Bcl6 and Maf Cooperate To Instruct Human Follicular Helper CD4 T Cell Differentiation

Mark A. Kroenke, Danelle Eto, Michela Locci, Michael Cho, Terence Davidson, Elias K. Haddad and Shane Crotty

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Follicular helper CD4 T (Tfh) cells are a specialized subset of T cells that provide help to B cells (1). The defining characteristics of Tfh cells are their ability to colocalize with B cells in the follicle and their ability to provide specialized help to B cells in the form of specific cytokines and cell surface molecules. Tfh cells are required for germinal centers (GCs). In the absence of Tfh cells, severe reductions are seen in the development of Ag-specific IgG responses, memory B cells, and memory plasma cells (1). A long-term Ab response is the central attribute of most successful human vaccines (2, 3); therefore, understanding Tfh cells is important for developing truly rational vaccine-development strategies. Furthermore, Tfh cells are potentially useful biomarkers in human vaccine clinical trials. However, to harness this biology, the transcriptional control of human Tfh cell differentiation and function must be understood. Finally, several autoimmune diseases are characterized by the presence of increased GCs and autoantibodies (4, 5), which are dependent on Tfh cells in multiple models (6, 7). Therefore, understanding the transcriptional regulators of Tfh cells is also valuable for developing therapeutics against major autoimmune diseases.

Bcl6 was originally identified as a human B cell oncogene. A significant percentage of individuals with follicular lymphoma or diffuse large B cell lymphoma have mutations in Bcl6 (8, 9). Bcl6 also plays an important role in acute lymphoblastic leukemia (10), and it is a critical transcription factor for GC B cells (8, 11, 12). Extensive studies on Bcl6 in B cells revealed that it regulates GC B cell survival, cell cycle control, and somatic hypermutation (8, 10, 13–15). Recently, it was determined that Bcl6 is important in mice for Tfh cell differentiation (16–18). However, it is not known whether and how Bcl6 controls Tfh cell differentiation of human T cells (1). Importantly, there is limited evidence about which proteins in CD4 T cells are explicitly regulated by Bcl6 in any species, because there are no obvious parallels between Bcl6-expressing B cells and Bcl6-expressing CD4 T cells (13). How specific transcription factors imbue different differentiated CD4 T cell types with distinct functional characteristics has been a topic of great interest in immunology (19, 20). In this article, we describe that Bcl6 instructs core modules of Tfh cell biology in human CD4 T cells, but Bcl6 does not regulate all human Tfh cell features. The transcription factor Maf contributes significantly to IL-21 production by Tfh cells. Furthermore, Maf and Bcl6 work together to maximally induce important Tfh cell characteristics.

Materials and Methods

Human samples

Fresh human tonsils were obtained from the University of San Diego Hillcrest Medical Center, the National Disease Resource Interchange, or the Children’s Hospital of Orange County. The majority of tonsils were from adults. Informed consent was obtained from all donors. Tonsils were homogenized using wire mesh and passed through a cell strainer to make a single-cell suspension. Mononuclear cells were isolated using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). All protocols were approved by the La Jolla Institute for Allergy and Immunology (LIAI) and National Disease Resource Interchange, LIAI and University of San Diego, or LIAI and Children’s Hospital of Orange County Institutional Review Boards.

Bcl6 and Maf Cooperate To Instruct Human Follicular Helper CD4 T Cell Differentiation

Mark A. Kroenke,* Danelle Eto,* Michela Locci,* Michael Cho, † Terence Davidson, ‡ Elias K. Haddad, § and Shane Crotty*

Follicular helper CD4 T (Tfh) cells provide B cells with signals that are important for the generation of high-affinity Abs and immunological memory and, therefore, are critical for the protective immunity elicited by most human vaccines. Transcriptional regulators of human Tfh cell differentiation are poorly understood. In this article, we demonstrate that Bcl6 controls specific gene modules for human Tfh cell differentiation. The introduction of Bcl6 expression in primary human CD4 T cells resulted in the regulation of a core set of migration genes that enable trafficking to germinal centers: CXCR4, CXCR5, CCR7, and EBI2. Bcl6 expression also induced a module of protein expression critical for T–B interactions, including SAP, CD40L, PD-1, ICOS, and CXCL13. This constitutes direct evidence for Bcl6 control of most of these functions and includes three genes known to be loci of severe human genetic immunodeficiencies (CD40L, SH2D1A, and ICOS). Introduction of Bcl6 did not alter the expression of IL-21 or IL-4, the primary cytokines of human Tfh cells. We show in this article that introduction of Maf (c-Maf) does induce the capacity to express IL-21. Surprisingly, Maf also induced CXCR5 expression. Coexpression of Bcl6 and Maf revealed that Bcl6 and Maf together to maximally induce important Tfh cell characteristics. The Journal of Immunology, 2012, 188: 000–000.

*Division of Vaccine Discovery, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037; †Children’s Hospital of Orange County at Mission Hospital, Mission Viejo, CA 92691; ‡Division of Otolaryngology, University of California San Diego, San Diego, CA 92103; and §Vaccine and Gene Therapy Institute of Florida, Port St. Lucie, FL 34987

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Address correspondence and reprint requests to Dr. Shane Crotty, Division of Vaccine Discovery, La Jolla Institute for Allergy and Immunology, 9420 Athena Circle, La Jolla, CA 92037. E-mail address: shane@liai.org

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Abbreviations used in this article: GC, germinal center; LIAI, La Jolla Institute for Allergy and Immunology; LV, lentivirus; MFI, mean fluorescence intensity; ORF, open reading frame; SAP, SLAM-associated protein; SEB, staphylococcal enterotoxin B; Tfh, follicular helper CD4 T.
Flow cytometry and sorting
For intracellular staining, cells were restimulated with 25 ng/ml PMA and 1 μg/ml ionomycin in the presence of 10 μg/ml brefeldin A for 3 h. Cells were fixed with BD PhosFlow Fix Buffer I and permeabilized with BD PhosFlow Perm/Wash Buffer I. All cells were stained using a BD FACSAria. All Tfh cell sorts were initially gated on CD4^{+}CD19^{-} 7-AAD^{-} cells. CD45RO^{-} cells were confirmed to be naive by co-staining for CD45RA. Naive B cells were CD19^{+} CD20^{-} CD3^{-} IgD^{+}. The following anti-human Abs were used: CD45RO (clone UCHL1), CD45RA (clone HI100), CD19 (clone HIB19), CD38 (clone HFT2), CD20 (clone 2H7), CD27 (clone O323), PD-1 (clone J105), ICOS (clone ISA-3), CXCR4 (clone 12G5), CD40L (clone 24-31), CXCR5 (clone 2F7), and CD4 (clone RPA-T4) (eBioscience, San Diego, CA); CXCR5 (clone RF8B2), CCR7 (clone 3D12), Bcl6 (clone K112-91), and IgG (clone I6-2-6) (BD Biosciences, Franklin Lakes, NJ); and SAP (clone 1D12) (Cell Signaling Technology, Danvers, MA) (21). For intracellular staining, the following Abs were used: IL-4 (clone MP4-25D2; BioLegend, San Diego, CA); IL-21 (clone 64DEC17), and IFN-γ (clone 4S.B3) (eBioscience); and CXCL13 (clone 53610; R&D Systems, Minneapolis, MN) (22). Anti-CD19 (clone HIB19) was used as the mouse IgG1 isotype for the CXCL13 stain.

B/T coassay culture
Sorted tonsil CD4^{+} T cell subsets CD45RO^{-} CXCR5^{-}, CD45RO^{+} CXCR5^{+}, CD45RO^{+} CXCR5^{hi}, and CD45RO^{+} CXCR5^{*} were cocultured with autologous naive B cells (4 \times 10^{7} cells/well) in the presence of staphylococcal enterotoxin B (SEB; 250 ng/ml; Sigma-Aldrich) in 96-well V-bottom plates. In blocking experiments, endogenous IL-21 was neutralized by the inclusion of IL-21-RF (20 μg/ml; R&D Systems) or an isotype-matched control. Secretion of IgM was determined by ELISA after 7 d in culture. Ninety-six-well Poly-lysor microtiter plates (Nunc) were coated overnight with monoclonal anti-human IgM (1 μg/ml; AbbVie) in PBS. All samples were run in duplicate, and IgM from the coculture supernatant was detected with HRP-conjugated human IgM Fc (Hybridoma Reagent Laboratory).

T cell cultures
Sorted cells were stimulated with anti-CD3/CD28 Dynabeads (Invitrogen, Carlsbad, CA) in 96-well flat-bottom plates at a starting density of 7.5 \times 10^{4} cells/well. Beads were used at a concentration of 1 μl/well. RPMI 1640 medium with 10% FCS was supplemented with 2 ng/ml recombinant human IL-7. Cells were split as necessary.

Lentiviral vectors and transductions
pHAGE vector containing a PGK promoter, a multiple cloning site, and ZsGreen downstream of an IRES (Invitrogen, Carlsbad, CA) in 96-well flat-bottom plates at a starting density of 7.5 \times 10^{4} cells/well. Beads were used at a concentration of 1 μl/well. RPMI 1640 medium with 10% FCS was supplemented with 2 ng/ml recombinant human IL-7. Cells were split as necessary.

T cell cultures
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Profile vectors containing a PGK promoter, a multiple cloning site, and ZsGreen downstream of an IRES (Invitrogen, Carlsbad, CA) in 96-well flat-bottom plates at a starting density of 7.5 \times 10^{4} cells/well. Beads were used at a concentration of 1 μl/well. RPMI 1640 medium with 10% FCS was supplemented with 2 ng/ml recombinant human IL-7. Cells were split as necessary.

RT-PCR
RNA was isolated by QIAGEN RNeasy spin columns and reverse-transcribed into cDNA using Superscript II Reverse Transcriptase (Invitrogen). Quantitative real-time PCR of GAPDH, EBI2, and MAF was performed using Taqman follow-up primers: GAPDH forward 5'-GAGGGCGGATTTTCTG3', GAPDH reverse 5'-GTGATGTTAGGCTAAGGGG-3', EBI2 forward 5'-AACATGCCACATTTAAACTTCT-3', EBI2 reverse 5'-GAGGGCGGATTTTCTG3'; and MAF forward 5'-CAAG-

CTAGAGCGCCCC-3', MAF reverse 5'-AGTTCTGTAGGCATTTCTCCCTG-3'. Real-time PCR was set up with BioRad iQ SYBR Green Supermix.

Statistical analysis
A two-tailed, paired t test was used to analyze phenotypic differences between the CXCR5^{hi}, CXCR5^{*}, and CXCR5^{*} CD4 T cells, shown in Fig. 1. Based on the data in Fig. 1, a clear prediction of the phenotype of a Bcl6^{hi} Tfh cell could be made; consequently, one-tailed, paired t tests were used to analyze differences in Bcl6-transduced versus control-transduced cells. For the intracellular cytokine staining done in transduced cells (Fig. 6), a two-tailed, paired test was used. For ectopic expression of Maf + Bcl6, two-tailed paired t tests were used. For correlative analyses between Bcl6 and other markers, linear-regression analysis was performed, and R^{2} was calculated using Prism 5.0 (GraphPad, La Jolla, CA).

Results
Bcl6 protein is expressed by human Tfh and GC Tfh cells
Bcl6 mRNA is present in CXCR5^{hi} CD4 T cell subsets isolated from human tonsil (18, 25, 26). However, Bcl6 mRNA levels frequently do not correlate with Bcl6 protein expression (13). Bcl6 protein has been detected in human GC CD4 T cells by immunofluorescence (26, 27). To address the importance of Bcl6 in human Tfh cell differentiation, we first examined Bcl6 protein expression in human CD4 T cells at the single-cell level by flow cytometry. Human tonsil is a lymphoid tissue rich in GCs. When tonsillar B cells were stained with a Bcl6 mAb, expression was limited to CD20^{*}CD38^{hi} GC B cells, as expected (Supplemental Fig. 1). Tonsillar CD4 T cells were gated on CD45RO^{*} (memory/effector) or CD45RO^{*} (naive), and CD45RO^{*} were subsequently divided into CXCR5^{hi}, CXCR5^{*}, and CXCR5^- gates (Fig. 1A). At least two distinct populations of CXCR5-expressing Tfh cells exist: one population within the GC and another population outside the GC (1, 28). In mice, these two populations have been termed GC Tfh and Tfh cells, respectively, and they can be distinguished on the basis of PD-1 expression (29). Given the dynamic nature of CD4 T cells entering and exiting GCs (30, 31), it is likely that Tfh and GC Tfh cells represent two interconverting activation states of the same Tfh cell type captured at different moments in time (1). Although human GC Tfh cells are sometimes defined by CD57 expression (32, 33), CD57 is not exclusively expressed on GC Tfh cells (25, 34). These CXCR5^{hi} Tfh and CXCR5^{*} GC Tfh cell populations are more precisely delineated by coexpression with ICOS (34, 35) (Fig. 1B) or by their dramatically high level of PD-1 (18, 25) (Fig. 1A). We found that both CXCR5^{hi} and CXCR5^{*} cells expressed significantly more Bcl6 protein compared with CXCR5^{*} CD45RO^{hi} cells (p = 0.0014 and 0.0038, Fig. 1B, 1C). CXCR5^{hi} cells also expressed very high levels of PD-1, ICOS, CXCR4, and SLAM-associated protein (SAP) compared with CXCR5^{*} or CXCR5^{-} cells (Fig. 1B, 1C). CXCR5^{hi} Tfh cells also express more canonical Tfh cell markers but at lower levels than CXCR5^{*} cells (Fig. 1B, 1C). We further characterized the different tonsillar CD4 T cell subsets by comparing their B cell help capabilities in coculture with B cells. When cultured in the presence of autologous naive B cells and SEB, both CXCR5^{*} and CXCR5^{*} CD4 T cells induced substantial IgM production (Fig. 1D), consistent with both CXCR5^{*} and CXCR5^{*} CD4 T cells being related populations of Tfh cells. The majority of the Ab secretion was dependent on IL-21 (Fig. 1D), consistent with previous reports (25, 35, 36).

Bcl6 instructs the conversion of Tfh cells to GC Tfh cells
To determine whether high Bcl6 expression was sufficient to induce the human GC Tfh cell phenotype, we constructed a lentiviral expression vector, Bcl6-LV, that constitutively expresses Bcl6 and a fluorescent protein (ZsGreen). CXCR5^{hi} Tfh cells were sorted from tonsil (Fig. 2A), stimulated with anti-CD3/CD28–coated beads,
and transduced with either Bcl6-expressing viral vector (Bcl6-LV) or an equivalent vector with no Bcl6 insert (Ctrl-LV) (Fig. 2B). Bcl6-LV drove a nearly 10-fold increase in Bcl6 protein expression compared with Ctrl-LV (Fig. 2C). Importantly, this increase in Bcl6 expression led to strong upregulation of CXCR5, the central marker of Tfh cells (p = 0.0011, Fig. 2D). The mean fluorescence intensity (MFI) of CXCR5 exhibited a strikingly strong linear relationship with the MFI of Bcl6 (R² = 0.97) (Fig. 2K).

Bcl6 expression also drove the expression of another major chemokine receptor, CXCR4 (p = 0.0016) (Fig. 2E). Detailed analysis revealed that CXCR4 levels exhibited a tight linear correlation with Bcl6 expression (R² = 0.96, Fig. 2K). Cells that expressed intermediate levels of ZsGreen and Bcl6 exhibited the same changes as the top 5–10% but to a lesser extent (Supplemental Fig. 2). CCR7 was consistently downregulated in the presence of Bcl6 (p = 0.0172, Fig. 2F; R² = 0.81, Fig. 2L). Downregulation of CCR7 is critical for T cell entry into the B cell follicle (37). Bcl6 also negatively regulated the G protein-coupled receptor EBI2 (Fig. 2M, p = 0.004). EBI2 downregulation is critical for movement of B cells from the outer follicle into the GC (38–41), but there is no known role for EBI2 in CD4 T cells. In this study, we show that Bcl6 mediates repression of EBI2, which is likely important for entry of Tfh cells into the GC. CXCR4 expression is important for correct GC architecture and is associated with localization of GC B cells to the dark zone (42). Therefore, Bcl6 regulates the migratory capacity of human Tfh cells for appropriate localization to, and within, GCs by controlling CXCR5, CXCR4, EBI2, and CCR7 expression.
SAP is an intracellular SH2 domain-signaling molecule that transmits signals for SLAM family receptors, and mutations in the gene encoding SAP (SH2D1A) are the cause of the severe human genetic immunodeficiency X-linked lymphoproliferative disease (43). SAP is critically required for GC and memory B cell development in both mice and man (30, 44, 45), and murine GC Tfh cells require SAP expression (29). No connection has been made between Bcl6 and SAP. Therefore, we examined whether SAP expression is regulated by Bcl6 in human CD4 T cells. SAP was uniformly upregulated by Bcl6 expression ($p = 0.0002$, Fig. 2G).

SAP mRNA was also upregulated in Bcl6-expressing cells (data not shown). The data in this study now directly connect these two central regulators of T cell help to B cells.

In addition to Bcl6 directing the upregulation of SAP, the cell surface signaling molecule PD-1 was upregulated by Bcl6 ($p = 0.0013$, Fig. 2H). PD-1 expression by Tfh cells may be important for regulating Tfh cell proliferation and signaling to GC B cells. ICOS is also central to Tfh cell interactions with B cells. Humans with ICOS deficiencies exhibit severe Tfh cell and GC defects and have minimal response to vaccines (46–48). Tfh cells depend on ICOS ligand signals from B cells to maintain Bcl6 expression (49). Therefore, we examined whether ICOS expression is regulated by Bcl6 in human CD4 T cells. ICOS expression was modestly increased by expression of Bcl6 ($p = 0.04$, Fig. 2I).

Surprisingly, CD40L was upregulated by expression of Bcl6 ($p = 0.04$) (Fig. 2J). CD40L is one of the most critical molecules for T cell help to B cells. Mice deficient in CD40-CD40L signaling have neither GCs nor plasma cells (50–55). Defective CD40–CD40L signaling in humans causes the severe immunodeficiency hyper-IgM syndrome (56). Therefore, human Bcl6 controls both the central components of Tfh cell migration (CXCR5, CXCR4, CCR7, EBI2) and multiple central components of T–B interactions (SAP, PD-1, ICOS, CD40L). Collectively, these data indicate that Bcl6 instructs Tfh cell differentiation by regulating a program of gene expression that allows the T cells to colocalize with B cells and provide critical help functions.

**Bcl6 can convert non-Tfh cells to Tfh cells**

Next, we set out to determine whether Bcl6 expression was sufficient to drive the differentiation of effecter CD4 T cells.
We transduced CD45RO+CXCR5+CD4 T cells with either Bcl6-LV or Ctrl-LV and analyzed the cells on day 7 (Fig. 3A, Supplemental Fig. 2A). Expression of Bcl6 was confirmed by flow cytometry (Fig. 3B). Activation of human CD4 T cells was sufficient to induce some expression of CXCR5 (57) [this is different from murine CD4

FIGURE 3. Bcl6 converts human CXCR5+CD4 T cells to Tfh cells. (A) CD45RO+CXCR5+ CD4 T cells were sorted from human tonsil. (B) Expression of Bcl6 was measured based on the top gates shown in Supplemental Fig. 2A. (C-I) Expression of Tfh cell-associated molecules by Bcl6-LV and Control-LV (Ctrl-LV) groups based on gates used in Supplemental Fig. 2A. A representative plot is shown (left panel) alongside the complete data set with five or six individual donors (right panel). (J and K) ZsGreen-expressing cells were divided into five equal gates based on ZsGreen MFI. Bcl6, CXCR5, CXCR4, PD-1 (J), and CCR7 (K) MFIs were determined for each ZsGreen gate. Data shown in (J) and (K) are representative of six individual donors. *p < 0.05, **p < 0.005.

FIGURE 4. Bcl6 instructs CXCL13 production by human Tfh cells. (A) Selective production of CXCL13 by human Tfh cells. Unstimulated tonsil cells were incubated for 3 h with brefeldin A before intracellular staining for CXCL13. Left panel, CXCL13 expression shown in total CD4 T cells. Activated CD4 T cell subsets were gated (middle panel), and CXCL13 levels for each population are shown versus isotype control (right panel). Isotype control was gated on total CD4 T cells. MFI for each population is indicated. (B) CXCR5int Tfh cells were sorted from tonsil, stimulated with anti-CD3/CD28 beads, and transduced with Bcl6-LV or Control-LV. Five days posttransduction, CXCL13 present in the supernatant was analyzed by ELISA. Data are representative of four total donors from two independent experiments. *p < 0.05, **p < 0.01.

(CD45RO+CXCR5+) to Tfh cells. We transduced CD45RO+CXCR5+ CD4 T cells with either Bcl6-LV or Ctrl-LV and analyzed the cells on day 7 (Fig. 3A, Supplemental Fig. 2A). Expression of Bcl6 was confirmed by flow cytometry (Fig. 3B). Activation of human CD4 T cells was sufficient to induce some expression of CXCR5 (57) [this is different from murine CD4

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Nevertheless, expression of Bcl6 in human CXCR5− effector Th cells led to a much greater and sustained increase in CXCR5 expression (p = 0.0099, Fig. 3C). Furthermore, Bcl6+ cells specifically exhibited significantly increased expression of SAP, PD-1, and CD40L (p = 0.001, 0.002, and 0.02, respectively, Fig. 3F, 3G, 3I), as well as downregulation of CCR7 (p = 0.01, Fig. 3E; R² = 0.80, Fig. 3K). A small increase in ICOS was observed (Fig. 3H, p < 0.05). A robust correlation between Bcl6 and both CXCR5 and CXCR4 expression was observed (R² = 0.88 and 0.97, Fig. 3J). Similar effects were seen when starting with naive (CD45RO−CXCR5−) CD4 T cells (Supplemental Fig. 4). Overall, these data demonstrate that Bcl6 controls a program of Th cell gene expression in human CD4 T cells, and Bcl6 expression is sufficient to convert previously Ag-experienced human CD45RO+ CXCR5− CD4 T cells into Thf cells.

**Bcl6 induces CXCL13 production**

Thf cells are well known as important producers of the helper cytokines IL-4 and IL-21 (1). Human Thf cells, but not murine, also specifically express the chemokine CXCL13 (32, 34, 36), a B cell-attracting molecule usually made by stromal cells (59). We hypothesized that CXCL13 expression by Thf cells may be regulated by Bcl6. Unmanipulated human GC Thf cells are a significant source of CXCL13 protein, as determined at the single-cell level (Fig. 4A). Impressively, when Bcl6 was constitutively expressed in human CXCR5int Thf cells, thereby inducing a GC Thf cell phenotype (Fig. 2), >10-fold increases in CXCL13 protein were detected in culture supernatants compared with control cells (60–100 pg/ml, p < 0.05, Fig. 4B). CXCL13 mRNA was also upregulated in Bcl6-expressing cells (data not shown). This spe-
cialized production of CXCL13 by GC Tfh cells may help to properly organize the GC and may also provide cytokine-like signals to GC B cells.

**Helper cytokines IL-4 and IL-21**

A population of human GC Tfh cells produce both IL-4 and IL-21 (Fig. 5). These cytokines have been implicated in the maintenance of GC B cells and the differentiation of long-lived plasma cells (1, 60). IL-4 is known to provide prosurvival signals to B cells (61, 62). Mice dually deficient in both IL-21 and IL-4 signals had significantly reduced B cell responses (63). GC Tfh cells exhibited considerably more IL-4 expression than did Tfh cells or even the effector CD4 T cell (effector Th) population that includes Th2 cells (Fig. 5). Virtually all of the GC Tfh IL-4+ cells also expressed IL-21, the canonical Tfh cell cytokine (Fig. 5) (6, 26, 49, 64). The frequency of IL-4+IL-21+ cells in the CXCR5hi GC Tfh cell population was significantly higher than in the CXCR5int cell population ($p = 0.005$) or the effector Th subsets ($p = 0.003$, Fig. 5).

The Tfh cell program is perhaps best conceptualized as a developmental program that can coexist with Th1, Th2, or Th17 gene expression to a limited extent (1). Therefore, we examined the capacity of human Tfh and GC Tfh cells to produce Th1 or Th17 cytokines. GC Tfh cells secreted significantly less IL-17 compared with Tfh or effector Th cells (Fig. 5). A modest reduction in IFN-$\gamma$+ cells was observed for GC Tfh cells compared with effector Th cells ($p = 0.225$ and 0.239, respectively) (Fig. 5). A considerable percentage of human CXCR5int Tfh cells produced IL-17 or double produced IFN-$\gamma$ and IL-17, consistent with CXCR5int Tfh cells being a less polarized and more heterogeneous cell type than CXCR5hi GC Tfh cells (Fig. 5).

Given that Bcl6 controls both Tfh cell migration and Tfh:B interaction genes, we set out to determine whether Bcl6 also instructs GC Tfh cell cytokine expression. Unexpectedly, constitutive expression of Bcl6 did not significantly increase expression of IL-4 or IL-21 in any CD4 T cell subset examined (Fig. 6). Furthermore, the IL-4+IL-21+ population characteristic of GC Tfh cells was not observed (Fig. 6). Therefore, although Bcl6 instructs many aspects of the human Tfh cell phenotype, the production of B cell help cytokines requires additional signals.

**Maf induces IL-21 secretion in CD4 T cells**

Maf is a transcription factor involved in IL-21 (65, 66) and IL-4 (67) production in mouse CD4 T cells. Maf is expressed at a higher level in CXCR5hi and CXCR5int human Tfh cells than in naive or CXCR5int CD45RO+ cells (Fig. 7A). Maf expression can be induced by ICOS signaling (68, 69). Consequently, we set out to test the hypothesis that Maf contributes a complementary role to Bcl6 in Tfh cell differentiation by inducing secretion of helper cytokines. To test this hypothesis, we generated a viral expression vector containing human Maf. Transduced cells were visualized by their expression of ZsGreen (Maf) or a truncated, nonsignaling human nerve growth factor receptor (dNGFR; Bcl6). Transduction of CD45RO+CXCR5int CD4 T cells with Maf did not result in a notable change in the percentage of cells secreting IL-4 upon restimulation (Supplemental Fig. 3). However, Maf expression...
resulted in a marked increase in the percentage of IL-21–pro-
ducing CD4 T cells compared with control-transduced or Bcl6-
transduced cells (\(p=0.0009\) and 0.016, respectively, Fig. 7B, 7C).
Dual transduction with both Maf and Bcl6 (Fig. 7D) did not in-
crease IL-21 production above the percentage induced by Maf
alone (Fig. 7C). Dual transduction with both Maf and Bcl6 did not
enhance IL-4 production (Supplemental Fig. 3). Maf is expressed
by Th17 cells (65, 69); however, no significant change in IL-17
expression was observed in Maf-transduced human CD45RO+
CXCR5int CD4 cells (data not shown). Naive CD4 T cells that
were activated and then transduced with Maf also did not express
IL-17 (data not shown). These data demonstrate that, although
Bcl6 is critical for T cell positioning within the follicle and direct
T–B interactions, Maf, but not Bcl6, can enhance IL-21 produc-
tion by CD4 T cells.

Maf cooperates with Bcl6 to instruct human Tfh cell
differentiation

We examined whether Maf regulates additional Tfh cell-associated
proteins. Surprisingly, Maf induced a significant increase in
CXCR5 expression, and this effect was particularly notable when
starting with CD45RO+ CXCR5+ naive CD4 T cells (\(p=0.0002\),
Fig. 8A, 8B). This was not due to Maf induction of Bcl6 (Fig. 8C).
Of note, ectopic expression of Bcl6 induced a modest, but sig-
nificant, increase in Maf expression (Fig. 8D). Cotransduction of
naive CD4 T cells with Bcl6 and Maf did not further enhance
CXCR5 expression. In contrast, ICOS and PD-1 expression both
increased significantly when Bcl6 and Maf were ectopically
expressed together compared with cells expressing Bcl6 alone
(\(p=0.02\) and 0.002, Fig. 8E, 8F). CXCR4 was also significantly
induced by Maf at levels comparable to that seen with Bcl6, and
further increased CXCR4 levels were observed in doubly trans-
duced cells (\(p=0.03\), Fig. 8G). Unlike Bcl6, Maf did not have an
impact on CD40L expression (Fig. 8H). Taken together, these data
show that Bcl6 and Maf both play a role in human Tfh cell dif-
ferentiation. Bcl6 controls modules of genes important for Tfh
cell localization and T–B interactions. Complementing Bcl6, Maf
induces IL-21 secretion and CXCR5 expression and enhances ex-
pression of a number of Bcl6-regulated Tfh cell-associated mole-
cules, such as ICOS and PD-1.

Discussion

In-depth knowledge of the pathways of human CD4 T cell differ-
entiation is critical for understanding how to accomplish rational
vaccine design against the myriad pathogens that humans remain
unprotected against. In addition, understanding human CD4 T cell
differentiation is critical for understanding most human autoim-
mune diseases. These feats can only be accomplished with a clear
understanding of the transcription factors that regulate human CD4
T cell differentiation. In this study, we demonstrate that Bcl6
is a central regulator of human Tfh cell differentiation, and we
demonstrate that Bcl6 accomplishes this task by controlling two
major modules of Tfh cell gene expression. In human B cells, Bcl6
was generally shown to repress gene transcription (14, 15). It is
likely that upregulation of many Tfh cell genes is an indirect effect
brought about by Bcl6-mediated repression of specific transcription
factors and chromatin remodelers, such as histone-modification
enzymes (9, 10). However, the possibility that Bcl6 binding to
some genes may positively influence gene expression must also
be considered.

There has been only a limited understanding of how Bcl6
controls CD4 T cells, and little data have been available on Bcl6
function in human CD4 T cells (18, 25). Ectopic expression of
Bcl6 in murine CD4 T cells in vivo induced a Tfh cell phenotype

FIGURE 7. Maf instructs IL-21 production by CD45RO+ CXCR5+ CD4 T cells. (A) Maf mRNA expression was measured by quantitative PCR on
different tonsillar CD4 T cell subsets (defined as in Fig. 1A). (B–D) CD45RO+ CXCR5+ CD4 T cells were sorted from human tonsil, stimulated with anti-
CD3/CD28 beads, and either left unmanipulated or transduced on day 2 with Bcl6-LV (Bcl6), Maf-LV (Maf), Bcl6-LV and Maf-LV (both), or a Control-LV
dNGFR-LV [C1]; ZsGreen-LV [C2]). Five days posttransduction, cells were restimulated with PMA and ionomycin in the presence of brefeldin A and
stained. (B) Representative plots showing IL-21 expression. (C) IL-21 expression data from eight donors. (D) Representative plot of Bcl6-dNGFR and Maf-
ZsGreen RV double transfection. *\(p<0.05\), ***\(p<0.001\).
but it had surprisingly limited activity in purified murine CD4 T cells in vitro, with minimal Tfh cell-associated protein changes (17, 18, 70). Importantly, our in vitro human CD4 T cell system allowed us to identify downstream targets of Bcl6 regulation, without the confounding effects of non-Bcl6 signals present in the in vivo mouse models that also contribute to Tfh cell differentiation. We demonstrate that introduction of Bcl6 into human CD45RO+ CD4 T cells converts those cells to a Tfh-like cell phenotype in vitro, and the degree of conversion strongly correlates with the level of Bcl6 expression (Figs. 2, 3). We show that Bcl6 regulates distinct modules of the Tfh cell program: one Bcl6-dependent module is genes critical for Tfh cell migration (CXCR5, CXCR4, CCR7, EBI2), and the second Bcl6-dependent module is a set of genes important for T–B interactions (SAP, PD-1, CD40L, ICOS, CXCL13), including two genes known to be critical for contact-dependent B cell help (SAP and CD40L).

Therefore, Bcl6 is a true nexus for human Tfh cell differentiation and functions. Perhaps the most striking finding from this study is that Bcl6 regulates all three of these critical human genes, highlighting its powerful role in defining Tfh cell functionality.

The data in this study also show that PD-1 is explicitly regulated by Bcl6. Therefore, the high level of PD-1 on Tfh cells is not simply a byproduct of TCR stimulation but is a specific component of the Tfh cell gene program. In the absence of PD-1 in mice, increased GC B cell death and a defective plasma cell response were observed in one study (71), whereas excessive Tfh cell proliferation was seen in another study (72). PD-1 is a potent negative regulator of T cell proliferation. We propose that PD-1 is an important negative regulator of Tfh cells, most likely by dissociating Tfh cell TCR signaling from proliferation. The purpose of GCs is the rapid evolution of BCR affinity through rapid GC B cell proliferation and hypermutation. Tfh cells are critical for this process and must preferentially select the “best” GC B cells for further rounds of proliferation and mutation via sensing quantitative differences in peptide:MHC complexes between different GC B cells. This must require highly sensitive TCR signaling. At the same time, GC Tfh cell TCR signaling should be dissociated from proliferation; although the GC B cells must undergo rapid proliferation for selection of novel mutations, GC Tfh cell numbers remain relatively static for the duration of the GC. Therefore, Bcl6 induction of PD-1 on Tfh cells is likely
important for restriction of Tfh cell proliferation in the presence of continuous Ag stimulation.

The role of Bcl6 in human Tfh cell differentiation differs from murine Tfh cell differentiation in several key ways. Direct induction of CXCR5 protein expression on purified CD4 T cells is one example. Bcl6 regulation of CXCL13 is a second example. CXCL13 is expressed by human Tfh cells but not mouse Tfh cells. Production of CXCL13 by Tfh cells may serve two purposes. First, CXCL13 is a chemoattractant and will recruit B cells to the location of the Tfh cells. This may be important in the face of significant reductions in CXCL13 expression by lymphoid tissue stromal cells during viral infections (73). Second, CXCL13 binding to CXCR5 on B cells induces LTα1β2 expression (74). As such, Tfh cell expression of CXCL13 may orchestrate GC architecture. This CXCL13 expression by Tfh cells may also drive tertiary lymphoid neogenesis in autoimmune diseases (4). CXCL13 and LTα1β2 are important components of lymphoid organ neogenesis and the formation of ectopic lymphoid aggregates (75). It is feasible that CXCL13 binding to CXCR5 on GC B cells induces other nonchemotactic signaling responses that are important for GC B cell survival or differentiation.

IL-21 is the most important known cytokine for T cell help to B cells, both through maintenance of GCs and for induction of plasma cell differentiation. In this study, we found that Bcl6 has minimal influence on IL-21 production, whereas Maf does drive IL-21 production. More surprisingly, Maf also controls other components of human Tfh cell function, including expression of CXCR5. Intriguingly, coexpression of Bcl6 and Maf exhibited additive effects on gene expression for some Tfh cell-associated genes (e.g., PD-1 and ICOS), whereas, in other cases, Bcl6 and Maf appear to have parallel, nonadditive capacities (e.g., CXCR5).

Altogether, these findings reveal that Bcl6 and Maf collaborate to orchestrate a suite of genes that define nearly all of the core characteristics of human Tfh cell biology. We demonstrated that Bcl6 and Maf synergistically induce human Tfh cell differentiation and function. As such, manipulation of Bcl6 and/or Maf activity in a positive way in human CD4 T cells may have an important impact for human vaccines, resulting in enhanced Tfh cell number or functionality and long-term B cell immunity. Alternatively, inhibition of Bcl6 and Maf in CD4 T cells is an outstanding candidate for ameliorating autoimmune diseases associated with Tfh cell dysregulation.

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Disclosures

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References

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