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ORIGINAL ARTICLE

B Cells Increase Myocardial Inflammation by Suppressing M2 Macrophage Polarization in Coxsackie Virus B3-Induced Acute Myocarditis

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Abstract— The role of B cells in viral myocarditis (VMC) remains controversial. In order to establish a role and mechanism of action for B cells in acute VMC, we established an acute VMC mouse model by intraperitoneal injection of Coxsackie virus group B type 3 (CVB3). At day 7, mice were analyzed using myocardial histopathology, and the presence of M2 macrophages in spleen and heart. Mice were divided into four groups, all having a C57BL/6 background: control group; wild-type (WT) VMC; mMt/mMt (−/−) VMC (BKO), and BKO + B cell VMC. A role for B cells was demonstrated by a significant reduction in myocardial pathological score and an increase in the frequency of M2 macrophages in the BKO group, when compared to the WT group. Once BKO mice underwent B cell reconstitution with isolated WT B cells, the myocardial pathological score was increased significantly, while the frequency of M2 macrophages decrease. Our findings demonstrate that B cells increase myocardial inflammation by suppressing M2 polarization in acute VMC *in vivo*.

KEY WORDS: B cells; macrophage; polarization; viral myocarditis.

INTRODUCTION

Viral myocarditis (VMC) is a common cardiovascular disease in adolescents, which is often caused by Coxsackie virus group B type 3 (CVB3), and can lead to heart failure and sudden death [1, 2]. Direct myocardial injury induced by virus infection, and a prolonged inflammatory response, is the main pathology [3]. However, the mechanism of the immune inflammatory response in VMC is still unclear. Therefore, the exact pathogenesis of viral myocarditis needs further clarification in order to find efficient therapeutic options.

The immune system becomes activated in response to myocardial damage [4]. In VMC, the myocardium is infiltrated by a variety of immunocytes, including T cells, macrophages, and B cells. Many studies have confirmed an important role for $CD4^+$ T cells, and macrophages in the pathogenesis of VMC [5–8]. Previous studies have demonstrated that B cells secrete immunoglobulins with anti-inflammatory effects in VMC, while secreting anti-myocardial antibodies to promote myocardial injury [3, 9]. In addition to humoral immunity, B cells participate in T cell activation and tissue inflammation *via* antigen presentation and cytokine production [10]. B cells can directly damage the myocardium by producing cytokines or antibodies, and therefore, may be responsible for the increased myocardial injury seen in VMC. However, the role of B cells in VMC has been rarely studied.

Our research found that in a mouse model of VMC induced by CVB3, B cells secrete a variety of proinflammatory cytokines (IFN-γ, TNF-α, IL-6) and promote Th1 and

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Th17 cell differentiation (unpublished observation). Recent research has found that in acute myocardial infarction, B cells increase myocardial injury by secreting the chemokine (C-C motif) ligand 7, and recruiting Ly6C^{hi} monocytes to local myocardial tissue [11]. In the inflammatory microenvironment, Ly6C^{hi} monocytes differentiated into classically activated macrophages (M1), and increased tissue damage. Therefore, B cells may promote myocardial injury by modulating macrophage function in VMC. In this study, we show that B cells caused myocardial injury by suppressing M2 polarization in acute VMC induced by CVB3.

MATERIALS AND METHODS

Mouse Model of VMC Induction

Pathogen-free, 5-week-old male C57BL/6 mice (WT), muMt/muMt (−/−) mice (B cell-knockout (BKO), B6.129S2-Ighm^{tm1Cgn}/J, Stock No: 002288, Jackson Laboratories, ME, USA), and BKO mice reconstructed with B cells (BKO + B cells), were used to establish an acute VMC model. The mouse model of acute VMC was established as described previously [12]. WT mice injected intraperitoneally with phosphate buffer solution (PBS) were used as controls. All surviving animals were sacrificed on day 7. The hearts and spleens were removed aseptically for further analyses. The protocols of animal experiments were approved by the Guangxi Medical University Animal Ethics Committee.

B Cell Reconstitution

WT mice underwent the VMC protocol, sacrificed on day 7, and the spleens removed aseptically. The spleens were ground into a single-cell suspension, and filtered through a 30-μm cell strainer, and centrifuged at 300*g* for 5 min. The single-cell suspension was then treated with lysing buffer (BD Biosciences, San Diego, CA, USA) to remove red blood cells, and washed twice with 1 mL of PBS. After centrifugation at 300*g* for 5 min, the pellet was resuspended in 1 mL of separation buffer (Miltenyi Biotec, Bergisch Gladbach, Germany). B cell isolation was performed on the cell suspension using a B cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's instructions. The purified B cells were then diluted in PBS, and injected intraperitoneally into BKO mice 1 day before induction of VMC, at a density of approximately 2×10^6 cells per mice. Flow cytometry analysis of B cells in spleen confirmed B cell reconstruction.

Histological Analysis

Hearts were removed and transversely sectioned midheart, and the base portions fixed in 4% paraformaldehyde, paraffin embedded, and cut into 5-μm sections. Hematoxylin and eosin staining was used to determine the level of myocardial inflammation. The pathological scores were determined using a light microscope (magnification × 400) and by two independent investigators, according to the semi-quantitative scale: grade 0, no cardiac inflammation; grade 1, < 25%; grade 2, 25–50%; grade 3, 50–75%; grade 4, > 75% [13].

Cardiac Tissue Mononuclear Cell Preparation

Hearts were minced, digested, and processed into single-cell suspensions as previously described [14, 15]. Briefly, minced heart tissue was incubated in 0.1% collagenase II (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37 °C. A single-cell suspension was obtained by filtration through a nylon mesh and combined with lysing buffer to remove red blood cells. Finally, cells were resuspended in PBS for further analyses.

Flow Cytometry

Spleens were removed and prepared as described above (B cell reconstitution protocol). For cell labeling, spleen cells and cardiac tissue mononuclear cells were stained and incubated for 30 min at 4 °C in the dark with mouse anti-CD11b-PerCP-cyanine5.5-conjugated mAbs (BD Biosciences, San Diego, CA, USA, clone: M1/70), anti-F4/80-FITC-conjugated mAbs (eBioscience, San Diego, CA, USA, clone: BM8), anti-CD206-APC-conjugated mAbs (Invitrogen, San Diego, CA, USA clone: MR6F3). Isotype control antibodies (eBioscience, San Diego, CA, USA) were used to eliminate background staining due to nonspecific antibody binding to cells. Samples were analyzed by fluorescence activated cell sorting Canto II using fluorescence activated cell sorting Diva software (BD Biosciences, San Diego, USA). Data was analyzed using a Flowjo 7.6 software package.

Quantitative Real-Time PCR

Total RNA extraction, cDNA synthesis, and real-time fluorescence quantitative PCR was performed as previously described [12]. The primers used were as follows: β-actin (sense:CATCCGTAAAGACCTCTATGCC AAC, antisense: ATGGAGCCACCGATCCACA) [12]. arginase (sense:TG CTCACACTGACATCAACACTCC, antisense: TCTACGTCTCGCAAG CCAATGTAC). Amplification conditions were as follows: denaturation at 95 °C for 30 s,

40 cycles of extension at 95 °C for 5 s, 58 °C for 30 s, and 72 °C for 30 s. Data from the gene expression study was analyzed using the ΔΔCt method.

Statistical Analysis

Data are represented as mean ± SD. Analysis was performed using GraphPad Prism software (La Jolla, CA, USA). ANOVA with post hoc Tukey's test was used when analyzing more than three groups. A P value of ≤ 0.05 was considered statistically significant.

RESULTS

B Cells Aggravated Myocardial Inflammation in Acute VMC

Intraperitoneal injection of C57BL/6 mice with CVB3 successfully established the mouse acute VMC model. There was no inflammatory cell infiltration in the control group. The myocardial tissue displayed obvious infiltration of inflammatory cells in the WT, BKO, and BKO + B groups. The myocardial pathological scores (summarized in Fig. 1) were markedly reduced in the BKO group, compared to the WT group ($P \leq 0.001$). After adoptive B cell transfer to BKO mice, myocardial inflammation was increased significantly when compared to the BKO group ($P \leq 0.01$). The results showed that B cells increased myocardial inflammation in acute VMC mice.

B Cells Suppressed M2 Macrophages Polarization in Acute VMC *in vivo*

To determine the number of macrophages in acute VMC, flow cytometry was performed on spleen cells. The number of macrophages ($F4/80^{+}CD11b^{+}$) was markedly reduced in the WT group, when compared to control group ($P \leq 0.01$) and BKO group ($P \leq 0.001$). After adoptive B cell transfer to BKO mice, the number of macrophages

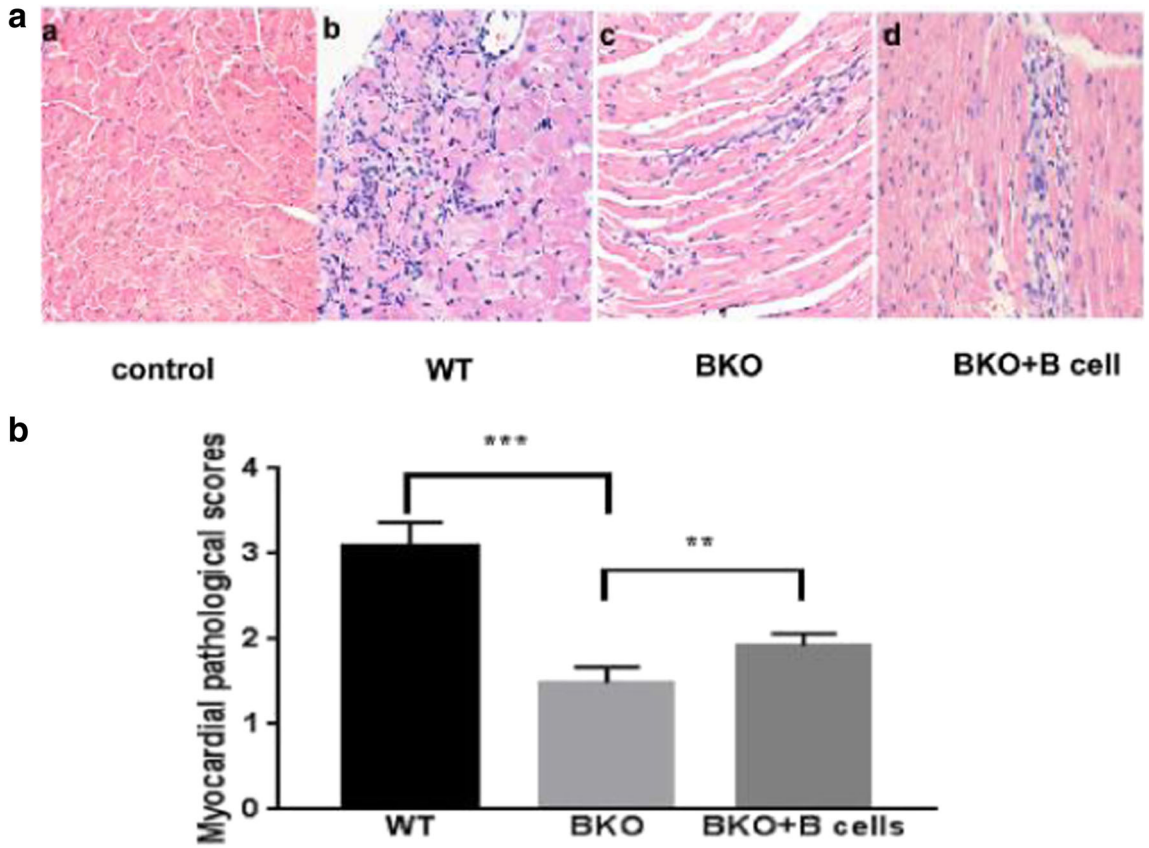

Fig. 1. Pathological changes of myocardial tissue in different myocarditis mice. **a** Representative images of myocardial pathology of myocarditis (day 7) in control group (a), WT VMC (b), BKO VMC (c), and BKO + B cells VMC (d). The heart sections of mice were stained with hematoxylin and eosin, original magnification × 400. **b** The results of myocardial pathological scores in different groups. $N = 8$ mice/group. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. Values are means ± SD.

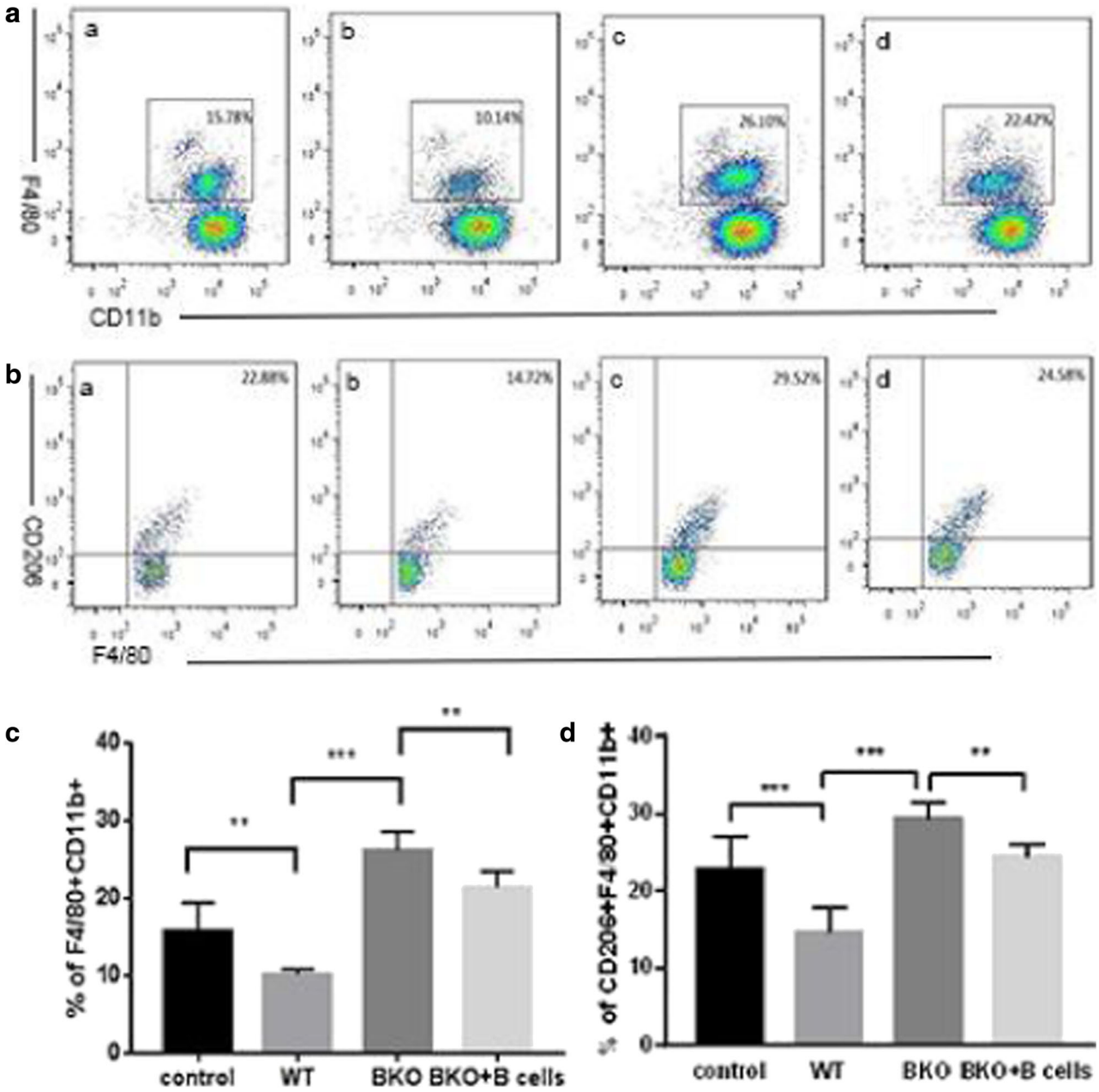

Fig. 2. The changes of splenic macrophages and M2 macrophages in different groups. **a** Representative pictures for the frequency of macrophage in splenic monocytes from mice (day 7) in control group (a), WT group (b), BKO group (c), and BKO + B cells group (d). **b** Representative pictures for the frequency of M2 macrophages in splenic macrophage from mice (day 7) in control group (a), WT group (b), BKO group (c), and BKO + B cells group (d). **c** The results of the statistical analysis of the frequency of macrophage. **d** The results of the statistical analysis of the frequency of M2 macrophage. $N = 8$ mice/group. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

decreased significantly in the BKO + B cell group, when compared to the BKO group ($P \leq 0.001$). CD206 is a characteristic surface marker of M2 macrophage. Therefore, to investigate whether B cells modulate macrophage polarization, we detected the frequency of $CD206^+$ macrophages in spleen cells. After 7 days of infection with CVB3, the number of M2 was decreased significantly in the WT group, when compared to the control group ($P \leq 0.001$) and BKO group ($P \leq 0.001$). Reconstituted B cells in BKO mice showed a

significantly lower number of M2, when compared to the BKO group ($P \leq 0.01$, summarized in Fig. 2).

B Cells Suppressed M2 Macrophages Polarization in Myocardial Tissue in Acute VMC

Macrophages were involved in myocardial immune inflammation mainly through changes in their polarization. To investigate these changes in macrophage polarization in VMC, we determined the number of M2 ($CD206^+F4/80^+CD11b^+$) in myocardial tissue by flow cytometry (summarized in Fig. 3). The results showed that the number of M2 in myocardial tissue increased significantly in the WT group, when compared to the control group ($P \leq 0.001$). An absence of B cells lead to an increase in the frequency of M2 in myocardial tissue, when compared to the WT group ($P \leq 0.001$). After adoptive B cell transfer to BKO mice prior to infection with CVB3, the frequency of M2 decreased significantly, when compared to the BKO group ($P \leq 0.05$). These results demonstrate that B cells suppress M2 macrophages' polarization in the myocardial tissue of acute VMC.

B Cells Reduce Arginase mRNA Expression in the Myocardial Tissue in Acute VMC

Since arginase is predominantly synthesized and secreted by M2, we examined a role for B cells in the expression of arginase messenger RNA (mRNA) in heart tissue. As shown in Fig. 4, arginase mRNA expression in the heart tissue of the BKO group, was significantly up-regulated, when compared to the WT group ($P \leq 0.001$) and BKO + B cell group ($P \leq 0.01$). Our results clearly

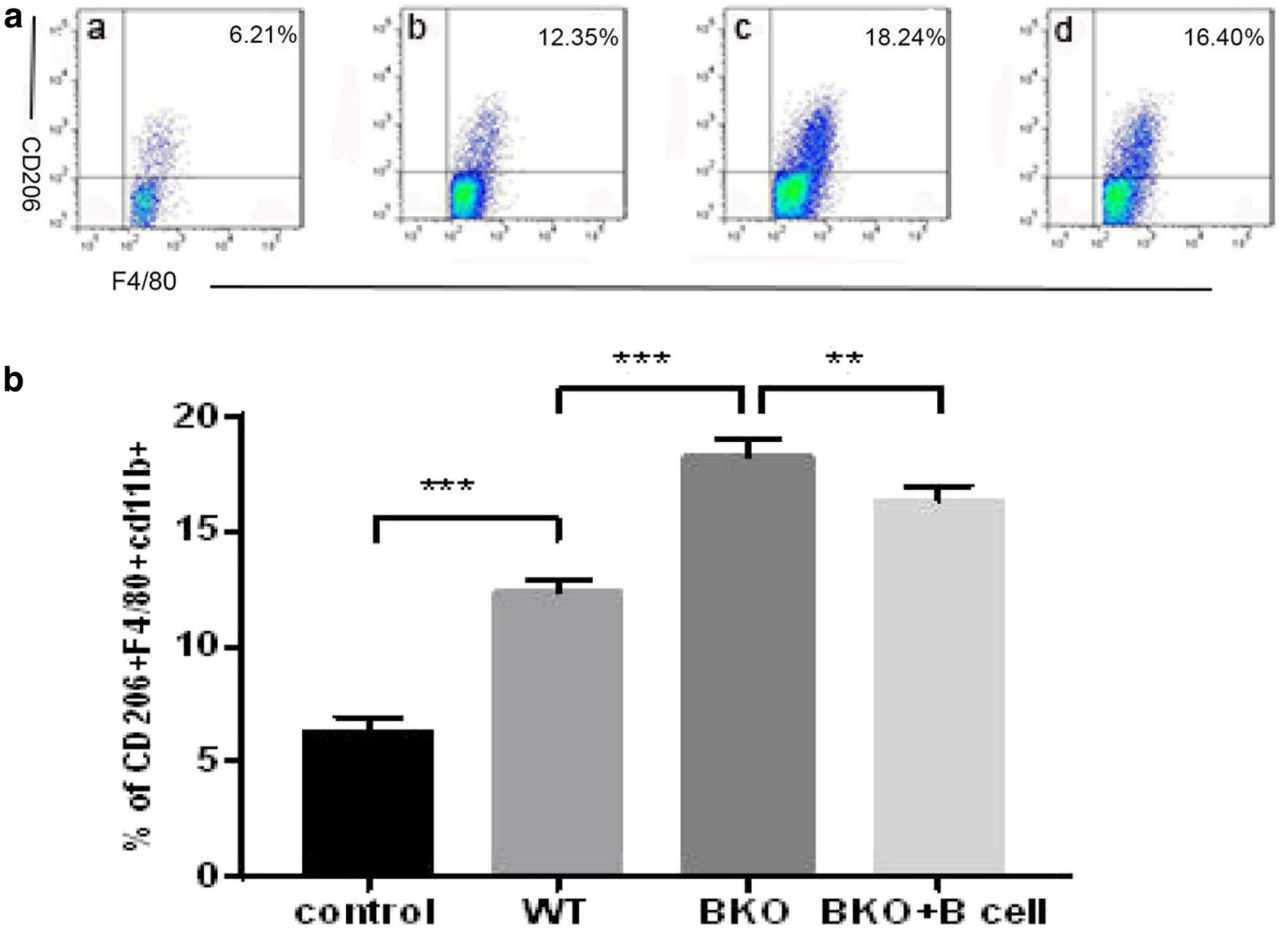

Fig. 3. The changes of cardiac M2 macrophages in different groups. **a** Representative pictures for the frequency of M2 in macrophage in the heart from mice (day 7) in control group (a), WT group (b), BKO group (c) and BKO + B cell group (d). **b** The results of the statistical analysis of the frequency of M2 in the heart. $N = 8$ mice/group. $^{**}P \leq 0.01$. $^{***}P \leq 0.001$.

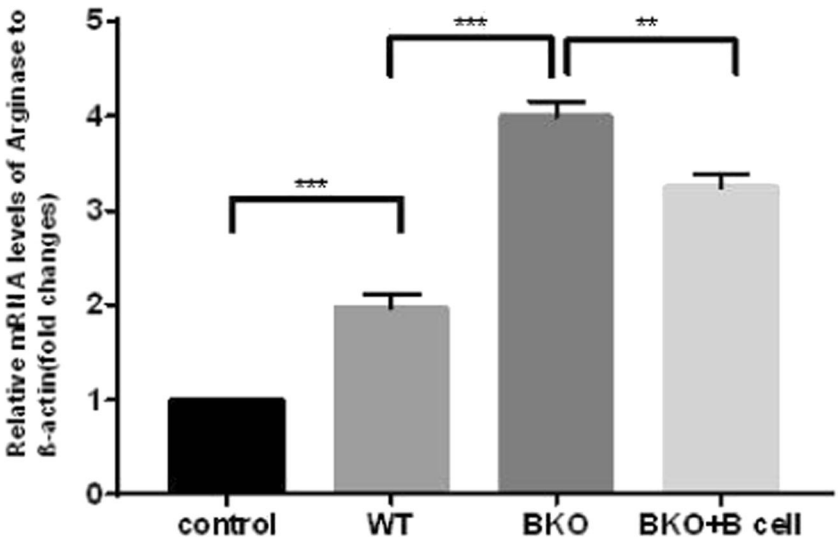

Fig. 4. The levels of arginase mRNA expression in the myocardial in different groups. $N = 8$ mice/group. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

indicate that B cells reduce arginase mRNA expression in myocardial tissue.

DISCUSSION

Previous studies have demonstrated a role for B cells in the pathogenesis of VMC, through the secretion of cytokines. In patients with dilated cardiomyopathy, TNF-α-secreting B cells are significantly increased. The number of TNF-α-secreting B cells was found to be negatively correlated with left ventricular ejection fraction, but positively correlated with procollagen type III in dilated cardiomyopathy patients [16]. B cells are involved in the myocardial fibrosis process in dilated cardiomyopathy by secreting TNF-α. In VMC mice, IL-10 secreting B cells are significantly increased [17]. IL-10 secreting B cells alleviated tissue injury by suppressing CD4 + T cell responses [18]. However, it is not clear whether B cells affect myocardial injury or inhibit myocardial inflammation in VMC. In this study, we used BKO mice to explore the role of B cells in acute VMC. When B cells are absent, myocardial inflammation is significantly alleviated. To further define a role for B cells in VMC, we used B cell transfer to BKO mice before CVB3 infection and found that the myocardial pathological scores were increased. Hence, B cells can increase myocardial injury in acute VMC.

Macrophages play an important role in VMC and are plastic and pluripotent cell types [19]. According to current research, there are two different types of macrophage: classically activated macrophages (M1) and alternatively activated macrophage (M2). M1 has a role in promoting inflammation, while M2 has a more anti-inflammatory role [20, 21]. In VMC, myocardial damage in male mice was more severe than in females. Myocardial infiltrating macrophages from CVB3-infected male mice expressed high levels of M1 markers, whereas high levels of M2 markers were seen in female mice. Adoptive transfer of *ex vivo* programmed M1 macrophages, significantly increased myocardial inflammation in male mice. Remarkably, transfer of M2 macrophages into susceptible male mice alleviated myocardial inflammation [22]. Macrophage polarization is regulated by cytokines, such as IFN-γ and TNF-α, which cause macrophage polarization into M1, whereas IL-4, IL-13, and IL-10 promote macrophage polarization into M2 [23]. Recent studies have demonstrated that B cells promote myocardial injury by secreting proinflammatory factors such as IFN-γ, and TNF-α [24], and promoting Th1 and Th17 differentiation, thereby amplifying the inflammatory response [25]. In malignant tumor tissues, B1 cells promoted the polarization of macrophages into M2 by secreting IL-10 [26]. Therefore, we propose that B cells may regulate macrophage polarization in VMC.

To clarify a role for B cell modulation of macrophage polarization in VMC, we studied M2 formation in the spleens and hearts of VMC mice. Notably, the frequency of M2 increased significantly in BKO mice when compared to WT mice, both in the spleen and heart. After adoptive B cell transfer to BKO mice, M2 decreased significantly. The change in M2 conflicted with that of the myocardial pathological scores in acute VMC. To further demonstrate that B cells regulate macrophage polarization, we examined the mRNA expression of arginase in the heart. After induced

myocarditis, macrophages proliferated significantly [27]. Therefore, the mRNA expression of arginase was significantly upregulated in VMC. However, the mRNA expression of arginase was higher in myocardial tissue from BKO mice than from WT mice. Therefore, B cells increase myocardial inflammation by suppressing M2 polarization in acute VMC.

B cells secrete a variety of cytokines and participate in the pathogenesis of various diseases, such as multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus [28–30]. Specific therapies using cell-depleting antibodies targeting B cells have been shown to be effective. In this study, we applied mature B cell defective mice to demonstrate B cell aggravated myocardial inflammation in acute VMC. However, there is much still to learn regarding the pathogenesis of B cells. We have found that IFN-γ-secreting B cells and TNF-α-secreting B cells were significantly increased in VMC mice. However, in VMC, it is not clear which B cell subset secrete these proinflammatory factors. CD20 and CD22 are specific surface molecules of mature B cells. In VMC mice, the mature B cells ($CD22^+CD19^+$) significantly increased in spleen and peripheral blood by more than 60% (unpublished data). Mature B cells are the principle B cell subset in VMC. Recently, Fleischer have indicated that Epratuzumab, an anti-CD22 monoclonal antibody, inhibited the production of the proinflammatory cytokines IL-6 and TNF-α in B cells [31]. Therefore, mature B cells may regulate polarization of macrophages by secreting various proinflammatory cytokines in VMC. The co-culture of B cells and macrophages *in vitro* is needed for further verification. Meanwhile, prospective studies are essential to further clarify B cell-induced cardiac injury by regulating other immune cells, such as $CD8^+$ T cells, dendritic cells, or NK cells, and identify which B cell subsets are pathogenic in VMC.

To our knowledge, this is the first study to show that B cells increase myocardial inflammation by suppressing M2 polarization in acute VMC. Our findings support immunosuppressive therapy for B cells as a novel therapeutic option in VMC.

FUNDING INFORMATION

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflicts of interest.

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