BCL6 controls contact-dependent help delivery during follicular T-B cell interactions

Highlights
- Both copies of Bcl6 gene are required for efficient follicular T-B cell entanglement
- BCL6 is required for calcium signaling in Tfh cells and CD40L delivery to B cells
- Expression of Tfh markers responds to acute perturbation of T-B cell interactions
- Defective functions of BCL6-insufficient T cells are rescued by CD40L overexpression

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In brief
BCL6 is required for Tfh cell development. Liu et al. show that BCL6 controls calcium signaling of Tfh cells, follicular T-B cell entanglement, and efficient T cell-to-B cell CD40L delivery and promotes Tfh cell maintenance partly in a T cell non-autonomous manner.
BCL6 controls contact-dependent help delivery during follicular T-B cell interactions

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SUMMARY

BCL6 is required for development of follicular T helper (Tfh) cells to support germinal center (GC) formation. However, it is not clear what unique functions programmed by BCL6 can explain its absolute essentiality in T cells for GC formation. We found that ablation of one Bcl6 allele did not appreciably alter early T cell activation and follicular localization but inhibited GC formation and Tfh cell maintenance. BCL6 impinged on Tfh calcium signaling and also controlled Tfh entanglement with and CD40L delivery to B cells. Amounts of BCL6 protein and nominal frequencies of Tfh cells markedly changed within hours after strengths of T-B cell interactions were altered in vivo, while CD40L overexpression rectified both defective GC formation and Tfh cell maintenance because of the BCL6 haploinsufficiency. Our results reveal BCL6 functions in Tfh cells that are essential for GC formation and suggest that BCL6 helps maintain Tfh cell phenotypes in a T cell non-autonomous manner.

INTRODUCTION

The germinal center (GC) is the primary site in which high-affinity, long-lived plasma cells and memory B cells develop (Berek et al., 2000; Coico et al., 1983; Jacob et al., 1991; MacLennan, 1994). GC development requires activation of antigen-specific helper T cells and their provision of contact-dependent help signals such as the CD40 ligand (CD40L) to drive optimal clonal expansion, survival, and differentiation of antigen-specific B cells (Banchereau et al., 1994; Victoria and Nussenzweig, 2012). While all helper T cells synthesize CD40L and can in principle promote B cell proliferation in vitro (Koguchi et al., 2012; Koguchi et al., 2007), follicular T helper (Tfh) cells, a subset of effector CD4+ T cells localized in the follicle (Campbell et al., 2001; Schaerli et al., 2000), are specialized in promoting the GC development in vivo (Crotty, 2011; Qi, 2016; Vinuesa et al., 2016b).

Following antigen activation, the initial encounter between antigen-specific helper T cells and B cells takes place in the interfollicular region and at the border between the T cell zone and the follicle (Garside et al., 1998; Kerfoot et al., 2011). Here, these cells form mobile but stable contacts that can last for tens of minutes to hours, invariably with migratory B cells leading the conjugate pair (Okada et al., 2005; Qi et al., 2008). As antigen-specific B cells clonally expand on helper signals provided by T cells, they migrate back into the follicle in preparation for GC formation (Cyster, 2010). A fraction of activated helper T cells concomitantly migrate into the follicle, in a manner that is independent of the cognate B cell partner (Vinuesa and Cyster, 2011). Follicular localization of activated helper T cells depends on the chemokine receptor CXCR5 (Arnold et al., 2007; Haynes et al., 2007), which senses the follicle-derived directional cue CXCL13 (Forster et al., 1996; Gunn et al., 1998), and inducible costimulator molecule (ICOS), which promotes follicular T cell motility upon engagement by ICOS ligand (ICOSL) displayed on bystander B cells (Xu et al., 2013).

BCL6 is a transcription repressor that is required for the development and maintenance of Tfh cells and Tfh cell-supported GC formation (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). However, little is known about biological processes that are both controlled by BCL6 and absolutely essential for GC formation. Chromatin-binding and transcriptomic analyses have revealed many genes that are potentially subject to BCL6-mediated suppression (Hatzis et al., 2015; Liu et al., 2016b). These include signaling molecules and transcription factors that promote terminal effector T cell differentiation and chemotactic receptors that are incompatible with follicle-directed movement.
and localization. CD4+ T cells genetically deficient in BCL6 cannot normally express CXCR5 and do not relocalize into the follicle. Although the transcription factor ASCL2 instead of BCL6 directly binds to and regulates Cxcr5 gene expression (Hatzi et al., 2015; Liu et al., 2014; Liu et al., 2016b), BCL6 can suppress the expression of the transcription factor Id2 and thus release Id2-mediated suppression of E proteins, which can positively drive Cxcr5 expression (Liu et al., 2014; Shaw et al., 2016). These results that indicate follicular localization is a key aspect for which BCL6 is required in order to promote Tfh cell development and function (Qi, 2016). However, whereas CXCR5 ablation or prevention of CCR7 downregulation in T cells causes a defect in follicular recruitment, GCs can still form inside the follicle with the help of CXCR5 null Tfh cells and suffer a relatively mild 2-fold reduction in magnitude (Arnold et al., 2007; Haynes et al., 2007). This is in sharp contrast to the outcome of T cell-specific Bcl6 ablation, which completely abrogates GC formation (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). Therefore, beyond follicular recruitment, BCL6 must regulate additional aspects of T cell biology that are uniquely important for helping B cells (Qi et al., 2014). One unique aspect of T cell help for GC formation is its dependence on physical cell-cell contact, which takes on highly dynamic forms in vivo (Liu et al., 2015; Okada et al., 2005; Qi et al., 2008; Shulman et al., 2014). Such contacts require active signaling through the T cell antigen receptor (TCR), which is further enhanced by accessory molecules such as SAP and ICOS (Cannons et al., 2010; Chu et al., 2014; Liu et al., 2015; Qi et al., 2008). It has been found that, when deprived of B cell-mediated antigen presentation for several days, frequencies of Tfh cells would decrease, even though no unique instructional signals from B cells, other than antigen presentation, are required for maintaining these Tfh cells (Baumjohann et al., 2013; Deenick et al., 2010). The simplest explanation is that, in a cell-autonomous manner, BCL6 instructs a stable program of Tfh cell differentiation, albeit requiring periodic TCR stimulation to maintain, polarize, and/or manifest (Crotty, 2014; Vinuesa et al., 2016a).

In this study, we combined static and intravital imaging analyses to investigate how BCL6 regulates cell biology of Tfh cells and found that BCL6 impinges on calcium signaling of T cells, regulates dynamics of T-B cell interactions and efficiency of help delivery to B cells, and promotes Tfh cell maintenance partly in a T cell non-autonomous manner.

RESULTS

BCL6 is haploinsufficient for recruitment-independent helper functions

Antigen-activated BCL6-deficient T cells exhibit an intrinsic defect in follicular localization, as demonstrated in mixed bone-marrow chimera (Yu et al., 2009), presumably because of their failure to properly shift the chemo-sensing preference by upregulating CXCR5 and downregulating CCR7 (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). Lack of follicular localization of BCL6-deficient T cells precludes analysis of potentially important functions for BCL6 in the follicular phase, which appears likely given the phenotypic discrepancy in GC formation between T cell-intrinsic BCL6 deficiency and CXCR5 deficiency. To circumvent this problem, we first tested T cells isolated from heterozygous Bcl6+/− mice. As shown in Figure 1A, Bcl6+/− and Bcl6+/+ OT-II T cells expressed grossly similar amounts of CXCR5 and were recruited into the follicle with comparable efficiencies 3–4 days after NP-OVA immunization (Figures 1B and 1C), as measured by the ratio between the follicular and the T zone densities of donor OT-II T cells (the follicle recruitment index, FRI; Figure S1). These data indicate that one intact allele of the Bcl6 gene is sufficient for ensuring follicular recruitment of helper T cells. Importantly, however, Bcl6+/− OT-II T cells remained significantly less competent in promoting GC formation (Figure 1D) or maintaining the associated CXCR5+ Tfh cell phenotype over a longer term, a defect that manifested 8 days after immunization (Figure 1E). Therefore, both alleles of the Bcl6 gene must be expressed to normally orchestrate helper functions inside the follicle to maintain Tfh cell phenotype and support GC formation.

BCL6 is not required for maintaining stable T-B cell interactions at the T zone-follicle border

Stable T-B cell interactions, in the form of long-lasting conjugate pairs at the T zone-follicle border (Okada et al., 2005; Qi et al., 2009) or entangled couples inside the follicle (Liu et al., 2015; Shulman et al., 2014), are required for GC formation and maintenance. To explore whether BCL6 would impinge on cognate T-B cell interactions in vivo, we first visualized Bcl6+/− and Bcl6+/+ OT-II T cells interacting with MD4 B cells 36–48 h after HEL-OVA immunization by 2-photon intravital microscopy. As previously reported, this is a time point when activated T cells form long-lasting conjugate pairs with cognate B cells at the T zone-follicle border (Okada et al., 2005; Qi et al., 2009). We found that both Bcl6+/− and Bcl6+/+ OT-II T cells formed comparably stable conjugates with MD4 B cells (Figure S2; Video S1 and S2). Therefore, in the early phase of the immune response at the T zone-follicle border, one intact Bcl6 allele is sufficient for activated T cells to maintain long-lasting contacts with cognate B cells.

BCL6 is required for T cells to maintain entangled contacts with cognate B cells

As the response progresses into the follicular stage, antigen-specific T-B cell interactions become shortened in duration but extensive in area of contact, frequently assuming the entangled configuration (Liu et al., 2015). To determine whether one intact Bcl6 allele is still sufficient for maintaining entangled contacts with antigen-specific B cells inside the follicle, CFP-expressing control Bcl6+/+ OT-II cells and dsRed-expressing Bcl6+/− or Bcl6+/− OT-II cells were co-transfered together with GFP-expressing MD4 B cells into B6 recipients that were subsequently immunized with HEL-OVA. We conducted intravital imaging 96 h post immunization, when large numbers of OT-II T cells and MD4 B cells could be seen in the follicle. As shown in Videos S3 and S4 and Figures 2A–2C, contacts between Bcl6+/− OT-II T cells and MD4 B cells were comparable in duration to those between Bcl6+/+ OT-II T cells and the same cohort of MD4 B cells; however, the normalized surface area of contacts, or the extent of entanglement measured by the surface engagement index (SEI) as previously defined (Liu et al., 2015) (Figure S3), was significantly reduced for Bcl6+/− OT-II T cells, revealing an haploinsufficient role for BCL6 in maintaining efficient T-B cell entanglement.
T-B cell entanglement is associated with heightened calcium signaling in T cells (Liu et al., 2015). We thus conducted calcium imaging of Bcl6+/− or Bcl6+/+ OT-II T cells interacting with MD4 B cells in the follicle after HEL-OVA immunization, using a fluorescence resonance energy transfer (FRET)-based calcium reporter YC-Nano-50CD (Horikawa et al., 2010), as previously used in Liu et al. (2015). As exemplified in Figure 3A and Videos S5 and S6, contacts between Bcl6+/− OT-II T cells and MD4 B cells were more frequently associated with pronounced calcium signaling than those between Bcl6+/+ T cells and MD4 B cells. The fractional change in the normalized FRET signal intensity, $\Delta R_t/R_0$, reflects the change in intracellular calcium signaling at time $t$ as compared with the no-contact reference state $0$ of the same T cell prior to a contact. The mean $\Delta R_t/R_0$ measures the average calcium signaling intensity of a T cell in contact with a B cell. Quantitatively, Bcl6+/− OT-II T cells exhibited significantly lower mean $\Delta R_t/R_0$ values than control T cells (Figure 3B; mean ± SEM of mean $\Delta R_t/R_0$ values for 65 Bcl6+/+: 134 ± 34; Bcl6+/−: 28 ± 13; $p < 0.01$). To gauge the overall productivity of contact events involving the two T cell types, we used the time-integrated calcium-response index (TICRI) as previously described (Liu et al., 2015), which is calculated by multiplying mean $\Delta R_t/R_0$ of each contact with its duration. As shown in Figure 3C, TICRIs of Bcl6+/− cells were lower by ~3-fold (Bcl6+/+: 134 ± 34; Bcl6+/−: 28 ± 13; $p < 0.01$). Combined together, these data reveal a haploinsufficient role for BCL6 in promoting optimal calcium signaling and T-B cell entanglement inside the follicle.

The absence of BCL6 impairs CD40L externalization

During T-B cell entanglement in the follicle or GCs, calcium-signaling-mediated CD40L externalization is a prerequisite for
helper defects can readily explain impairment of B cell expansion and GC formation. On the other hand, it is difficult to explain why BcI6+/− T cells exhibited grossly normal Tfh cell development at the beginning but could not maintain their Tfh cell phenotype over time. In particular, the effect of missing one copy of BcI6 gene in T cells appeared to have a more immediate and pronounced repercussion on B cells being helped rather than on the helper cells per se.

Our use of OT-II and MD4 receptor-transgenic cells, while necessary for intravitral imaging experiments to examine cell-cell interaction dynamics, might reveal functional defects that do not normally manifest. Moreover, germline BcI6 ablation might have unintended consequences. To ameliorate these concerns, we bred Cd4-cre and BcI6fl/fl mice to create T cell-specific Bcl6-deficient animals. In replicating our observations with the heterozygous germline BcI6+/− (Figure 1), we found that Cd4-cre × BcI6fl/fl OT-II T cells were able to express similar amounts of CXCR5 following 4 days of activation in vivo but were significantly less competent in promoting GC formation or maintaining the CXCR5+ phenotype by day 8 (Figure S5). Next, we transferred splenocytes from Cd4-cre, Cd4-cre × BcI6fl/fl, or Cd4-cre × BcI6fl/+ mice into sub-lethally irradiated CD45.1 SAP-deficient mice and immunized these recipients with NP-KLH (Figure 5A). In this system, normal polyclonal B cells would respond to NP-KLH with cognate T cell help provided by exogenous wild-type (Cd4-cre), Bcl6-insufficient (Cd4-cre × BcI6fl/fl), or Bcl6-deficient (Cd4-cre × BcI6fl/fl) T cells from a polyclonal repertoire. The endogenous T cells cannot support GC formation because of the SAP deficiency (Qi et al., 2008). By day 13, when the NP-KLH-induced GC response typically peaked in wild-type mice, the group with Bcl6-deficient T cells had far fewer T cells exhibiting the Tfh phenotype and almost no detectable GCs, as expected; with Bcl6-insufficient T cells as helper T cells, the magnitude of the GC response was halved, while the frequency of T cells exhibiting the Tfh phenotype was barely reduced (Figures 5B and 5C). These data indicate that, in a polyclonal response to immunization, both copies of the BcI6 gene are required for normal helper T cell activities to support GC formation, which is, again, more sensitive to the Bcl6 insufficiency in T cells than in T cell development into Tfh cells per se (appearance of CXCR5+PD-1+ T cells).

Next, to look for signs that Bcl6-insufficient T cells may not efficiently deliver CD40L to B cells in a polyclonal response in vivo, we transferred wild-type (Cd4-cre) or Bcl6-insufficient (Cd4-cre × BcI6fl/fl) OT-II T cells into SAP-deficient mice and immunized these mice with NP-OVA (Figure S6A). By day 3 post immunization, we sort-purified NP-binding B cells that were developing into GCs (Figure S6B) and conducted RNA sequencing (RNA-seq) analyses of their transcriptome. Using a gene expression signature induced by CD40 signaling in B cells (Basso et al., 2004) for enrichment analysis, we found such CD40-induced gene set expression was significantly deprived in those NP-binding B cells helped by Cd4-cre × BcI6fl/+ T cells (Figure S6C). These data suggest Bcl6 insufficiency in T cells leads to impaired CD40L signaling to B cells.

BCL6 and other Tfh cell markers depend on acute T-B cell interactions

It has been reported that, when Tfh cells are deprived of reciprocal B cell stimulation for several days, Tfh cell maintenance
is severely impaired (Baumjohann et al., 2013; Deenick et al., 2010). Based on a model of BCL6-instructed Tfh cell differentiation and maintenance (Crotty, 2014; Vinuesa et al., 2016a), one interpretation is that BCL6 may initiate an epigenetic Tfh cell program (Hatzi et al., 2015; Kroenke et al., 2012; Li et al., 2018; Liu et al., 2016a; Liu et al., 2012; Lu et al., 2011) that is inheritable but requires intermittent antigen stimulation to enforce over a long period of time. Alternatively, because markers of Tfh cells (namely CXCR5, PD-1, and BCL6) can be rapidly upregulated upon antigen stimulation in vivo, at least for naive T cells (Chen et al., 2016a; Liu et al., 2012; Lu et al., 2011) that is inheritable but

Figure 3. BCL6 is required for optimal calcium signaling during T-B cell entanglement in vivo

(A–C) dsRed-expressing MD4 B cells and YC-Nano-50CD-transduced Bcl6+/+ or Bcl6−/− OT-II T cells were visualized in B6 recipients 4 days after immunization. (A) Image sequences showing a Bcl6+/+ and a Bcl6−/− OT-II T cell interacting with MD4 B cells. CFP fluorescence from the YC-Nano-50CD reporter identifies T cells in the fluorescence overlays (columns 1 and 3), and ratio-metric FRET signals are presented in the heatmap images (columns 2 and 4). Scale bar, 20 μm. See corresponding Videos S5 and S6. (B and C) Mean ΔR0/R0 (B) and time-integrated calcium index (C) of individual contacts between Bcl6+/+ (n = 65) or Bcl6−/− (n = 64) OT-II T cells and MD4 B cells. Lines denote the means. Data are pooled from four experiments. **p < 0.01.

Figure 4. BCL6 is required for efficient CD40L delivery

OT-II T cells of indicated genotypes, 7–8 days after being activated in vivo in adoptive B6 hosts immunized with NP-OVA, were subjected to the CD40L mobilization assay as described in the STAR Methods.

(A) Representative histograms of cell surface CD40L (left) or total CD40L measured by intracellular staining (right) of Bcl6+/+ and Bcl6−/− OT-II T cells.

(B) MFI s of CD40L staining for conditions in (A); each line represents one independent experiment for each comparison setup.

(C and D) CD40L mobilization by anti-CD3 in Bcl6+/+ and Bcl6−/− OT-II T cells; representative histogram overlays (C) and changes in surface CD40L MFI (ΔMFI) following indicated stimulation (D). Each line represents one independent experiment. n.s. not significant, **p < 0.01.
it is also plausible that, even in the follicular phase of the response, reduced T-B cell interactions have a rapid repercussion on expression of markers of Tfh cells within a short time frame. Either of these two models could explain the inability of Bcl6+/−/C0 T cells to maintain Tfh cell phenotype in the follicular phase. A key distinction, however, is the timescale by which manipulation of follicular T-B cell interactions can affect the Tfh cell phenotype.

To test whether BCL6 and other markers of Tfh cells can be rapidly up- or downregulated upon acute manipulation of follicular T-B cell interactions, we took two approaches. First, we injected a DEC205 antibody linked to the ovalbumin-derived peptide epitope OVA 323-339 (a DEC-OVA) 100 h post HEL-OVA immunization of B6 mice that received OT-II T cells and MD4 B cells. Because as antigen-activated B cells upregulate DEC-205 as they develop into GC B cells, the a DEC-OVA treatment preferentially delivers OVA epitope to those B cells (Vic-tora et al., 2010). Merely 15 h after a DEC-OVA injection, OT-II T cells in the spleen markedly upregulated CXCR5, PD-1, and BCL6 (Figure 6A), leading to doubling of the frequency of CXCR5hiPD-1hi cells (Figure 6B) without changing the overall OT-II abundance. BCL6 expression in cells already gated as CXCR5 hiPD-1hi were also markedly increased by 3-fold after a DEC-OVA injection (Figure 6C). Second, we injected a CD40L antibody to block CD40-CD40L interactions between follicular OT-II and MD4 B cells. Within the same 15 h period, amounts of BCL6, CXCR5, and PD-1 decreased on OT-II T cells (Figure 6A), with their nominal Tfh cell frequency being reduced by approximately one-third (Figure 6B). Those CXCR5+/PD-1−/−Tfh cells also showed a significant decrease in BCL6 expression (Figure 6C). These results suggest any BCL6-instructed, inheritable program that specifies and maintains the CXCR5+/PD-1+/−BCL6+/ Tfh cell phenotype would not be stable for longer than 15 h without exogenous input (e.g., B cell-mediated antigen presentation). Given how brief this 15 h period, it is plausible that BCL6 also helps maintain the CXCR5+/PD-1+/− phenotype in a T cell non-autonomous manner by simply promoting cognate T-B cell interactions (Figures 3 and 4).

**Defects in GC formation and Tfh cell maintenance associated with BCL6-compromised T cells can be rescued**

If there is a T cell non-autonomous mechanism of BCL6-dependent Tfh cell maintenance as deduced above, one would predict that if CD40L is made more abundantly available in BCL6-insufficient T cells to compensate for the impaired delivery of CD40L, both the failure of normal GC formation and Tfh cell maintenance would be rescued. On the other hand, if BCL6 only instructs and maintains a Tfh cell fate in a strictly T cell autonomous manner, CD40L overexpression should not rescue the Tfh cell defect of BCL6-insufficient cells, even if it might rescue defective GC formation. To test this, we overexpressed CD40L in Bcl6+/− and Bcl6+//+ OT-II T cells by retroviral transduction, transferred these cells together with MD4 B cells into SAP-deficient mice, and examined MD4 GC formation and OT-II Tfh cell development. As shown in Figure 7A, while control-transduced Bcl6+/− OT-II T cells were not able to support normal GC formation, Bcl6+/− OT-II T cells overexpressing CD40L now supported a similar magnitude of GC formation as compared with the Bcl6+/+ counterpart. Importantly, the reduced CXCR5+/PD-1−/− Tfh cell frequency of Bcl6+/−/−Tfh cells was rectified to a comparable magnitude of control-transduced Bcl6+/−/− cells (Figure 7B). Taken together, these data suggest that, in the follicular phase, the key biological process that BCL6 controls is contact-dependent help delivery to B cells, which is not only essential for GC formation but also for Tfh cell maintenance in a T cell non-autonomous manner.
BCL6 is required for normal expression of calcium-signaling-related genes

Finally, in trying to understand how BCL6 may regulate calcium signaling in Tfh cells, which is important for T-B cell entanglement and for CD40L delivery, we noted that regulatory regions of human STIM1 gene are bound by BCL6 in human Tfh cells (Hatzi et al., 2015). We conducted mRNA sequencing of BCL6-sufficient (Cd4-cre) and BCL6-insufficient (Cd4-cre × Bcl6fl/fl) OT-II T cells sort-purified 3 days after activation in vivo by NP-OVA immunization. As shown in Figure S7, when one copy of the Bcl6 gene was missing, CXCR5+PD-1+ Tfh cells differentially expressed many genes; among those downregulated were Stim1 and PIGC1 (which code for STIM1 and PLCγ1, respectively) that both impinge on calcium signaling downstream of TCR activation. Moreover, KEGG pathway analyses revealed that the calcium-signaling-related pathway was generally upregulated in wild-type as compared with BCL6-insufficient Tfh cells (Figure S7). Together, these data support the notion that BCL6 controls follicular T-B cell interactions by regulating multiple target genes involved in antigen-triggered calcium signaling in T cells.

DISCUSSION

Our study reveals a previously unappreciated role for BCL6 in enhancing antigen-triggered calcium signaling in Tfh cells and promoting their entangled interactions with and CD40L delivery to cognate B cells, all specifically in the follicular phase of a primary response. Combined with its obligate role in regulating CXCR5 expression and associated follicular recruitment (Liu et al., 2014; Shaw et al., 2016), our findings provide a more complete picture for biological processes that BCL6 actually controls in T cells to render its essentiality for GC formation. At least in the model of protein immunization used here, early CXCR5 upregulation and Tfh cell development proceeded normally as far as one copy of Bcl6 allele is intact, whereas follicular functions of BCL6 in T cells required both alleles intact. Given the fact that BCL6 is very abundantly expressed in normal Tfh cells, we speculate those target genes involved in BCL6-dependent calcium signaling might utilize low-affinity binding sites such that only a high concentration of nuclear BCL6, achievable with expression from both alleles, can ensure productive engagement and regulation.
Although the capacity to produce CD40L is not unique to the Tfh cell subset, by definition only Tfh cells are tasked to deliver CD40L to B cells inside the follicle. CD40L delivery requires cell-cell contacts of sufficient stability, strength, and quality. The follicular microenvironment is highly dynamic, with all cells being in constant and fast migration, increasing the threshold for any T-B cell contacts to be functionally productive. This is probably the main reason why two copies of the Bcl6 gene are not required for stable T-B cell interactions at the T zone-follicle border but become essential for those entangled contacts inside the follicle.

Expression of BCL6, CXCR5, and PD-1—markers universally used to identify Tfh cells and to assess the underlying developmental program—could markedly change within 15 h, depending the status of follicular T-B cell interactions in vivo. In additional work, zDEC-OVA was found to drive such changes within 7 h (unpublished data). Our results set the upper time limit by which manifestation of a Tfh cell program can be maintained in the follicular phase of a primary response following protein immunization. These results are also consistent with the fact that none of Tfh cell-associated genes are known to be epigenetically rendered open or shut across multiple mitoses in single Tfh cells by a BCL6-mediated mechanism, even though transcription of Tfh cell-associated genes may depend on BCL6, directly or indirectly.

It can be argued that because only three main markers of Tfh cells are examined in the current study, other Tfh cell-associated molecules or pathways, such as those underlying BCL6 follicular functions reported here, might be programmed in a more long-lasting mode. This possibility warrants further investigation. However, our results do caution interpreting changes in CXCR5, PD-1, and BCL6 markers, either in terms of expression intensities or frequencies of marker-positive cells (universally used to report Tfh cell frequencies in the literature), as demonstration of differentiation, trans-differentiation, or plasticity events being induced by any experimental perturbation—particularly when the perturbation is applied days or even weeks before reading the outcome. Such perturbation could be simply changing the strength of TCR stimulation and T-B cell interactions hours before the assay readout. At the same time, we should also note the fact that T cell CXCR5 expression, besides its extreme flexibility that is conditional upon T-B cell interactions in the follicle, might be subjected to long-lasting epigenetic regulation, as implied by the previous observation that some CXCR5+ T cells do not completely lose CXCR5 expression a month after being transferred into antigen-free hosts (Lüthje et al., 2012).

While how BCL6 precisely controls STIM1 and PLCγ1 expres-sion and promotes calcium signaling in Tfh cells remains to be further investigated, it is notable that BCL6 expression in T cells partly depends on the nuclear factor of activated T cell (NFAT) transcription factor (Martinez et al., 2016; Vaeth et al., 2016), which is activated by calcium signaling (Gwack et al., 2007). This pathway potentially explains how the amount of BCL6 protein in normal Tfh cells can rapidly increase in response.
to acute antigen stimulation in vivo. A positive feedback might operate inside Tfh cells between BCL6-promoted calcium signaling downstream of TCR stimulation and NFAT-facilitated BCL6 expression. When BCL6 becomes insufficient, even though the calcium signaling is not totally abolished, a reduced efficiency in T-B cell interactions would compromise help delivery, hamper Tfh cell maintenance, and reduce GC formation. In this scenario, development and maintenance of full Tfh cell features require intrinsic BCL6 expression but invoke both cell-autonomous and non-autonomous modes of action.

The role of BCL6 in regulating calcium signaling and T-B cell interactions is reminiscent of ICOS, which co-stimulates TCR-mediated calcium signaling and thereby promotes T-B cell interactions (Liu et al., 2015). It is also reminiscent of SAP, which protects proximal TCR signaling (Chu et al., 2014; Kageyama et al., 2012; Qi, 2012) and thereby promotes T-B cell interactions (Qi et al., 2008). It is probably not a coincidence that these three T cell-expressed factors essential for GC formation all impinge on antigen-triggered signaling, all regulate physical T-B cell interactions, and none demonstrably instruct a unique signaling pathway dedicated for Tfh cell development or maintenance, with the potential exception for ICOS-TBK1-associated signaling (Pedros et al., 2016), perhaps because sufficient CD40L delivery through physical T-B cell contacts is likely the only help factor that is absolutely essential and irreplaceable for GC formation in a T-dependent B cell response (Wan et al., 2019).

**Limitations of study**

Our multiple attempts to rescue the helper activity of BCL6-insufficient T cells with STIM1 overexpression did not yield consistent results, potentially because of strict requirement for appropriate stoichiometry of STIM and ORAI proteins, which is difficult to achieve using retroviral overexpression. The current study has not established a causal link between reduced STIM1 and/or PLC-γ1 expression and defective T-B cell interactions when T cells are BCL6 insufficient.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.immuni.2021.08.003.

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**AUTHOR CONTRIBUTIONS**

H.Q. conceptualized and supervised the study. D.L., J.Y., and J.S. conducted a majority of experiments. B.L. and X.S. conducted imaging and animal experiments, Y.L. and J.S. conducted RNA-seq analysis. H.Q. wrote the paper together with D.L. and B.L. All authors contributed to data interpretation.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| anti-CD19 APC-Cy7 (clone 1D3) | eBioscience | CAT# 25-0193-82 |
| anti-B220 APC-Cy7 (clone RA3-6B2) | eBioscience | CAT# 47-0452-82 |
| anti-CD4 AF700 (clone RM 4-5) | eBioscience | CAT# 56-0042-82 |
| anti-CD4 APC (clone GK1.5) | eBioscience | CAT# 17-0041-82 |
| anti-CD4 FITC (clone GK1.5) | eBioscience | CAT# 11-0041-82 |
| anti-CD4 AF700 (clone 1M7) | eBioscience | CAT# 56-0441-82 |
| anti-CD45.1 APC (clone A20) | eBioscience | CAT# 17-0453-82 |
| anti-CD45.2 eFlour450 (clone 104) | eBioscience | CAT# 48-0454-82 |
| anti-CD95 PE-Cy7 (clone Jo2) | BD Biosciences | CAT# 557653 |
| anti-GL7 eFlour450 (clone GL7) | eBioscience | CAT# 48-5902-82 |
| anti-PD-1 PE-Cy7 (clone RMP1-30) | BioLegend | CAT# 109110 |
| anti-IgM* PE (clone DS-1) | BD Biosciences | CAT# 553517 |
| anti-IgD FITC (clone 11-26c) | eBioscience | CAT# 11-5993-82 |
| anti-CXCR5 biotinylated (clone 2G8) | BD Biosciences | CAT# 551960 |
| anti-CD40L PE (clone MR1) | BD Biosciences | CAT# 553658 |
| Streptavidin PE | BD Biosciences | CAT# 554061 |
| Streptavidin APC | BioLegend | CAT# 554067 |
| NP-PE | Biosearch Technologies | CAT# N-5070-1 |
| 7-AAD | Biotium | CAT# 40084 |
| Anti-CD40L (clone MR1) | Bio X Cell | CAT# BP0017-1 |
| Anti-CD3e (clone 145-2C11) | Bio X Cell | CAT# BP0001-1 |
| Anti-CD28 (clone 37.51) | Bio X Cell | CAT# BE0015-1 |
| **Commercial reagents** |        |            |
| Lipopolysaccharides (Escherichia coli, serotype O111:B4) | Sigma-Aldrich | CAT# L2630-25MG |
| NP-OVA | Biosearch Technologies | CAT# N-5051-10 |
| NP-KLH | Biosearch Technologies | CAT# N-5060 |
| Imject Alum Adjuvant | Thermo Fisher | CAT# 77161 |
| Lysozyme from chicken egg white | Sigma-Aldrich | CAT# L4919 |
| Albumin from chicken egg white | Sigma-Aldrich | CAT# A2512 |
| RNase Inhibitor | Takara | CAT# 2313B |
| TE buffer | Invitrogen | CAT# 12090015 |
| Triton X-100 | Invitrogen | CAT# 15596018 |
| Oligo-dT | Takara | CAT# 3806 |
| dNTP | Invitrogen | CAT# 18427013 |
| SuperScript II reverse transcriptase | Invitrogen | CAT# 18064071 |
| Superscript II first-strand buffer | Invitrogen | CAT# 18064014 |
| DTT | Solarbio | CAT# D1070-5 |
| Betaine | Sigma | CAT# 107-43-7 |
| MgCl2 | Invitrogen | CAT# AM9530G |
| KAPA HiFi HotStart ReadyMix | KAPA | CAT# Ki2601 |
| DNA clean beads | Vazyme | CAT# N411-02 |
| Nucl ease-free water | Invitrogen | CAT# 10977015 |
| TruePrep DNA Library Prep Kit V2 for Illumina | Vazyme | CAT# TD501-02 |
| SDS | Takara | CAT# 3806 |

(Continued on next page)
Continued

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed and will be fulfilled by the lead contact Hai Qi (qihai@tsinghua.edu.cn).

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Q5 HF master mix    | NEB    | CAT# M0492L |
| Tris                | Amresco| N/A        |

**Critical commercial assays**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Cytoperm/Cytofix kit| BD Biosciences | CAT# 554714 |
| Foxp3/Transcription Factor Staining Buffer Set | eBioscience | CAT# 00-5523-00 |

**Deposited data**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| mRNA sequencing data of pre-GC B cells helped by Cd4-cre or Cd4-cre x Bcl6fl/+ T cells | This paper | GSE181081 |
| mRNA sequencing data of activated Cd4-cre or Cd4-cre x Bcl6fl/+ T cells | This paper | GSE181081 |

**Recombinant DNA**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Plasmid pMSCV       | N/A    | N/A        |
| Plasmid pIE          | N/A    | N/A        |
| Plasmid for DEC205-OVA | Gift from Dr. M. Nussenzweig | N/A |
| Plasmid for YC-nano50CD reporter | N/A | N/A |

**Experimental models: Organisms/strains**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse: C57BL/6J     | Jax 664|            |
| Mouse: Sap−/−       | Gift from Dr. P. Schwartzberg (Czar et al., 2001) | N/A |
| Mouse: Bcl6−/−      | Gift from Dr. L. Staudt (Dent et al., 1997) | N/A |
| Mouse: Bcl6fl/fl    | Gift from Dr. T. Takemori (Kaji et al., 2012) | N/A |
| Mouse: CD45.1       | Jax 002014|            |
| Mouse: CD4-Cre      | Jax 022071|            |
| Mouse: GFP-expressing | Jax 4353|            |
| Mouse: CFP-expressing | Jax 4218|            |
| Mouse: dsRed-expressing | Jax 6051|            |
| Mouse: OT-II        | Jax 4194|            |
| Mouse: MD4          | Jax 2595|            |

**Software and algorithms**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| FlowJo v10          | Tree Star | N/A        |
| ImageJ              | Wayne Rasband | N/A        |
| Imaris v9           | Bitplane | N/A        |
| Prism v6            | Graphplane | N/A        |
| GSEA                | Broad Institute | N/A |
| Adobe AfterEffect   | Adobe | N/A        |
| Adobe PhotoShop     | Adobe | N/A        |
| FastQC              | v0.11.9 |            |
| Hisat2              | version 2.2.1 |        |
| Samtools            | version 1.10 |            |
| HTSeq-count         | version 0.12.4 |        |
| DESeq2 software     | R | N/A |
| Other               | Illumina | N/A        |

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**Materials availability**

Supply of the following reagents and mice are subject to MTA agreements: DEC205-OVA (Dr. M. Nussenzweig); Bcl6-fl/fl mice (Dr. L. Staudt); Bcl6/fl mice (Dr. T. Takemori).

**Data and code availability**

mRNA sequencing data have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. Microscopy data reported in this paper will be shared by the lead contact upon request. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Animal experimental models**

C57BL/6 (Jax 664), CD45.1 (Jax 002014), CD4-Cre (Jax 022071), GFP-expressing (Jax 4353), CFP-expressing (Jax 4218), dsRed-expressing (Jax 6051), OVA323-339-specific TCR transgenic OT-II (Jax 4194) and HEL-specific Ig-transgenic MD4 (Jax 2595) mice were originally purchased from the Jackson Laboratory. Bcl6-fl/fl mice (Dent et al., 1997), Bcl6/fl mice (Kaji et al., 2012) and Sap-fl/fl mice (Czar et al., 2001) were gifts from Drs. L. Staudt, T. Takemori and P. Schwartzberg, respectively. Relevant alleles were interbred to obtain desired genotypes. All mice were maintained under specific-pathogen free conditions, and used according to institutional and governmental guidelines for animal welfare.

**METHOD DETAILS**

**Cell isolation, culture and retroviral transduction**

Naive OT-II T cells, polyclonal T cells, MD4 B cells or polyclonal B cells were isolated using the negative CD4 T cell or B cell isolation kit (Miltenyi Biotec). To obtain activated T cells in vitro, naive OT-II or polyclonal B6 CD4 T cells were cultured on plates coated with anti-CD3 (8 μg/mL) and anti-CD28 (8 μg/mL). Retroviral vectors that express relevant target genes were packaged with the Plat-E system, as previously described (Xu et al., 2013). For transduction, activated T cells were spin-infected at 2200 rpm with viral supernatants in the presence of 1 μg/mL polybren (Sigma) for 2 h at 32 °C. Infected T cells were then cultivated in the presence of 10 ng/mL human IL-2 until use for in vitro experiments or for adoptive transfer.

**Adoptive transfer, immunization, and perturbation of T-B cell interactions**

For various in vivo experiments, naive or in vitro activated and retrovirally transduced OT-II T cells were intravenously injected into B6 or SAP-deficient mice, alone or in combination with MD4 B cells. For certain experiments, total splenocytes were intravenously injected into sub-lethally irradiated CD45.1 SAP-deficient mice. In such experiments, the number of CD4 T cells in the splenocyte inoculum was kept constant among different groups. Typically, splenocytes from one donor mouse were split and transferred into three recipients. For subcutaneous immunization, each recipient mouse was given either 30 μg NP-OVA (Biosearch Technology) or 30 μg HEL-OVA antigen plus 0.5 μLPS in alum (Thermo Scientific). For intraperitoneal immunization, each recipient mouse was given 100 μg NP-OVA (Biosearch Technology) or 100 μg NP-KLH (Biosearch Technologies) or 50 μg HEL-OVA antigen plus 1 μLPS in alum (Thermo Scientific). For CD40L blockade in the early follicular phase, HEL-OVA-immunized B6 recipients of OT-II T cells and MD4 B cells were intravenously given 200 μg αCD40L antibody or PBS 50-60 h post immunization. For αDEC-OVA and CD40L blockade experiments, HEL-OVA-immunized B6 recipients of OT-II T cells and MD4 B cells were intravenously given 50 μg αDEC-OVA or 100 μg αCD40L antibody or PBS 100 h post immunization, a time point well into the follicular phase of the response.

**Flow cytometry**

Single-cell suspension of splenic or lymph node cells were incubated with 10% 2.4G2 culture supernatants to block Fc receptors and then stained with indicated antibodies in MACS buffer (PBS supplemented with 1% FBS and 5mM EDTA). Staining reagents include: APC-Cy7 anti-CD19 (1D3), APC-Cy7 anti-B220 (RA3-6B2), AF700 anti-CD4 (RM 4–5), APC anti-CD4 (GK1.5), FITC anti-CD4 (gk1.5), AF700 anti-CD44 (IM7), APC anti-CD45.1 (A20), eF450 anti-CD45.2 (104), PE-Cy7 anti-CD95 (Jo2), eFlour450 anti-GL7 (GL7), PE-Cy7 anti-PD-1 (RMP1-30), PE anti-IgMα (DS-1), FITC anti-IgD (11–26c) from eBioscience; biotinylated anti-CXCR5 (2G8), PE anti-CD40L (MR1), streptavidin PE (Cat 554061) and streptavidin APC (Cat 554067) from BD Biosciences. Dead-cell exclusion was based on 7-AAD staining (Biotium), and non-singlet events were excluded with FSC-H/FSC-W and SSC-H/SSC-W. Isotype-matched non-specific antibodies were purchased from the corresponding companies. Cells were stained with primary antibodies for 60-90 min, washed, and then with secondary reagents for 30 min on ice. For intracellular staining, cells were stained using Cytoperm/Cytofix kit (BD Biosciences) according to the manufacturer’s protocol. All flow-cytometry data were collected on an LSR-II or Aria III (BD).

**Immunohistochemistry**

To examine T cell distribution in the draining lymph node, tissues were fixed with paraformaldehyde, and sections were stained with EF450-anti-IgD and APC-anti-CD3, mounted with the ProlongGold Antifade reagent (Invitrogen), and imaged with an Olympus FV1000 upright microscopy using 20 × lens.
**CD40L mobilization of T cells ex vivo**

As described previously (Liu et al., 2015), OT-II T cells of indicated genotypes were transferred into B6 mice, which were subsequently immunized with NP-OVA intraperitoneally. 7 to 8 days later, splenocytes were stimulated by anti-CD3 (2 μg/mL) for 15 min in the presence of an anti-CD40L staining antibody (clone MR1) to capture externalized CD40L on the cell surface. Cells were further stained for additional surface markers before analyzed by flow cytometry.

**Intravital imaging**

Intravital two-photon imaging of mouse inguinal lymph nodes were done as described previously (Liu et al., 2015), including measurement of T-B cell contact extents by contact duration and SEI. To visualize T cell calcium signaling during T-B cell contacts in vivo, YC-nano50CD reporter was transduced into Bcl6+/+ or Bcl6+/− OT-II T cells. Ratiometric images of calcium signals were analyzed with the RatioPlus plugin of ImageJ. Quantitative analyses were conducted by the method established previously (Liu et al., 2015). Adobe Photoshop and AfterEffect were used to annotate and prepare image sequences and to make playback videos.

**RNA-seq and Gene Set Enrichment Analysis (GSEA)**

CD4-Cre × Bcl6+/+ or control CD4-Cre OT-II T cells were transferred into CD45.1 Sap−/− mice (5 × 10^5 per recipient), which were subcutaneously immunized with 40 μg NP-OVA plus 0.4 μg LPS in alum one day after the cell transfer. At day 3 post immunization, CXCR5hiPD-1hi Tfh cells were sort-purified from the draining lymph node and prepared for RNA-seq analysis. Two technical repeats of ~200 cells per sample from each of 3 mice were included. To analyze NP1IgDB220+ pre-GC B cells, cells from recipients of the same group were pooled to sort-purify 4 200-cell repeats. The SMART-Seq2 protocol for single-cell analysis was adapted and optimized for analyzing ~200 cells. All libraries were sequenced on a HiSeq × Ten sequencer (Illumina). After pre-processed using FastQC (v0.11.9), raw sequences were aligned to the Mus musculus reference genome (GRCm38) using Hisat2 (version 2.2.1) and then sorted with Samtools (version 1.10). Reads were counted in genes with the utilization of HTSeq -count (version 0.12.4). Gene expression was calculated with the DESeq2 software in R. Heatmaps and volcano plots were generated with pheatmap and ggplot2 R functions, respectively. GSEA analysis was carried out using the GSEA software from the Broad Institute.

**Statistical analysis**

Statistics and graphing were done with Prism (Graphpad). Unless indicated otherwise, two-tailed Student’s t test was used to compare end-point means of different groups.
Supplemental information

BCL6 controls contact-dependent help delivery during follicular T-B cell interactions

Dan Liu, Jiacong Yan, Jiahui Sun, Bo Liu, Weiwei Ma, Ye Li, Xingxing Shao, and Hai Qi
Figure S1. Definition of follicle recruitment index (FRI), related to Figure 1.

The follicle recruitment index (FRI) to quantify the efficiency of OT-II follicular localization. The experimental system and color code as in Figure 1B.
Figure S2. Comparable duration of contacts between MD4 B cells and $Bcl6^{+/+}$ or $Bcl6^{+/+}$ OT-II T cells at the T-B border, related to Figure 2.

B6 mice that received transfer of GFP-expressing MD4 B cells, control CFP-expressing $Bcl6^{+/+}$ and dsRed-expressing test $Bcl6^{+/+}$ or test $Bcl6^{+/+}$ OT-II T cells were immunized with HEL-OVA. Draining lymph nodes were intravitaly imaged 36-48 hours later, a time point at which T-B contacts were mainly seen at the T-B border. Contact durations were quantitated (see corresponding Movie S1 and S2). Each dot represents one contact. Data pooled from two independent imaging experiments, each of which involved at least 2 recipient mice.
Figure S3. Definition of surface engagement index (SEI), related to Figure 2.

In maximum-intensity projection, the proportion of T-cell perimeter (blue) that is engaged by an interacting B cell (line fragment in yellow) is traced over time and the maximum proportion reached during a continuous contact incidence is defined as the surface engagement index.

\[ \text{SEI} = \max \left( \frac{\text{Length of contact}}{\text{T-cell perimeter}} \right) \]
Figure S4. Follicular phase of CD40L delivery is required for GC formation, related to Figure 4.

B6 mice that received transfer of MD4 B cells and OT-II T cells were immunized with HEL-OVA and treated with αCD40L antibody (200 μg) or PBS 50-60 hours later. A, Representative FACS profile and frequency of FAS$^{hi}$ GL7$^{hi}$ GC B cells in IgM$^{++}$ CD19$^{+}$ B cells at 5 days post immunization. B, Frequencies of IgM$^{++}$ MD4 GC cells in total CD19$^{+}$ B cells. Each symbol represents one mouse, and lines denote means. One of three experiments with similar results is shown. *** $P < 0.001$. 
Figure S5. Defective helper functions due to BCL6 haploinsufficiency as demonstrated with T-cell-specific conditional BCL6 deficiency, related to Figure 5.

A, The experimental scheme. B, Representative histograms and MFIs of surface CXCR5 expression on OT-II T cells of indicated Bcl6 genotypes in draining lymph nodes 4 days after NP-OVA immunization. C,D, Representative flow-cytometry profiles and frequencies of FAS<sup>hi</sup>GL7<sup>hi</sup> GC B cells in total B220<sup>-</sup> B cells (C) or CXCR5<sup>hi</sup>PD-1<sup>hi</sup> Tfh cells in OT-II T cells (D) 8 days after NP-OVA immunization in CD45.1 Sap<sup>-/-</sup> recipients. Each symbol represents one mouse, and lines denote the means. Data are pooled from 2 independent experiments. n.s., not significant. * P<0.05.
Figure S6. Reduced CD40 signaling signature in antigen-activated B cells helped by BCL6-insufficient T cells, related to Figure 5.

mRNA sequencing of pre-GC B cells helped by CD4-Cre×Bcl60/+ or CD4-Cre OT-II T cells. A, The experimental scheme. B, Enrichment analysis comparing pre-GC antigen-specific B cells helped by CD4-Cre×Bcl60/+ (“HET”) or by CD4-Cre (“WT”) OT-II T cells for a gene set upregulated by CD40L stimulation of Ramos cells previously reported (Basso et al., 2004). NES, normalized enrichment score; FDR, false discovery rate.
Figure S7. Reduced calcium signaling signature in BCL6-insufficient Tfh cells, related to Figure 3.

mRNA sequencing analysis of CD4-Cre\textit{Bcl6}\textsuperscript{fl/+} or CD4-Cre OT-II Tfh cells 3 days post immunization. A, The experimental scheme. B, The gating strategy for sorting Tfh cells. C, A volcano plot highlighting genes differentially expressed (\textit{P}<0.05, fold change \textgeq 1.2) between CD4-Cre\textit{Bcl6}\textsuperscript{fl/+} and CD4-Cre Tfh cells. Genes upregulated in CD4-Cre\textit{Bcl6}\textsuperscript{fl/+} Tfh cells are in red, and downregulated are shown in blue. Circles highlight \textit{Stim1} and \textit{Pleck1}. D, Enrichment analysis comparing CD4-Cre\textit{Bcl6}\textsuperscript{fl/+} (“HET”) or CD4-Cre (“WT”) OT-II T cells for genes in the calcium signaling pathway as defined in the KEGG database. NES, normalized enrichment score; FDR, false discovery rate.