The role of B cells in regulation of Th cell differentiation in Coxsackievirus B3-Induced Acute Myocarditis

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Research Article

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Abstract

Background:

Viral myocarditis (VMC) is the major cause of sudden death in adolescents. To date, no effective treatment has been identified for VMC. Studies have shown that T helper (Th) cells such as Th1, Th2, Th17, and Th22 cells are involved in the pathogenesis of VMC. However, the role of B cells and their impact on Th cells in VMC is unclear. In this study, we investigated the role of B cells in Th cell differentiation in myocardial damage in an animal model of VMC.

Methods and Results:

C57BL/6 mice were infected with Coxsackievirus B3 (CVB3) intraperitoneally or injected with phosphate-buffered saline as a control condition. At day 7, samples from these mice were analyzed by histology, ELISA, flow cytometry, and gene expression assays. We found that TNF-α-, IL-6-, and IL-17-producing B cell numbers were significantly increased, while IL-4-producing B cell population was significantly reduced in acute VMC. Furthermore, we performed B cell knockout (BKO), SCID, and SCID+B cells reconstitution experiments. We found that BKO alleviated the cardiac damage following CVB3 infection, may hamper the differentiation of Th1 and Th17 cells, may promote the differentiation of Th2 cells, and proved ineffective for the differentiation of Th22 cells. In contrast, SCID+B cells reconstitution experiment exacerbated the cardiac damage. *Ex vivo* studies further revealed that B cells promote the differentiation of Th1 and Th17 cells and inhibit the differentiation of Th2 cells.

Conclusions:

Our study shows that B cells are activated and have strong abilities of antigen presentation and producing cytokines in VMC; B cells not only play a pathogenic role in VMC independent of T cells, but also promote Th1 and Th17 cell differentiation, and hamper Th2 cell differentiation in VMC.

Introduction

Myocarditis is an inflammatory disease of the heart muscle. It accounts for about one in nine cases of non-ischemic heart failure clinically, and remains the main reason for heart transplantation worldwide [1]. Coxsackievirus B3 (CVB3) is the most common cause of myocarditis. To date, no specific treatments for viral myocarditis (VMC) have been identified, owing to its poorly understood pathogenesis. Although studies have shown that $CD4^+$T helper (Th) cell-mediated (Th1, Th2, Th17, and Th22) immune response plays a crucial role in the pathogenesis of VMC [2–5], they fail to completely illuminate its regulatory mechanisms.

B lymphocytes (B cells) mainly mediate humoral immune response. Classically, the studies about B cells primarily focus on the production of immunoglobulins. Currently, some evidence suggests that B cells can regulate the course of the immune response through antigen presentation and cytokine production,

but not antibody production mechanisms. Harris et al. demonstrated that B cells have the ability to produce cytokines such as IL-2, IFN-γ, IL-12, IL-6, IL-4, and TNF-α, which are not normally thought to be produced by B cells [6]. In addition, Vazquez-Tello et al. showed that B cells can produce IL-17A and IL-17F cytokines after stimulation with transforming growth factor (TGF)-β, IL-23 or IL-6. This study revealed that by producing cytokines, B cells play a key role in a mouse model of chemically-induced asthma without the help of T cells [7]. Many studies have also shown that B cells play a pathogenic role in autoimmune diseases such as systemic lupus erythematosus (SLE) [8], multiple sclerosis [9], and rheumatoid arthritis (RA) [10].

In experimental autoimmune encephalomyelitis, IL-6 secreted by B cells promoted Th1 and Th17 cell differentiation[11]. In a mouse model of asthma, Wypych et al. showed that B cells efficiently primed naïve $CD4^+$T cells to differentiate into Th1 and Th17 cell subsets [12]. However, no study to date has focused on the role of B cells in regulation of Th cell differentiation in VMC.

In this study we investigated the role of B cells in Th cell differentiation in myocardial damage in an animal model of CVB3-induced acute myocarditis.

Materials And Methods

Mice

This study was performed with the approval of Guangxi Medical University Animal Ethics Committee. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23). C57BL/6 mice (wild type (WT)) and severe combined immunodeficient mice (SCID) were provided by the Hunan SJA Laboratory Animal Co., Ltd (Hunan, China). B cell-deficient (labeled as BKO, 002288) mice in a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed and maintained under specific pathogen-free conditions. Male mice at the age of 4–6 weeks (15–20 g body weight) were used for the experiments.

Virus and CVB3 infection

CVB3 (Nancy strain) was propagated in HEp-2 cells. Virus titer was routinely decided prior to infection by a 50% tissue culture infectious dose (TCID50) assay in HeLa cell monolayers following previously published protocols [13]. WT C57BL/6 mice, BKO mice and SCID mice were infected intraperitoneally (IP) with 0.1ml phosphate-buffered saline (PBS) containing approximately 10^5 plaque-forming units (PFU) of the virus on day 0. Control mice received the same dose of PBS. Surviving mice were euthanized on day 7. Tissue or blood samples were collected aseptically.

Groups

All mice were divided into five groups randomly as following: (1) Control group (n=8), WT C57BL/6 mice treatment with PBS (0.1ml/mouse); (2) WT group or VMC group (n=8), WT C57BL/6 mice treatment with CVB3 (0.1ml/mouse); (3) BKO (n=8), BKO mice treatment with CVB3 (0.1ml/mouse); (4) SCID (n=8), SCID mice treatment with CVB3 (0.1ml/mouse); (5) SCID +B cells group (n=8), seeing below "**B Cell Reconstitution"**.

Cell Isolation

Spleens from mice of WT and BKO groups were cut into pieces and gently grounded with a grinding rod. Single cell suspensions were filtered through a 30 μm cell strainer (Miltenyi Biotec, Bergisch Gladbach, Germany). B cells from WT group VMC mice were isolated using mouse CD19 MicroBeads (Miltenyi Biotec), following the manufacturer's instructions (catalog #130-052-021). Single cell suspensions from mice of WT and BKO groups were prepared as described above to isolate naïve $CD4^+$ T cells. Naive $CD4^+$T cells were isolated with the specific naïve $CD4^+$ T cell isolation kit (Miltenyi Biotec) following to the manufacturer's instructions (catalog #130-104-453). The purity of B cells or naïve $CD4^+$ T cells was determined by flow cytometry to be >97%.

BCell Reconstitution

Approximately 10^7 above purified B cells were injected IP into SCID mice. Three days after the IP injection, the SCID mice were inoculated with CVB3 (SCID group, n=8), and reconstituted B cells (SCID+B cells group, n=8). Surviving mice were euthanized 7 days after CVB3 infection. Tissue or blood samples were collected aseptically.

Histological Analysis

Mouse hearts were sectioned midheart. The base portions of the hearts were used for the polymerase chain reaction (PCR) studies, while the apex portions were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 μm thick) were stained with hematoxylin and eosin to determine the level of inflammation. Myocardial pathological scores were blindly graded by two independent researchers following a semi-quantitative scale [14]. Specifically, grade 0 was assigned to samples with no infiltration of inflammatory cells and necrosis, grade 1 to samples with the ratio of inflammatory cells and necrosis <25%, grade 2 to samples with the ratio of inflammatory cells and necrosis at 25–50%, grade 3 to samples with the ratio of inflammatory cells and necrosis at 50–75%, and grade 4 to samples with the ratio of inflammatory cells and necrosis >75%.

Flow Cytometry

Single cell suspensions from spleens were prepared as described above. The mouse antibodies were as follows: anti-CD4 (PerCP-Cy5.5), anti-CD19 (PerCP-Cy5.5), anti-CD40 (APC), anti-CD69 (PE), anti-CD80(APC), anti-CD86 (PE), anti-MHC-II (PE), anti-IFN-γ (FITC), anti-IL-6 (FITC), anti-IL-17 (PE), anti-IL-4 (PE), anti-TNF-α (APC), and anti-IL-22 (APC), and were purchased from either BD Pharmingen (San Diego,

CA, USA) or eBioscience (San Diego, CA, USA). For specific measurement details, please refer to the flow cytometry section of our previous study [15]. Flow cytometry was performed on a BD FACSCanto II and data were analyzed using FlowJo7.6 software.

Enzyme-Linked Immunosorbent Assay (ELISA)

Supernatant levels of IFN-γ, IL-4, IL-17, and IL-22 were detected using mouse IFN-γ, IL-4, IL-17, and IL-22 ELISA kits (RayBiotech, Atlanta,USA), while serum levels of cardiac troponin (cTn)T were determined using mouse cTn ELISA kit (CUSABIO, Wuhan, China). All protocols were performed according to the manufacturer's instructions.

Quantitative Real-Time PCR

Total RNA was extracted from the heart tissue samples with RNAiso Plus (TaKaRa, Dalian, China) and cDNA produced immediately using the PrimeScript™RT reagent kit (TaKaRa) following the manufacturer's protocols. Quantitative real-time PCR was performed using SYBR® Premix Ex Taq™ II (TaKaRa) with the following conditions: pre-denaturation at 95°C for 30 seconds, 40 cycles of 95°C for 5 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Quantitative real-time PCR was performed using the Step One Real-Time PCR System (Applied Biosystems). The primers are listed in Table 1. The mRNA expression levels were calculated using the comparative CT (ΔΔCT) method and normalized against β-actin mRNA levels.

Table 1
The primer sequences

Name Sequence (5'-3') Length (bp)
T-bet Sense: CTGCCTACCAGAACGCAGA 199
antisense: AAACGGCTGGGAACAGGA
GATA-3 Sense: AGAGATTTCAGATCTGGGCAATGG 96
antisense: CAGGGACTGATTCACAGAGCATGTA
ROR-γt Sense: TCTGCAAGACTCATCGACAAGG 80
antisense: CACATGTTGGCTGCACAGG
AHR Sense: GGATGAAGAAGGACGAGAGCA 137
antisense: TTTTGGTGCGTATTGGTAGGG
β-actin Sense: CATCCGTAAAGACCTCTATGCCAAC 99
antisense: ATGGAGCCACCGATCCACA

Ex Vivo Experiments

Purified naïve $CD4^+$T cells from WT or BKO mouse groups were divided into three subgroups: (1) WT group, the naïve $CD4^+$T cells from WT group VMC mice; (2) BKO group, the naïve $CD4^+$T cells from BKO group VMC mice; (3) BKO + B cells group, the naïve $CD4^+$T cells from BKO group VMC mice, and combined with above purified B cells from WT group VMC mice (1:1 ratio). All naïve $CD4^+$T cells (1×10^6 cells/mL) were placed in 24-well plates and maintained in RPMI1640 (Gibco, Grand Island, NY, USA) containing 10% FCS (Gibco) and stimulated with anti-CD3 (5 mg/mL; eBioscience) and soluble anti-CD28 (2 mg/mL; eBioscience). The differentiation conditions were as follows: Th1: IL-12 (20 ng/ml; eBioscience) and anti-IL-4 (10 μg/mL; eBioscience); Th2: IL-4(10ug/mL, eBioscience) and anti-IFN-γ mAb (10 μg/mL, eBioscience); Th17: IL-2 (10 ng/mL), IL-1β (10 ng/mL), IL-6 (20 ng/mL), IL-23 (20 ng/mL), TGF-β (2 ng/mL), anti-IL-4 mAb (10 μg/mL), and anti-IFN-γ mAb (10 μg/mL) (all purchased from eBioscience); Th22: IL-1β (10 ng/mL), IL-6 (20 ng/mL), IFN-γ (20ng/mL), and TNF-α (20 ng/mL) (all purchased from eBioscience). At days 2 and 4, cultures were also treated with IL-2 (10 ng/mL; eBioscience). After 7 days in culture, some cells were harvested and stimulated with PMA (50 ng/mL; Sigma-Aldrich) ionomycin (500 ng/mL; Sigma-Aldrich), and brefeldin A (10 μg/mL; Sigma-Aldrich) for 4 h. Some cells were subsequently stained with anti-IFN-γ, anti-IL4, anti-IL-17, and anti-IL-22 mAb as described above, while others were harvested for quantitative real-time PCR analysis. The supernatant was analyzed using ELISA.

Statistical Analysis

Data were analyzed with Prism 6 (GraphPad Software Inc., La Jolla, CA). Unpaired student t-test was used to compare two groups, and one-way ANOVA (Gaussian distribution) was used to compare more than two groups. Data were represented as the mean ± SD, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$, ns $p > 0.05$.

Results

B cells Activation and Antigen-presenting Ability Are Significantly Enhanced in Acute VMC

Because CD69 is a marker of early B cells activation, while CD40, CD80, CD86, and MHCII represent antigen-presenting ability of B Cells. We detected their expressions in B cells from spleen samples by flow cytometric. As shown in Fig. 1A-F, the expressions of CD69, CD40, CD80 and MHCII in B cells were markedly upregulated in VMC group compared to the control group (all $P < 0.0001$), but the expression of CD86 was significantly downregulated in VMC group compared to the control group ($P < 0.0001$), whereas the total number of B cells remains unchanged between VMC group and control group ($P > 0.05$). These results reveal B cells activation and antigen-presenting ability are significantly enhanced in acute VMC.

Cytokine-Producing B cells Were Significantly Increased in Acute VMC

To analyze cytokine-producing B cells, mice were treated with PBS or CVB3 on day 0 and sacrificed on day 7. Spleens were collected and performed by flow cytometric analysis. As shown in Fig. 2, the frequencies of TNF-α, IL-6, and IL-17 in B cells were significantly increased in VMC group compared to the

control group ($p < 0.0001$ for all), but the frequency of IL-4 in B cells was significant reduced ($p < 0.01$). This suggests that B cells have a strong capacity to produce pro-inflammatory cytokines in acute VMC.

B Cells Play a Pathogenic Role in Acute VMC Independent of T Cells

As shown in Fig. 3A, representative pictures of myocardial tissue sections stained for HE in control, WT, BKO, SCID and SCID + B cells groups. There was some infiltration of inflammatory cells and necrosis in WT, BKO, SCID, and SCID + B cells groups, while no cellular infiltration, necrosis or fibrosis was observed in the control group. The infiltration of inflammatory cells and necrosis in WT and SCID + B cells groups was significantly increased compared to BKO and SCID groups. As shown in 3B and 3C, the myocardial pathological scores and cTNT levels were significantly decreased in BKO group compared to WT group ($p < 0.0001$). To further clarify whether the role of B cells in VMC depends on T cells, we used SCID mice and B cells reconstitution. The myocardial pathological scores and cTNT levels were significantly reduced in SCID group compared with those in SCID + B cells group ($p < 0.0001$).While there were no statistical significance between WT and SCID + B cell groups or between SCID and BKO groups in myocardial pathological scores and cTNT levels ($P > 0.05$). However, these two indices for evaluation of the severity of VMC was decreased in BKO group compared to WT or in SCID group compared to SCID + B cells group, which suggests that B cells play a pathogenic role in VMC independent of T cells.

B Cells Influence Th cell and Their Corresponding Transcription Factors in Acute VMC *In Vivo*

To determine whether B cells have an effect on Th1, Th2, Th17, and Th22 cells, we detected the frequency of Th1, Th2, Th17, and Th22 in spleen cells via flow cytometry. As showed in Fig. 4A-B, the frequency of Th1 and Th17 cells was significantly reduced in BKO group compared to the WT group ($p < 0.0001$). However, the frequency of Th2 cells were higher than those in WT group ($p < 0.001$). There was no significant difference between Th22 cells in BKO and WT groups ($p > 0.05$). T-bet, GATA-3, RORγt, and AHR are the specific transcription factor of Th1, Th2, Th17, and Th22 cells, respectively, which are sufficient and necessary conditions in their differentiation. We examined their mRNA expression levels in the heart tissue with quantitative real-time PCR. The data for quantitative real-time PCR were consistent with flow cytometry result (Fig. 4C). These results suggest that B cells may affect Th Cell differentiation.

B Cells Affect Th Cell Differentiation *Ex Vivo*

To further elucidate the effects of B cells on Th1, Th2, Th17, and Th22 differentiation *ex vivo*, naïve CD4 + T cells were purified from BKO and WT groups mouse spleens and cultured with or without addition of B cells in different differentiation conditions. As presented in Fig. 5, the levels of IFN-γ and IL-17 in the supernatant were reduced in BKO group compared to the WT group ($p < 0.0001$), while the levels of IFN-γ and IL-17 in the supernatant were increased in BKO + B cells group compared to the BKO group ($p < 0.0001$). There was no significant difference between WT and BKO + B cells groups. Furthermore, the levels of IL-4 in the supernatant were increased in BKO group compared to the WT group ($p < 0.0001$), and downregulated in BKO + B cells group compared to the BKO group ($p < 0.0001$). There was no significant difference between WT and BKO + B cells groups. The levels of IL-22 in the supernatant were not

significantly different among BKO, WT, and BKO + B cells groups. The data for quantitative real-time PCR and flow cytometry were consistent with ELISA results. This suggests that B cells could regulate Th cell differentiation ex vivo.

Discussion

B cells are an important type of immune cell that are widely involved in the immune regulation. The traditional view suggests that B cells play a protective role in VMC through immunoglobulin secretion. However, more recent studies showed that B cells play a pathogenic role in cardiovascular diseases, with their exact role in VMC remaining unclear.

In this study, by performing *ex vivo* experiments and utilizing mice with different genetic modifications, we demonstrated that B cells are essential in the development of VMC. B cells cause myocardial damage by antigen presentation, producing cytokines and affecting Th cell differentiation in VMC. We thus found that B cells play a pathogenic role in a mouse model of acute VMC independent of T cells.

Acute VMC is characterized by a focal cellular infiltration with little necrosis or fibrosis [16]. Its principal causes are the viral infection and excessive immune response, which lead to myocardial damage. B cells play a vital role in immune response. More and more studies demonstrate that B cells play a pathogenic role in inflammatory or autoimmune diseases, such as asthma [12], systemic sclerosis [9], and rheumatic autoimmune disease [17]. In a clinical setting, B cell depletion therapy achieves satisfactory results in the treatment of refractory autoimmune rheumatic diseases, for example SLE [18] and RA [19]. Cordero-Reyes et al. [20] suggested that B cells play a crucial role in angiotensin-II-induced heart failure by influencing cytokine activation, immunoglobulin deposition, and activation of apoptosis.

In the current study, we found that expression of CD69 in B cells was elevated in VMC, which suggest B cells are activated in VMC; expressions of CD40, CD80, MHCII in B cells were increased in VMC, which reveal that B cell antigen presentation are enhanced in VMC. Although CD86 is an important costimulatory molecule, which has similar structure and function with CD80, they play distinct roles in VMC[15], which maybe explain the expression of CD86 in B cells was reduced in VMC. Meanwhile, we found that the expression of TNF-α, IL-6, and IL-17 in B cells was significantly increased in VMC group compared to control group, and that the expressions of IL-4 in B cells was significantly reduced. These suggest that B cells may play a crucial role by antigen presentation and producing pro-inflammatory cytokine in acute VMC.

To further demonstrate the role of B cells in acute VMC, we used BKO mice, SCID mice and B cells reconstitution. Our results show that B cells play a pathogenic role in VMC independent of T cells. The antigens recognized by B cells are mainly T cell-dependent antigens and some T cell-independent antigens. Most studies suggest that the role of B cells in the immune response requires T cell assistance. However, in this study, we found that B cells independently play a role in VMC without T cell assistance. Because antigen presentation requires the help of T cells, our study shows that the cytokines produced by

B cells may play a role in promoting myocardial damage in VMC. The pro-inflammatory cytokine-producing B cells directly cause myocardial injury.

Although the exact mechanism of VMC pathogenesis is still unclear, the immune response plays an important role. In particular, B and T cells exhibit meaningful interactions, relevant to this process. Previous studies showed that Th1, Th2, Th17, and Th22 cells are involved in the pathogenesis of VMC [3–5, 21], especially the Th1 and Th17 cells, which play a central role in VMC. In this study, we showed that the role of B cells in VMC is independent of T cells. However, whether B cells promote naïve T cell differentiation into Th1, Th2, Th17, and Th22 cells remains unknown. To illustrate this, we used B cells and naïve $CD4^+$T cells in co-culture, as well as BKO mice to observe B cell influence on the differentiation of Th cells. Our results showed that B cells are able to promote Th1 and Th17 differentiation, suppress Th2 differentiation, and have no influence on Th22 differentiation.

Naïve $CD4^+$T cells differentiate into effective T cells, such as Th1, Th2, and Th17 cells, and are dependent on co-stimulatory molecules and antigen-derived peptide complexes like major histocompatibility complex class II, which are presented with antigen-presenting cells, and cytokine stimulation. B cells can present antigens and produce cytokines, therefore, B cells have the capacity to prime naïve $CD4^+$T cells both *in vivo* and *ex vivo* [22, 23]. However, B cells promote naïve $CD4^+$T cells differentiation into different effective T cells in different diseases. Barr et al. showed that B cells induce Th1 differentiation in Salmonella enterica [24]. In a mouse model of asthma induced with house dust mite extracts, Wypych et al. revealed that B cells prime Th1 and Th17 differentiation [12]. In experimental autoimmune encephalomyelitis, B cell-derived IL-6 aggravated disease pathogenesis by promoting Th1 and Th17 cell differentiation [25, 26], León et al. also suggested that B cells regulate Th2 differentiation by antigen presentation [27]. In the current study, we found that B cell-derived pro-inflammatory cytokines were markedly elevated in VMC, which may be the reason for B cell promotion of Th1 and Th17 cell differentiation in VMC. However, B cell-derived IL-4 was decreased in VMC, which may explain why B cells limit Th2 cell differentiation. Th22 cell differentiation requires IL-6 and TNF-α. B cell-derived IL-6 and TNF-α were significantly up-regulated in this study. In theory, B cells promote Th22 cell differentiation in VMC and have no effect on Th22 cell differentiation. The reason for this effect remains to be discovered.

Previous studies have shown that Th1 and Th17 cells play a pathogenic role, while Th2 andTh22 cells have a protective effect in VMC. However, in this study, we did not only discover a pathogenic role for B cells, but also learned that B cells can promote Th1 and Th17 cell differentiation, while limiting Th2 cell differentiation. Therefore, depletion of B cells can alleviate the effects from the disease in two different ways in VMC. B cell immunotherapy thus might prove to be a novel therapeutic option in VMC.

Our study has some limitations. First, we could not determine which B cell function plays a role in VMC. Second, the specific mechanisms for B cell regulation of Th cell differentiation need to be studied further.

In conclusion, B cells are activated and have strong abilities of antigen presentation and producing cytokines in VMC. B cells not only play a pathogenic role in VMC independent of T cells, but also promote

Th1 and Th17 cell differentiation, and limit Th2 cell differentiation in VMC. Our current findings suggest that B cell depletion therapy might provide a novel therapeutic treatment for VMC.

Declarations

Ethics approval and consent to participate

This study was performed with the approval of Guangxi Medical University Animal Ethics Committee. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23).

Consent for publication

Not Applicable.

Availability of data and materials

Not Applicable.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' contributions

Zhihong Cen carried out experiments and prepared the manuscript.Yong Li, Bin Wei analyzed experimental results. Weifeng WU,Yanlan Huang conceived, designed, coordinated the study as well as reviewed the manuscript. Jing Lu carried out data

collection. All authors read and approved the final version of the manuscript.

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Not Applicable.

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Figures

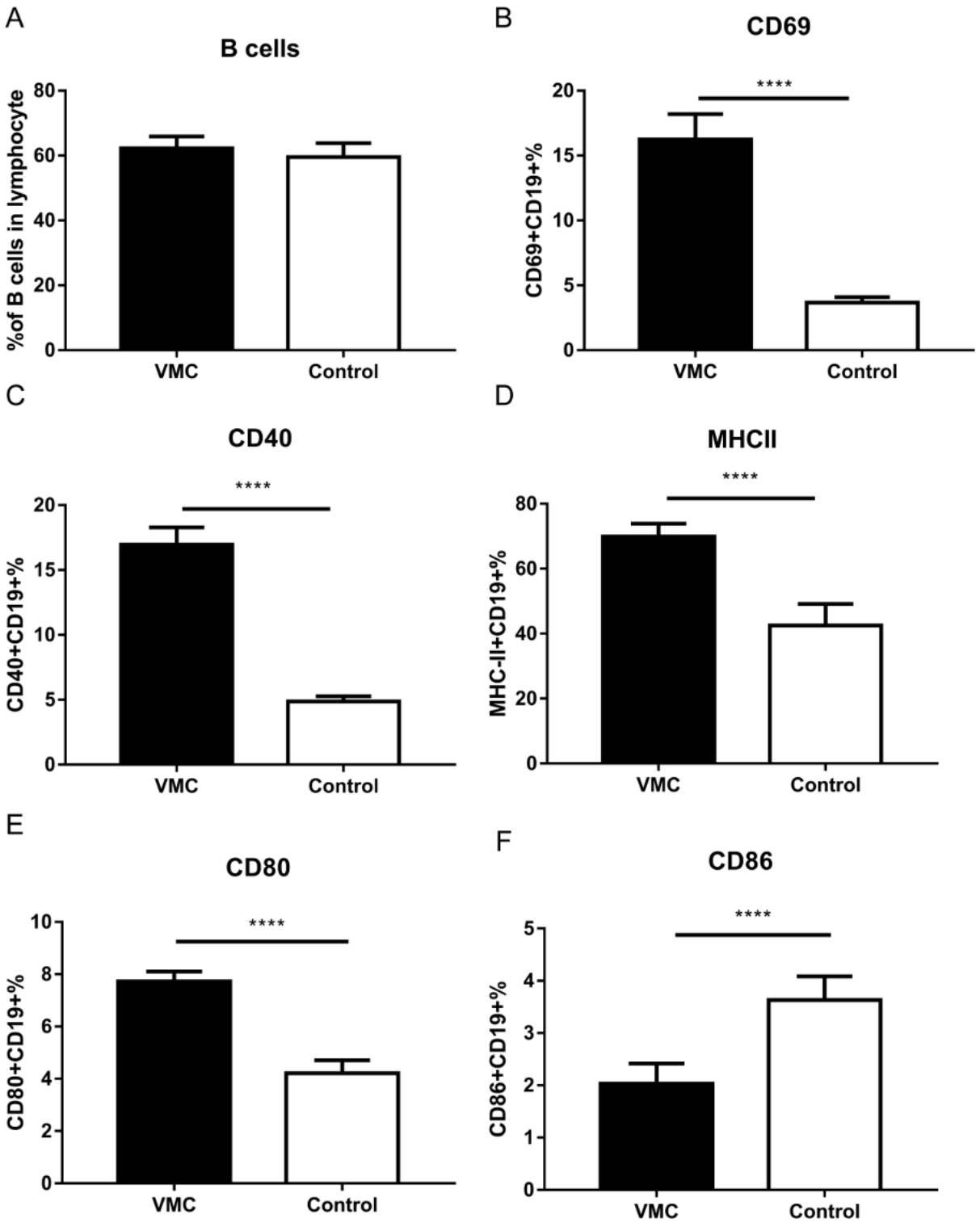

Figure 1

The changes of B cells surface markers in spleens in acute VMC. (A) The total number of B cells in VMC and control groups. (B) The expression of CD69 in B cells in VMC and control groups. (C) The expression of CD40 in B cells in VMC and control groups. (D) The expression of MHCII in B cells in VMC and control groups. (E) The expression of CD80 in B cells in VMC and control groups. (F) The expression of CD86 in B

cells in VMC and control groups. Data are represented as mean ± SD, where n = 8 mice/group, * p <0.05, ** p<0.01, *** p<0.001, and **** p<0.0001.

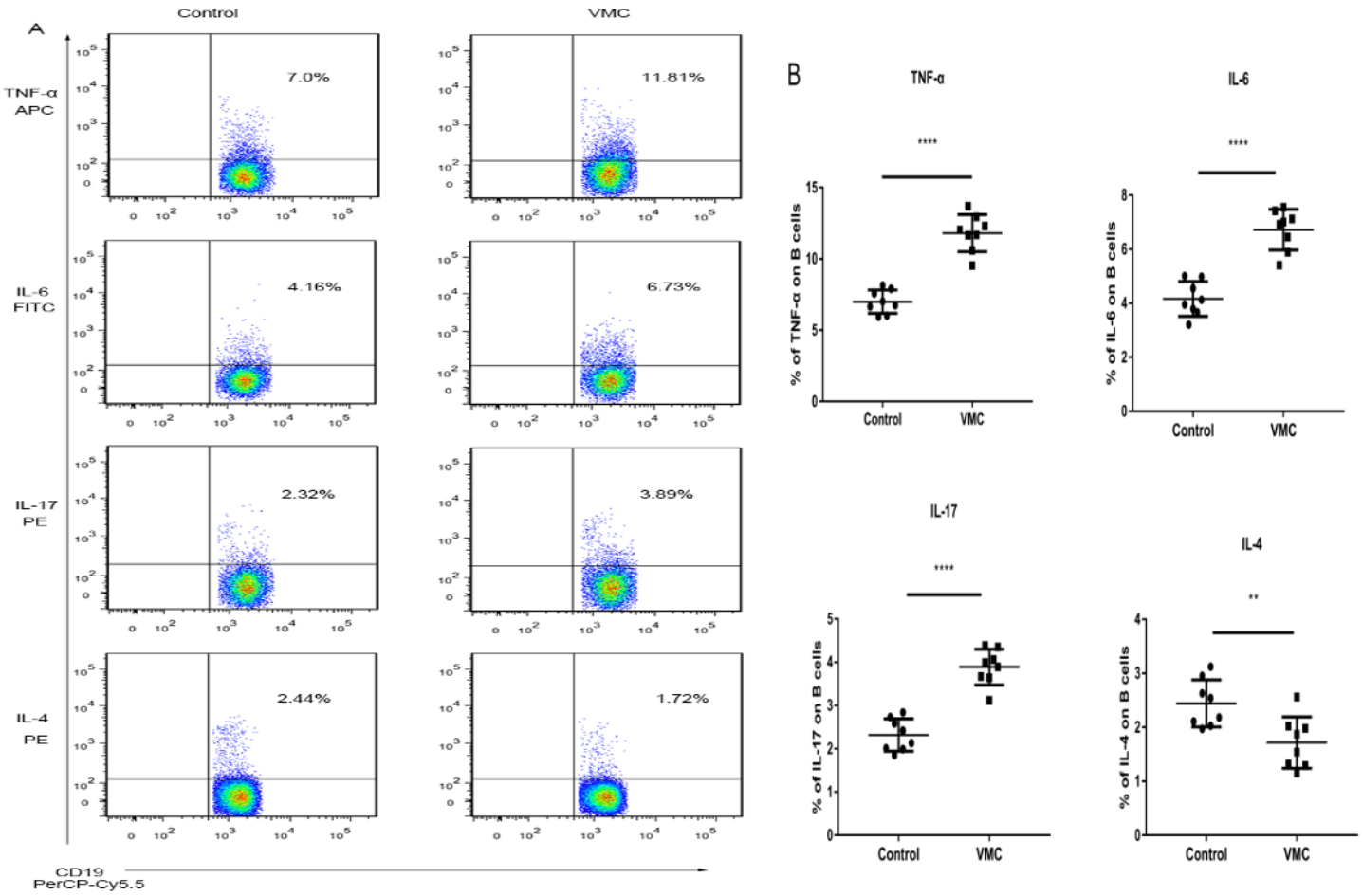

Figure 2

Cytokine-producing B cells from spleen cells are upregulated in acute VMC. (A) Representative images for the frequencies of TNF-α, IL-6, IL-17, and IL-4 in B cells from mice (day 7) in control group compared with VMC group. Numbers in the upper right quadrants represent the mean frequencies. (B) Statistical analysis results for the cytokine-producing B cells. Data are represented as mean ± SD, where n = 8 mice/group, * p <0.05, ** p<0.01, *** p<0.001, and **** p<0.0001.

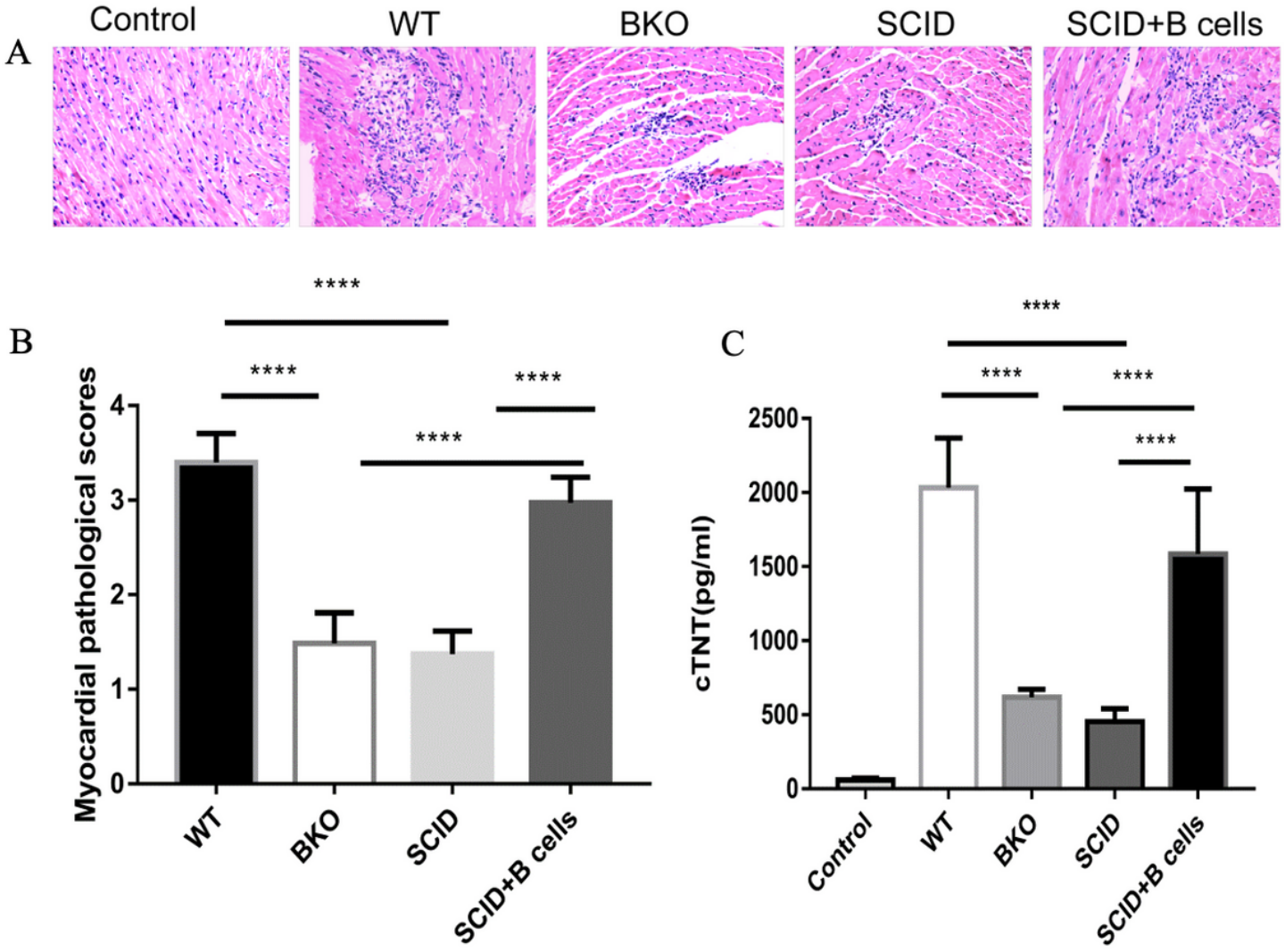

Figure 3

B cells exacerbate the severity of VMC in mice. (A) Representative images of histopathology (400x magnification) in heart tissue. (B) Myocardial pathological scores in different test groups. (C) The levels of serum cTnT serum in different groups. Data are represented as mean ± SD, where n = 8 mice/group, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$.

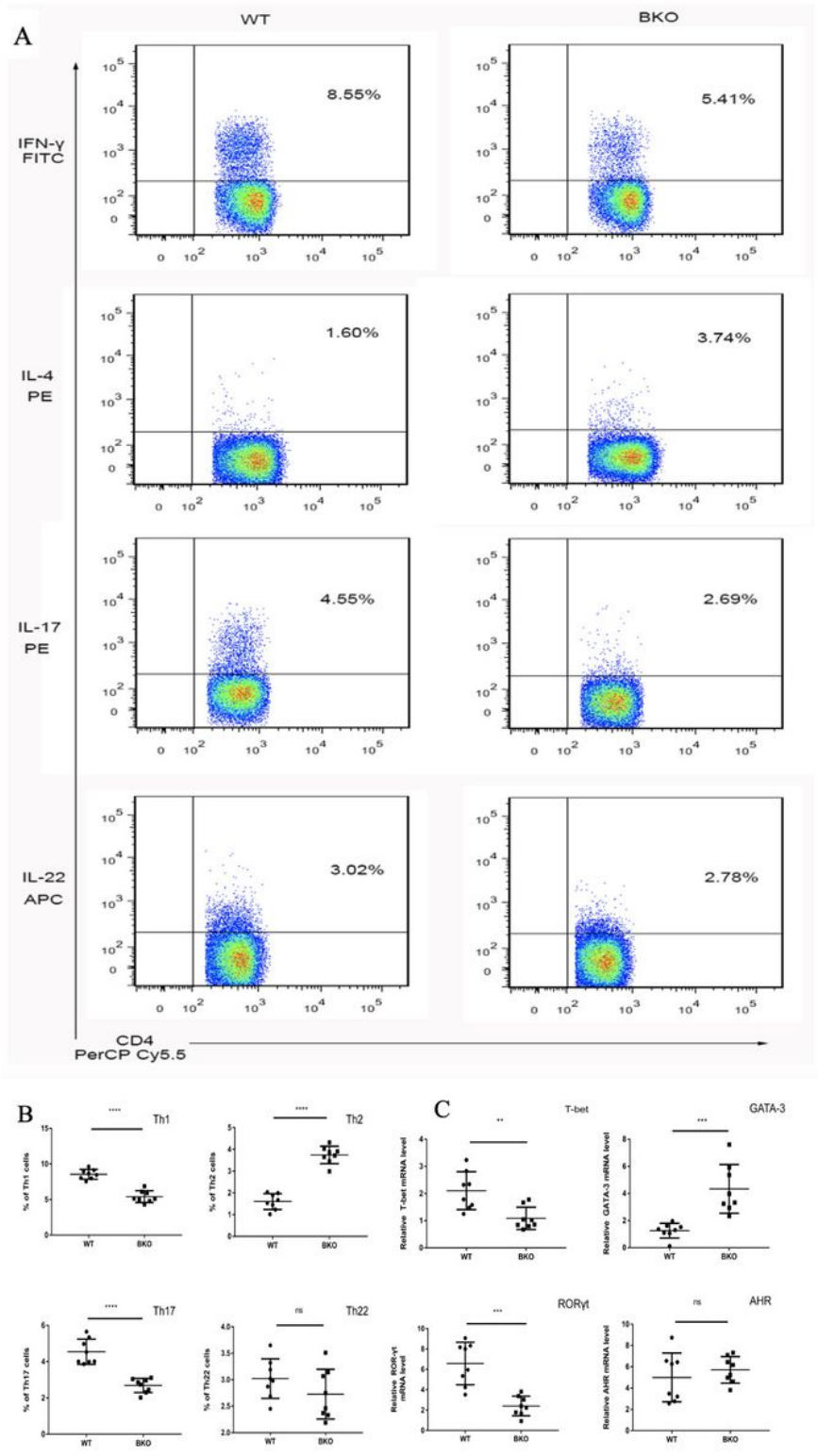

Figure 4

B cells influence Th cell and their corresponding transcription factors. (A) Representative images for the frequencies of Th1, Th2, Th17, and Th22 in WT group compared to BKO group. Numbers in the upper right quadrants represent the mean frequencies. (B) Statistical analysis for the Th1, Th2, Th17, and Th22 cells. (C) The mRNA expression levels of T-bet, GATA-3, RORγt, and AHR in heart tissue in WT group

compared to BKO group. Data are presented as mean ± SD, where n = 8 mice/group, * p <0.05, ** p<0.01, *** p<0.001, and **** p<0.0001, ns p>0.05.

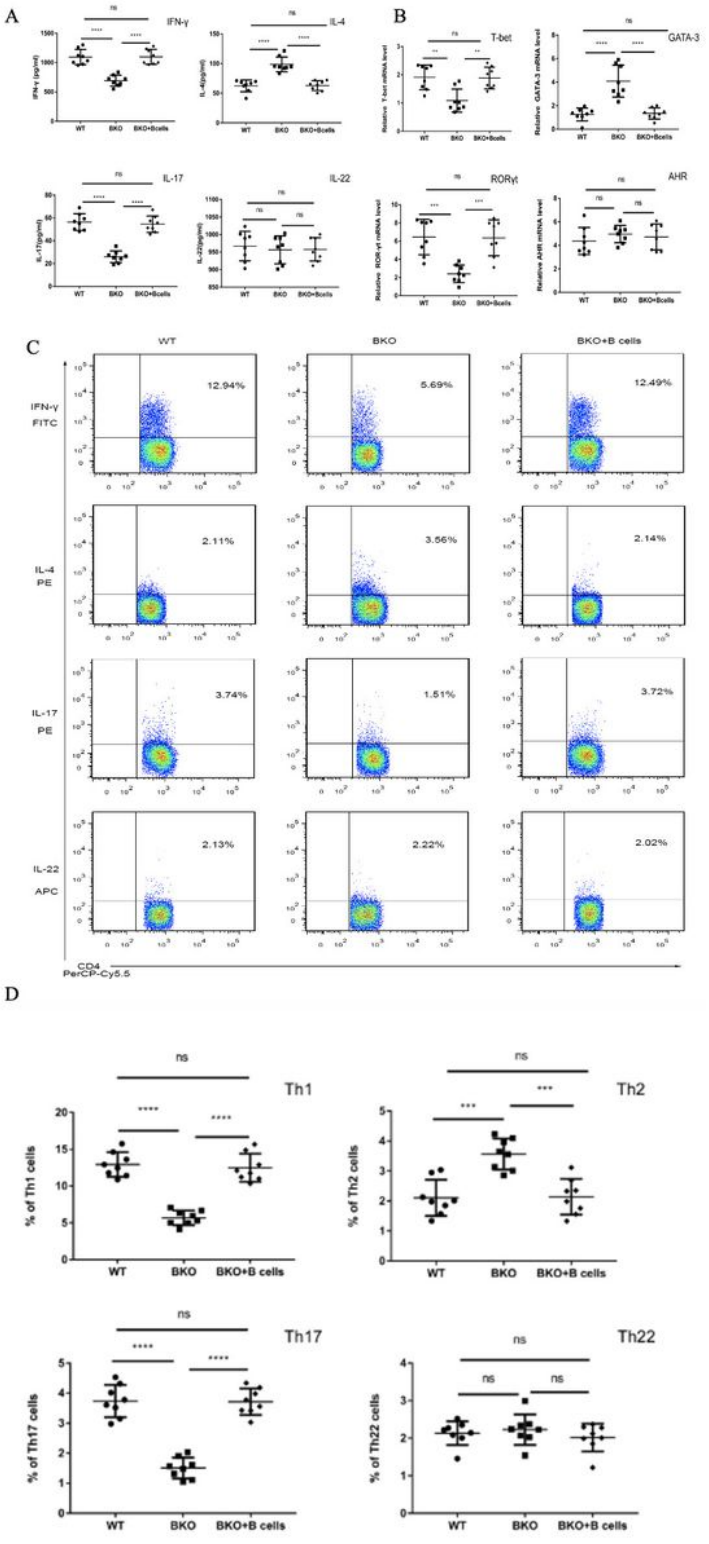

Figure 5

B cells affect Th cell differentiation ex vivo. (A) Supernatant levels of IFN-γ, IL-4, IL-17, and IL-22 in the WT, BKO, and BKO+B cells groups. (B) The mRNA expression levels of T-bet, GATA-3, RORγt, and AHR from cultured cells in WT, BKO, and BKO+B cells groups. (C) Representative images for the frequencies of

Th1, Th2, Th17, and Th22 in WT, BKO, and BKO+B cells groups ex vivo. Numbers in the upper right quadrants represent the mean frequencies. (D) Statistical analysis results for the Th1, Th2, Th17, and Th22 cells ex vivo in different groups. Data are presented as mean ± SD, where n = 8 mice/group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$, ns $p > 0.05$.