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BCL6 represses antiviral resistance in follicular T helper cells

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ABSTRACT

Follicular Th (Tfh) cells are a distinct subset of Th cells that help B cells produce class-switched antibodies. Studies have demonstrated that Tfh cells are highly prone to HIV infection and replication. However, the molecular mechanisms underlying this phenomenon are largely unclear. Here, we show that murine and human Tfh cells have diminished constitutive expression of IFN-stimulated genes (ISGs) inclusive of antiviral resistance factor MX dynamin-like GTPase 2 (MX2) and IFN-induced transmembrane 3 (IFITM3) compared with non-Tfh cells. A lower antiviral resistance in Tfh was consistent with a higher susceptibility to retroviral infections. Mechanistically, we found that BCL6, a master regulator of Tfh cell development, binds to ISG loci and inhibits the expression of MX2 and IFITM3 in Tfh cells. We demonstrate further that inhibition of the BCL6 BR-C, ttk, and bab (BTB) domain function increases the expression of ISGs and suppresses HIV infection and replication in Tfh cells. Our data reveal a regulatory role of BCL6 in inhibiting antiviral resistance factors in Tfh cells, thereby promoting the susceptibility Tfh cells to viral infections. Our results indicate that the modulation of BCL6 function in Tfh cells could be a potential strategy to enhance Tfh cell resistance to retroviral infections and potentially decrease cellular reservoirs of HIV infection. *J. Leukoc. Biol.* 102: 527–536; 2017.

Introduction

$CD4^+$ T cells, responding to a variety of environmental cues, can differentiate into several distinct subsets of Th that confer specific effector functions. Tfh cells are a newly identified CD4 Th subset that can help B cells form GCs and produce class-switched antibodies [1, 2]. Tfh cells produce the signature cytokine IL-21 and express key transcription factors, including BCL6, which is required for the generation, survival, expansion, and function of both murine and human Tfh cells [3–5]. Whereas overproduction of Tfh cells can lead to autoimmunity [6], Tfh cells are critical for the proper production of class-switched, high-affinity antibodies against various viral infections or in response to vaccination.

Interestingly, chronic viral infections, such as HIV-1 infection, promote the drastic expansion of Tfh cells, possibly as a result of constant antigenic stimulation [7–9]. Accumulation of Tfh cells is associated with increased frequency of activated GC B cells [7, 8] and is thought to contribute to the GC hyperplasia and lymphadenopathy during HIV infection. The production of class-switched BnAbs against HIV is thought to be dependent on Tfh cell function during HIV infection [9]. However, despite the fact that increased Tfh cells and GC reactions are observed in HIV patients, Tfh functional capacity is proposed to be dysregulated in viremic HIV-infected subjects that lose their ability to generate properly robust HIV-specific and de novo humoral responses to new infections or vaccinations [9].

Strikingly, the Tfh cell population in HIV patients contains the highest percentage of $CD4^+$ T cells having HIV DNA [10], and GC Tfh cells harbor much higher HIV RNA than non-Tfh cells in viremic individuals [10, 11]. These data raise the possibility that Tfh cells may serve as the major $CD4^+$ T cell compartment and reservoir for HIV infection, replication, and production. Indeed, in situ hybridization on LN sections from untreated, HIV-1-infected individuals revealed higher frequencies of HIV-1 RNA (+) cells in GCs [11]. Furthermore, Tfh cells are believed to be the major reservoir of SIV following ART [8, 12]. Consistent with these in vivo findings, ex vivo HIV infection in Tfh cells has demonstrated that Tfh cells are substantially more permissive to HIV infection than non-Tfh cells [10, 11]. Together, these data suggest that Tfh cells are highly susceptible to HIV infection and

Abbreviations: ART = antiretroviral therapy, BCL6 = B cell lymphoma 6, BCOR = B cell lymphoma 6-interacting corepressor, BnAb = broadly neutralizing antibody, BTB = Broad-Complex, Tramtrack and Bric-a-brac, ChIP = chromatin immunoprecipitation, CP = control peptide, DC = dendritic cell, GC = germinal center, IFITM = IFN-induced transmembrane, IFNAR = IFN-α/βR, IRB = Institutional Review Board, IRES = internal ribosome entry site,

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replication, but the associated molecular mechanisms that may act to favor greater viral infection and replication in this cell subset remain unknown.

BCL6 is a transcriptional repressor that is required for the lineage specification of Tfh cells. Specific deletion of BCL6 in T cells abrogates Tfh cell generation in vivo, whereas enforced expression of BCL6 promotes Tfh cell differentiation [2, 3, 13]. BCL6 consists of a zinc finger DNA-binding domain, a middle repressor domain (RD2), and an N-terminal BTB repressor domain. The BTB domain is responsible for the recruitment of various transcriptional corepressors, including SMRTs, NCOR, and BCOR [14]. Mice engineered to express a BCL6 BTB mutant that cannot bind to SMRT/NCOR/BCOR show impaired GC B cell phenotype development and diminished Tfh generation and function in vivo [15, 16]. Peptide mimics and small molecules that can disrupt the BCL6 BTB domain binding to SMRT/NCOR/BCOR are able to inhibit the BCL6-dependent growth of lymphoma in vitro and in vivo, suggesting that BCL6 BTB domain inhibitors could be useful targets to disrupt certain BCL6 functions for therapeutic interventions [17].

In this report, we have examined antiviral gene expression in Tfh and non-Tfh effector cells. We found that both murine and human Tfh cells exhibit diminished antiviral ISG expression compared with their counterparts, non-Tfh cells. We found that Tfh cells exhibited diminished sensitivity to type I IFN stimulation, most probably as a result of the direct inhibition of ISG expression by BCL6. We showed that both murine and human Tfh cells exhibit enhanced susceptibility to viral infections. Importantly, inhibition of BCL6 function enhanced ISG expression in Tfh cells and promoted Tfh cell resistance to HIV infection. Our data reveal that the Tfh master transcription factor BCL6 suppresses antiviral resistance in Tfh cells, thereby enhancing their susceptibility to viral infection.

MATERIALS AND METHODS

Ethics statement

Mouse studies were approved by the Animal Care and Use Program at IUSM (Protocol Number: 10006). IUSM is in compliance with all applicable federal regulations and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Daily care for the animals was provided by the trained staff from the Laboratory Animal Resource Center in IUSM. Collection of Tfh cells from human tonsils, LN, and spleens was approved by the IRBs for Human Research at the IUSM (IRB Approval Numbers: 1501544805 and 1402507097). Written, informed consent was provided by each participant directly or by his or her parent or guardian.

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ISG = IFN-stimulated gene, IU = Indiana University, IUSM = Indiana University School of Medicine, LN = lymph node, MLN = mediastinal lymph node, MSCV = murine stem cell virus, MX2 = MX dynamin-like GTPase 2, NCOR = nuclear receptor corepressor-1, PD-1 = programmed cell death 1, r = recombinant, RD2 = repression domain 2, RI-BPI = retroinverso B cell lymphoma 6 peptide inhibitor, SAMHD1 = sterile alpha motif domain and HD domain-containing protein 1, SLFN11 = Schlafen family member 11, SMRT = silencing mediator for retinoid or thyroid hormone receptors, Tfh = follicular Th, Tfr = follicular regulatory T, WT = wild-type

Murine Tfh cell isolation from secondary lymphoid tissues

C57BL/6 mice were infected with influenza A/X-31 (H3N2; ~150 PFU/mouse) for 8–10 d. $CD4^+$ T cells were enriched from MLNs with CD4 microbeads (Miltenyi Biotec, San Diego, CA, USA). The enriched $CD4^+$ T cells were then stained with antibodies to define Tfh cells, as described in flow methods below.

Human Tfh cell isolation from secondary lymphoid tissues

Inflamed human tonsil, LN, and spleen tissues were obtained from Riley Hospital for Children at IU Health or IU Hospital (Indianapolis, IN, USA) and processed immediately in a sterile biosafety hood under the Biosafety Level 2 conditions. In brief, tissues were cut into small pieces, removed of any fat components, homogenized in a sterile cell strainer in PBS, digested in 0.5 μg/ml collagenase D for 10 min at a 37°C incubator with pipetting up and down, filtered through a 70 μm sterile mesh filter, and washed twice with culture medium. Excess cells were frozen in freezing medium for later experiments after counting the cell number. Approximately 100 million total tonsil cells were used to isolate total T cells by using a human T cell enrichment kit from Thermo Fisher Scientific (Waltham, MA, USA). T cells were then surface stained for Tfh cell markers before sorting. $CD4^+CD45RO^+PD\text{-}1^{hi}CXCR5^{hi}$ cells were defined as Tfh cells, whereas $CD4^+CD45RO^+CXCR5^{-/lo}PD\text{-}1^{-/lo}$ cells were defined as non-Tfh cells. Tfh/non-Tfh cells were characterized for their expressions of antiviral genes at the both mRNA and protein levels and also tested for their susceptibility to HIV-1 infection ex vivo.

Flow cytometry

Antibodies conjugated with appropriate fluorochromes were purchased from BioLegend (San Diego, CA, USA), eBioscience (San Diego, CA, USA), or BD PharMingen (San Diego, CA, USA). For murine experiments, enriched LN $CD4^+$ T cells were stained with antibodies against mouse CD4, CD44, PD-1, and CXCR5 (BioLegend) and sorted into Tfh ($CD4^+CD44^+PD\text{-}1^{hi}CXCR5^{hi}$) or non-Tfh ($CD4^+CD44^+PD\text{-}1^{-/low}CXCR5^{-/low}$) cells using FACSAria (BD Biosciences, San Jose, CA, USA). To detect mouse IFNAR1 on the Tfh or non-Tfh cells, $CD4^+$ T cells were stained with antibodies against mouse CD4 (Clone RM4-5), CD44 (Clone IM7), PD-1 (Clone 29F.1A12), CXCR5 (Clone L128D7), or IFNAR1 (Clone MAR1-5A3). After gating on $CD4^+CD44^+PD\text{-}1^+CXCR5^+$ or $CD4^+CD44^+PD\text{-}1^-CXCR5^-$ cell populations, IFNAR1 expression was measured for human experiments: anti-human CD4 (Clone RPA), CD45RO (Clone UCHL1), PD-1 (Clone J105), CXCR5 (Clone MUSUBEE), CCR5 (Clone T21/8), and BCL6 (Clone Bcl-UP). Anti-CXCR4/CD184 mAb (Clone 12G5) was purchased from Thermo Fisher Scientific. Anti-HIV-1 mAb (Clone CK57) was purchased from Beckman Coulter (Brea, CA, USA). Appropriate numbers of human tonsil (T cell enriched) cells were surface stained for Tfh cell markers first, fixed/permeabilized with Fix/Perm buffer (eBioscience), and stained with anti-human BCL6 or anti-HIV-1 antibody in the infection experiments. FACS data were acquired by LSR II or FACSCanto (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

Western blot analysis

Sorted murine Tfh and non-Tfh cells were washed with cold PBS. Cell pellets were lysed in sample lysis buffer (Cell Signaling Technology, Danvers, MA, USA) with a protease inhibitor cocktail (Thermo Fisher Scientific) and incubated on ice for 30 min. Lysed sample was sonicated and centrifuged. Proteins in the supernatants were heat denatured, separated by 4–12% gradient Bis-Tris gel (Thermo Fisher Scientific), and transferred into a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membrane was blocked with 5% of non-fat milk in TBST for 1 h at room temperature. After washing with TBST, the membrane was incubated with the following primary antibodies overnight: rabbit anti-BCL6 (Cell Signaling Technology), rabbit anti-IFITM3 (Sigma-Aldrich, St. Louis, MO, USA), and mouse anti-actin (Santa Cruz Biotechnology, Dallas, TX, USA). The

membrane was then rinsed with TBST and incubated with the HRP-conjugated secondary antibodies for 1 h at room temperature. ECL kit was used for film development (Thermo Fisher Scientific).

ChIP assay

Murine total $CD4^+$ T cells isolated from MLNs of X-31-infected mice, or $CD4^+$ T cells cultured under Tfh conditions were used for ChIP assay. In brief, cells were cultured with 100 ng/ml mouse rIFN-α4 (BioLegend) and fixed with 1% formaldehyde. Then cells were lysed by SDS lysis buffer, including 1% SDS, 10 mM EDTA, and 50 mM Tris (pH 8.1). Anti-mouse BCL6 antibody (Clone N-3; Santa Cruz Biotechnology) was used for immunoprecipitation with DNA/protein complexes. After overnight of immunoprecipitation, DNA was purified, and BCL6-binding DNA fragments were measured by real-time PCR with ChIP primers (shown in **Table 1**) for mouse IFITM3 or MX2. Antibody-binding value was normalized by the amount of input DNA.

In vitro T cell culture

Murine bone marrow-derived DCs were generated as described previously [18]. Naïve $CD4^+$ T cells were isolated from WT or BCL6-fl/fl CD4-Cre mice (BCL6 deficient). $CD4^+$ T cells were mixed with DCs at the ratio of 1:10 in the presence of 0.1 μg/ml soluble αCD3 under Tfh condition with rIL-12 (5 ng/ml), rIL-21 (20 ng/ml), anti-IL-2 (20 μg/ml, Clone S4B6; Bio-X-Cell, West Lebanon, NH, USA), and anti-IFN-γ antibodies (20 μg/ml, Clone XMG1.2; Bio-X-Cell) for 4 d. T cells were washed and treated with rIFN-α for 6 h for the determination of antiviral genes.

Real-time PCR

RNA was extracted from cells indicated in the text with a Total RNA Miniprep Kit (Sigma-Aldrich). RNA RT and real-time PCR were performed as described previously [18]. Data were generated with the comparative threshold cycle method by normalizing to hypoxanthine phosphoribosyltransferase or GAPDH.

MSCV retroviral expression

Sorted murine Tfh or non-Tfh cells from MLNs of influenza-infected mice were spin -infected with MSCV-IRES-GFP retrovirus at 2500 rpm for 90 min. After retroviral infection, the cells were cultured with 20 U human IL-2 for 2 d. GFP^+ cells were measured by flow cytometry. In some experiments, cells were precultured with CP or BCL6 inhibitor RI-BPI (Bio-Synthesis, Lewisville TX, USA) for 1 h.

$HIV\text{-}1_{NL4\text{-}3}$ viral stock preparation and ex vivo infection. HIV-1 viral stock was prepared as described in our recent report [19]. In brief, viral DNA pNL4-3 (plasmid construct harbering full-length HIV genome NL4-3) was obtained from the NIH AIDS Reagent Program (Germantown, MD, USA). After expansion (by transformation to DH5α) and purification, pNL4-3 was transfected into human embryonic kidney 293T cells by lipofectamine. Cell-free supernatants, 48 h post-transfection, were collected as infectious virions by centrifugation and followed by passing through a 0.2 μm filter. Viral stocks were stored at −80°C after measuring virus titer using quantitating p24-Gag (HIV-1 core protein) ELISA. HIV-1 infections were carried out by adding 20 ng/ml p24 of virus solutions to the cells and incubating at 37°C for 2 h. Infected cells were cultured in complete RPMI-1640 medium at 37°C, with or without peptide treatment, for 2 or 4 d in various experiments. Viral RNA or p24-Gag was quantitated as a marker of HIV-1 replication.

BCL6 inhibitor peptide RI-BPI, CP, and IFN-α2b. CPs and RI-BPI were synthesized from Bio-Synthesis (Lewisville, TX). IFN-α2b was purchased from R&D Systems (Minneapolis, MN, USA). After sorting, human Tfh cells were treated with 10 μM CP or BCL6 inhibitory peptide RI-BPI in the presence or absence of IFN-α (100 ng/ml) at 37°C for 12 h. In HIV-1 infection experiments, peptides remained in culture during the infection (48 h).

Statistical analysis

Graphs were generated by GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Statistical significance was evaluated by calculating *P* values using one-way ANOVA or Student's *t* test. Significance between the groups was judged based on $P < 0.05$ (two-tailed).

RESULTS

Diminished ISG antiviral gene expression in Tfh cells

To determine antiviral gene expression in Tfh and non-Tfh cells, we first analyzed ISG expression in a publicized microarray data set of murine Tfh cells (GEO #GSE40068) [20]. We found that compared with non-Tfh cells ($CD44^+CXCR5^-BCL6^-$), Tfh ($CD44^+CXCR5^+BCL6^{hi}$) cells exhibit diminished expression of a number of ISGs (**Fig. 1A**). To confirm these microarray data, we sorted murine Tfh and non-Tfh cells from the draining MLNs of day 8 influenza X-31-infected WT mice. We sorted Tfh cells as $CD44^+CXCR5^+PD\text{-}1^{hi}$ and non-Tfh cells as $CD44^+CXCR5^-PD\text{-}1^-$ (Supplemental Fig. 1A). As expected, Tfh cells expressed higher levels of the transcription factor BCL6 compared with non-Tfh cells (Supplemental Fig. 1A). We then examined ISG expression in Tfh and non-Tfh cells by quantitative real-time RT-PCR. We found that a number of ISGs, including IFITMs, MX2, and SAMHD1, were lower in Tfh than non-Tfh cells (Fig. 1B). Western blot analysis confirmed enhanced BCL6 and diminished IFITM3 protein

TABLE 1. ChIP primers

Sites	Forward primer	Reverse primer
	Ifitm3 gene sites	
A	5′-GCTCCCCCCCTTACTCTCTA-3′	3′-CGGTGGCTATGCAGTCATAT-5′
B	5′-CCGTTCATCCCACCTGTCTA-3′	3′-CAAATTACTCCAGGGCAGG-5′
C	5′-GTTTGGGGGCTGTCCTCCAC-3′	3′-CCGCAGGCTTTTTAGATCCC-5′
	Mx2 gene sites	
A	5′-CATCCGTGGTGGAGGAAACC-3′	3′-GGTGTGGTAACCACCAGGTC-5′
B	5′-TCCCCCATCCCTGGCACAGT-3′	3′-AAGAGTGTGTGAGACAGGGG-5′
C	5′-ATTCCAGCTTCCCTCCAGCT-3′	3′-GGTTCCTGGCATACAATG-5′
D	5′-GCTTAGTGAAAAACTGGCCC-3′	3′-ATGGACACCTCTGGCCCCAA-5′
E	5′-GTAGACAGAGGGAGAGCACA-3′	3′-GCCATTGCTCCAGCCTCCAA-5′

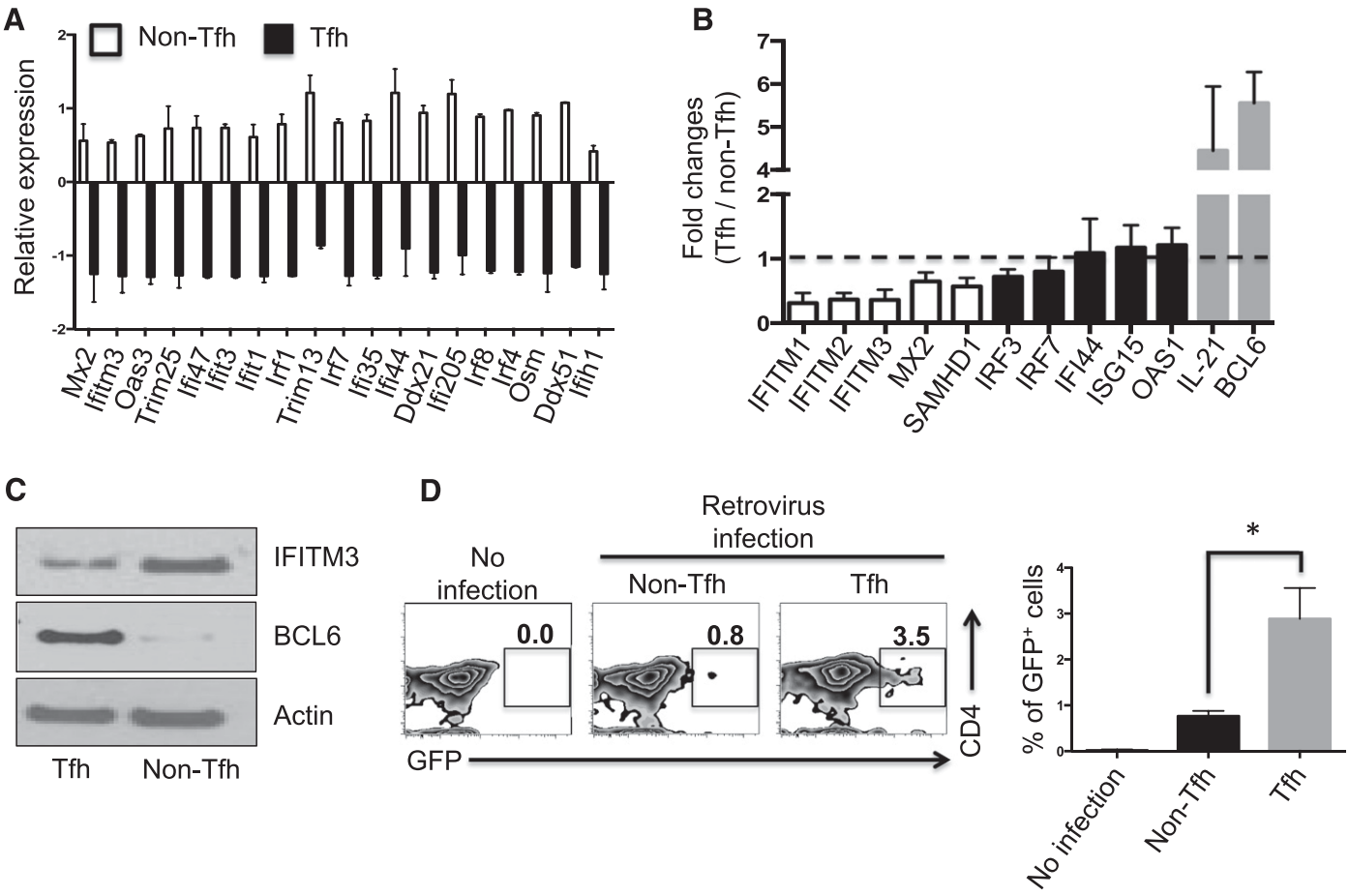

Figure 1. Murine Tfh cells exhibit diminished antiviral gene expression and enhanced retroviral infection. (A) Relative antiviral ISG expression in published microarray data (GEO #GSE40068) Tfh (BCL6^{hi}CXCR5^{+}) and non-Tfh (BCL6^{-}CXCR5^{-}) cells isolated from keyhole limpet hemocyanin/CFA immunized mice. (B) Tfh or non-Tfh cells were sorted from MLNs of X-31-infected mice at day 8 postinfection. Antiviral genes were determined by real-time PCR. (C) IFITM3 and BCL6 protein levels were measured by Western blot in sorted Tfh and non-Tfh cells. (D) Sorted Tfh and non-Tfh cells were infected with nonreplicating MSCV-IRES-GFP retrovirus in vitro, and GFP^{+} cells were measured by flow cytometry at 2 d postinfection. Data are representative of 2 experiments or pooled from 3 (B and D) independent experiments. *$P < 0.05$ significant differences.

expression in Tfh cells compared with non-Tfh cells (Fig. 1C). Taken together, these data suggest that murine Tfh cells exhibit diminished antiviral ISG expression compared with non-Tfh effector cells. Next, we spin infected Tfh cells and non-Tfh cells isolated from influenza-infected mice with nonreplicating MSCV retrovirus with a GFP reporter. We then determined GFP expression in Tfh and non-Tfh cells as a surrogate of infection. Consistent with diminished antiviral ISG expression, we found that Tfh cells exhibited enhanced susceptibility to retroviral infection, as evidenced by the higher percentages of cells expressing GFP in Tfh cells (Fig. 1D). Thus, these results suggested that Tfh cells have attenuated antiviral resistance and show enhanced susceptibility to retroviral infection when compared with non-Tfh cells.

BCL6 regulates expression of ISG antiviral genes

To probe the potential mechanisms by which Tfh cells exhibit lower antiviral ISG expression in vivo, we first investigated whether murine Tfh cells exhibit diminished sensitivity to type I IFN treatment. Therefore, we measured the expression of ISGs (MX2, IFITM1, and IFITM3) in sorted Tfh and non-Tfh cells following ex vivo IFN-α treatment. Our results showed that Tfh cells exhibited diminished MX2 and IFITM3 expression following IFN-α stimulation compared with non-Tfh cells, suggesting that Tfh cells have lower sensitivity to type I IFNs (**Fig. 2A**). We also cultured naïve or BCL6-deficient CD4^{+} T cells under Tfh conditions and treated the cells with IFN-α (Fig. 2B). We found that in the absence of BCL6, CD4^{+} T cells exhibited enhanced ISG expression following IFN-α treatment, suggesting that BCL6 may suppress IFN-α sensitivity in CD4^{+} T cells. As the diminished sensitivity of Tfh cells to type I IFNs could result from decreased type I IFNR expression, we examined IFNAR1 expression on Tfh and non-Tfh cells. However, we found that Tfh and non-Tfh cells have comparable IFNR expression (Fig. 2C). BCL6 has been shown to regulate Stat1 expression in osteoblasts [21]. However, we found that Tfh and non-Tfh cells showed

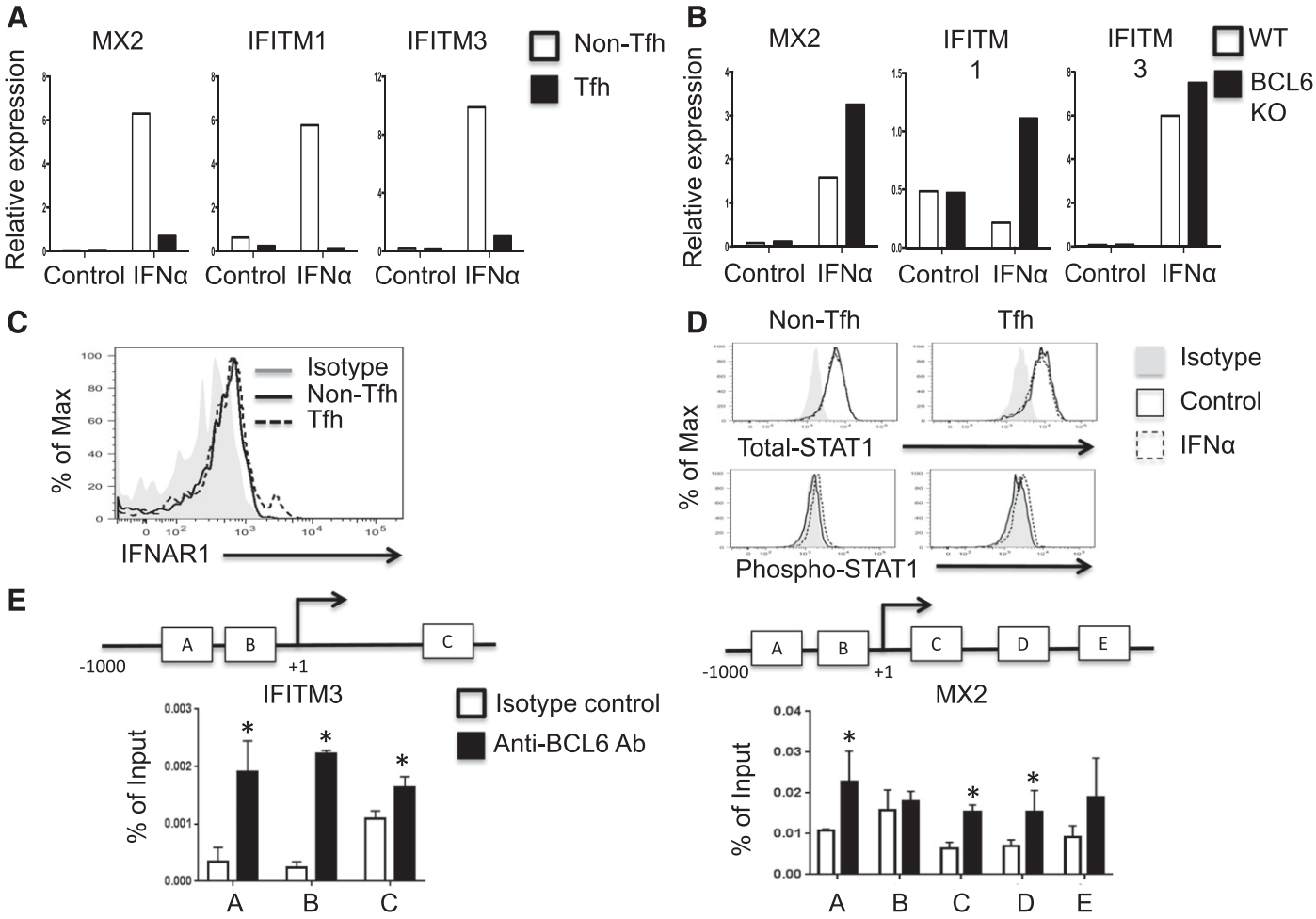

Figure 2. BCL6 binds to antiviral gene loci. (A) Sorted Tfh and non-Tfh cells were treated with IFN-α in vitro. Antiviral genes were detected 6 h after treatment. (B) Naïve WT and BCL6-deficient [knockout (KO)] $CD4^+$ T cells were cultured under the Tfh condition for 3 d. Cells were cultured in the absence or presence of IFN-α. Antiviral genes were determined 6 h following treatment. (C) IFNAR1 surface expression on WT Tfh and non-Tfh cells from influenza-infected mice was measured by flow cytometry. (D) Phospho- or total-STAT1 expression was detected in WT Tfh and non-Tfh cells from day 8 influenza-infected mice in the presence or absence of IFN-α. (E) BCL6 binding to IFITM3 or MX2 in $CD4^+$ T cells following IFN-α treatment was determined by ChIP. Data are representative of 2–3 independent experiments. $*P < 0.05$ significant differences.

similar levels of total STAT1 expression (Fig. 2D). Furthermore, Tfh cells exhibited comparable STAT1 phosphorylation following IFN-α stimulation compared with non-Tfh cells. BCL6 is a transcriptional repressor that can bind directly to specific DNA-binding sequences to repress target gene expression [14]. We reasoned that BCL6 may directly bind to ISG loci to inhibit their expression in Tfh cells. Consistent with this idea, we found that MX2 and IFITM3 loci contain several potential BCL6 binding sites. Indeed, the BCL6 ChIP assay confirmed that BCL6 could bind to these sites, including the promoter and 3′ conserved untranslated region (Fig. 2E and Supplemental Fig. 2). Together, these data suggest that BCL6 directly binds to ISG loci to repress their expression.

Inhibition of BCL6 BTB domain activity enhances Tfh cell resistance to viral infection

The BCL6 BTB domain is required for the recruitment of corepressors to inhibit target gene transcription, which can be abrogated by a peptide inhibitor, RI-BPI [17]. To determine whether the BCL6 BTB domain is responsible for the enhanced susceptibility of viral infection in Tfh cells, we infected WT Tfh and non-Tfh cells in the presence of CP or RI-BPI. We found that RI-BPI treatment did not significantly alter T cell CD69 expression and their survival in vitro (Supplemental Fig. 3). However, RI-BPI treatment decreased the level of retroviral infection of Tfh cells but not of non-Tfh cells (**Fig. 3**). These results suggest that the BCL6 BTB domain is responsible for dampening the resistance of Tfh cells to viral infection.

Human Tfh cells exhibit diminished antiviral gene expression and enhanced susceptibility to HIV infection

We next examined whether human Tfh cells have similar features of diminished antiviral gene expression compared with non-Tfh cells. Analysis of published microarray data sets (GEO #GSE50391) of tonsil Tfh cells revealed that human Tfh cells exhibit diminished expression of several anti-HIV restriction

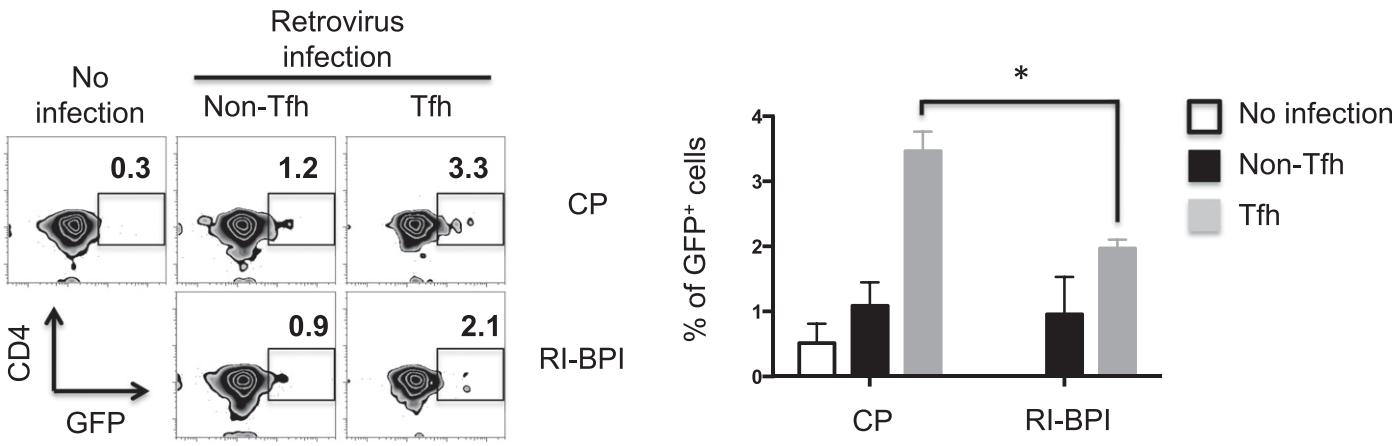

Figure 3. Inhibition of BCL6 BTB domain function decreases the susceptibility of murine Tfh cells to retroviral infection. Sorted Tfh and non-Tfh cells were cultured with CP or RI-BPI (a BCL6 BTB domain inhibitor) and infected with retrovirus. Percentages of GFP^+ cells as a surrogate of retroviral infection were measured by flow cytometry. Data are representative of 3 independent experiments. $*P < 0.05$ significant differences.

factors, including MX2, IFITMs, SAMHD1, and SLFN11 (**Fig. 4A**) [9], which are the ISGs that have been shown to inhibit HIV infection/replication [22, 23]. To confirm these results, we isolated tonsil Tfh and non-Tfh cells from 6 donors. We identified human Tfh cells as $CD4^+CD45RO^+PD\text{-}1^{hi}CXCR5^{hi}$ cells and non-Tfh cells as $CD4^+CD45RO^+CXCR5^{-/lo}PD\text{-}1^{-/lo}$ (Supplemental Fig. 1B). Similar to murine Tfh cells, we found that human Tfh expressed higher levels of BCL6 compared with non-Tfh cells (Supplemental Fig. 1B). We determined antiviral gene expression in sorted Tfh and non-Tfh cells by real-time RT-PCR. Our data confirmed that Tfh cells exhibited diminished expression of major anti-HIV genes, including IFITM3, MX2, SAMHD1, and SLFN11 (Fig. 4B). To determine whether human Tfh cells have enhanced susceptibility to HIV infection, we isolated Tfh and non-Tfh cells from human secondary lymphoid tissues (tonsil, LN, and spleen) and infected the isolated cells with $HIV\text{-}1_{NL4\text{-}3}$. We then analyzed viral gene expression levels in Tfh versus non-Tfh cells. Consistent with the previous reports that Tfh cells are prone to HIV-1 infection/replication [10, 11], we detected higher viral Gag RNA levels in Tfh cells compared with non-Tfh cells (Fig. 4C). We have also assessed HIV-1 core protein (p24-Gag) expression levels by intracellular staining with a Gag-specific antibody. Consistent with the viral Gag RNA levels, we observed higher levels of HIV-1 core protein in Tfh cells compared with non-Tfh cells (Fig. 4D). Notably, in agreement with a previous report [11], we found that Tfh and non-Tfh cells exhibited comparable expression levels of HIV-1 coreceptors CXCR4 and CCR5 (Supplemental Fig. 4). Together, these data indicate that human Tfh cells have diminished anti-HIV innate immunity and enhanced susceptibility to HIV infection and replication.

BCL6 inhibition leads to enhanced anti-HIV gene expression and host resistance to HIV infection

We reasoned that the inhibition of the BCL6 BTB domain function may increase antiviral gene expression in human Tfh cells. To this end, we treated tonsil Tfh cells with RI-BPI in the absence or presence of IFN-α. We found that RI-BPI treatment augmented anti-HIV ISG expression, with and without IFN- α treatment (**Fig. 5A**). To investigate further the regulatory function of BCL6 on HIV-1 infection/replication in human Tfh cells, we infected human tonsil Tfh cells with HIV in the presence of CP or RI-BPI. As shown in Fig. 5B, we detected diminished levels of HIV-1 RNA in RI-BPI-treated cells compared with CP-treated cells. Thus, inhibition of BCL6 BTB domain function resulted in enhanced anti-HIV ISG expression and diminished HIV infection/replication in human Tfh cells.

DISCUSSION

In this report, we provided evidence indicating that both murine and human Tfh cells exhibit diminished expression of antiviral ISG ex vivo. We further showed that the Tfh lineage-defining transcription factor BCL6 binds directly to antiviral ISG loci and potentially represses ISG gene expression. Importantly, inhibition of the BCL6 BTB domain function enhanced ISG expression in Tfh cells and increased their resistance to viral infection. Therefore, our results have provided a molecular mechanism by which Tfh cells are prone to HIV/SIV infection and also offer novel insight into developing methods to boost anti-HIV resistance in Tfh cells potentially to decrease HIV cellular reservoirs following ART.

HIV and SIV infection is associated with great expansion of Tfh cells in the host [7–9]. Indeed, Tfh cells were shown to have multifaceted roles during HIV and SIV infection. On one hand they are essential in regulating the development of BnAb responses, and on the other hand, chronic Tfh cell activation may contribute to the immunopathology of HIV and SIV infection. Furthermore, multiple lines of evidence suggest that Tfh cells are highly permissive to HIV or SIV infection in vitro and are major cellular targets of HIV infection in vivo [11, 24–26]. Previous findings have suggested that HIV-1 chemokine coreceptor expression and Tfh immune activation were not linked to the permissivity of Tfh cells to ex vivo HIV infection [11]. In accordance with these findings, we also found that Tfh cells (or GC-Tfh, as referred to by the other report [11]) in our

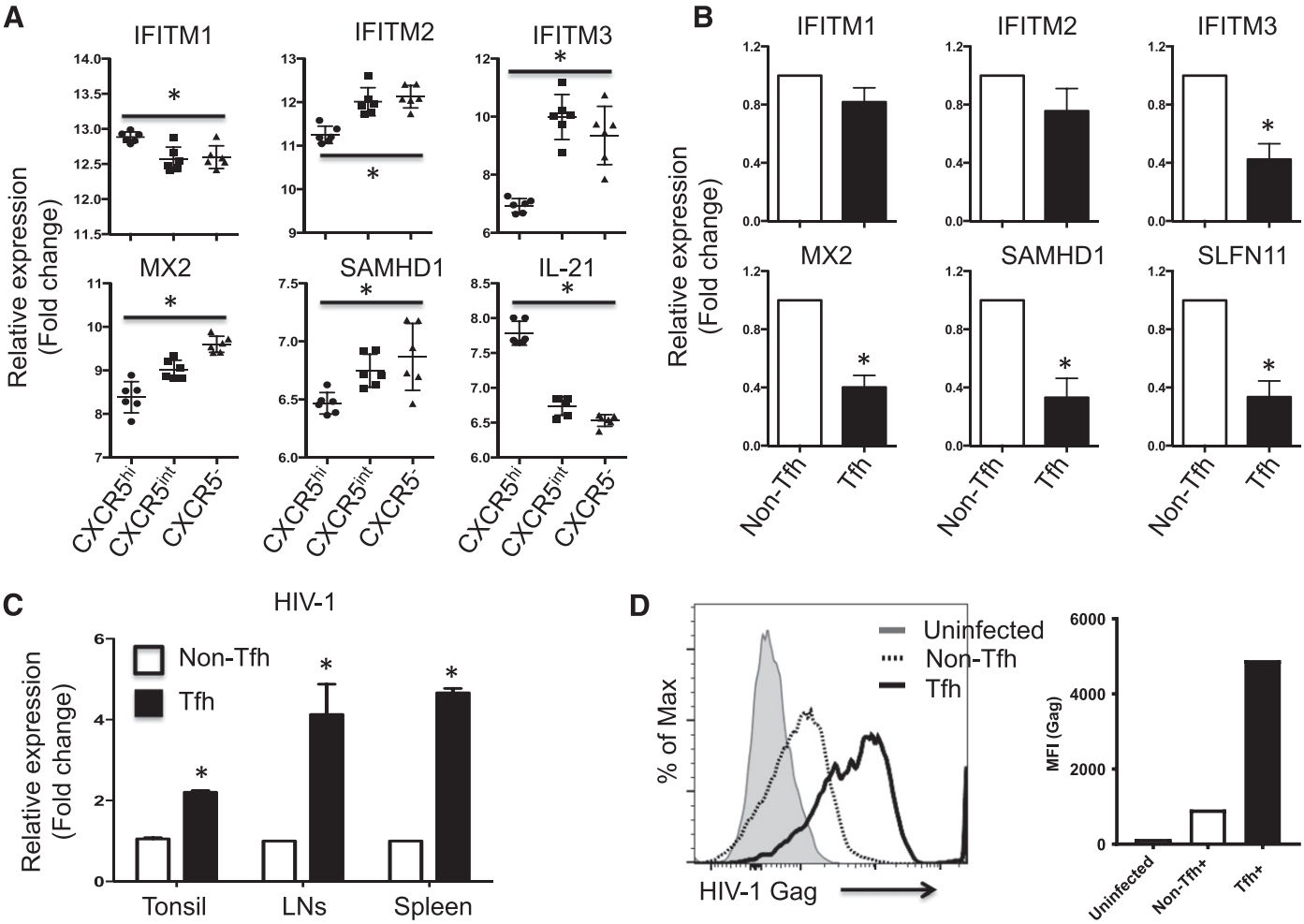

Figure 4. Human Tfh cells exhibit lower levels of antiviral factors and are more prone to HIV-1 infection. (A) Antiviral gene expression in published microarray data (GEO #GSE50391) from tonsil Tfh (PD-1^{high}CXCR5^{high}) or non-Tfh (PD-1^{inter}CXCR5^{inter} or PD-1^{-}CXCR5^{-}) populations. (B) Antiviral gene expression in sorted human tonsil Tfh and non-Tfh cells was determined by real-time RT-PCR. (C and D) Human Tfh and non-Tfh cells were sorted from the secondary lymphoid organs, including tonsil, LNs, and spleen, and subjected to HIV-1 (NL_{4-3} strain) infection. (C) HIV-1 Gag RNA levels were determined by quantitative real-time RT-PCR on day 4 postinfection. (D) HIV-1 core (Gag) protein levels in tonsil Tfh and non-Tfh cells were determined by flow cytometry. Data are from 1 (A) or 3 experiments (B–D) with total sample numbers from 3 (C and D) to 6 (A and B). MFI, Mean fluorescence intensity. *$P < 0.05$ significant differences.

tonsil cell populations exhibit comparable expression of CXCR4 and CCR5, suggesting that the enhanced HIV infection and replication are not a result of the differential expression of these two HIV coreceptors.

SAMHD1 is an important HIV restriction factor in CD4^{+} T cells, and Tfh cells exhibited lower levels of SAMHD1 expression [27]. Likewise, we found that Tfh cells exhibited lower expression of SAMHD1. In addition, we found that murine Tfh cells exhibited impairment in the expression of a variety of antiviral genes downstream of type I IFN, suggesting that Tfh cells have a global defect in innate resistance to viral infection. Consistent with this idea, human Tfh cells exhibit impaired expression of IFITM3, MX2, and SLFN11, 3 major HIV restriction factors identified recently that can block HIV infection and/or replication at various stages. IFITM3 blocks HIV infection, possibly through the disruption of viral fusion [28–30], whereas MX2 is a postentry inhibitor of HIV by antagonizing nuclear accumulation of viral cDNAs [31–33]. SLFN11 is upregulated in HIV-1 elite controllers and is thought to inhibit viral protein synthesis in HIV-infected cells by means of codon-bias discrimination [34, 35]. The relative contributions of impaired expression of SAMHD1, IFITM3, MX2, or SLFN11 in the susceptibility of Tfh cells to HIV infection were not tested in this study. However, BCL6 inhibition failed to augment SAMHD1 expression but still improved Tfh resistance to HIV infection. One possible explanation is that IFITM3, MX2, and/or SLFN11 may supplement SAMHD1 function in this regard. In addition, we did not firmly establish that diminished ISG expression is responsible for the enhanced HIV infection in our study. It is possible that other factors, including the differential activation status of Tfh and non-Tfh, could underlie their differential susceptibility to HIV infection. Further studies are warranted to determine whether Tfh susceptibility to HIV infection is regulated through the impaired expression of a single factor or a

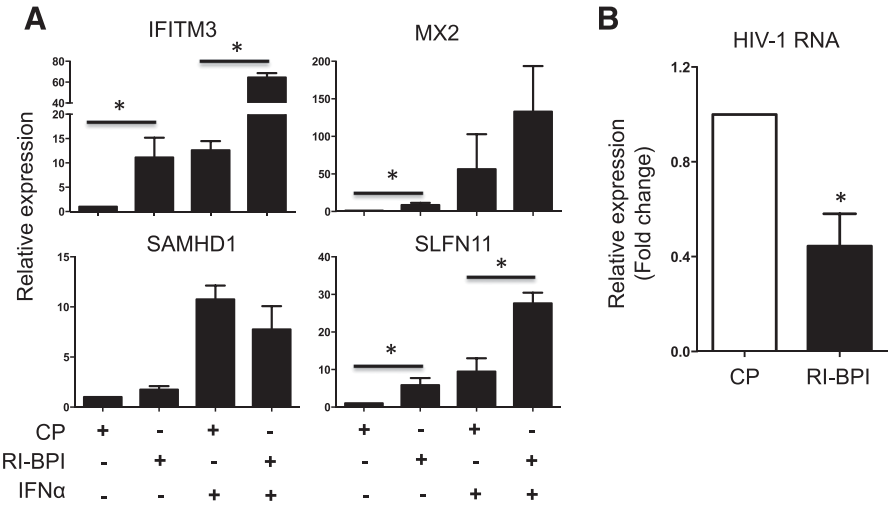

Figure 5. Inhibition of BCL6 BTB domain function increases antiviral gene expression and suppresses HIV-1 replication. (A). Tfh cells were sorted from tonsil cells and treated with CP or RI-BPI in the presence or absence of IFN-α. The expression of antiviral factors was analyzed by real-time RT-PCR. (B) Sorted tonsil Tfh cells were infected with HIV in the presence or absence of CP or RI-BPI. HIV-1 viral RNA levels were quantitated by RT-PCR on day 2 postinfection. Normalized data are pooled from 3 experiments with total sample numbers of 3. $*P < 0.05$ significant differences.

combination of multiple factors. Notably, type I IFN treatment inhibits cells to produce infectious HIV virions [36]. Thus, it is of interest to investigate in the future whether Tfh cells are able to produce more infectious virus as a result of their lower levels of ISG expression compared with non-Tfh cells.

An interesting question raised from our study is why Tfh cells down-regulate their antiviral gene expression compared with non-Tfh cells. BCL6 was shown to be required for Tfh cell survival [37]. Many ISGs are proapoptotic and/or can arrest the cell cycle to inhibit virus dissemination [38]. Thus, the survival and/or proliferation of Tfh cells may require the function of BCL6 to antagonize expression or function of ISGs. To this end, type I IFN signaling, which leads to the expression of ISGs, has been shown to interfere with both murine and human Tfh cell development and expansion [39, 40], suggesting that ISGs downstream of IFN signaling could hamper the development or maintenance of Tfh cells. Consistent with the idea, we found that Tfh cells were less capable of up-regulating ISGs following type I IFN treatment. Of note, 1 recent study has shown that the anti-apoptotic protein BCL2 expression was enhanced in Tfh cells compared with non-Tfh cells, presumably to help Tfh survival, but may contribute to HIV propagation in B cell follicles in vivo [41]. The relationship and functional intersection of BCL6 and BCL2 in promoting Tfh survival and HIV propagation in vivo require future studies.

Tfr cells are recently identified FOXP3+CXCR5+PD-1+ regulatory T cells that are specialized to control GC antibody responses [42]. As a result of technical challenges of isolating live Tfr cells, we were not able to examine ISG expression in those cells. Given Tfr cells express high levels of BCL6 [42] similar to Tfh cells, it could be possible that Tfr cells exhibit lower ISG expression compared with other effector and/or regulatory cell subsets. This hypothesis warrants future investigations.

Interestingly, although BCL6 has been found to regulate STAT1 expression in osteoblasts [21], we found that Tfh cells exhibited similar levels of type I IFNR and STAT1 and were able to up-regulate similar levels of phosphorylated STAT1 expression when compared with non-Tfh cells. As BCL6 is a transcriptional repressor, we had hypothesized that BCL6 directly inhibits ISG expression in Tfh cells following type I IFN exposure. Consistent with this idea, BCL6 could bind to ISG loci, strongly indicating that its mechanism of action includes direct inhibition of ISG expression in Tfh cells. Genomic studies have revealed that many BCL6-bound loci were found to be present with a STAT-binding motif, suggesting that BCL6 may globally antagonize the functions of STATs [16]. BCL6 may act as a negative-feedback mechanism for type I IFN signaling, as STAT1 can also up-regulate BCL6 expression in T cells [43]. Thus, a regulatory balance of STAT1–BCL6 may determine the outcome of ISG expression and its downstream effects on viral susceptibility in Tfh cells. Notably, we did not compare comprehensively the type I IFN downstream signaling events between Tfh cells and non-Tfh cells other than STAT1 phosphorylation in our study. Thus, it remains possible that other STAT1-independent defects of type I IFN signaling contribute to the impaired ISG expression in Tfh cells. Such a possibility requires further studies.

BCL6 contains two repressor domains: a middle RD2 and an N-terminal BTB domain [14]. Each of these domains can associate with specific corepressor complexes in B cells and macrophages. Both the RD2 domain and BCL6 BTB domain have been shown to be important in Tfh cell function in vivo [44–46]. We found that RI-BPI, a peptide inhibitor that can disrupt the association of the BCL6 BTB domain with its corepressors, was able to up-regulate Tfh ISG expression, suggesting the BCL6 BTB domain is critically important in suppressing ISG expression in Tfh cells. A recent report has also suggested that inhibition of the BCL6 BTB domain could boost type I IFN expression in myeloid cells [47]. Thus, the inhibition of the BCL6 BTB domain function may offer a unique opportunity to boost both type I IFN production and sensitivity to type I IFNs in IFN-responding cells. The targeting of BCL6 may be a promising approach to decrease HIV reservoirs in vivo. This possibility clearly deserves further future investigation.

AUTHORSHIP

T. A. and Y.M.S. participated in research design, performance of research, data analysis, and the writing of the paper. L.J., I.S.C., and S.H. participated in performance of research and data

analysis. S.K.G. and A.L.D. participated in data analysis. L.J.M., Q.Y., and J.S. participated in research design, data analysis, and the writing of the paper. All authors read and approved the final manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

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KEY WORDS:
HIV infection · Tfh cell · IFITM3 · MX2

BCL6 represses antiviral resistance in follicular T helper cells

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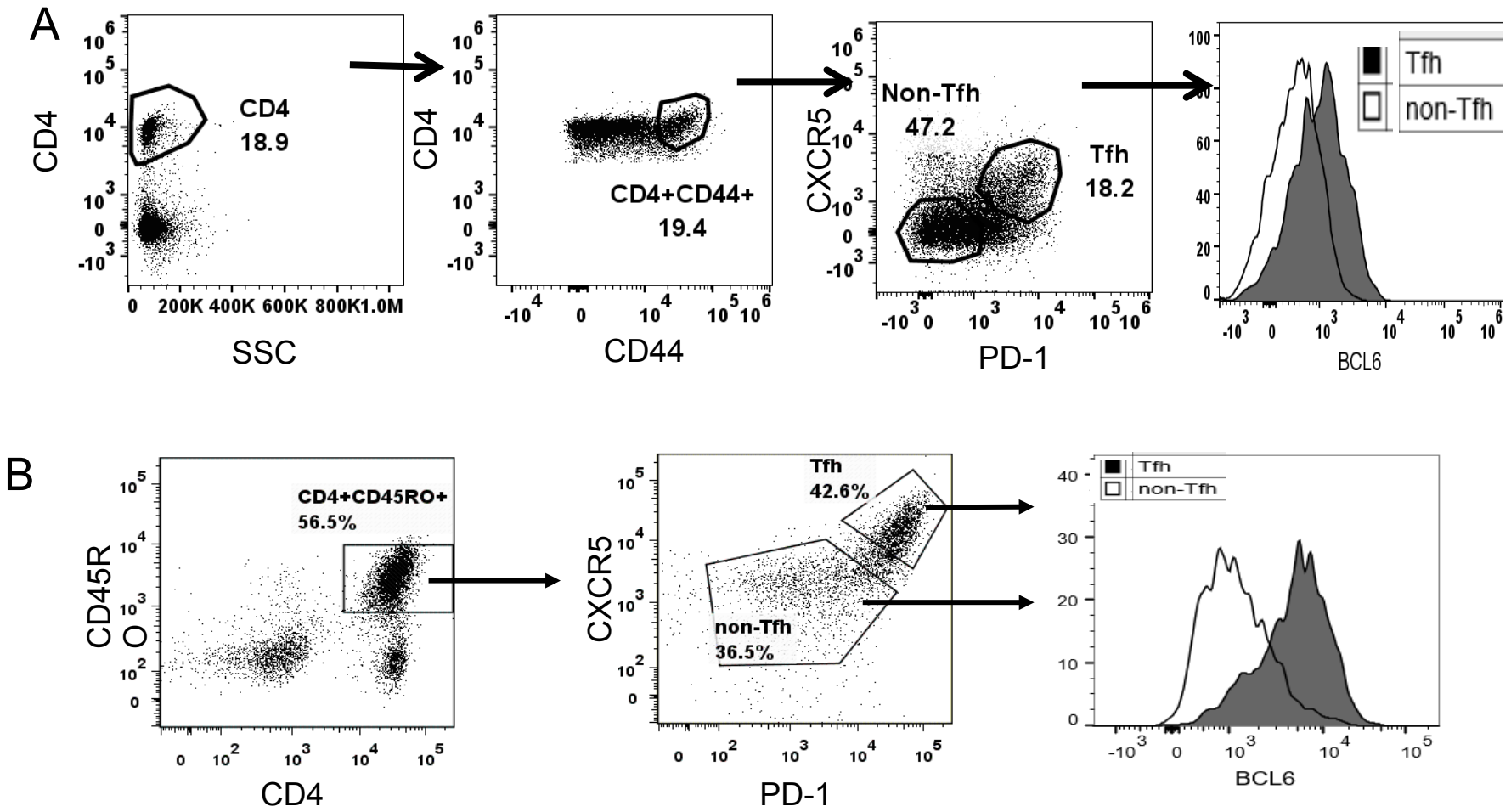
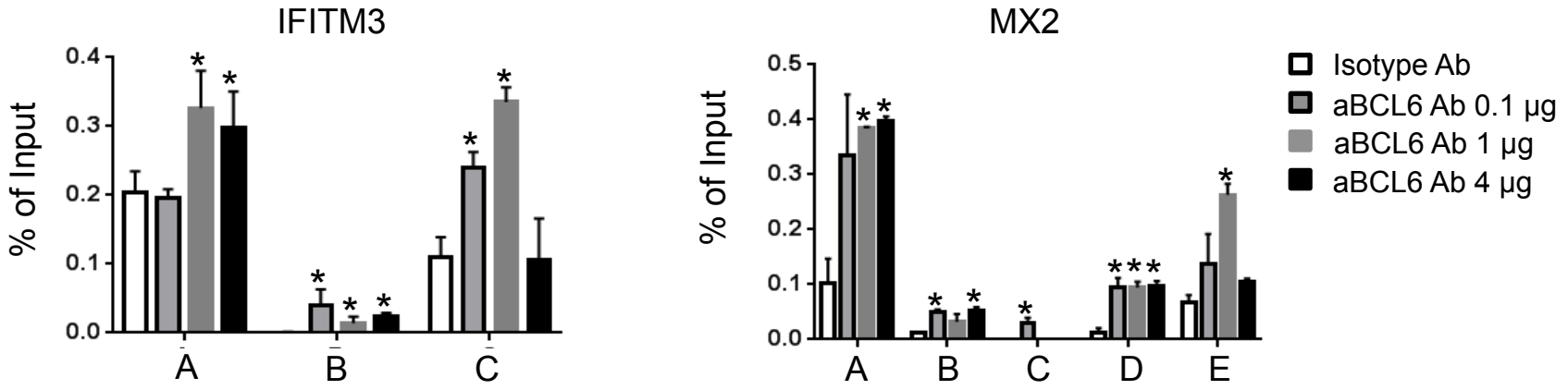
Supplementary Figure 1



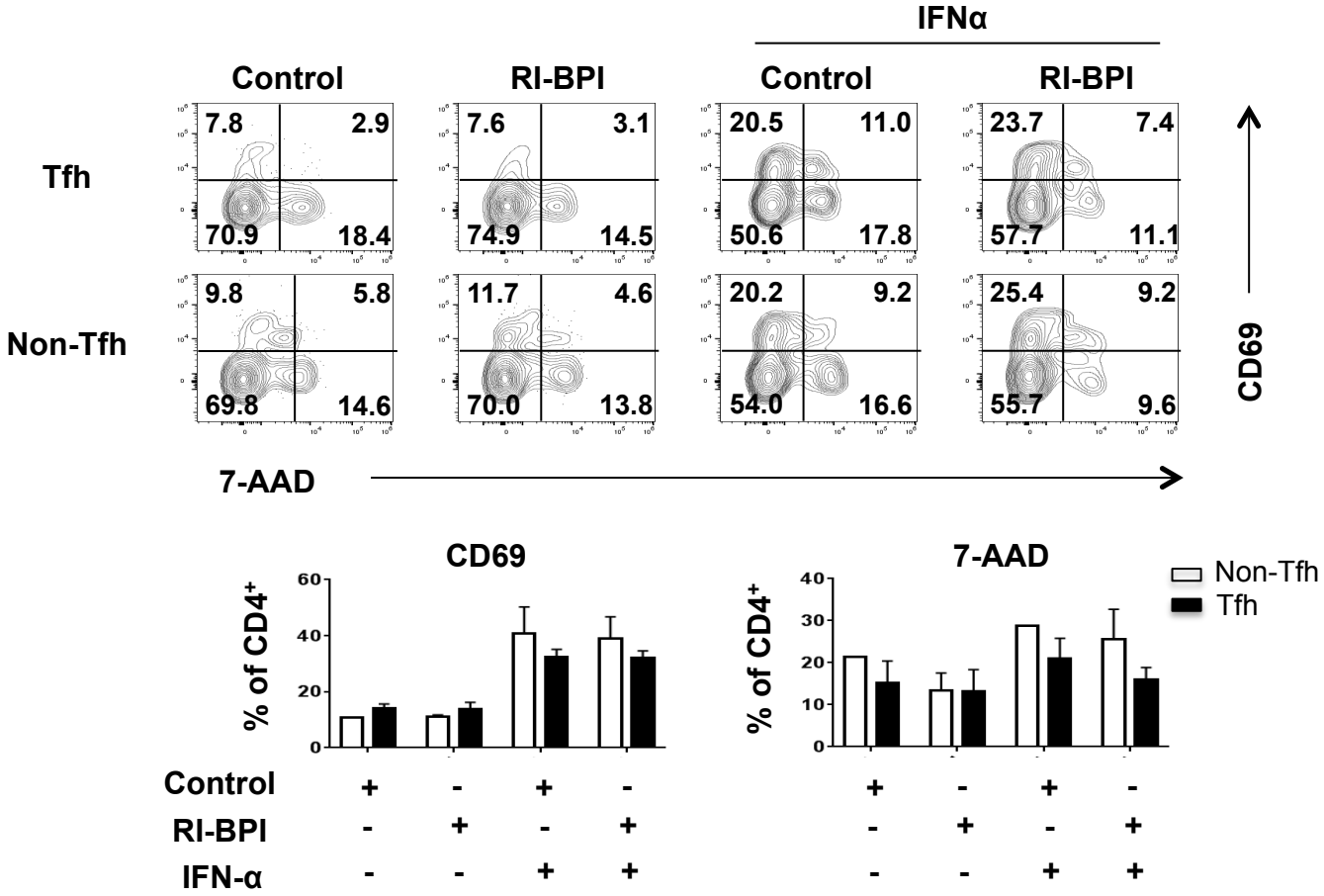
Supplementary figure 1. Characterization of mouse and human Tfh and non-Tfh cells. A. Gating strategy for the identification of mouse Tfh cells from LN cells of influenza-infected mice. LN cells were stained with CD4, CD44, PD-1, CXCR5 and BCL6 and subjected to flow cytometry analysis. B. T cell-enriched human tonsil cells were stained with CD4, CD45RO, PD-1, and CXCR5 and BCL6 and subjected to flow cytometry analysis.

Supplementary Figure 2



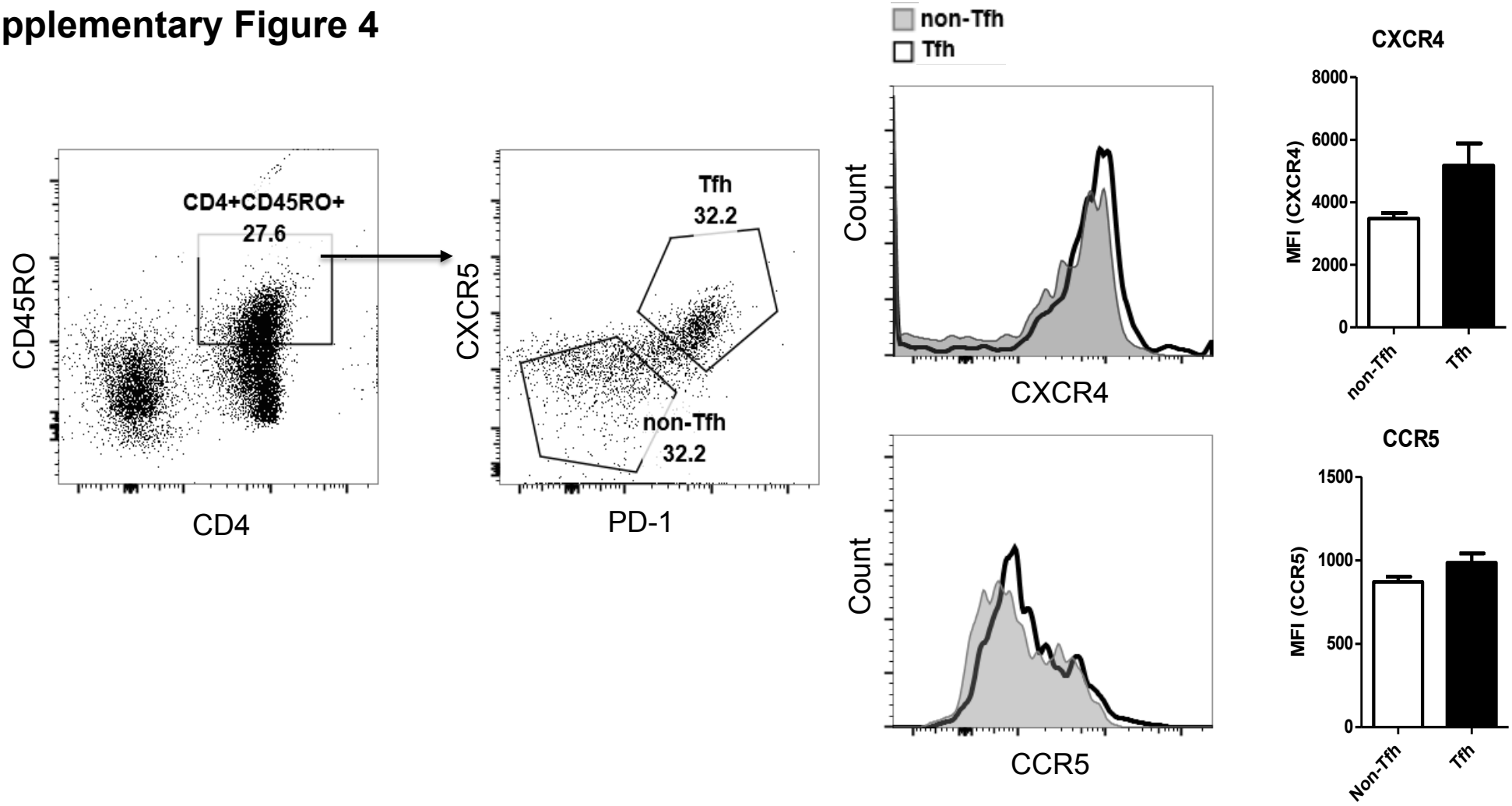
Supplementary figure 2. BCL-6 binds to ISG loci in CD4⁺ T cells cultured under Tfh condition.
Naïve CD4 T cells were cultured with Tfh condition for 4 days. Cells were then stimulated with IFN-α for 6 hrs. BCL-6 binding to *Ifitm3* and *Mx2* loci were assessed through ChIP assay with increasing amount of anti-BCL-6 Ab (0.1, 1, 4 ug) used. Representative data were obtained from two independent experiments. * indicates significant differences ($P < 0.05$).

Supplementary Figure 3



Supplementary figure 3. RI-BPI treatment did not alter CD69 expression and cell survival.
Tfh or non-Tfh cells sorted from influenza-infected mice were treated with control peptide or RI-BPI in the presence or absence of IFN-α for 1 day. CD69 expression and cell viability (7-AAD staining) were determined through flow cytometry. Representative data are obtained from two independent experiments.

Supplementary Figure 4



Supplementary figure 4. Tfh and non-Tfh cells exhibit similar levels of HIV-1 co-receptors. Human tonsil T cells were stained with CD4, CD45RO, PD-1, CXCR5, CXCR4 and CCR5. The expression levels of CXCR4 and CCR5 were compared in gated Tfh versus non-Tfh cell populations by flow cytometry. Representative data were obtained from two independent experiments.