Chromosoma (2023) 132:257–268
https://doi.org/10.1007/s00412-023-00799-2

RESEARCH

The regulation of Tfh cell differentiation by β-hydroxybutyrylation modification of transcription factor Bcl6

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Received: 23 February 2023 / Revised: 25 April 2023 / Accepted: 10 May 2023 / Published online: 25 May 2023

Abstract

Transcriptional repressor B cell lymphoma 6 (Bcl6) is a major transcription factor involved in Tfh cell differentiation and germinal center response, which is regulated by a variety of biological processes. However, the functional impact of post-translational modifications, particularly lysine β-hydroxybutyrylation (Kbhb), on Bcl6 remains elusive. In this study, we revealed that Bcl6 is modified by Kbhb to affect Tfh cell differentiation, resulting in the decrease of cell population and cytokine IL-21. Furthermore, the modification sites are identified from enzymatic reactions to be lysine residues at positions 376, 377, and 379 by mass spectrometry, which is confirmed by site-directed mutagenesis and functional analyses. Collectively, our present study provides evidence on the Kbhb modification of Bcl6 and also generates new insights into the regulation of Tfh cell differentiation, which is a starting point for a thorough understanding of the functional involvement of Kbhb modification in the differentiations of Tfh and other T cells.

Keywords β-Hydroxybutyrylation · Bcl6 · Tfh cells · Post-translational modifications

Introduction

Follicular helper T (Tfh) cells are a special subset of $CD4^+$ T cells. They are mainly located in the germinal center (GC) and play a key role in regulating T cell-dependent B cell responses by providing signals such as CD40L and IL-21 (Crotty 2014; Awe et al. 2015). Through directly interacting with B cells, Tfh cells induce B cell proliferation and differentiation into memory B cells and plasma cells (Awe et al. 2015) and participate in antibody production and Ig isotype switching and affinity maturation processes (Vinuesa et al. 2016; Purwada and Singh 2017). The primary role of Tfh cells is protecting body against infectious diseases (Crotty 2014). For example, Tfh cells have been shown to play an important role in controlling chronic lymphocytic choriomeningitis virus (LCMV) and human immunodeficiency virus (HIV) infection (Crotty 2014; Miles et al. 2016; Zander et al. 2022). During chronic LCMV infection, naïve $CD4^+$ T cells deviate from Th1 and differentiate towards Tfh cells. Cytokine IL-21 is the key to maintaining the antiviral function of $CD8^+$ effector T cells (Zander et al. 2022). In the early and middle stages of HIV infection, the number of Tfh increases significantly. Correspondingly, Tfh-hosted HIV will proliferate to drive disease progression and lead to impaired Tfh and B cell function, which may be the main reason of the failure in neutralizing antibody production for many infected individuals (Miles et al. 2016; Cirelli et al. 2019). The recovery of Tfh cell function may be particularly important for individual immune reconstruction and the development of antibodies and vaccines against HIV (Cirelli et al. 2019). On the other hand, studies have shown that the abnormal immune response of the body to autoantibodies is closely related to the development of autoimmune diseases (Tsokos 2020). In many autoimmune diseases, Tfh cells also play a central role (Kim et al. 2010). The upregulation of

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Tfh cells was observed in patients with Sjögren's syndrome (Pontarini et al. 2020; Verstappen et al. 2019), juvenile dermatomyositis (Morita et al. 2011), and systemic lupus erythematosus (He et al. 2016; Blanco et al. 2016). In addition, Tfh cells may also relate to cancer. The secreted cytokine IL-21 promotes $CD8^+$ T cell-mediated tumor killing and prevents T cell exhaustion (Zander et al. 2022). The importance of Tfh cells in tumor immunity has been demonstrated in lung cancer (Cui et al. 2021), pancreatic cancer (Lin et al. 2021), breast cancer (Garaud et al. 2019), and so on.

Transcriptional repressor B cell lymphoma 6 (Bcl6), as a key transcription factor of Tfh cells, is selectively highly expressed in mouse and human Tfh cells (Qi et al. 2014). The absence of Bcl6 prevents the polarization from naive $CD4^+$ T cells to Tfh cells (Johnston et al. 2009). After antigen stimulation, dendritic cells (DCs) will first act as antigen-presenting cells (APCs) to differentiate naïve $CD4^+$ T cells into early Tfh cells. The expression of inducible T cell costimulator (ICOS) and Bcl6 will be up-regulated during this process. At the same time, IL-6 and IL-21 will bind to the corresponding receptors on the surface of Tfh cell and assist its differentiation through the STAT3 pathway (Crotty 2014). The up-regulated Bcl6 will further promote the expression of C-X-C motif chemokine receptor 5 (CXCR5). Early Tfh cells can interact with B cells in the T cell zones of peripheral lymphoid organs through CXCR5 and ICOS, and then, B cells replace DC cells to further migrate early $CXCR5^+$Tfh cells toward the interior of B cell follicles while forming GCs (Yu et al. 2009; Vinuesa et al. 2016). In the GC, Bcl6 and CXCR5 reach to maximize expression, and Tfh cells can perform their B cell helper functions through highly expressing ICOS, CD40L, and IL-21, which has great significance for GC development and the sustained humoral immunity (Vogelzang et al. 2008; Yu et al. 2009).

The complexity and diversity of biological systems are largely depended on the regulation of protein functions. After translation, proteins need to undergo various chemical modifications, called post-translational modifications (PTMs). As a significant session in the regulation of protein function, protein PTMs play an important regulatory role in the occurrence and development of various biological processes and diseases (Araki and Mimura 2017; Bao et al. 2019). Mediated by BHB, lysine β-hydroxybutyrylation (Kbhb) as a novel protein post-translational modification has been discovered in recent years. Histone Kbhb has been detected in yeast, drosophila, mouse, and human cells, with a total of 44 histone Kbhb sites identified in human cells and mouse liver (Xie et al. 2016). Among non-histone proteins, only p53 has been reported to have Kbhb modifications at lysines 120, 319, and 370 that reduce cell growth arrest and apoptosis under p53-activated conditions (Liu et al. 2019). A variety of post-translational modifications have been reported in Bcl6 protein, such as acetylation (Cortiguera et al. 2019; Bereshchenko et al. 2002), ubiquitination (Mena et al. 2018), and phosphorylation (Niu et al. 1998). But the function of Kbhb in this important transcriptional repressor has not been fully elucidated.

In this study, we first proved that Bcl6 can undergo Kbhb process in vitro and in vivo and then determined the lysine residues of Kbhb in Bcl6. Then, verify the effect of Bcl6 Kbhb on Tfh differentiation in vitro. Moreover, the interaction proteins of Bcl6 were confirmed by mass spectrometry (MS), which laid a groundwork for subsequent research. Our work further supplements the role of post-translational modifications in regulating the human immune system and provides novel ideas for the diagnosis and treatment of related diseases.

Materials and method

Reagents

Purified anti-human CD3 (OKT3) and purified anti-human CD28 (CD28.2) were from eBioscience. Anti-human Bcl6 (EP529Y) was from abcam (Cambridge, MA, USA). Anti-CBP (D6C5) and anti-P300 (E6D1T) were from Cell Signal Technology (USA). Anti-β-actin (AB21800) and HRP-conjugated anti-rabbit and anti-mouse IgG (ABL3012 and ABL3032) were from Absci (Baltimore, MD, USA). Anti-β-hydroxybutyryllysine antibody (PTM-1204) was from PTMbio (China). Protein A/G-PLUS-agarose beads (37478) were from Cell Signaling Technology (Danvers, USA). Fluorochrome-conjugated anti-CD4 (RPA-T4), anti-human CD185 (RF8B2), anti-human CD279 (MIH4), and anti-human CD278 (DX29) were from BD Bioscience. Recombinant human IL-12, human TGFβ, human IL-23, and human IL-6 were from R&D Systems.

Cell culture and transfection

HEK 293T cells were purchased from the Chinese Academy of Science (Shanghai, China) and cultured in DMEM containing 10% FBS and penicillin/streptomycin at 37 °C under 5% CO_2. For plasmid transfection, the cells were transfected with Flag-Bcl6, Flag-Bcl6-KKYR, Flag-Bcl6-KRYK, Flag-Bcl6-RKYK, and Flag-Bcl6-3KR recombination plasmid or pcDNA3.1 using JetPRIME (Polyplus Transfection, USA) according to the manufacturer's instructions; transfection efficiency is verified by fluorescence intensity. After transfection, cells were treated with BHB according to different groups. Then, the cells were harvested for Western blot detection.

Isolation of PBMCs and Tfh cell differentiation assays

Blood was acquired and diluted with PBS in a ratio of 1:1. The diluted blood was laid carefully above lymphocyte separation solution (Cedarlane Laboratories, Hornby, Ontario) and then centrifuged at 400×g for 30 min; the peripheral blood mononuclear cells (PBMCs) were collected from the interphase. Then, wash once with PBS and centrifuge at 300×g for 10 min.

Naïve $CD4^+$ T cells were enriched from PBMCs by a magnetic cell isolation kit (Miltenyi Biotec, Germany). Subsequently, the cells were activated by plate-bound anti-CD3(2 μg/mL) and soluble anti-CD28 (1 μg/mL) and cultured with anti-IL-12/23 p40 antibody (10 ng/mL), rhIL-12 (1 ng/mL), rhTGFβ (5 ng/mL), rhIL-23 (10 ng/mL), and rhIL-6 (10 ng/mL) in RPMI 1640 medium containing 10% FBS (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin. The cells were cultured at 37 °C, in a 5% CO_2 incubator for 6 days for further analysis.

Western blotting

The cells were harvested and washed with PBS twice and then homogenized with RIPA lysis buffer (Merck Millipore, Billerica, MA, USA) containing protease inhibitor (1:100, I3786, Sigma-Aldrich, St. Louis, MO, USA) and PMSF (1 mM). The cell lysate supernatants were examined by Western blot according to standard protocols.

Immunoprecipitation (IP)

The transfected 293T cells were harvested and lysed in RIPA lysis buffer. The cell lysates were first incubated with 2-mL anti-Flag antibody for 1 h at 4 °C. Then, 20-μL protein A/G-PLUS-agarose beads (for Flag-Bcl6) were incubated in the mixture at 4 °C overnight. Collect immunoprecipitates by centrifugation at 2500 rpm for 5min at 4 °C. Then, carefully aspirate and discard the supernatant. Wash the beads four times with 1-mL RIPA buffer, each time repeating the centrifugation step above. After the final wash, discard the supernatant and resuspend beads in 40 μL of 1× electrophoresis sample buffer. The following steps are the same as Western blotting.

CCK8 assay

HEK 293T and PBMCs were inoculated 5000 cells/well in 96-well plate and then stimulated with different concentrations of BHB at 37 °C under 5% CO_2 for 24 h. Then, add 10-μL CCK8 solution to each well and continue to culture for 1–4 h. The absorbance was read at 450 nm. Cell viability was calculated by the following formula: cell viability (%) = [(As-Ab)/(Ac-Ab)] × 100 and inhibition (%) = [(Ac-As)/(Ac-Ab)] × 100 (As, absorbance of experimental well (contains cells, medium, CCK-8, and compound); Ab, absorbance of blank well (contains medium and CCK-8); Ac, absorbance of control well (contains cells, medium, and CCK-8)).

Flow cytometry analysis

The stimulated PBMCs were stained with anti-CD4 at 4 °C for 30 min, and these cells were then fixed and permeabilized using a fixation/permeabilization buffer (BD Biosciences, San Jose, CA, USA). Intracellular staining was performed using anti-human CD185, anti-human CD279, and anti-human CD278. All samples were acquired on a BD Accuri C6 flow cytometer (BD Biosciences), and the data were analyzed using FlowJo software (TreeStar, America).

RNA extraction and quantitative real-time PCR

To determine the mRNA expression levels of the transcription factors T-bet, total RNA was extracted from the PBMCs with an RNAprep Pure Cell/Bacteria kit (TIANGEN, Beijing, China). Then, cDNA was synthesized with a FastQuant RT Super Mix (TIANGEN, Beijing, China) according to the manufacturer's protocol. qPCR was performed in technical triplicate using a ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) with a LightCycler® 96 analysis system (Roche, Basel, Switzerland)). The thermocycler program was as follows: 30 s at 95 °C for denaturation and then 40 cycles (10 s at 95 °C and 30 s at 60 °C) for PCR amplification, followed by amplicon melting analysis to evaluate the specificity of the reaction and identify the presence of primer dimers. All primers were purchased from GenScript Biotech Corp. The primers were as follows: hβ-actin, 5′-TGG ACT TCG AGC AAG AGA TG-3′ and 5′-GAA GGA AGG CTG GAA GAG TG-3'; hBcl6, 5′-ACA CAT CTC GGC TCA ATT TGC-3′ and 5′-AGT GTC CAC AAC ATG CTC CAT-3′; and hPrdm1: 5′-TAA AGC AAC CGA GCA CTG AGA-3′ and 5′-ACG GTA GAG GTC CTT TCC TTTG-3′. The RNA expression level of each gene of interest was normalized to that of β-actin, and the results were expressed using the $2^{-\Delta\Delta Ct}$ method.

Mass spectrometric analysis

The band of interest was excised from the gel and rinsed three times with Milli-Q water. The band was then cut into approximately 1-mm^2 pieces and dried. The gel slices were reduced with 30 μL of 10-mM DTT at 56 °C for 60

min and cooled down to RT. Sixty microliters of 100-mM iodoacetamide was added and the protein was alkylated for 45 min at RT in the dark. Subsequently, the gel slices were completely dried and added to a trypsin solution to incubate at 37 °C overnight. After the reaction was cooled down to RT, the supernatant was removed and saved. The gel was subsequently extracted with 100 µL 0.1% and 5% TFA in 50% ACN by gently mixing and incubated at RT for 15 min, respectively. Each wash was combined with the saved supernatant, and the resulting solution was lyophilizated for further MALDI-TOF/TOF (MALDI-7090, Shimadzu Kratos) analysis. The peptide mass fingerprints and peptide ion MS/MS spectra were acquired on MALDI-7090. The total MS/MS data was searched against SwissProt database using the following parameters: trypsin digestion allowing up to 2 missed cleavages, fixed modifications of cysteine (carbamidomethylation), variable modifications of methionine (oxidation) and lysine (β-hydroxybutyrylation), precursor peptide tolerance of 0.05 Da, and MS/MS tolerance of 0.2 Da. Search results with *e* values less than 0.01 were judged as positive identifications.

Interacting protein assays

Bcl6 protein was isolated by SDS-PAGE after IP enrichment. The targeted gel was obtained for in-gel tryptic digestion. Gel pieces were destained in 50-mM NH4HCO3 in 50% acetonitrile (v/v) until clear. Gel pieces were dehydrated with 100 µL of 100% acetonitrile for 5 min, the liquid was removed, and the gel pieces were rehydrated in 10-mM dithiothreitol and incubated at 56 °C for 60 min. Gel pieces were again dehydrated in 100% acetonitrile, liquid was removed, and gel pieces were rehydrated with 55-mM iodoacetamide. Samples were incubated at room temperature, in the dark for 45 min. Gel pieces were washed with 50-mM NH4HCO3 and dehydrated with 100% acetonitrile. Gel pieces were rehydrated with 10-ng/µL trypsin resuspended in 50-mM NH4HCO3 on ice for 1 h. Excess liquid was removed and gel pieces were digested with trypsin at 37 °C overnight. Peptides were extracted with 50% acetonitrile/5% formic acid, followed by 100% acetonitrile. Peptides were dried to completion and resuspended in 2% acetonitrile/0.1% formic acid. The follow-up analysis was performed by LC-MS/MS.

Enzyme-linked immunosorbent assay

According to the agreement of the manufacturer, the levels of IL-21 were determined by using a specific ELISA kit (MULTISCIENCES, Hangzhou, China). All cytokines were quantified using the specific standard curve of recombinant cytokines provided by the corresponding enzyme-linked immunosorbent assay kit.

Statistical analysis

Measurement data are represented by mean ± SEM. Statistical comparisons between groups were evaluated by the Student's unpaired (2-tailed) *t*-tests. The Spearman test was used to analyze the correlation between two continuous variables. The resulting MS/MS data were processed using Proteome Discoverer 1.3. All statistical analyses were performed using GraphPad Prism software version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). A p value less than 0.05 (two-tailed) was considered statistically significant and labeled with *. p values less than 0.01 were labeled with **, and p values less than 0.001 were labeled with ***, respectively.

Results

Bcl6 Kbhb occurs in the presence of β-hydroxybutyrate

β-Hydroxybutyrate (BHB), as the main component of ketone bodies, is an important energy source for the heart and brain during starvation (Wei et al. 2021). In addition, BHB exerts neuroprotective effects in some neurodegenerative disease models such as Parkinson's disease and Alzheimer's disease (Kashiwaya et al. 2000). BHB has been reported to induce histone Kbhb (Liu et al. 2019). To explore whether the Bcl6 protein will undergo the Kbhb process, we first enriched and purified the Bcl6 protein from 293T cells which overexpressed the Flag-tagged Bcl6 protein. Bcl6 eukaryotic expression plasmid map is shown in Fig. 1A. The positive cloned plasmid was extracted for double enzyme digestion, and a band with a size of 2118 bp was obtained, which was consistent with the target position (Fig. 1B). Green fluorescence was observed 6 h after transfection (Fig. 1C), and the overexpression of Bcl6 protein was detected by Western blotting (Fig. 1D). Immunoblot analysis was performed with anti-Bcl6 and anti-panβ-hydroxybutyryl-lysine antibody (BHB-K). In the presence of BHB, the degree of Kbhb for the enriched Bcl6 protein was significantly increased, proving that Bcl6 protein undergoes Kbhb in the presence of BHB (Fig. 1D). We further verified whether Kbhb modification exists in endogenous Bcl6 protein on the primary human in vitro Tfh differentiation model (Fig. 1F). Endogenous Bcl6 protein undergoes Kbhb, and the level of Kbhb was significantly increased at 10 mM (Fig. 1G). According to previous reports, the histone acetyltransferase CBP and p300 can catalyze Kbhb in p53 (Liu et al. 2019) and histones (Huang et al. 2021). We observed a significant increase in p300 and CBP at 10 mM, proving that CBP and p300 may be involved in the Kbhb process of Bcl6 protein (Fig. 1F).

Finally, to rule out the potential cytotoxic effect of BHB, we used CCK8 assay to detect the cytotoxic effect of BHB on cells in primary human cells and HEK 293T cells. The results

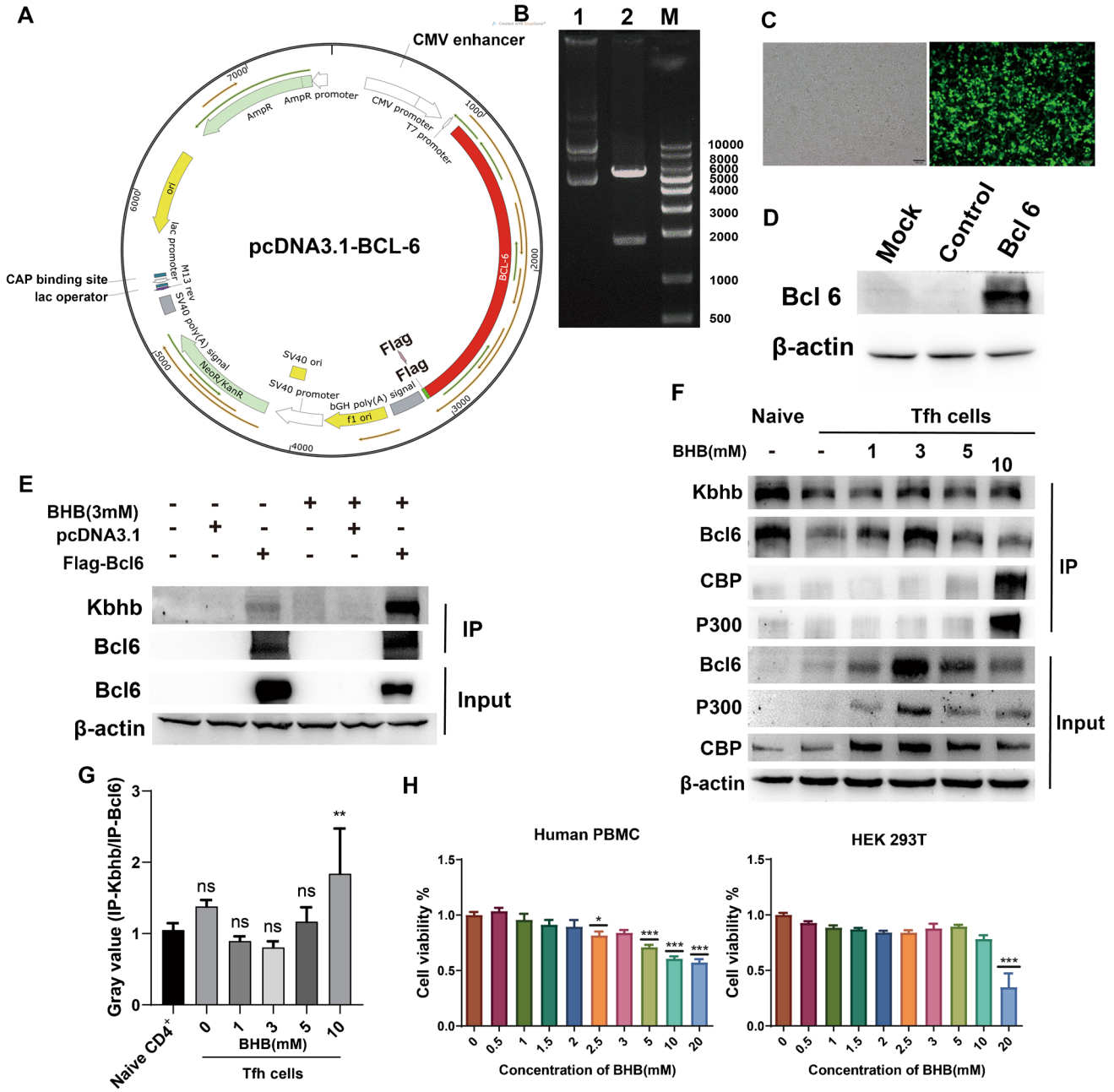


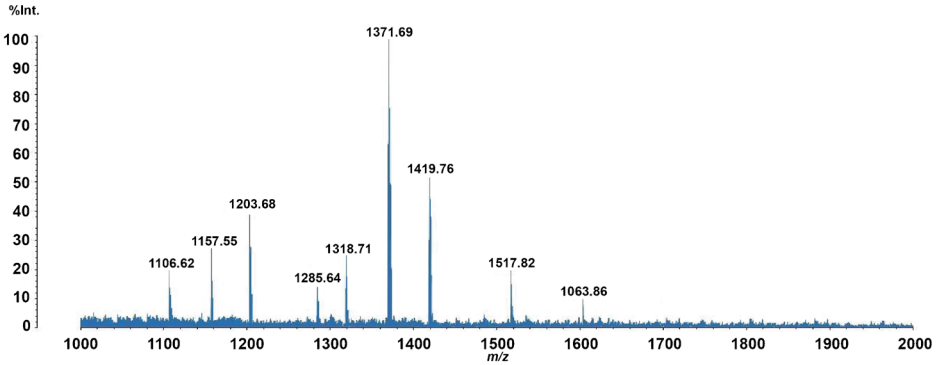
Fig. 1 Bcl6 undergoes Kbhb in vitro. **A** Map of Bcl6 gene plasmid vector. **B** Double enzyme digestion verified the correctness of Bcl6 sequence (lane 1: plasmid; lane 2: plasmid digested by EcoRI and HindIII; lane M: KB ladder). **C** The fluorescence was detected after the overexpression of Bcl6 in 293T cells to determine the transfection efficiency. **D** The overexpression of Bcl6 in 293T cells was detected by Western blotting. **E** After overexpression Bcl6 and 3-mM BHB treatment in 293T cells, the Kbhb modification of Bcl6 protein was detected by Western blotting. **F** The Kbhb modification of Bcl6 and CPB/P300 expression in vitro differentiated Tfh cells were measured by Western blotting. **G** Gray-scale scanning for **F**. The x-axis indicates the name of each experimental group, and the y-axis indicates the level of Kbhb on the Bcl-6 protein with IP-Kbhb/IP-Bcl6. **H** Human primary PBMCs and HEK 293T cells were treated with the indicated concentrations of BHB. After 24 h of exposure, cell viability was determined via CCK8 assays. Data are from three independently replicated experiments. All data were representative of mean ± SEM (compared with BHB = 0 mM; ns, no significance; $*p < 0.05$ and $***p < 0.001$ by Student's unpaired *t*-test)

showed that BHB had a little effect on the cell viability of hPBMCs within the concentration range used in the experiment but had no significant cytotoxicity on 293T cells (Fig. 1H).

Taken together, the above results demonstrate that both exogenous Bcl6 and endogenous Bcl6 proteins undergo the Kbhb modification process.

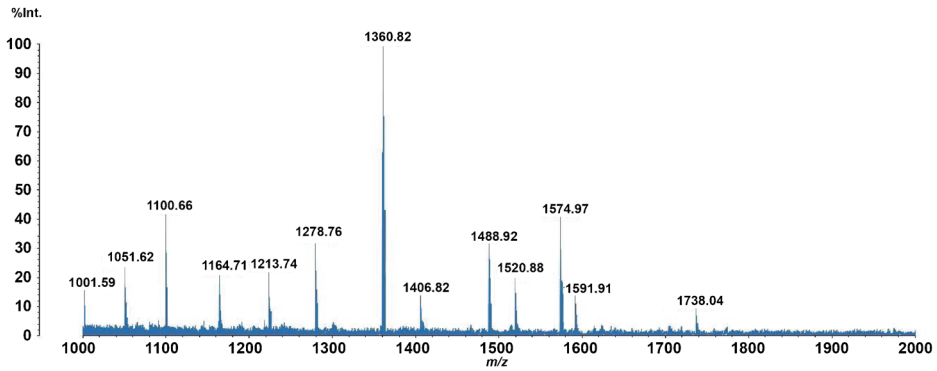
A

Source: B-cell lymphoma 6 protein	BCL6_ HUMAN			
Product ion sequence	m/z (cal)	m/z (exp)	Position	Modifications
SPTDPKA#CNW*K*K	1603.79	1603.86	C373; K376/K377	#carbamidomethylation; *β-hydroxybutyrylation
SPTDPKA#CNW*KK	1517.75	1517.82	C373; K376	#carbamidomethylation; *β-hydroxybutyrylation
TDPKA#CNW*K*K	1419.70	1419.76	C373; K376/K377	#carbamidomethylation; *β-hydroxybutyrylation
SPTDPKA#CNW*K	1371.63	1371.69	C373; K376	#carbamidomethylation; *β-hydroxybutyrylation
DPKA#CNW*K*K	1318.65	1318.71	C373; K376/K377	#carbamidomethylation; *β-hydroxybutyrylation
SPTDPKA#CNWK	1285.59	1285.64	C373	#carbamidomethylation
PKA#CNW*K	1203.63	1203.68	C373; K376	#carbamidomethylation; *β-hydroxybutyrylation
SPTDPKA#CNW	1157.5	1157.55	C373	#carbamidomethylation
KA#CNW*K*K	1106.57	1106.62	C373; K376/K377	#carbamidomethylation; *β-hydroxybutyrylation



B

Source: B-cell lymphoma 6 protein	BCL6_ HUMAN			
Product ion sequence	m/z (cal)	m/z (exp)	Position	Modifications
Y*KFIVLNSLNQNAK	1737.96	1738.04	K379	*β-hydroxybutyrylation
Y*KFIVLNSLNQNA	1591.84	1591.91	K379	*β-hydroxybutyrylation
*KFIVLNSLNQNAK	1574.90	1574.97	K379	*β-hydroxybutyrylation
Y*KFIVLNSLNQN	1520.81	1520.88	K379	*β-hydroxybutyrylation
KFIVLNSLNQNAK	1488.86	1488.92		
Y*KFIVLNSLNQ	1406.76	1406.82	K379	*β-hydroxybutyrylation
FIVLNSLNQNAK	1360.76	1360.82		
YKFIVLNSLN	1278.71	1278.76	K379	*β-hydroxybutyrylation
IVLNSLNQNAK	1213.69	1213.74		
Y*KFIVLNSL	1164.66	1164.71	K379	*β-hydroxybutyrylation
VLNSLNQNAK	1100.61	1100.66		
Y*KFIVLNS	1051.58	1051.62	K379	*β-hydroxybutyrylation
LNSLNQNAK	1001.54	1001.59		



◂**Fig. 2** Identification of Kbhb modification sites in Bcl6 protein via mass spectrometry analysis. Purified Bcl6 proteins were enriched by immunoprecipitation and then detected the Kbhb modification sites by mass spectrometry analysis. **A** The MS/MS data (upper) and spectra (lower) of peptide SPTDPKACNWKK (derived from amino acids 366-377). **B** The MS/MS data (upper) and spectra (lower) of peptide YKFIVLNSLNQNAK (derived from amino acids 378-391)

Bcl6 Kbhb occurred at lysines 376, 377, and 379

To further investigate the specific Kbhb modification site, Bcl6 was overexpressed in HEK-293T cells and enriched by immunoprecipitation assay. Possible modification sites of Bcl6 protein were detected by MS. The results are shown in Fig. 2. Bcl6 undergoes Kbhb at lysine residues 376, 377, and 379. To verify these findings, we further mutated these possible modification sites to arginine to mimic the diacylation (K376R/Bcl6, K377R/Bcl6, K379R/Bcl6, K376, K377, and K379R/Bcl6 (3KR/Bcl6), respectively). First, 3KR/Bcl6 was transfected into 293T cells, and the immunoprecipitation results showed that the level of Bcl6 Kbhb was significantly reduced compared to the control group, and the Kbhb level did not increase significantly in the presence of BHB (Fig. 3A). Based on the above results, we preliminarily concluded that Kbhb occurs on K376/K377/K379. Further compare the changes of Kbhb after mutating three sites, respectively. Compared with the control and other experimental groups, the Kbhb decreased significantly after the K376 mutation (Fig. 3B). In conclusion, K376 is the main site of Bcl6 Kbhb.

To further explore the possible mechanism, the enriched protein was searched for possible interacting proteins. Analysis of the MS results revealed several possible interacting proteins, including three ATP-dependent RNA helicases, seven subunit proteins of the chaperone-containing T-complex (TRiC), RACK1, and ILF2/ILF3 (Table 1).

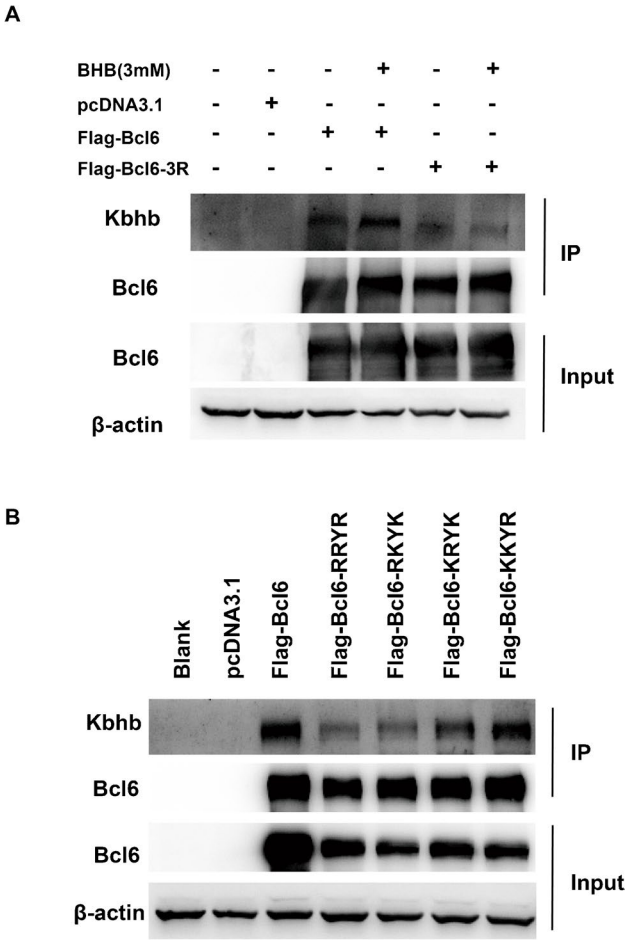


Fig. 3 Bcl6 Kbhb mainly occurred at lysines 376. **A**, **B** Effect of modification site mutation on Bcl6 Kbhb. Western blot analysis was performed on wild-type and mutant Flag-Bcl6 expressed in 293T cells treated with 3-mM BHB for 24 h. Data are from three independently replicated experiments

Bcl6 Kbhb inhibits in vitro differentiation of Tfh cells

Bcl6 is a key transcription factor for Tfh differentiation, and Bcl6 can block the expression of Blimp-1 to promote Tfh differentiation (Johnston et al. 2009). To further explore the effect of Bcl6 Kbhb modification on its function to promote Tfh cell differentiation, we used flow cytometry to detect the population of Tfh cells after BHB administration in the primary Tfh cells. The results are shown in Fig. 4A and B. The percentage of Tfh cells in human undifferentiated PBMCs (Th0 group) was 3.082 ± 0.095%. The percentages of Tfh at BHB concentrations of 0 mM, 1 mM, 3 mM, 5 mM, and 10 mM were 11.68 ± 0.942%, 5.486 ± 0.239%, 2.986 ± 0.095%, 1.448 ± 0.048%, and 0.4655 ± 0.0463%, respectively. With the increase of BHB concentration, the proportion of Tfh cells gradually decreases in a dose-dependent manner.

Next, we examined the mRNA expression levels of key transcription factors in Tfh cells by Q-PCR. As shown in the Fig. 4C, D and E, with the increase of BHB concentration, the mRNA expression levels of *Bcl6* and *IL-21* decreased, while the expression of *PRDM1*, which functioned opposite to Bcl6, increased first and then decreased. At the same time, the expression of IL-21, the main cytokine of Tfh cells, was detected by ELISA. Compared with the control group, the expression of IL-21 decreased with the increase in BHB concentration (Fig. 4F). These results demonstrated that the function of Bcl6 in promoting Tfh differentiation is undermined under Kbhb modification.

Discussion

As one of the most important processes in organisms, protein PTM is involved in the occurrence and development of various diseases. More than 20 protein PTMs such as ubiquitination, phosphorylation, methylation, and acetylation

Table 1 Bcl6 interacting protein identified by mass spectrometry analysis

Accession	Protein names	Gene names	MW (kDa)	Protein score	Sequence coverage (%)	Unique peptides	Peptides	PSMs
P50990	T-complex protein 1 subunit theta	CCT8	59.58	177.82	10.95	5	5	5
P78371	T-complex protein 1 subunit beta	CCT2	57.45	142.98	10.65	4	4	4
Q99832	T-complex protein 1 subunit eta	CCT7	59.33	135.27	4.79	2	2	2
P49368	T-complex protein 1 subunit gamma	CCT3	60.50	107.53	4.04	2	2	2
P50991	T-complex protein 1 subunit delta	CCT4	57.89	78.10	5.57	2	2	2
P48643	T-complex protein 1 subunit epsilon	CCT5	59.63	49.76	2.96	1	1	1
P40227	T-complex protein 1 subunit zeta	CCT6A	57.99	46.25	1.88	1	1	1
Q12906	Interleukin enhancer-binding factor 3	ILF3	95.28	92.16	3.13	3	3	3
Q12905	Interleukin enhancer-binding factor 2	ILF2	43.04	85.87	5.38	2	2	2
O00571	ATP-dependent RNA helicase DDX3X	DDX3X	73.20	340.12	17.67	9	9	9
P17844	Probable ATP-dependent RNA helicase DDX5	DDX5	69.10	114.00	6.03	2	3	3
Q92841	Probable ATP-dependent RNA helicase DDX17	DDX17	80.22	159.88	5.21	2	3	3
P63244	Receptor of activated protein C kinase 1	RACK1	35.05	87.01	8.52	3	3	3

have been found in eukaryotic cells (Zhao et al. 2010). As a novel post-translational modification discovered in recent years, Kbhb was found to be involved in the occurrence and development of depression and related to the active gene expression of multiple metabolic pathways in response to starvation (Goudarzi et al. 2020; Xie et al. 2016; Kashiwaya et al. 2000). Histone β-hydroxybutyrylation modification is a reversible process. Huang et al. found that p300 can catalyze histone Kbhb both in vitro and in vivo (Huang et al. 2021). A previous study has shown that p300 can bind to the short-chain acyl-CoA family and catalyze various short-chain lysine acylation reactions (Kaczmarska et al. 2017). With reference to histone acetylation, it is assumed that p300 catalyze Kbhb by binding a molecule of bhb-CoA and subsequently catalyzing the transfer of the moiety to a lysine residue and the depletion of Kbhb is mainly mediated by HDAC1 and HDAC2 (Huang et al. 2021). For non-histones, Kbhb has been shown to reduce cell growth arrest and apoptosis in cells cultured under p53-activating conditions (Liu et al. 2019). Recently, Kbhb was found to improve the stability of COVID-19 antibodies to the proteasome and heat treatment (Li et al. 2022), which may extend the protection of antibodies against COVID-19.

We observed that Bcl6 also undergoes Kbhb process in the presence of BHB. BHB is one of the main components of the ketone body and is the precursor of Kbhb modification (Xie et al. 2016). After overexpressing Bcl6 in 293T cells, the level of Kbhb in the enriched Bcl6 protein increased with the increase of BHB concentration, indicating that there is indeed Kbhb modification in Bcl6 protein. Next, we further observed the similar changes in hPBMCs. As observed on 293T cells, a rise in Kbhb levels with BHB administration was also observed on the human Tfh differentiation model, with significant enrichment of the acylation-modifying enzymes CBP and P300 at 10 mM. The above results demonstrate that Bcl6 undergoes Kbhb and preliminarily suggest that CBP/P300 may be a Bcl6 protein Kbhb-modified acylase, but this hypothesis still needs to be further confirmed. Through MS analysis, we found that Bcl6 may be modified at lysine residues 376, 377, and 379. Finally, through a series of point mutation plasmids, K376 was preliminarily determined as the main site of Kbhb.

The three Kbhb modification sites identified by MS came to our attention because they were consistent with sites of acetylation on the Bcl6 protein (Bereshchenko et al. 2002). The modified KKYK motif is located in the middle domain of Bcl6 and is essential for the binding of metastasis-associated protein 3 (MTA3) (Fujita et al. 2004; Nance et al. 2015). MTA3, a subunit of the Mi-2/NuRD complex, binds to the middle domain of Bcl6 as a corepressor and contributes to the transcriptional repression of Bcl6 (Fujita et al. 2003; Fujita et al. 2004; Nance et al. 2015). Acetylation, on the other hand, prevents the binding of MTA3 and abolishes the transcriptional repression ability of Bcl6 (Nance et al. 2015). We speculate that Kbhb may affect the function of Bcl6 in a similar way, but the specific mechanism needs to be further explored.

The effects of other post-translational modifications on Bcl6 function have also been reported besides acetylation; for example, MAPK-mediated phosphorylation leads to its inactivation by accelerating the degradation of Bcl6 (Niu et al. 1998); ubiquitination leads to its degradation by the proteasome. However, the role of Kbhb on Bcl6 remains unknown. We conducted a series of studies around the effect of Kbhb on Bcl6 protein function.

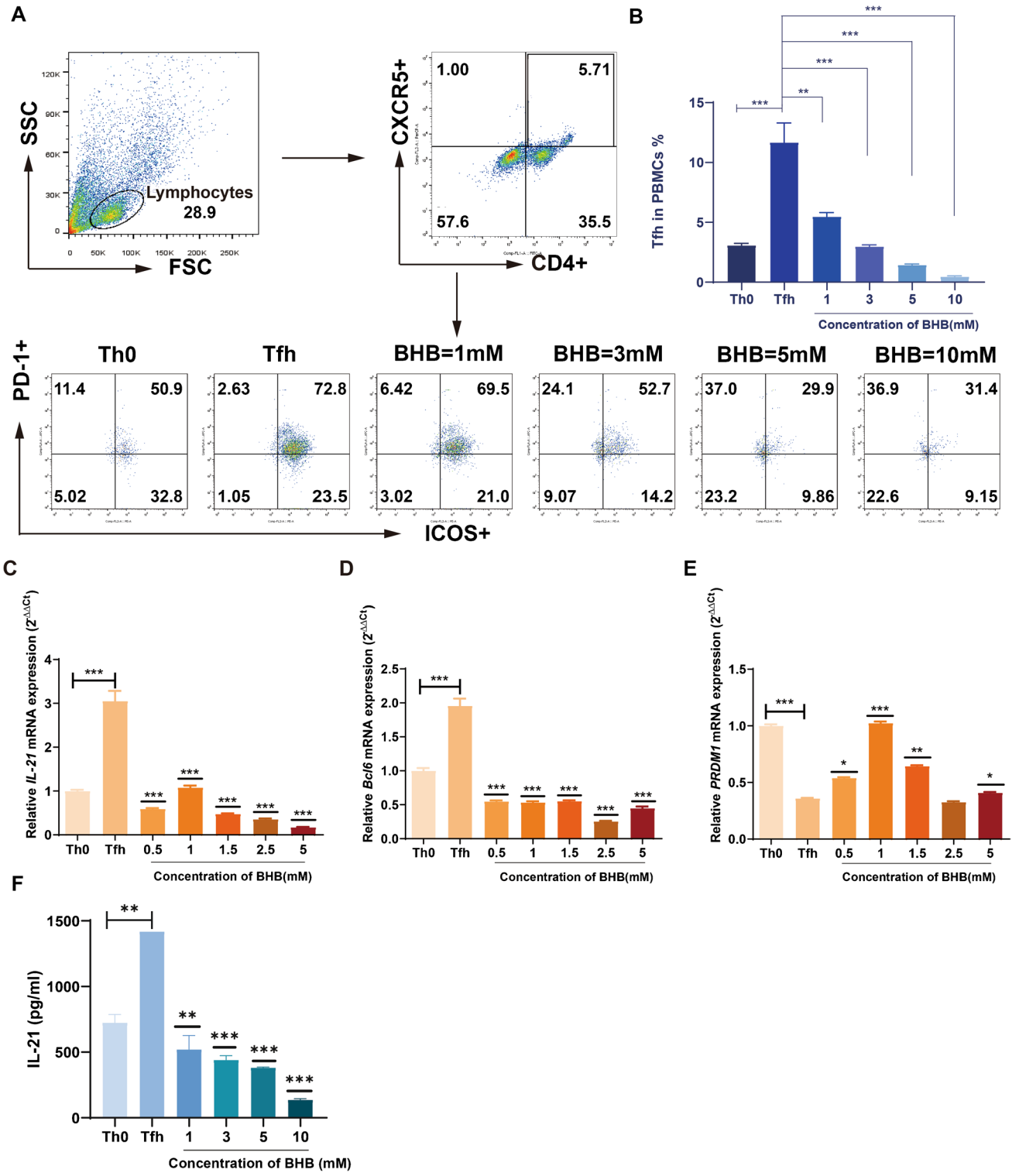


Fig. 4 Bcl6 Kbhb inhibits the in vitro differentiation of Tfh cells. **A**, **B** Flow cytometry analysis of Tfh cells in total PBMCs after treated with different BHB concentrations. **C, D, E** After treated with BHB, *IL-21*, *Bcl6*, and *PRMD1* mRNA expressions in Tfh cells were determined by qPCR. **F** After treated with BHB, IL-21 expression was determined by ELISA. Data are from three independently replicated experiments. All data were representative of mean ± SEM (compared with Th0 group; ns, no significance; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ by Student's unpaired *t*-test)

Bcl6 protein is the key player in establishing and maintaining the immune memory of B cells in the GC by controlling the development of Tfh cells (Yang et al. 2020; Yang et al. 2015). To further explore the effect of Bcl6 Kbhb on Tfh cell differentiation, a dose-dependent decrease in the ratio of Tfh cells was observed after BHB treatment on the in vitro Tfh cell differentiation model. The mRNA expression levels of Bcl6 and IL-21 were decreased while prdm1, which was opposite to Bcl6 function, increased first and then decreased. The secretion of IL-21 is decreased. These results further support that BHB treatment decreased the level of Tfh cells. Our results demonstrate that BHB-induced Kbhb attenuates the activity of the Bcl6 in inducing differentiation of Tfh cells. At the same time, given that BHB is an important product in fatty acid metabolism (Youm et al. 2015), the association between metabolism and immunity deserves further exploration.

Tfh cells play a crucial role in regulating T cell-dependent B cell responses. The upregulation of CXCR5 allows Tfh cells to home to the interface between the T cell and B cell regions of the lymph nodes, where they interact with newly activated B cells through antigen presentation (Cui et al. 2021; Crotty 2014; Crotty 2011). B cells provide ICOS and IL-6 to Tfh cells, while Tfh cells provide CD40L and IL-21 to B cells (Awe et al. 2015; Jogdand et al. 2016). Through sequential interactions with B cells, Tfh cells promote survival, proliferation, Ig class switching, maturation, and differentiation of B cells to memory B cells and antibody-producing plasma cells (Olson et al. 2019; Purwada and Singh 2017). Since many autoimmune diseases are characterized by the presence of self-reactive autoantibodies. In human autoimmune diseases, expansion of circulating Tfh cells has been observed to be closely associated with disease progression in patients with (Morita et al. 2011), rheumatoid arthritis (Tang et al. 2017; Zhang et al. 2019), systemic lupus erythematosus (He et al. 2016; Choi et al. 2015; Blanco et al. 2016), and Sjögren's syndrome (Pontarini et al. 2020; Verstappen et al. 2019). Regulating and restoring the human normal immune system from the perspective of post-translational modification may provide a new perspective for understanding the pathogenesis of such diseases and seeking new diagnostic methods.

Several interacting proteins that may react with Bcl6 were also identified in MS analysis, including DDX3X, DDX5, DDX17, CCT2, CCT3, CCT4, CCT5, CCT8, CCT6A, CCT7, ILF2, ILF3, and RACK1. DDX3X, DDX5, and DDX17 are involved in various steps in the transcription process, including pre-mRNA splicing, ribosomal RNA processing, miRNA processing, and transcriptional regulation (Song and Ji 2019; Huang et al. 2018; Kao et al. 2019). For example, DDX3X positively regulates CDKN1A/WAF1/CIP1 transcription and inhibits cell growth in a P1-dependent manner (Chao et al. 2006), as well as DDX5 cooperates with DDX17 to activate MYOD1 transcriptional activity and participates in skeletal muscle differentiation (Terrone et al. 2022). Based on these results, we hypothesized that these proteins might further regulate cell differentiation by interacting with Bcl6 to regulate transcriptional processes. CCT2, CCT3, CCT4, CCT5, CCT8, CCT6A, and CCT7 are seven subunit proteins that make up the chaperone-containing T-complex (TRiC) (Grantham 2020). The TRiC is abundant in cells and is thought to perform specific functions during protein folding and assembly (Grantham 2020). In our work, we assume that the possible function of CCT proteins is involved in the process of Bcl6 modification; however, the exact role still needs further investigation. Homologous to the β subunit of G proteins, RACK1 has a 7-bladed propeller structure that binds signaling molecules from different transduction pathways and plays the role of a multifunctional adaptor protein in a variety of different biological events (Johnson et al. 2019). The role of RACK1 in the regulation of post-translational modifications drew our attention. In non-small-cell lung cancer, RACK1 forms a ternary complex with PP2A and Akt to promote Akt dephosphorylation, which suppresses tumor metastasis (Fei et al. 2017). Therefore, we speculate that RACK1 may play a role in promoting Bcl6 Kbhb. ILF3 involves mRNA stabilization and translational repression and biogenesis of noncoding RNAs by binding to different cellular RNAs (Vrakas et al. 2019). In addition, ILF3 also forms heterodimers with ILF2, promoting the formation of a stable DNA-dependent protein kinase holoenzyme complex (Bremer et al. 2018). Since there is no clear report, it is hypothesized that these proteins may interact with Bcl6 to affect the process of cell differentiation, which preliminary lays a foundation for our subsequent exploration.

Taken together, our results reveal an important role for Kbhb modification of the Bcl6 protein, a key transcription factor of Tfh cells, in directing the differentiation of Tfh lineages. Our evidence suggests that Bcl6 Kbhb hinders the differentiation of naïve $CD4^+$ T cells into Tfh cells, which shed new light on the factors affecting Tfh differentiation and the immunomodulatory mechanisms in vivo.

Acknowledgements Thanks are due to Yu Zeng, Dong Wu, Mengjiao Zhu, and Ziran Zhou for assistance with the experiments and to Yinhua Sun for valuable discussion.

Author contributions Yimeng Wang and Jingtian Guo conceptualized and designed the study, collected and processed experimental results, and interpreted data set results; Jingtian Guo drafted the initial manuscript; Wei Guo conceptualized and designed the study; Wei Guo and Xiaomeng Jiang critically reviewed the manuscript for its content; Lei Tang, Tiejun Tang, Zhuolan Li, Mengyuan Li, Liming Wang, Aizhong Zeng, Yuxiao Ma, and Shihao Huang coordinated and supervised data collection.

Funding This study was supported by the National Natural Science Foundation of China (81973221), the "Double-First Class" University Project (CPU2018GF08), and Chinese foundation for hepatitis prevention and control—TianQing liver disease research fund subject (TQGB20210089).

Data availability Data are available on request to the authors.

Declarations

Ethics approval Not applicable

Consent to participate Not applicable

Consent for publication Not applicable

Competing interests The authors declare no competing interests.

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