Transcription factor achaete-scute homologue 2 initiates follicular T-helper-cell development

Xindong Liu1,2, Xin Chen1, Bo Zhong1, Aibo Wang1,2, Xiaohu Wang1,2, Fuliang Chu3, Roza I. Nurieva4, Xiaowei Yan4, Ping Chen5, Laurens G. van der Flier6, Hiroko Nakatsukasa7, Sattva S. Neelapu1, Wanjun Chen8, Hans Clevers8, Qiang Tian4, Hai Qi1, Lai Wei6 & Chen Dong1,2

In immune responses, activated T cells migrate to B-cell follicles and develop into follicular T-helper (TFH) cells, a recently identified subset of CD4+ T cells specialized in providing help to B lymphocytes in the induction of germinal centres. Although Bcl6 has been shown to be essential in TFH-cell function, it may not regulate the initial migration of T cells or the induction of the TFH program, as exemplified by C-X-C chemokine receptor type 5 (CXCR5) upregulation. Here we show that expression of achaete-scute homologue 2 (Ascl2)—a basic helix-loop-helix (bHLH) transcription factor—is selectively upregulated in TFH cells. Ectopic expression of Ascl2 upregulates CXCR5 but not Bcl6, and downregulates C-C chemokine receptor 7 (CCR7) expression in T cells in vivo, as well as accelerating T-cell migration to the follicles and TFH-cell development in vivo in mice. Genome-wide analysis indicates that Ascl2 directly regulates TFH-related genes whereas it inhibits expression of T-helper cell 1 (Th1) and TFH17 signature genes. Acute deletion of Ascl2, as well as blockade of its function with the Id3 protein in CD4+ T cells, results in impaired TFH-cell development and germinal centre response. Conversely, mutation of Id3, known to cause antibody-mediated autoimmunity, greatly enhances TFH-cell generation. Thus, Ascl2 directly initiates TFH-cell development.

The development of TFH cells is initiated by and dependent on their movement out of the T-cell zone and into the B-cell follicle. This migration process is regulated by upregulation of CXCR5 as well as downregulation of both CCR7 and P-selectin glycoprotein ligand 1 (PSGL1)2-6. TFH cells have unique developmental regulation and Bcl6 was reported to be selectively expressed in TFH cells4,5. However, although Bcl6 potentiates TFH-cell generation in vivo, recent data suggest that it may not regulate CXCR5 upregulation by activated T cells or their migration to B-cell follicles in vivo4,6. Hence, the transcriptional mechanisms underlying initial TFH-cell commitment remain unclear.

Recently, we observed that a bHLH-domain-containing transcription factor, Ascl2, was highly expressed in CXCR5+ Bcl6− cells in comparison with CXCR5− Bcl6− T cells1. Interestingly, CXCR5+ Bcl6+ T cells also exhibited upregulation of Ascl2 messenger RNA expression4, suggesting that its upregulation may precede that of Bcl6. Moreover, the Ascl2 gene locus was marked with active chromatin marker tri-methylated histone H3 lysine 4 (H3K4me3) in TFH and, to a much less extent, Tfh2 cells, but not in other T-cell subsets, whereas the other TFH-regulating genes Bcl6 (refs 7–9), Maf10, Batf11,12 and Irf4 (ref. 13) were uniformly associated with H3K4me3 in all T-cell subsets (Extended Data Fig. 1a). To validate these results, we sorted three subpopulations of cells (CXCR5+RFP−, CXCR5−RFP− and CXCR5−RFP+), RFP, red fluorescent protein) from Bcl6−RFP reporter mice immunized with keyhole limpet haemocyanin (KLH) emulsified with complete Freund’s adjuvant (CFA) (Fig. 1a), and found that Ascl2 was highly expressed in TFH cells at both mRNA and protein levels (Fig. 1b and Extended Data Fig. 1b). Also, Ascl2 expression was closely correlated with that of CXCR5 (Fig. 1b) and was higher in TFH cells than in other T-cell subsets (Fig. 1c). In human T cells, expression of Ascl2 as well as CXCR5 and Bcl6 was found in human tonsil CXCR5+PF1 TFH cells (Fig. 1d, e). Collectively, these results suggest that Ascl2 is highly expressed in TFH cells and that its expression may precede that of Bcl6.

Bcl6 and Batf are necessary in TFH-cell development13,14, whereas Stat5 inhibits TFH-cell development4,15. Overexpression of Bcl6 or Batf, or Stat5 deficiency, failed to increase Ascl2 expression (Extended Data Fig. 1c). None of the known stimuli, including anti-CD3, anti-CD28, anti-ICOS, interleukin (IL)-6 and IL-21, nor their combination, upregulated Ascl2 expression in T cells (Extended Data Fig. 1d). Ascl2 was previously shown to be a target of canonical Wnt signalling in intestinal stem cells16, and we also found that Ascl2 and CXCR5 but not Bcl6-expression in CD4+ T cells could be upregulated by TWS119 (ref. 16; Fig. 1f, g). Hence, our findings suggest that Ascl2 is unique in its ability to induce follicular homing ability and decrease IL-2 signalling in TFH cells17,18.

In the development of TFH cells, retroviral overexpression of Ascl2 was conducted in CD4+ T cells, leading to substantial induction of CXCR5 expression in over 30% of transduced cells, whereas overexpression of Bcl6, Batf or Maf in purified T cells did not (Fig. 2a and Extended Data Fig. 2a). Ascl2 overexpression increased Cxcr5 mRNA expression by ~60 fold (Fig. 2b), without blocking Bcl6, Prdm1, Batf, Il2rb1a, Cd40lg, Icos, Lap1a, Bta and Il21 expression (Fig. 2c). CXCR5 expression was equally induced by Ascl2 in wild-type, Bcl6−/− and Batf−/− CD4+ T cells in vitro (Fig. 2d). Thus, our findings suggest that Ascl2 is unique in its ability to induce CXCR5 protein expression in CD4+ T cells in vitro. CCR7 and PSGL1 (Selpg) as well as CD25 (Il2rα) and CD122 (Il2rb) expression were downregulated in Ascl2-overexpressing T cells (Fig. 2e and Extended Data Fig. 2b), probably accounting for increased follicular homing ability and decreased IL-2 signalling in TFH cells17,18.

We then assayed the role of Ascl2 in vivo by transferring Ascl2-transduced OT-II cells. In Tcrb−/− recipient mice, at day 2 after immunization with 4-hydroxy-3-nitrophenyl (NP)-ovalbumin (OVA) in CFA, neither CXCR5 nor Bcl6 expression were detectable in the vector-transduced control group, whereas Ascl2 overexpression strongly increased CXCR5 (Fig. 2f, g). By contrast, ectopic expression of Bcl6 did not promote TFH-cell generation at this time point (Extended Data Fig. 2d, e). At day 6 after immunization, Ascl2 overexpression induced a higher percentage of CXCR5hiBcl6hi TFH cells (Fig. 2f, g). Accordingly, germinal centre B cells and the total area of the germinal centre at day 8 in C57BL/6 mice receiving Ascl2-transduced T cells were significantly increased (Fig. 2h, i). Anti-NP immunoglobulin (Ig)M, IgA, IgG1 as well as IgG3 titres were increased in Tcrb−/− mice, whereas

1Tsinghua University School of Medicine, Beijing 100084, China. 2Department of Immunology, MD Anderson Cancer Center, Houston, Texas 77054, USA. 3Department of Lymphoma and Myeloma, MD Anderson Cancer Center, Houston, Texas 77054, USA. 4Institute for Systems Biology, Seattle, Washington 98103, USA. 5Laboratory of Immunology, National Eye Institute, NIH, Bethesda, Maryland 20892-1858, USA. 6Hubrecht Institute-KNAW and University Medical Center Utrecht, Uppsalalaan 6, 3584 CT Utrecht, the Netherlands. 7National Institute of Dental and Craniofacial Research, NIH, Bethesda, Maryland 20892-2190, USA. 8State Key Laboratory of Ophthalmology, Sun Yat-sen University, Guangzhou 510275, China. 9Present addresses: College of Life Sciences, Wuhan University, Wuhan 430072, China (B.Z.); Somantix B.V., Padualaan 8, 3584 CH Utrecht, the Netherlands (L.G.v.d.F.).

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Ascl2 is selectively expressed in both mouse and human T<sub>FH</sub> cells. a. Three populations of CXCR5<sup>+</sup>Bcl6–RFP<sup>hi</sup> (red), CXCR5<sup>+</sup>Bcl6–RFP<sup>lo</sup> (blue) and CXCR5<sup>+</sup>Bcl6–RFP<sup>–</sup> (black) cells were sorted from dLNs in Bcl6-RFP mice immunized with KLH emulsified in CFA subcutaneously. b. Ascl2, CXCR5 and Bcl6 transcriptional expression in sorted cells. c. Ascl2 mRNA expression among in-vivo-generated T<sub>FH</sub>, naïve, T<sub>H1</sub>, T<sub>H2</sub> and T<sub>H17</sub> cells by quantitative polymerase chain reaction with reverse transcription (RT–PCR). d. Flow cytometric analysis of human tonsil CD4<sup>+</sup> T cells by CXCR5 and PD1 staining. e. The expression of Ascl2, Cxcr5, Bcl6 and Gata3 mRNA in sorted cells. f. CXCR5 expression in CD4<sup>+</sup> T cells activated by anti-CD3/anti-CD28 in the presence of TWS119 (1 μM) (an inhibitor of glycogen synthase kinase 3β (GSK-3β)) for 3 days. All experiments were repeated at least three times with similar results. b, c, e. Bar graphs show the relative level of mRNA as mean ± standard deviation (s.d.), n = 3 per group. *P < 0.05, **P < 0.01, two-tailed t-test.

IgG2a and IgG2b were not affected by Ascl2-overexpressing T cells (Fig. 2k), consistent with IgG2a switching being primarily mediated by extrafollicular T cells.<sup>3</sup>

We next assessed whether Ascl2 could promote T-cell follicular homing in vivo. Ascl2-overexpressing OT-II cells preferentially accumulated in follicles (Fig. 2l), and even in the germinal centre (Fig. 2j), in comparison with control vector-infected T cells. Given that Bcl6 overexpression does not affect early T<sub>FH</sub>-cell generation (Extended Data Fig. 2d, e) and T<sub>FH</sub>-cell migration<sup>4</sup>, these observations collectively demonstrate that, by contrast with Bcl6, Ascl2 promotes T-cell migration to the follicles and the initiation of T<sub>FH</sub>-cell development.

To investigate the mechanism of Ascl2-controlled T<sub>FH</sub>-cell generation, we performed microarray analysis, and found that the expression of 293 genes was changed by more than twofold by Ascl2 overexpression. Cross-referencing the current data set of Ascl2 versus vector with our previous T<sub>FH</sub>-cell versus non-T<sub>FH</sub>-cell data<sup>4</sup> revealed that 85 of the 293 genes affected by Ascl2 were directly associated with T<sub>FH</sub>-cell differentiation: 22 genes were upregulated and 63 genes were downregulated<sup>4</sup> (Fig. 3a, b and Supplementary Table 1). The chemokine receptors CXCR5 and CXCR4, which are germinal centre T<sub>FH</sub>-related receptors<sup>11</sup>, were at the top of the upregulated gene list, whereas T<sub>H1</sub>-related genes (Il12rb1, Tbx21, Ifng and Gzmb) and the T<sub>H17</sub>-related aryl hydrocarbon receptor (Ahr) gene were greatly suppressed by Ascl2 (Fig. 3a). When comparing Ascl2-RV–GFp-infected (GFp, green fluorescent protein; RV, retroviral vector) CXCR5<sup>+</sup> Cd40lg (<i>Il12rb1</i>) and the TH17-related gene expression in sorted cells.

Ascl2-induced CXCR5<sup>+</sup> T cells and CXCR5<sup>–</sup> T cells with in-vivo-generated T<sub>FH</sub> and non-T<sub>FH</sub> cells<sup>4</sup>, we found that Ascl2-induced CXCR5<sup>+</sup> T cells were more similar in gene expression to T<sub>FH</sub> cells (Extended Data Fig. 2f, g), with ~350 genes commonly expressed in these cells (Extended Data Fig. 2h).

We further examined the effect of Ascl2 on T<sub>H1</sub>, T<sub>H2</sub> and T<sub>H17</sub>-cell differentiation. As shown in Extended Data Fig. 3a, overexpression of Ascl2 suppressed both T<sub>H1</sub> and T<sub>H17</sub> differentiation and induced CXCR5 expression. Ascl2 had no effect on TGF-β-induced Foxp3 expression but induced CXCR5<sup>+</sup> regulatory T (T<sub>reg</sub>) cells, suggesting that it may also be related to follicular T regulatory (T<sub>FH</sub>)-cell generation<sup>28</sup>. Under T<sub>H2</sub>-polarized conditions, Ascl2 enhanced IL-4 expression, whereas it inhibited the expression of Gata3, IL-5 and IL-13 (Extended Data Fig. 3a–c), in agreement with recent studies that showed IL-4 but not Gata3, IL-5 or IL-13 expression in T<sub>FH</sub> cells<sup>21</sup>. Also, we observed that Ascl2 increased IL-4 but not IL-21 production in vivo (Extended Data Fig. 3d, e). Therefore, Ascl2 promotes T<sub>FH</sub> gene expression and inhibits T<sub>H1</sub>-, T<sub>H2</sub>- and T<sub>H17</sub>-related gene expression.

We next assessed Ascl2 target genes by chromatin immunoprecipitation with high-throughput sequencing (ChIP-seq). The analysis revealed a total of 10,028 Ascl2-binding peaks, among which 41% and 36% were enriched in intronic and intergenic regions, respectively (Fig. 3c). Only 20% of Ascl2-binding sites were located at promoter regions (Fig. 3c). Further comparison of global Ascl2-binding sites with the Ascl2-regulated gene list showed that 145 among 4,374 Ascl2-bound genes were transcriptionally regulated by Ascl2 (Fig. 3d).

In particular, the Cxcr5 locus was found to have multiple Ascl2-binding sites in the conserved non-coding sequence (CNS) regions (Fig. 3f, g). Moreover, these Ascl2-binding sites at the Cxcr5 locus were confirmed in in-vivo-generated T<sub>FH</sub>: two strong binding sites at intronic regions (CNS5 and CNS4-3), and three at distal promoter region (CNS1-1, CNS1 and CNS1-4) (Fig. 3f–h). Of note, the strongest Ascl2-binding peak was in the CNS5 region, which is consistent with the E47- (bHLH family member) binding site at the Cxcr5 locus<sup>22</sup> (Fig. 3f–h), implying a potentially redundant role of E2A in transcriptional regulation of the Cxcr5 gene<sup>23</sup>.

To examine the functional significance of Ascl2 binding in regulating Cxcr5 expression, we introduced Id3, an inhibitor of the E-box protein<sup>20</sup>, into Ascl2-overexpressing OT-II cells by retroviral infection, and observed a substantial reduction in Ascl2-regulated Cxcr5 expression (Extended Data Fig. 4a); this reduction was due to the inhibition of Ascl2 binding at the Cxcr5 locus, as revealed by ChIP assay (Extended Data Fig. 4b). Additionally, a luciferase reporter assay showed that CNS5 and CNS1 were responsive to Ascl2 binding (Extended Data Fig. 4c, d). Together, these results provide evidence that Ascl2 is involved in the direct control of T<sub>FH</sub>-cell programming.
Figure 2 | Ascl2 expression induces the Tm program. a, Flow cytometry analysis of surface CXCR5 expression from empty-RV-GFP, Ascl2-RV-GFP and Bc6-RV-GFP retrovirus-infected T cells, respectively. b, Sorted GFP+ T cells were subjected to the measurement of Cxcr5 mRNA by quantitative RT–PCR. c, Measurement of gene expression, including Cxcr5, Bc6, Prdm1, Batf, Sh2d1a, Cd40lg, Icos, Pdcd1, Btag and Il21. d, Surface CXCR5 expression in Ascl2-RV-GFP- and vector-virus-infected wild-type (WT), Bc6−/− and Batf−/− T cells. e, CCR7, PSGL1, CD25 and CD122 expression by flow cytometry analysis. Dashed line, isotype; solid line, Ascl2-RV-GFP; shaded area, empty-RV-GFP. f–i, Ascl2-RV-GFP- or empty-RV-GFP-transduced GFP+ OT-II cells were transferred into naive Tcrb−/− mice subsequently immunized with NP-OVA/CFA. f, At day 2 and day 6, flow cytometry analysis of donor cells was undertaken with staining for CXCR5 and Bc6 (n = 4). g, Quantification of CXCR5+ and Cxcr5− Bc6+ donor-derived T cells. h, Germinal centre B cells (GL7hiFAShi) in recipient mice (n = 4). i, Quantification of germinal centre (GC) B cells. j, Ascl2-RV-GFP- or empty-RV-GFP-transduced GFP+ OT-II cells were transferred into congenic mice. Four days later, each mouse was immunized subcutaneously with OVA (30 μg)/Alum/LPS. At day 8, dLNs were collected and subjected to histochromoscopy analysis. Green, GFP; red, Bc6; blue, anti-IgD; scale bar, 100 μm). k, Graphs show data as mean ± s.d. empty-RV-GFP, n = 5; Ascl2-RV-GFP group (n = 12). l, Titres of NP-specific antibodies in serum from mice on day 8 after immunization (n = 4). m, Distribution of Ascl2-RV-GFP- and vector-infected GFP+ OT-II donor cells in IgD+B-cell follicles from dLNs in mice immunized with OVA/Alum/LPS for 4 days. Scale bar, 100 μm. Dot graph represents distribution with the ratio of donor cells in the B-cell follicle (fol.) versus the T zone, displayed as mean ± s.d. empty-RV-GFP, n = 21; Ascl2-RV-GFP, n = 15. All experiments were repeated at least two times with similar results. b, c, g, i–j. Graphs show data as mean ± s.d., two-tailed t-test. NS, not significant.
As ClrA, Batf1,2,3, and IRF4 (ref. 13) are required in Tfh-cell differentiation, we compared genome-wide occupancy of these transcriptional factors, and found that Ascl2-bound genes hardly correlated with those bound by Maf (Extended Data Fig. 5a). For instance, IL-21 is directly regulated by Maf4, but not by Ascl2 (Extended Data Fig. 5b). Additionally, there was no binding site for Maf at the Ascl2 locus (data not shown), or vice versa (Extended Data Fig. 5b), suggesting that Ascl2 and Maf are functionally independent in Tfh cells. A large proportion of Ascl2 occupancy colocalized with Batf/IRF4-binding sites (Extended Data Fig. 5c), including at the CNS of gene loci including Cxcr5, Cxcr4, Ccr7, Selplg1, Il2ra, Il2rb, Ifng, Tbx21, Il2 and Rorc. This data strongly support Ascl2 acting as a specific regulator in Tfh cells.

Tfh cells provide important help to B cells in the induction of efficient anti-virus antibodies during viral infection5-26. To address the functional roles of Ascl2, we generated Ascl2fl/fl/CD4-Cre mice, in which T cells were developmentally intact (data not shown), and assessed the requirement of Ascl2 in Tfh-cell development in vivo with influenza virus infection. After intranasal infection, Ascl2fl/fl/CD4-Cre mice developed gradual body weight loss from day 3 to day 9, whereas control littermate mice recovered after day 8 (Extended Data Fig. 6a). At day 9 post-infection (d.p.i.), viral haemagglutinin (HA) mRNA expression in the lungs of Ascl2fl/fl/CD4-Cre mice was over fivefold higher than that in control mice (Extended Data Fig. 6b), whereas Cxcr5+ Bcl6+ Tfh cells in lung draining lymph nodes (dLNs) had
Ascl2 gene deletion is performed using a Cre-expressing viral vector and examined for Tfh cell differentiation. Ascl2 fl/fl CD4-Cre mice show a reduced Tfh cell population in lymph nodes compared to control mice. This reduction is consistent with increased E47 activity in a recent report. Ascl2 seems to induce Id3-induced TFH blockage. Furthermore, these data suggest that Ascl2 deletion at an early developmental stage has an earlier function than Bcl6 in Tfh-cell development. To overcome the inducible compensation mechanism, we deleted the Ascl2 gene acutely using a Cre-expressing retrovirus and examined Tfh-cell differentiation in chimaeric mice reconstituted with Ascl2 fl/fl/ERT2-Cre bone marrow cells. As shown in Extended Data Fig. 7c, d, in chimaeric mice, Ascl2 deficiency reduced TCR-β CD69 mature T cells, whereas mice receiving only Ascl2 fl/fl/CD4-Cre cells had fewer defects in T-cell maturation. Accordingly, after immunization with KLH/CFA, Tfh-cell generation was inhibited in the Ascl2 fl/fl/CD4-Cre counterpart from mixed chimaeric mice. These data suggest that Ascl2 deletion at an early developmental stage induces compensatory mechanisms to allow T-cell maturation.

To overcome the inducible compensation mechanism, we deleted the Ascl2 gene acutely using a Cre-expressing retrovirus and examined Tfh-cell differentiation. As shown in Fig. 4b, Ascl2 deficiency in this case resulted in an absolute impairment in Tfh development in vivo. By contrast, Bcl−/− OT-II cells showed intact Cxcr5 expression and homing ability at day 3 after immunization (Extended Data Fig. 8a–e). Moreover, Id3 overexpression impaired Tfh-cell generation and germinal centre responses (Fig. 4c and Extended Data Fig. 9a–c). Conversely, Id3 deficiency enhanced the Tfh-cell population (Fig. 4d). Together with the observation that Bcl6 overexpression could not rescue Id3-induced Tfh blockage (Extended Data Fig. 9d), Ascl2 seems to have an earlier function than Bcl6 in Tfh-cell development.

Because Ascl2 can form heterodimers with three other bHLH family members, including E2-2 (also known as TCF4), E47 (also known as TCF3) and HEB (also known as TCF12) in human cells, this suggests that the partial defect in Tfh-cell differentiation in Ascl2 fl/fl/CD4-Cre mice might be caused by compensation from other bHLH members. Indeed, Tfh cells and germinal centre responses were normal in Ascl2 fl/fl/CD4-Cre mice after immunization with KLH in CFA (data not shown). A substantial enhancement of E47 expression was noticed in naive T, Tfh and even non-Tfh cells from Ascl2 fl/fl/CD4-Cre mice compared with control mice (Extended Data Fig. 7a), and expression of E47 also increased Cxcr5 expression in CD4+ T cells (Extended Data Fig. 7b), consistent with a recent report that increased E47 activity in Id3−/− mice is associated with increased Tfh-like cells. Furthermore, we examined T-cell maturation and Tfh-cell differentiation in chimaeric mice receiving both Ascl2 fl/fl/CD4-Cre and Ascl2 fl/fl/CD4-Cre, or only Ascl2 fl/fl/CD4-Cre bone marrow cells. As shown in Extended Data Fig. 7c, d, in chimaeric mice, Ascl2 deficiency reduced TCR-β CD69 mature T cells, whereas mice receiving only Ascl2 fl/fl/CD4-Cre cells had fewer defects in T-cell maturation. Accordingly, after immunization with KLH/CFA, Tfh-cell generation was inhibited in the Ascl2 fl/fl/CD4-Cre counterpart from mixed chimaeric mice (Extended Data Fig. 7e–g). These data suggest that Ascl2 deletion at an early developmental stage induces compensatory mechanisms to allow T-cell maturation.
To confirm this, we examined T<sub>FH</sub>-cell differentiation in either mixed chimaeric mice receiving equal numbers of Ascl2<sup>fl/fl</sup>/ETR2-Cre and Ascl2<sup>+/−</sup>/ETR2-Cre bone marrow cells (Fig. 4c), or intact Ascl2<sup>fl/fl</sup>/ETR2-Cre mice (data not shown). After tamoxifen treatment and KLH/CFA immunization, Ascl2 deletion inhibited T<sub>FH</sub>-cell differentiation and germinal centre response in vivo (Fig. 4e and data not shown). Therefore, these data verify that Ascl2 is intrinsically necessary for T<sub>FH</sub>-cell differentiation.

We have identified a new player—the Ascl2 transcription factor—that is crucial for T<sub>FH</sub>-cell development and function. On the one hand, similar to Bcl6, Ascl2 acts as a novel suppressor of T<sub>FH1</sub>-, T<sub>FH2</sub>- and T<sub>FH7</sub>-cell differentiation. On the other hand, Ascl2 uniquely regulates T<sub>FH</sub>-cell migration and development by increasing CXCR5 and CXCR4 expression, and suppressing CCR7 and PSGL1 expression, and IL-2 signalling. Our data indicate that Ascl2 and Bcl6 act to program T<sub>FH</sub>-cell generation. Activated T cells by antigen-presenting cells gain Ascl2 expression, which allows their migration towards B-cell follicles. At the T–B border, cognate B cells provide another signal for precursor T<sub>FH</sub> cells to increase Bcl6 expression, which completes T<sub>FH</sub> polarization and germinal centre formation. Therefore, Id3 and Ascl2 may serve as an early checkpoint during T<sub>FH</sub>-cell development in promoting appropriate antibody responses to infection while keeping autoimmune diseases in check (Extended Data Fig. 10). Further investigation into this axis may offer new ways to modulate antibody responses in infection and autoimmunity.

**METHODS SUMMARY**

**Mice, immunization and infection.** Ascl2<sup>fl/fl</sup> mice were generated previously and had been backcrossed with C57BL/6 for at least six generations. Mice were immunized with antigens emulsified in CFA subcutaneously (100 μl each mouse). Influenza virus A/Puerto Rico/8 (PR8, H1N1) was purchased from Charles River Laboratories. The output of the Solexa Analysis Pipeline was converted to browser-extensible data (BED) files, and the data were viewed in the UCSC genome browser.
METHODS

Mice. Mice were housed in specific pathogen-free animal facilities at the MD Anderson Cancer Center and Tsinghua University, and were used according to protocols approved by the Institutional Animal Care and Use Committee. Six to eight week old mice were used for all experiments, and were randomly allocated into treatment groups. C57BL/6 mice were from the National Cancer Institute. ETR2-Cre, CD4-Cre, OT-II, B6SJL, Tcrb
\(^{-/-}\), Tcrb
\(\beta\)-/-, Rag1
\(-/-\) and Batf
(-/-) mice were from Jackson Laboratories. Stat5
\(-/-\) (ref. 15), Id3
\(-/-\) (ref. 29), Bcl6-RFP (ref. 4) and Bcl6
\(-/-\) (ref. 8) mice were previously described.

The Ascl2
\(-/-\) mice were generated previously and had been backcrossed with C57BL/6 for at least six generations. Ascl2
\(-/-\) mice were bred with CD4-Cre mice to generate Ascl2
\(-/-\)/CD4-Cre and Ascl2
\(-/-\)/CD4-Cre control mice. Ascl2
\(-/-\) mice were crossed with ETR2-Cre mice to generate Ascl2
\(-/-\)/ETR2-Cre and Ascl2
\(-/-\)/ETR2-Cre control mice. Ascl2 deletion in Ascl2
\(-/-\)/ETR2-Cre cells was achieved by administering 200 μmol tamoxifen (5 mg mL
\(^{-1}\)) in sunflower seed oil subcutaneously or intraperitoneally (i.p.) every other day for a total of 5 days.

KLH, NP-KLH, OVA and NP-OVA Immunization. Mice and their wild-type controls (6–8 weeks old; three per group) were immunized with antigen (0.5 mg mL
\(^{-1}\)) emulsified in CFA (0.5 mg mL
\(^{-1}\)) subcutaneously (100 μl per mouse). After immunization, these mice were killed and analysed individually. Germline centre B cells were determined by staining with FITC-labelled anti-GL7, PE-labelled anti-FAS and PerCP-labelled anti-B220 monoclonal antibody (Pharmingen). T911 cells were determined by staining with PerCP-labelled anti-CD4 monoclonal antibody and biotinylated anti-CXCR5 monoclonal antibody (Pharmingen), followed by APC-labelled streptavidin (Jackson ImmunoResearch Laboratories) and surface staining by PE-labelled anti-PD1 monoclonal antibody or intracellular staining by PE-labelled anti-Bcl6 monoclonal antibody (Pharmingen). Sera from immunized mice were collected, and antigen-specific IgM, IgA, IgG1, IgG3, IgG2a and IgG2b antibodies were measured by using ELISA. Briefly, isotype-specific antibodies to NP were measured in plates coated with NP-2 BSA using the SBA Clonotyping System (Southern Biotech). Titres were presented as the maximum serum dilution exceeding 1:5-fold above the background average. KLH- or OVA-specific titres were measured in a threefold serial dilution onto plates pre-coated with 100 μg mL
\(^{-1}\) KLH or OVA.

Influenza virus infection. Influenza virus A/Puerto Rico/8 (PR8, H1N1) was purchased from Charles River Laboratories. Six to eight week old Ascl2
\(-/-\)/CD4-Cre mice (3–5 mice per group) were anaesthetized by i.p. injection with ketamine and infected intranasally with a dose of PR8 influenza virus
\(^{-12}\) lethal to 50% of animals tested (LD
\(_{50}\)) of 0.5. Mice were monitored daily, and weight loss was recorded. To analyse influenza-virus-specific germinal centre responses, T911 and germinal centre B cells from lung mediastinal lymph nodes and spleens were analysed by flow cytometry. BALF and sera were collected from virus-infected mice on 9 d.p.i. Virus-specific IgG antibodies were measured using ELISA. Briefly, serum samples were added in a threefold serial dilution onto plates pre-coated with heat-inactivated virus. Bound antibodies were detected by the incubation of horseradish peroxidase-conjugated anti-mouse total IgG (1:10,000, Southern Biotech) antibodies. Lung viral titre was monitored by examining influenza virus HA and neuraminidase (NA) gene expression using real-time RT–PCR as previously described
\(^{27}\).

Retroviral transduction and T-cell differentiation. Naive CD4
\(^{+}\) CD25
\(^{-}\)/CD44
\(^{low}\) CD62L
\(^{high}\) T cells from Ascl2
\(\beta\)-/-/OT-II, OT-II or C57BL6 mice were FACs sorted and activated with plate-bound anti-CD3e (clone, 2C11) and anti-CD28 (clone, 37.51) under neutral conditions. Thirty-six hours after activation, cells were infected by retroviruses Ascl2-RV-GFP, Bcl6-RV-GFP, Batf-RV-GFP, MaF-RV-GFP, Id3-RV-GFP, Cre-RV-GFP, E47-RV-CFP (a gift from Y. Zhuang) or control empty vector (empty-RV-GFP or empty-RV-CFP). One day after infection, GFP cells were FACs sorted for adoptive transfer, or followed by re-stimulation with pre-coated anti-CD3e. Also, virus-infected cells were polarized under Th1 (IL-12, anti-IL-4), Th2 (IL-4, anti-INF-γ), Th17 (TGF-β, IL-6, IL-23, anti-IL-4, anti-INF-γ) and Treg (TGF-β) conditions. Four days after culture, cells were re-stimulated with PMA and ionomycin in the presence of Golgi-stop for 4 h, after which IFN-γ, IL-4/IL-5/IL-13, IL-17- and Foxp3-expressing cells were analysed using intracellular staining. Cytokines including IL-4, IL-5 and IL-13 from Ascl2-RV-GFP-infected Th2 and Th1 cells were measured by ELISA. Cell transfer numbers for different cells were as follows: 1 × 10
\(^{6}\) for GFP+ T cells; 8 × 10
\(^{6}\) for bone marrow cells.

Mixed chimaeric bone marrow mice. To generate mixed bone marrow chimaeras, T-cell-depleted bone marrow cells were obtained from Ascl2
\(-/-\)/CD4-Cre or Ascl2
\(-/-\)/ETR2-Cre and their respective congenic wild-type (CD45.1 and CD45.2) mice, and mixed at a ratio of 1:1 before being transferred into irradiated Rag1
\(-/-\) (750 rad) mice. Six to eight weeks later, the reconstituted mice were subject to immunization and analysis as described earlier.

Microarray and ChiP-seq. Total cellular RNA was extracted from cells transduced with vector or Ascl2-expressing retrovirus that were purified by their expression of GFP marker with TRIZol reagent (Invitrogen). DNA microarray labelling and analysis were performed by microarray core at the Institute for System Biology. Approximately 10 μg of RNA was labelled and hybridized to GeneChip Mouse Gene 1.0 ST Array (Affymetrix) according to the manufacturer’s protocols. Expression values were defined with GeneChip Operating Software (GCOSS) software.

ChiP-seq was performed as described previously
\(^{29}\). Briefly, sorted T cells were fixed by 1% paraformaldehyde, and this was followed by digestion with Mnsae cocktail (Active motif). Chromatin from 5 × 10
\(^{6}\) cells was used for each ChiP experiment. Antibodies against H3K4me3 (catalogue no. 07–473, Millipore), H3K27me3 (catalogue no. 07–449, Millipore) and Ascl2 monoclonal antibody (clone 7E2, Millipore) were used. The pull-down DNA fragments were blunt-end ligated with Solexa adaptors, and amplified for sequencing. The DNA fragments were sequenced with an Illumina 1G Analyzer at the Institute for System Biology. The output of the Solexa Analysis Pipeline was converted to browser-extensible data (BED) files, and the data were viewed in the UCSC genome browser.

Immunohistochemistry. The protocol for immunohistochemical staining was as described previously
\(^{30}\). Staining reagents included biotinylated PNA (Vector), AlexaFluor 647 anti-B220, biotinylated anti-IgD, AlexaFluor 488 anti-CD45.2, and streptavidin AlexaFluor 568 (Invitrogen). All stained slides were mounted with ProlongGold antifade reagents (Invitrogen) and examined with an Olympus FV1000 confocal system.

Real-time RT–PCR analysis. Real-time RT–PCR analysis of Bcl6, Batf, CXCR5, and streptavidin AlexaFluor 568 (Invitrogen). All stained slides were mounted with ProlongGold antifade reagents (Invitrogen) and examined with an Olympus FV1000 confocal system.

Statistics. Unless specifically indicated otherwise, comparison between two different groups was done with unpaired two-tailed Student’s t-tests or two-way analysis of variance (ANOVA). All P values below 0.05 were considered significant. Statistical analysis was performed with Graphpad Prism 6.

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Extended Data Figure 1 | Ascl2 exhibits unique epigenetic regulation in Tfh cells, and its expression is dependent on Wnt signalling.  

a, Genome-wide histone modifications (H3K4me3, permissive marker; H3K27me3, suppressive marker) across Bcl6, Maf, Batf, Irf4 and Ascl2 loci in T-cell subsets (in vivo Tfh and non-Tfh data sets were newly generated; the rest were derived from the Gene Expression Omnibus database (accession GSE14254)28).  
b, Flow cytometric analysis of Ascl2 expression in three populations of activated CD44^hi CD4^+ T cells in dLNs from Bcl6-RFP mice: CXCR5^hi Bcl6^hi (red), CXCR5^lo Bcl6^lo (blue) and CXCR5^lo Bcl6^lo (black) cells.  
c, Quantitative RT–PCR measurement of Ascl2, Bcl6 and Batf expression in Bcl6-RV-GFP, Batf-RV-GFP and control vector-infected CD4^+ T cells; wild-type (WT) and Stat5^2/2 naive CD4^+ T cells were cultured under Tfh conditions, or together with IL-6, respectively. Ascl2, Bcl6 and Batf transcriptional expression was measured by qRT–PCR. d, Quantitative RT–PCR measurement of Ascl2 in CD4^+ T cells cultured under indicated conditions. e, Quantitative RT–PCR measurement of Cxcr5 and Bcl6 in control or TWS119- (1 μM) treated T cells. All experiments were repeated at least three times with similar results. Bar graphs show the relative level of mRNA as mean ± s.d., n = 3 per group. *P < 0.05, **P < 0.01, two-tailed t-test. NS, not significant.
Extended Data Figure 2 | Ascl2 regulates a selective subset of Tfh-relevant genes. a, Flow cytometry analysis of CXCR5 expression in CD4+ T cells transduced with vector control, Ascl2-RV-GFP, Bcl6-RV-GFP, Batf-RV-GFP and Maf-RV-GFP. Data are representative of two independent experiments. b, Transcriptional expression of Ccr7, Pogl1, Il2ra and Il2rb in Ascl2-RV-GFP- or control-vector-infected T cells was measured by quantitative RT–PCR. Data are representative of two independent experiments. Bar graphs show the relative level of mRNA as mean ± s.d., n = 3, two-tailed t-test. c, Transcriptional expression of Ccr7, Pogl1, Il2ra and Il2rb in Ascl2-RV-GFP-or control-viral-vector-infected GFP+ OT-II cells were adoptively transferred into naive congenic mice, followed by subcutaneous OVA/CFA immunization. At day 2 after immunization, flow cytometry analysis of donor-derived cells in dLNs was carried out with CXCR5 and PD1 staining. Data are representative of two independent experiments (n = 3). e, Quantification of donor-derived Tfh cells. Bar graphs show mean ± s.d., n = 3, two-tailed t-test. NS, not significant. f, Vector-transduced GFP+CXCR5−CD4+ T (RV) cells, Ascl2-RV-GFP-infected GFP+CXCR5− (CXCR5−) and GFP+CXCR5+ (CXCR5+) CD4+ T cells were sorted and subjected to microarray assay. g, Hierarchical clustering and principal component analysis (PCA) were applied on seven microarray data sets including RV, CXCR5− and CXCR5+, as well as Tfh and non-Tfh cells (derived from GSE40068). h, The clustered heatmap of ~350 genes from RV, CXCR5−, CXCR5+, Tfh and non-Tfh cells. The colour coding applies to gene expression level (log2) with 0 as a median.
Extended Data Figure 3 | Regulation of T<sub>H</sub>-cell differentiation by Ascl2.

**a**, Naive CD<sup>4</sup><sup>+</sup> T cells from C57BL6 mice were activated under neutral conditions and infected with Ascl2-RV-GFP or control vector (empty-RV-GFP) virus, followed by continuous culture under TH1, TH2, TH17, inducible (i)T<sub>r</sub>reg, and T<sub>FH</sub>-like conditions for 3–4 days. Quantification of signature genes by intracellular staining and real-time RT–PCR. 

**b**, c, Ascl2-RV-GFP- or control-vector-transduced T cells were cultured under TH2 conditions for 4 days. **b**, After re-stimulation with PMA and ionomycin for 5 h, TH2-related gene expression, including IL-4, IL-5 and IL-13, was measured by flow cytometric analysis. **c**, GFP<sup>+</sup> T cells were sorted and re-stimulated by plate-bound anti-CD3, and transcriptional expression of IL-4, IL-5 and IL-13 was measured by quantitative RT–PCR; cytokines in supernatants from re-stimulation were subjected to ELISA analysis. 

**d**, e, Ascl2-RV-GFP- or control-vector-transduced OT-II cells were adoptively transferred into naive congenic mice, followed by subcutaneous OVA/CFA immunization for 7 days. After re-stimulation with OVA, flow cytometry analysis of donor-derived cells from dLNs was carried out with intracellular IL-4 and IL-21 staining. **e**, GFP<sup>+</sup> donor-derived T cells were sorted from dLNs, re-stimulated with anti-CD3, and subjected to quantitative RT–PCR measurement of Il21 and Il4 mRNA expression. All data are representative of two independent experiments. 

**a**–**c**, e, Bar graphs show mean ± s.d., n = 3, two-tailed t-test. NS, not significant.
Extended Data Figure 4 | CXCR5 expression is directly mediated by Ascl2. a, Naive CD4⁺ T cells were pre-activated and transduced with empty-RV-GFP, Id3-RV-GFP, Ascl2-RV-GFP or Ascl2-RV-GFP, together with Id3-RV-GFP retrovirus. a, Flow cytometry analysis of CXCR5 expression in retrovirus-infected T cells. Data are representative of two independent experiments.

b, GFP⁺ cells were sorted from Ascl2-RV-GFP or Ascl2-RV-GFP plus Id3-RV-GFP retrovirus-infected T cells, and subjected to Ascl2-binding analysis of the Cxcr5 gene locus using a ChIP assay. Primer information is listed in Supplementary Table 2. Data are representative of two independent experiments. c, Luciferase reporter assay of enhancer activity for the Ascl2-bound region of the Cxcr5 locus. CNS-containing PGL3 plasmid was transfected with either empty-RV-GFP or Ascl2-RV-GFP into the EL4 T-cell line. Bar graph shows mean ± s.d., n = 3, **P < 0.01, two-tailed t-test. d, Map of Cxcr5 gene locus and Ascl2-binding peaks at the Cxcr5 locus. Arrows indicate the Ascl2-responsive CNS region.
Extended Data Figure 5 | Coordinated function of Ascl2 and Batf/IRF4 in regulating TFH-related genes. a–g, ChIP-Seq data for Maf, Batf and IRF4 were derived from GSE40918 (ref. 25). a, Venn diagram of ChIP-seq peaks from Ascl2 and Maf. b, Distribution of ChIP-seq peaks by Ascl2, Batf and IRF4 on gene loci including Bcl6, Prdm1, Ascl2 and Maf. c, Venn diagram of ChIP-seq peaks from Ascl2, Batf and IRF4. d, Distribution of ChIP-seq peaks by Ascl2, Batf and IRF4 on gene loci including Cxcr5, Cxcr4, Ccr7, Selplg1, Il2, Il2ra and Il2rb. Blue frame represents the colocalization of peaks. 

f, Distribution of ChIP-seq peaks by Ascl2, Batf and IRF4 on TH1-related Tbx21 and Ifng gene loci. g, Distribution of ChIP-seq peaks by Ascl2, Batf and IRF4 on TH17-related Rorc, Il17a, Il17f and Il21 gene loci. ChIP-seq assay of Ascl2 was performed on Ascl2-overexpressing T cells cultured under TH0 conditions. ChIP-seq assays of Maf, Batf and IRF4 were performed on TH0 cells by D. R. Littman’s group, derived from GSE40918 (ref. 25).
Extended Data Figure 6 | Loss of Ascl2 in CD4⁺ T cells leads to impairment of germinal centre responses during influenza virus infection. a–j, Control and Ascl2⁻/⁻/CD4-Cre mice were infected intranasally with influenza virus A/PR8. a, The body weight of control and Ascl2⁻/⁻/CD4-Cre mice was monitored daily after infection. b, Mice were killed at day 9 after infection, and viral titre in the lungs was assessed by measurement of active HA gene expression with quantitative RT–PCR. c, Virus-specific total IgGs in the sera were measured by ELISA. d, Flow cytometry analysis of germinal centre B cells (GL7⁺/FAS⁺) in lung dLNs from influenza-infected control and Ascl2⁻/⁻/CD4-Cre mice. e, Frequencies of Tfh cells in spleens from influenza-infected control and Ascl2⁻/⁻/CD4-Cre mice. f, Frequencies of germinal centre B cells in spleens from influenza-infected control and Ascl2⁻/⁻/CD4-Cre mice. g, After 9 d.p.i., the CD4⁺/CD8⁺ T-cell ratio was measured in the lung, BALF, spleen and dLNs from control and Ascl2⁻/⁻/CD4-Cre mice. h, Flow cytometry analysis of granzyme B and IFN-γ production from both CD4⁺ and CD8⁺ T cells in dLNs. i, j, Mixed chimaeric mice were reconstituted with both Ascl2⁺/⁺/ETR2-Cre and Ascl2⁻/⁻/ETR2-Cre bone marrow cells at a ratio of 1:1. Eight weeks later, chimaeric mice were administered 200 μl tamoxifen (5 mg ml⁻¹) in sunflower seed oil i.p. every other day for a total of 5 days, and this was followed by influenza virus A/PR8 infection. At 9 d.p.i., the CD4⁺/CD8⁺ T-cell ratio and Tfh-cell generation was measured in dLNs (i) and spleens (j). All data are representative of three independent experiments. Graphs display mean ± s.d., n = 5 per group. *P < 0.05, **P < 0.01, two-way ANOVA (a, c), two-tailed t-test (b, e, f, g–j). NS, not significant.
Extended Data Figure 7 | In the absence of Ascl2, bHLH family member E47 may have a redundant role in TFH-cell differentiation. a, Quantitative RT–PCR measurement of E47 expression in naive CD4^+ T cells from Ascl2^{fl/fl}/CD4-Cre and littermate control mice; T<sub>FH</sub> and non-T<sub>FH</sub> cells were obtained from dLNs of Ascl2^{fl/fl}/CD4-Cre and littermate control mice immunized with KLH in CFA, and the expression of E47 was measured by real-time RT–PCR. b, Flow cytometry analysis of CXCR5 expression in T cells infected with E47-RV-CFP or control vector retrovirus. c–g, T-cell-depleted bone marrow cells were obtained from Ascl2^{+/+}/CD4-Cre (CD45.1^+ CD45.2^-) and Ascl2^{fl/fl}/CD4-Cre (CD45.2^+) mice and mixed at a ratio of 1:1 or 0:1 before being transferred into irradiated Rag1^{-/-} recipient mice (8 × 10^6 cells per mouse). c–g, Eight weeks later, mice were either used for measurement of thymic T-cell maturation (c, d), or immunized with KLH in CFA for monitoring peripheral T<sub>FH</sub>-cell differentiation (e–g). c, Flow cytometry analysis of T-cell maturation in thymus of mixed chimaeric mice containing both Ascl2^{+/+}/CD4-Cre and Ascl2^{fl/fl}/CD4-Cre bone marrow cells. d, Flow cytometry analysis of T-cell maturation in thymus of chimaeric mice containing only Ascl2^{fl/fl}/CD4-Cre bone marrow cells. e, Seven days after immunization, T<sub>FH</sub> cells in dLNs of mixed chimaeric mice (Ascl2^{+/+}/CD4-Cre and Ascl2^{fl/fl}/CD4-Cre) were measured by flow cytometry. f, Flow cytometry analysis of T<sub>FH</sub> cells in dLNs of chimaeric mice (Ascl2^{fl/fl}/CD4-Cre). g, The percentages of both CD4^+ T cells and B220^+ B cells in dLNs of mixed chimaeric mice (Ascl2^{+/+}/CD4-Cre and Ascl2^{fl/fl}/CD4-Cre). All data above are representative of two independent experiments. Graphs show mean ± s.d., n = 3 per group, two-tailed t-test. NS, not significant.
Extended Data Figure 8 | Loss of Bcl6 in CD4⁺ T cells does not affect early T<sub>FH</sub>-cell homing ability in vivo. Equal amounts of Cre-RV-GFP-transduced wild-type (WT)/OT-II, Bcl6<sup>−/−</sup>/OT-II and Ascl2<sup>fl/fl</sup>/OT-II cells were transferred into congenic mice, and this was followed by subcutaneous OVA/CFA immunization. a, At day 3 after immunization, flow cytometry analysis of donor-derived T<sub>FH</sub>-cell generation was carried out with CXCR5 and PD1 staining. b, Quantification of donor-derived CXCR5⁺ T cells. c, At day 8 after immunization, donor-derived T<sub>FH</sub>-cell generation was examined using CXCR5 and PD1 staining. d, Quantification of donor-derived CXCR5⁺PD1⁺ T<sub>FH</sub> cells. e, At day 3 after immunization, dLNs were isolated and subjected to histochemical staining of B-cell follicles and donor T cells. Green, GFP; red, B220; scale bar, 100 μm, n = 4. All data are representative of two independent experiments. Graphs display mean ± s.d., n = 4, two-tailed t-test, NS, not significant.
Extended Data Figure 9 | Ectopic expression of Id3 inhibits TFH-cell generation in vivo. a–c, Naive OT-II CD4^+ T cells were activated and transduced with Id3-RV-GFP or control viral vector (empty-RV-GFP) for 3 days. GFP^+ T cells were then sorted and transferred into naive congenic mice that were subsequently immunized with OVA/CFA. a, At day 4 after immunization with OVA/Alum/LPS, immunohistochemical staining of section slides of dLNs was carried out. Red, IgD^+ B cells; green, GFP^+ donor-derived OT-II cells. Data are representative of two independent experiments, n = 6. Scale bar, 100 μm. b, Quantification of GFP^+ OT-II cell distributions in dLNs. Data are representative of two independent experiments. Dot graph shows mean ± s.d., n = 17, two-tailed t-test. c, Optical density (OD) values of OVA-specific antibodies in serum from mice on day 7 after immunization with OVA/CFA, measured by threefold serial dilution in OVA- (100 μg ml^-1) coated plates. Data are representative of two independent experiments. Graphs show mean ± s.d., n = 6. *P < 0.05, **P < 0.01, one-way ANOVA. d, Naive CD4^+ OT-II cells were pre-activated and co-transduced with empty-RV-GFP/empty-RV-hCD2, empty-RV-GFP/Bcl6-RV-hCD2, Id3-RV-GFP/Bcl6-RV-hCD2, or Id3-RV-GFP/empty-RV-hCD2. Sorted hCD2^+ GFP^+ OT-II cells were transferred into congenic mice, followed by subcutaneous OVA/CFA immunization for 7 days. Measurement of donor-derived TFH cells was carried out with CXCR5 and PD1 staining. Data are representative of two independent experiments. Bar graphs show mean ± s.d., n = 3, two-tailed t-test.
Extended Data Figure 10 | Schematic model of the sequential roles of Ascl2/Id3 and Bcl6/Blimp1 during TFH-cell differentiation. Ascl2 expression plus simultaneous Id3 reduction in activated CD4\(^+\) T cells orchestrates T cells to migrate towards B-cell follicles and initiate the TFH program by inducing expression of the chemokine receptors CXCR5 and CXCR4, and suppressing expression of CCR7 and PSGL1 and the IL-2 signal pathway, as well as TH1 and TH17 differentiation. Upon interacting with cognate B cells at the T–B border, CXCR5\(^+\) T cells begin to increase Bcl6 expression, which eventually facilitates TFH maturation in B follicles and germinal centre formation.