are required to induce differentiation of CD8$^+$ T cells into suppressor cells, which, in turn, downregulated CD4$^+$ T cell activity, inhibiting antibody responses [40–43]. These CD4 cells are reported to express Qa-1-associated peptides [39]. Unlike, Qa-1–Qdm complexes, the Qa-1–Vβ complexes on activated CD4$^+$ T cells are thought to bind to NKG2A receptors on CD8$^+$ T cells with only low affinity. NKG2A-mediated inhibition of CD8$^+$ T cells is thereby avoided, and TCR signaling drives the cells into Vβ-specific effector cells.

On the basis of the hypothesis proposed above, the role of Qa-1 in CD8-mediated suppression of immune responses was tested recently in Qa-1 'knock-in' mutant mice that were generated to contain a Qa-1 amino acid exchange mutation that disrupted Qa-1 binding to the TCR/CD8 co-receptor but had no effect on the engagement of the inhibitory NKG2A receptor on CD8 T cells and NK cells [43,44]. Mice made genetically deficient in Qa-1 do not develop spontaneous autoimmune disease [42]; whereas Qa-1 mutant mice exhibited increased total IgG, Ig deposition in renal glomeruli, glomerulonephritis, autoantibodies against nuclear antigen, increased Tfh phenotype cells, increased GC area and GC B cells [43]. Interestingly, in the absence of activation by antigen, Tfh cells expressed high levels of Qa-1, whereas other T helper subsets expressed barely detectable levels, suggesting that Tfh cells could be a direct target of CD8-mediated suppression [43].

In a series of transfer experiments into immunodeficient host mice, CD8$^+$ T cells from mice immunized with 4-hydroxy-3-nitrophenylacetyl hapten conjugated to keyhole limpet hemocyanin (NP-KLH) were capable of suppressing both primary and secondary antibody responses to NP. When transferred with B cells, suppressor CD8$^+$ T cells caused a >90% reduction in the anti-NP response induced by Qa-1 wild type (WT) Tfh cells, but not Qa-1 mutant Tfh cells, after immunization [43]. These CD8 suppressor cells were CD44hi, ICOSL$^+$, were dependent upon IL-15 and their ability to induce suppression was dependent, at least partially, upon perforin [43]. The study demonstrated also that CD8$^+$ T cells that expressed CXCR5 were capable of migrating into lymphoid follicles after adoptive transfer [43].

In contrast to the spontaneous inflammation observed in Qa-1 mutant mice, the production of antibody in response to immunization with NP-KLH or infection with LCMV was similar in Qa-1 mutant and WT mice [43]. However, infection of mutant mice with LCMV exacerbated auto-antibody production (including anti-DNA antibodies), inflammatory cell infiltration into the liver, dermis, epidermis, glomerulonephritis and induced death 25–30 days post-infection [43]. The investigators concluded that the pathological features observed were due to the expression of the mutant Qa-1 on Tfh cells, which rendered them

resistant to suppression by $CD8^+$ T cells expressing the NKG2A inhibitory receptor. This study indicates that suppressor $CD8^+$ T cells interact directly with Qa-1 on Tfh cells to regulate their function and are consistent with the notion that dysregulated Tfh cells induce autoimmunity [45], although alternative explanations remain possible [46,47].

FoxP3$^+$ CD25hi CD4$^+$ T regulatory cells in the germinal center reaction

The ability of $CD4^+FoxP3^+CD25hi$ T regulatory (Treg) cells to suppress immune responses is well known [48,49]. Mice lacking Treg cells because of mutations in *Foxp3* develop spontaneous germinal centers and have markedly elevated serum antibody levels, including autoantibodies, compared to those of wild type mice [50,51]. Treg cells are found within the thymus and secondary lymphoid tissues, but the potential for Treg cells residing within the B cell follicle to modulate antibody responses has emerged only recently. A subset of $CD4^+$ $CD25^+$ $CD69^-$ Treg cells was observed in intrafollicular areas and GC of human tonsils that displayed potent suppressive activities towards GC Th cell-dependent antibody production and AID expression in B cells [52]. Stimulation through the TCR upregulated CXCR5 and downregulated CCR7, suggesting that once reactivated these cells were poised to migrate into the B cell follicle [52].

Three recent studies have demonstrated the presence of a subset of naturally occurring Treg cells in the GC that have the capacity to regulate Tfh cells (Figure 1) [53–55]. These cells were $FoxP3^+$ Treg cells of thymic origin, but were phenotypically similar to Tfh cells in exhibiting high expression of CXCR5 and Bcl-6 [53,54]. The density of Treg cells residing within GC peaked at day 12 after immunization, the time when the density of Tfh cells was reduced and the contraction phase of the GC was initiated [55]. Transfer of Foxp3 Treg cells with TCR transgenic OTII CD4+ T cells reduced the magnitude of the GC response to cognate ovalbumin antigen and antibody production. CXCR5 expression on Treg cells was observed to be important for this effect, because mice transferred with Treg cells from *Cxcr5*$^{-/-}$ donors had greater GC and greater serum titers of switched antibody [55]. Together, these studies identified a novel GC resident Treg population with Tfh characteristics and support the notion that Treg cells acquire characteristics of specific $CD4^+$ effector T cell subsets, including the expression of chemokine receptors that enable intimate regulation of T cell responses [56].

Treg cells are dependent upon the cytokine growth factor IL-2 [57,58]. However, the source of IL-2 for the survival of Treg cells within the GC remains unknown. Earlier studies showed that *Il2* mRNA is found in abundance in the T cell zone but there is little *Il2* mRNA within either the B cell follicle or in the GC [59–61]. In addition, Tfh cells produce only small amounts of IL-2 [11,62]; indeed, ICOS, which is highly expressed on Tfh cells, lacks the equivalent site in the cytoplasmic domain of CD28 that is necessary for binding of Grb2 family adaptor proteins and IL-2 production [63]. Upon upregulation of CCR7 and upregulation of CXCR5, Tfh cells leave the T cell zone, where they are in contact with interdigitating dendritic cells and large amounts of IL-2, and move into B cell-rich areas where ICOS–ICOSL interactions and Stat3-activating cytokines could become increasingly important for Tfh cell survival. IL-2 is a strong activator of Stat5, which downregulates the expression of Bcl6, resulting in loss of Bcl6 repression of its target genes [64]. These findings suggest that although IL-2 is critical for Treg survival, it could have a negative influence on Tfh cells. Instead, Tfh cells require signals that activate Stat3, such as the cytokines IL-6, IL-21 and IL-27 [65–67]. Analogous to the balance of Stat3 and Stat5 in regulating the generation of Th17 cells [68], Stat3 signaling cytokines may provide an important counterbalance to the effect of Stat5 on Bcl-6 expression during Tfh cell development.

Several mechanisms have been reported to account for T-regulation of immune responses, including consumption of IL-2 [69]. Thus, the observation that Treg-depleted mice exhibited higher levels of total antibody but lower levels of affinity-matured antibody after secondary immunization [53] may result from the removal of Treg-mediated inhibition of antibody production combined with the increased inhibition of Bcl-6 by IL-2 induced-activation of Stat5 [64]. By contrast, a similar study that identified the presence of a Bcl-6^+CXCR5^+ subset of Treg cells within the GC following immunization with KLH in complete Freund's adjuvant (CFA), demonstrated that these Treg cells had a crucial role in controlling Tfh cells, reducing both affinity-matured antibody and plasma cell differentiation [54]. Further examination will be required to reconcile these different observations on the influence of Bcl-6^+CXCR5^+ Treg cells on affinity-matured antibody.

Suppressor B cells

Much of the focus on immune regulation has been devoted to T cells but B cells can influence an immune response in an antigen-specific manner by generating antibody, which shuts down the response through negative feedback [70,71]. In addition, B cells can inhibit antibody production non-specifically; for instance, through the production of inhibitory cytokines. The existence of suppressor B cells was first noted in 1976 [72]. Subsequent studies showed that suppressor B cells can be induced by antigens, immune complexes, microorganisms and polyclonal stimulators in immune responses both *in vivo* and *in vitro*. Suppressor B cells have been detected in the bone marrow and spleen of non-manipulated mice and are implicated in the suppression of autoimmunity in NZB mice [71,73–80]. The mechanisms explaining the ability of B cells to suppress humoral immune responses remain largely unknown but a recent study has identified Tfh cells as a target of plasma cell-mediated negative feedback.

Plasma cell-mediated negative feedback of Tfh cells

Plasma cells are terminally differentiated effector B cells that retain a specialized ability to secrete antibody. These cells lose surface expression of immunoglobulin and exhibit a relatively poor capacity to present antigen to T cells. The transcriptional repressor Blimp-1 initiates the development of plasma cells in both pathways and exerts its

control through repression of Bcl-6 and the transcription factor Pax5 [4].

Using a model of NP-KLH immunization to detect recently formed antigen-specific, antibody-secreting plasma cells (IgM^- and $CD138^+$), plasma cells were observed to exhibit continued high expression of MHCII and the co-stimulatory molecules CD80 and CD86 [81]. Contrary to expectations, antigen-specific plasma cells harbored molecules required for antigen processing and presentation in the specialized MHCII peptide-loading pathway and were able to activate antigen-specific T helper cells *in vitro* and promote the expression of Blimp-1 in favor of Bcl-6 and IL-21 in responding T helper cells [81]. In B cell-specific *Blimp1*$^{-/-}$ mice, antigen-specific primary and memory B cell responses develop without production of antibody-secreting cells [82]. In these mice, $CXCR5^+$ $PD\text{-}1^+$ Tfh phenotype cells accumulated to large numbers in the draining and distal lymphoid tissues after protein immunization of mice lacking B cell-expressed Blimp-1 [81]. The transfer of spleen and lymph node cells from WT mice four days after secondary immunization restored Tfh cell numbers to normal levels in the spleen of B cell *Blimp1*$^{-/-}$ mice [81]. These findings suggest loss of a plasma cell-controlled negative feedback loop on Tfh cells, although the molecular mechanism explaining this effect awaits future studies.

Plasma cells appear to imprint a distinct program on responding Tfh cells because, in contrast to the adoptive transfer of antigen-pulsed dendritic cells, transfer of antigen-pulsed plasma cells diminished the expression of Bcl6 and IL-21 (but not GATA3 or IL-4) in antigen-specific Tfh cells *in vivo* [81]. Plasma cells were observed interspersed with Th cells at the T cell–B cell borders and throughout lymphoid tissue during the second week after antigen priming with evidence of contact between Th and recently formed plasma cells. In B cell *Blimp1*$^{-/-}$ mice, the primary GC reaction was significantly larger with more GC B cells [81], which may reflect the removal of the ability of Blimp-1 to repress Bcl-6. In this regard, it would be of interest to determine whether the increased numbers of GC B cells contributed to Tfh cell expansion in B cell *Blimp1*$^{-/-}$ mice.

Concluding remarks

In our efforts to understand the function of Tfh cells in immune responses, cell–cell interactions are currently viewed without a clear understanding of the context of opposing regulatory influences. The emerging focus on regulation of Tfh cells is an important step toward appreciating how Tfh cells can be manipulated to improve the design of effective immunotherapies. Tfh cells are crucial for affinity-matured antibody generated during the establishment of both immunity and autoimmunity. Thus, targeting Tfh cells could be useful in improving antibody production during immunodeficiency or for improved vaccination as well as for the inhibition of antibody production during antibody-mediated autoimmune diseases such as systemic lupus erythematosus (SLE). The observation that $CD8^+$ T cells, $CD4^+$ T cells and plasma cells exert regulatory effects on Tfh cells underscores the dynamic and interactive nature of immune cells that share common activation kinetics and pathways for migration through lymphoid tissues. In the future, it will be important to determine the influence of negative regulation of Tfh cells in terms of elimination of infected cells during immune responses to infection and failure of self tolerance during autoimmune disease. Lastly, despite significant advances in the field, the molecular mechanisms explaining competition between class I-restricted and class II-restricted responses as well as Treg and B cell suppression of Tfh cells and antibody responses remain important unanswered questions.

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