Bcl6 and Maf cooperate to instruct human follicular helper CD4 T cell (Tfh) differentiation

Mark A. Kroenke*, Danelle Eto*, Michela Locci*, Michael Cho^, Terence Davidson†, Elias Haddad§, and Shane Crotty*,1

*Division of Vaccine Discovery, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037
†Division of Otolaryngology, University of California School of Medicine, San Diego, CA 92103-8895
§Vaccine & Gene Therapy Institute of Florida, Port St. Lucie, FL 34987
^Mission Hospital and Children’s Hospital of Orange County (CHOC), Mission Viejo, CA 92691

Abstract

Follicular helper CD4 T cells (Tfh) provide B cells with signals important for the generation of high-affinity antibodies and immunological memory, and are therefore critical for the protective immunity elicited by most human vaccines. Transcriptional regulators of human Tfh cell differentiation are poorly understood. Here we demonstrate that Bcl6 controls specific gene modules for human Tfh differentiation. The introduction of Bcl6 expression in primary human CD4 T cells resulted in 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 is the first 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, ICOS). Introduction of Bcl6 did not alter expression of IL-21 or IL-4, the primary cytokines of human Tfh cells. We show here that introduction of Maf (c-Maf) does induce the capacity to express IL-21. Surprisingly, Maf also induced CXCR5 expression. Co-expression of Bcl6 and Maf together revealed that Bcl6 and Maf cooperate in the induction of CXCR4, PD-1, and ICOS. Altogether, these findings reveal that Bcl6 and Maf collaborate to orchestrate a suite of genes that define core characteristics of human Tfh cell biology.

Introduction

Follicular helper CD4 T cells (Tfh) are a specialized subset of T cells that provide help to B cells (1). The defining characteristics of Tfh cells are their ability to co-localize 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. In the absence of Tfh cells, severe reductions are seen in the development of antigen-specific IgG responses, memory B cells, and memory plasma cells (1). A long-term antibody response is the central attribute of most successful human vaccines (2, 3), and understanding Tfh cells is therefore important for developing truly rational vaccine development strategies. Furthermore, Tfh cells are potentially useful biomarkers in human vaccine clinical trials. In order to harness this biology, however, the transcriptional control of human Tfh cell

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Correspondence to: Shane Crotty, Division of Vaccine Discovery, La Jolla Institute for Allergy and Immunology, 9420 Athena Circle, La Jolla, CA 92037. Phone: 858-752-6816, Fax: 858-752-6993, shane@liai.org.
differentiation and function must be understood. Finally, several autoimmune diseases are characterized by the presence of increased germinal centers and autoantibodies (4, 5), which are dependent on Tfh cells in multiple models (6, 7). Understanding the transcriptional regulators of Tfh cells is therefore 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). Bcl6 is a critical transcription factor for germinal center B cells (8, 11, 12). Extensive studies on Bcl6 in B cells have revealed that Bcl6 regulates germinal center B cell survival, cell cycle control, and somatic hypermutation (8, 10, 13–15). Recently it has been determined that Bcl6 is important in mice for Tfh cell differentiation (16–18). However, it is not known if and how Bcl6 controls Tfh differentiation of human T cells (1). Importantly, there is limited evidence of which proteins in CD4 T cells are explicitly regulated by Bcl6 in any species, as 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). Here 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 (UCSD) Hillcrest Medical Center, the National Disease Resource Interchange (NDRI), or the Children’s Hospital of Orange County (CHOC), Mission Viejo. 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 LIAI and NDRI, LIAI and UCSD, or LIAI and CHOC Institutional Review Boards.

**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 hours. Cells were fixed with BD Phosflow Fix Buffer I and permeabilized with BD Phosflow Perm/wash Buffer I. All cells were sorted using a BD FACS Aria. All Tfh cell sorts were initially gated on CD4+ CD19− 7-AAD− cells. CD45RO− cells were confirmed to be naïve by co-staining for CD45RA. Naïve B cells were CD19+CD20+CD3−CD27−IgD+. The following anti-human antibodies were used: CD45RO (clone UCHL1), CD45RA (clone HI100), CD19 (clone HB19), CD38 (clone HIT2), CD20 (clone 2H7), CD27 (clone O323), PD-1 (clone J105), ICOS (clone ISA-3), CXCR4 (clone 12G5), CD40L (clone 24–31), CD3 (clone OKT3), and CD4 (clone RPA-T4) (eBioscience, San Diego, CA); CXCR5 (clone RF8B2), CCR7 (clone 3D12), Bcl6 (clone K112-91), and IgD (clone IA6-2) (BD Biosciences, Franklin Lakes, NJ); SAP (clone 1D12) (Cell Signaling Technology, Danvers, MA) (21). For intracellular staining, the following antibodies were used: IL-4 (clone MP4-25D2) (Biolegend, San Diego, CA); IL-21 (clone 3A3-N2), IL-17 (clone 64DEC17), and IFNγ (clone 4S.B3).
Sorted tonsil CD4+ T cell subsets CD45RO−CXCR5−, CD45RO−CXCR5int and CD45RO−CXCR5hi were co-cultured with autologous naïve B cells (4×10^4 cells/well) in the presence of 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-21R/Fc (20µg/mL; R&D Systems) or an isotype-matched control. Secretion of IgM was determined by ELISA after 7 days in culture. 96-well Polysorp microtiter plates (Nunc) were coated overnight with monoclonal anti-human IgM (1µg/ml, Abbiotech) in PBS. All samples were run in duplicate, and IgM from the co-culture supernatant was detected with HRP-conjugated human IgM Fc (Hybridoma Reagent Laboratory).

**T cell cultures**

Sorted cells were stimulated with anti-CD3/CD28 Dyna beads (Invitrogen, Carlsbad, CA) in 96 well flat bottom plates at a starting density of 7.5×10^4 cells/well. Beads were used at a concentration of 1 µl per well. RPMI medium with 10% fetal calf serum 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, was obtained from Dr. Richard Mulligan (23). Bcl6 LV (Bcl6-LV) was constructed by cloning the human Bcl6 ORF BC150184 (purchased from Open Biosystems, Huntsville, AL) into the NotI and BamHI sites of pHAGE, with an optimal Kozak sequence. A modified vector pHAGE-PGK-IRESDNGFR (pHPN) was made by introduction of a non-signaling version of the human NGFR gene (24). For the double transduction experiments, the Bcl6 ORF was cloned into pHAGE. Maf LV (Maf-LV) was constructed by cloning a codon optimized human MAF ORF into pHAGE, with an optimal Kozak sequence. 293T cells were transfected with lentivirus along with four expression vectors encoding the packaging proteins Rev, Tat, Gag/Pol, and the G-protein of vesicular stomatitis virus (VSV). Supernatant was collected at 24 and 48 hours and passed through a 0.22 µm filter. Virus was concentrated by centrifugation at 24,000 rpm for 90 minutes at 10 °C. Lentiviral preparations were titered on 293T cells, and CD4 T cells were transduced at an MOI of 2. Cells were transduced 36–48 hours after the start of culture by spinning at 1,500 rpm for 90 minutes at 37° C. Analysis was performed 5 days post-transduction for each experiment. Co-transduction of both control vectors (ZsGreen-LV + dNGFR-LV) was tested in pilot experiments and no difference in cell phenotype was observed when compared to single transduction with either control vector alone (ZsGreen-LV or dNGFR-LV) (data not shown).

**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 and EBI2 was performed using the following primers: GAPDH forward, 5’-ACATCGCTCAGACACCATG-3’, GAPDH reverse, 5’-TGTAGTTGAGGTCAATGAAGGG-3’, EBI2 forward, 5’-AACATGCCACAGTAAAACCTACT-3’, EBI2 reverse, 5’-GAGGGCGGAGTTATGGTTTG-3’, MAF forward, 5’-CAAGCTAGAAGCGCCCC-3’, MAF reverse 5’-AGTTTCTGATGCCCCATTCTCCTG-3’. Real-time PCR was set up with BioRad iTaq SYBR Green Supermix.
A two-tailed, paired t test was used to analyze phenotypic differences between the CXCR5\textsuperscript{hi}, CXCR5\textsuperscript{int}, and CXCR5\textsuperscript{−} CD4 T cells, shown in Figure 1. Based on the data in Figure 1, a clear prediction of the phenotype of a Bcl6\textsuperscript{hi} Tfh cell could be made and 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 together, 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, CA).

Results

Bcl6 protein is expressed by human Tfh and GC Tfh

Bcl6 mRNA is present in CXCR5\textsuperscript{+} CD4 T cells 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 germinal center CD4 T cells by immunofluorescence (26, 27). In order 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 germinal centers. When tonsillar B cells were stained with a Bcl6 monoclonal antibody, expression was limited to CD20\textsuperscript{+} CD38\textsuperscript{+} germinal center B cells, as expected (Suppl. Fig. S1).

Tonsillar CD4 T cells were gated on CD45RO\textsuperscript{+} (memory/effector) or CD45RO\textsuperscript{−} (naïve), and CD45RO\textsuperscript{+} were subsequently divided into CXCR5\textsuperscript{hi}, CXCR5\textsuperscript{int}, and CXCR5\textsuperscript{−} gates (Fig. 1A). At least two distinct populations of CXCR5-expressing Tfh cells exist, one population within the germinal center (GC) and another population outside (1, 28). In mice, these two populations have been termed GC Tfh and Tfh cells, respectively, and can be distinguished on the basis of PD-1 expression (29). Given the dynamic nature of CD4 T cells entering and exiting germinal centers (30, 31), it is likely that Tfh and GC Tfh cells represent two interconverting activation states of the same cell type captured at different moments in time (1). While 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\textsuperscript{int} Tfh and CXCR5\textsuperscript{hi} GC Tfh cell populations are more precisely delineated by co-staining with ICOS (34, 35) (Fig. 1B) or by their dramatically high level of PD-1 (18, 25) (Fig. 1A). We found that both CXCR5\textsuperscript{hi} and CXCR5\textsuperscript{int} cells expressed significantly more Bcl6 protein compared to CXCR5\textsuperscript{−} CD45RO\textsuperscript{+} cells ($p=0.0014$ and $0.0038$, Fig. 1B,C). CXCR5\textsuperscript{hi} cells also expressed very high levels of PD-1, ICOS, CXCR4, and SLAM-associated protein (SAP) when compared to CXCR5\textsuperscript{int} or CXCR5\textsuperscript{−} cells (Fig. 1B,C). CXCR5\textsuperscript{int} Tfh cells also express most canonical Tfh cell markers but at lower levels than CXCR5\textsuperscript{hi} cells (Fig. 1B,C).

We further characterized the different tonsillar CD4 T cell subsets by comparing their B cell help capabilities in co-culture with B cells. When cultured in the presence of autologous naïve B cells and SEB, both CXCR5\textsuperscript{int} and CXCR5\textsuperscript{hi} CD4 T cells induced substantial IgM production (Fig. 1D), consistent with both CXCR5\textsuperscript{int} and CXCR5\textsuperscript{hi} CD4 T cells being related populations of Tfh cells. The majority of the antibody secretion was dependent on IL-21 (Fig. 1D), consistent with previous reports (25, 35, 36).

Bcl6 instructs the conversion of Tfh to GC Tfh

In order to determine if high Bcl6 expression was sufficient to induce the human GC Tfh phenotype, we constructed a viral expression vector, Bcl6-LV, that constitutively expresses Bcl6 and a fluorescent protein (ZsGreen). CXCR5\textsuperscript{int} 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). The Bcl6-LV drove a nearly 10-fold increase in Bcl6 protein expression when
compared to the 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 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 (Suppl. Fig. S2). 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 to 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 germinal center (38–41), but there is no known role for EBI2 in CD4 T cells. Here we show that Bcl6 mediates repression of EBI2, which is likely important for entry of Tfh cells into the germinal center. CXCR4 expression is important for correct germinal center 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, germinal centers 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 (XLP) (43). SAP is critically required for germinal center and memory B cell development in both mice and man (30, 44, 45), and murine GC Tfh 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 here 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 both 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 ICOSL 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. 2l). Surprisingly, CD40L was upregulated by expression of Bcl6 (p=0.04) (Figure 2J). CD40L is one of the most critical molecules for T cell help to B cells. Mice deficient in CD40-CD40L signaling have neither germlinal centers nor plasma cells (50–55). Defective CD40-CD40L signaling in humans causes the severe immunodeficiency hyper IgM syndrome (HIGM) (56). Human Bcl6 therefore 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 differentiation by regulating a program of gene expression that allows the T cells to co-localize with B cells and provide critical help functions.

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

Next, we set out to determine if Bcl6 expression was sufficient to drive the differentiation of effector CD4 T cells (CD45RO⁺ CXCR5⁻) to Tfh cells. We transduced CD45RO⁺ CXCR5⁻
CD4 T cells with either Bcl6-LV or Ctrl-LV and analyzed the cells at day 7 (Fig. 3A and Suppl. 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 than murine CD4 T cells (58)). 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,G,I), and 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 CXCCL4 expression was observed (R²=0.88 and R²=0.97, Fig. 3J). Similar effects were seen when starting with naïve (CD45RO−CXCR5−) CD4 T cells (Sup Fig. 4).

Overall, these data demonstrate that Bcl6 controls a program of Thf gene expression in human CD4 T cells, and Bcl6 expression is sufficient to convert previously antigen-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 phenotype (Fig. 2), greater than 10-fold increases in CXCL13 protein were detected in culture supernatants when compared to control cells (60–100 pg/mL; p<0.05, Fig. 4B). CXCL13 mRNA was also upregulated in Bcl6 expressing cells (data not shown). This specialized production of CXCL13 by GC Thf cells may help properly organize the germinal center, and may also provide cytokine-like signals to GC B cells.

Helper cytokines IL-4 and IL-21

A population of human GC Thf 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). Human GC Thf cells exhibited considerably more IL-4 expression than Thf cells, or even the effector CD4 T cell (effector Th) population that includes Th2 cells (Fig. 5).

Virtually all of the GC Thf IL-4+ cells also expressed IL-21, the canonical Thf cytokine (Fig. 5) (6, 26, 49, 64). The frequency of IL-4+ IL-21+ cells in the CXCR5int GC Thf 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 Thf program is perhaps best conceptualized as a developmental program that can co-exists with Th1, Th2, or Th17 gene expression to a limited extent (1). Therefore, we examined the capacity of human Thf and GC Thf cells to produce Th1 or Th17 cytokines. GC Thf cells secreted significantly less IL-17 when compared to Thf or effector Th cells (Fig. 5). A modest reduction in the IFNγ+ cells was observed for GC Thf cells in comparison to effector Th cells (p=0.225 and p=0.239, respectively) (Fig. 5). A considerable percentage of human CXCR5int Thf cells produced IL-17 or double produced IFNγ and IL-17, consistent with CXCR5int Thf cells being a less polarized and more heterogeneous cell type than CXCR5hi GC Thf cells (Fig. 5).

Given that Bcl6 controls both Thf cell migration and Thf:B interaction genes, we set out to determine if Bcl6 also instructs GC Thf 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, while 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 higher level in CXCR5hi and CXCR5int human Tfh cells than in naïve or CXCR5−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. In order 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, non-signaling human nerve growth factor receptor (dNGFR; Bcl6). Transduction of CD45RO− CXCR5− CD4 T cells with Maf did not result in a notable change in the percentage of cells secreting IL-4 upon restimulation (Suppl. Figure S3). However, Maf expression resulted in a marked increase in the percentage of IL-21 producing CD4 T cells compared to control transduced or Bcl6 transduced cells (p=0.0009 and 0.016, respectively, Fig. 7B and 7C). Dual transduction with both Maf and Bcl6 (Fig. 7D), did not increase IL-21 production above the % induced by Maf alone (Fig. 7C). Dual transduction with both Maf and Bcl6 did not enhance IL-4 production (Suppl. Figure S3). 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). Naïve CD4 T cells that were activated and then transduced with Maf also did not express IL-17 (data not shown). These data demonstrate that while Bcl6 is critical for T cell positioning within the follicle and direct T:B interactions, Maf but not Bcl6 can enhance IL-21 production 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− naïve CD4 T cells (p=0.0002, Fig. 8A,B). This was not due to Maf induction of Bcl6 (Fig. 8C). Of note, ectopic expression of Bcl6 induced a modest but significant increase in Maf expression (Fig. 8D). Co-transduction of naïve CD4 T cells with Bcl6 and Maf did not further enhance CXCR5 expression. In contrast, ICOS and PD1 expression both increased significantly when Bcl6 and Maf were ectopically expressed together compared to cells expressing Bcl6 alone (p=0.02 and 0.002, Fig. 8E,F). CXCR4 was also significantly induced by Maf at levels comparable to that seen with Bcl6, and further increased CXCR4 levels in doubly transduced cells was observed (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 differentiation. 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 expression of a number of Bcl6 regulated Tfh associated molecules such as ICOS and PD1.

Discussion

In depth knowledge of the pathways of human CD4 T cell differentiation is critical for understanding how to accomplish rational vaccine design against the myriad pathogens that humans remained unprotected against. In addition, understanding human CD4 T cell
differentiation is critical for understanding most human autoimmune diseases. These feats can only be accomplished with a clear understanding of the transcription factors that regulate human CD4 T cell differentiation. Here 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 gene expression. In human B cells, Bcl6 has generally been shown to repress gene transcription (14, 15). It is likely that upregulation of many Tfh 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.

To date there has been only a limited understanding of how Bcl6 controls CD4 T cells, and little data has 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 (17), but had surprisingly limited activity in purified murine CD4 T cells in vitro, with minimal Tfh associated protein changes (17, 18, 70). Importantly, our in vitro human CD4 T cell system has 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). Here we have shown for the first time that Bcl6 regulates distinct modules of the Tfh 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, PD1, 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 differentiation and functions. Perhaps the most striking finding from this study is that Bcl6 specifically regulates CD40L, SAP and ICOS. From the perspective of human immunology, the CD40L, SH2D1A and ICOS genes are three loci of severe immunodeficiencies of adaptive immunity. Genetic lesions in CD40L and SH2D1A are lethal due to a resulting susceptibility to a range of infectious diseases. Extreme losses in responsiveness to vaccines and failure to develop B cell memory are prominent characteristics of these genetic diseases. Deletion of the human ICOS gene also results in immunodeficiency, susceptibility to infections, and a failure to respond to vaccines (46–48), consistent with the importance of ICOS for Tfh differentiation (49). Here we find that Bcl6 regulates all three of these critical human genes, highlighting the powerful role of Bcl6 in defining Tfh functionality.

The data here 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 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), while 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 germinal centers 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, for while 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 germinal center. Therefore, Bcl6 induction of PD-1 on Tfh cells is likely important for restriction of Tfh cell proliferation in the presence of continuous antigen stimulation.
The role of Bcl6 in human Tfh differentiation differs from murine Tfh 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 chemotactant 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 germinal center 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 non-chemotactic 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 germinal centers and for induction of plasma cell differentiation. Here we find 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, co-expression of Bcl6 and Maf exhibited additive effects on gene expression for some Tfh associated genes (e.g., PD-1 and ICOS), whereas in other cases Bcl6 and Maf appear to have parallel, non-additive, capacities (e.g., CXCR5). Altogether these finding 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 have demonstrated that Bcl6 and Maf synergistically induce human Tfh 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 autoantibody production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References


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Figure 1.
Two stages of human Tfh cell differentiation, associated with two levels of Bcl6 and Tfh associated genes. **A**, Human tonsil cells were gated on CD45RO\(^+\) memory/effector or CD45RO\(^-\) naïve CD4 T cells (top). CD45RO\(^+\) cells were subsequently divided into CXCR5\(^-\), CXCR5\(^{\text{int}}\), and CXCR5\(^{\text{hi}}\) gates (bottom). **B**, Representative phenotypic analysis of CD4 T cell subsets based on CXCR5 expression, compared to naïve CD4 cells. **C**, Data from 5 individual donors. Data are shown as fold change in MFI comparing CXCR5\(^-\), CXCR5\(^{\text{int}}\), or CXCR5\(^{\text{hi}}\) to naïve CD4\(^+\) T cells from the same donor. **D**, Tonsillar naïve, CXCR5\(^-\), CXCR5\(^{\text{int}}\), and CXCR5\(^{\text{hi}}\) CD4 T cell subsets were co-cultured with autologous naïve B cells in the presence of SEB (250 ng/mL). 20 µg/mL IL-21R/Fc or an isotype-matched antibody control was added to each well. Secretion of IgM was determined after 7 days.
days of culture. Duplicate samples from each of two donors is shown. * p < 0.05, ** p < 0.005, *** p < 0.0005.
Figure 2.
Human Tfh cell differentiation is instructed by Bcl6 expression. A, CD45RO+ CXCR5^int CD4 T cells were sorted from human tonsil. B, Cells were stimulated with anti-CD3/CD28 beads and either left unmanipulated or transduced on day 2 with Bcl6-LV or Control-LV (Ctrl-LV). Data shown is representative. C, Expression of Bcl6 was measured using a monoclonal antibody to Bcl6 based on the gates shown in B. D–J, Expression of Tfh associated molecules by Bcl6-LV and Ctrl-LV groups, based on the gates shown in B. A representative FACS plot is shown (left) alongside the complete data set with 5–6 individual donors (right). K–L, ZsGreen expressing cells were divided into 5 equal gates based on ZsGreen MFI. Bcl6, CXCR5, CXCR4, PD1 (K), and CCR7 (L) MFIs were determined for each ZsGreen gate. Data shown in K and L are representative of 6 individual donors. M, EBI2 mRNA levels, normalized to GAPDH. * p < 0.05, ** p < 0.005
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 Figure S2A. C–I, Expression of Tfh associated molecules by Bcl6-LV and Control-LV (Ctrl-LV) groups based on gates used in Supplemental Figure S2A. A representative plot is shown (left) alongside the complete data set with 5–6 individual donors (right). J–K, ZsGreen expressing cells were divided into 5 equal gates based on ZsGreen MFI. Bcl6, CXCR5, CXCR4, PD1 (J), and CCR7 (K) MFIs were determined for each ZsGreen gate. Data shown in J and K are representative of 6 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 hours with brefeldin A before intracellular staining for CXCL13. (left) CXCL13 expression shown in total CD4 T cells. (center and right) Activated CD4 T cell subsets were gated (center) and CXCL13 levels for each population are shown versus isotype control (right). Isotype control was gated on total CD4 T cells. MFI for each population is indicated. **B**, CXCR5<sup>int</sup> Tfh cells were sorted from tonsil, stimulated with anti-CD3/CD28 beads, and transduced with Bcl6-LV or Control-LV. 5 days post-transduction, CXCL13 present in the supernatant was analyzed by ELISA. Data is representative of 4 total donors from 2 independent experiments. * p < 0.05, ** p < 0.01.
Figure 5. IL-21 and IL-4 cytokine production by tonsillar CD4 T cells. A, Representative intracellular staining of CD4 T cells isolated from a human tonsil and restimulated for 3 hours with PMA and ionomycin. B, Intracellular staining data from 5 individual donors. * p < 0.05, ** p < 0.005.
Figure 6.
Bcl6 expression is not sufficient for helper cytokine regulation. CD45RO$^+$ CXCR5$^{\text{int}}$ (A), CD45RO$^+$ CXCR5$^-$ (B), or CD45RO$^-$ CXCR5$^-$ (C) CD4 T cells were sorted from human tonsil. Cells were stimulated with anti-CD3/CD28 beads and either left unmanipulated or transduced on day 2 with Bcl6-LV or Control-LV (Ctrl-LV). Five days post-transduction, cells were restimulated with PMA and ionomycin in the presence of brefeldin A and stained. Representative data (left) and pooled data from 5 individual donors (right) are gated on the top 5–10% of ZsGreen$^+$ or dNGFR$^+$ CD4 T cells in either the Bcl6-LV or Ctrl-LV groups. Quadrants are based on cells cultured with brefeldin A alone. * p < 0.05.
Figure 7. Maf instructs IL-21 production by CD45RO\(^{-}\) CXCR5\(^{-}\) CD4 T cells. 

A, Maf mRNA expression was measured by qPCR 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’), both (‘both’, Bcl6-LV and Maf-LV), or a Control-LV (dNGFR-LV, ‘C1’; ZsGreen-LV, ‘C2’). Five days post-transduction, 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 8 donors. D, Representative plot of Bcl6-dNGFR and Maf-ZsGreen RV double transfection (left). * p < 0.05, *** p < 0.001.
Figure 8. Maf enhances Tfh-associated gene expression in CD4 T cells. CD45RO^- CXCR5^- CD4 T cells were sorted from human tonsil, stimulated with anti-CD3/CD28 beads, and transduced with Bcl6-LV (‘Bcl6’), Maf-LV (‘Maf’), Bcl6-LV & Maf-LV (‘both’), or Control-LV (dNGFR-LV, ‘C1’. ZsGreen-LV, ‘C2’). 5 days post-transduction, top 5% of transduced cells were assessed by FACS for expression of Tfh-associated molecules. A, CXCR5 surface expression in naïve CD4 T cells. B, A representative CXCR5 histogram from a single donor. C, Naïve CD4 T cells transduced with Maf-LV, Bcl6-LV, or Ctrl-LV were stained for Bcl6 protein and percent of Bcl6^+ cells is shown. D, Maf mRNA from Ctrl-LV and Bcl6-LV transduced cells. E–H, ICOS (E), PD1 (F), CXCR4 (G), and CD40L (H) expression in naïve CD4 T cells transduced with Ctrl-LV, Bcl6-LV, Maf-LV, or both. Data is representative of 6–9 individual donors. * p < 0.05, ** p < 0.005, *** p < 0.0005.