Life Sciences 235 (2019) 116838

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

IL-10-producing B cells attenuate cardiac inflammation by regulating Th1 and Th17 cells in acute viral myocarditis induced by coxsackie virus B3

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ARTICLE INFO

Keywords:
IL-10-producing B cells
Th1 cells
Th17 cells
Acute viral myocarditis
Regulation
Cardiac inflammation

ABSTRACT

Aims: This work aimed to evaluate the regulatory function of IL-10-producing B cells in viral myocarditis (VMC).
Main methods: We adoptively transferred purified IL-10-producing B cells to VMC mice via the tail vein. We observed the inflammatory responses and cardiac lesions by histological analysis, examined the proportions of spleen Th1 and T17 cells by flow cytometry and expression levels of related transcription factors (T-bet and RORγt) by reverse transcription polymerase chain reaction (RT-PCR), and calculated the cardiac pathological scores and the mean survival times.
Key findings: IL-10-producing B cells were found to be T cell-dependent in the pathogenesis of VMC. They mainly downregulated T-bet and RORγt mRNA levels to decrease the proportions of Th1 and Th17 cells, thereby restraining the inflammation and damage in the myocardium in B cell-deficient VMC mice. Adoptive transfer of IL-10-producing B cells before VMC induction also normalized the inflammatory responses and prolonged the survival time in wild-type (WT) VMC mice. While the transfer of IL-10-producing B cells on day 3 of VMC alleviated the severity of disease, it did not extend the mean survival time of VMC mice. By contrast, IL-10-producing B cells showed no effect on day 7 of VMC. In conclusion, IL-10-producing B cells downregulate the proportion of Th1 and Th17 cells to alleviate inflammatory damage in the myocardium during VMC before the induction or the early phase of disease.
Significance: These findings suggest that IL-10-producing B cells may be a new therapeutic target for modulating the immune response in VMC.

1. Introduction

Viral myocarditis (VMC) is an inflammatory disease of the myocardium and is caused by a type of virus that preferentially gains entry to the heart [1]. The pathogenesis of VMC is involved not only in direct damage to the cardium by pathogens, but also in cell-mediated immunity [2]. It was shown that T cells play a vital role in the pathogenesis of VMC. Particularly, Th1 cells participate in the cardiac inflammatory responses by IFN-γ production, whereas Th17 cells aggravate cardiac damage and promote fibrosis by IL-17 production [3–5]. Recently, increasing attention has been paid to the regulatory mechanisms of T cell responses in the heart.

Regulatory B cells (Bregs) are a recently reported subset of B cells that exert potent regulatory functions different from those of traditional antibody (Ab)-producing B cells [6]. One of the most important Breg subtypes is IL-10-producing B cells [7]. These cells depend on the production of IL-10, a potent regulatory cytokine [8], to exert an effect on other cells, especially T cells, on a cellular level [9–14]. Bregs are confirmed to weaken immune responses and reduce inflammation in a variety of autoimmune diseases and inflammatory diseases [10,11,15,16]. It was previously reported that IL-10-producing B cells in the spleen are elevated in VMC, particularly in the acute phase of the disease [17,18]. However, their precise function in acute VMC remains unclear. Therefore, in this study, we generated a murine model of VMC induced by coxsackie B3 virus (CVB3) to further confirm the regulatory function of IL-10-producing B cells involved in the pathogenesis of acute VMC.

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https://doi.org/10.1016/j.lfs.2019.116838
Received 11 June 2019; Received in revised form 30 August 2019; Accepted 3 September 2019
Available online 04 September 2019

2. Materials and methods

2.1. Animals

Male 4-week-old BALB/c mice were purchased from Hunan Silaikejingda Laboratory Animal Co. Ltd., Hunan, China. Male 4-week-old SCID mice were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd., Beijing, China. All animals were bred in a specific pathogen-free barrier facility at the Laboratory Animal Center of Guangxi Medical University. All animal studies were performed in accordance with the Laboratory Animal Ethics Committee of Guangxi Medical University and the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). CVB3 was kindly supplied by the Institute of Immunology of Guangxi Medical University.

2.2. Animal models

To produce VMC models, both BALB/c and SCID mice were inoculated intraperitoneally (i.p.) with 0.1 mL of phosphate-buffered saline (PBS) containing 5×10^5 PFU of CVB3. Controls received the same dose of PBS. The day of first injection was considered day 0.

To produce a B cell-deficient VMC model, SCID mice were administered 1×10^7 $CD4^+$ T cells by tail vein and were inoculated i.p. with 5×10^7 PFU of CVB3 at the same time. The day of first injection was considered day 0.

2.3. Adoptive transfer experiments

After IL-10-producing B cells (8×10^5) from normal or VMC mice were sorted by magnetic-activated cell sorting, they were administered intravenously through tail vein to BALB/c or SCID mice one day before VMC induction. In some experiments, 8×10^5 IL-10-producing B cells obtained from BALB/c mice on day 7 of VMC were transferred into BALB/c mice on day 3 or day 7 of VMC induction. The control group (no transfer) was administered physiological saline. The survival time of recipient mice was noted for 14 days. Some of them were randomly chosen to be sacrificed on day 14.

2.4. IL-10-producing B cells and $CD4^+$ T cell isolation

IL-10-producing B cells were sorted using the Regulatory B Cell Isolation Kit (No. 130-095-873, Miltenyi Biotec) according to the manufacturer's instructions. In brief, isolation of IL-10-producing B cells was performed in three steps. First, B cells from normal BALB/c mice or from BALB/c mice on day 7 of VMC induction were pre-enriched by depletion of non-B cells. Second, the pre-enriched B cells were stimulated for 5 h in culture with lipopolysaccharide (LPS; *Escherichia coli* serotype 0111:B4, 10 μg/mL; Sigma-Aldrich), phorbol myristate acetate (PMA; 50 ng/mL; Sigma-Aldrich), and ionomycin (500 ng/mL; Sigma-Aldrich) to induce IL-10 secretion. Third, viable IL-10-producing cells were specifically isolated by using the Cytokine Secretion Assay technology. The purity of IL-10-producing B cells was > 90%.

$CD4^+$ T cells were sorted using the EasySep™ Mouse $CD4^+$T Cell Isolation Kit (Catalog #19852, STEMCELL) according to the manufacturer's instructions. In brief, the isolation of $CD4^+$ T cells was performed in five steps. First, a single-cell leukocyte suspension from the spleens of BALB/c mice on day 7 of VMC induction was prepared at the indicated cell concentration within the volume range. Second, Rat Serum and Isolation Cocktail was added to the sample. Third, samples were added to the respective tube, followed by the addition of RapidSpheres™ and incubation. Fourth, the tube was placed into a magnet and incubated. Fifth, the magnet was picked up, the magnet along with the tube was inverted, and the enriched cell suspension was separated into a new tube. The purity of $CD4^+$ T cells was > 95%.

2.5. Single-cell leukocyte suspension preparation

The spleen tissues were removed and dissected aseptically from mice. They were then minced gently and filtered through a 75-μm mesh. The collected cells including red blood cells were lysed, washed with PBS, and suspended in complete medium.

2.6. In vitro stimulation of B cells and T cells

Single-cell leukocyte suspensions were cultured at a density of 1×10^6 cells/mL in complete medium at 37 °C with 5% CO_2 atmosphere for 5 h. Complete medium was supplemented with LPS (10 μg/mL), PMA (50 ng/mL), ionomycin (500 ng/mL), and Brefeldin A (10 μg/mL) to stimulate B cells or was supplemented with PMA (50 ng/mL), ionomycin (500 ng/mL), and Brefeldin A (10 μg/mL) to stimulate T cells.

2.7. Analysis of IL-10-producing B cells, Th1 (IFN-γ^+CD4^+) cells, and Th17 (IL-17^+CD4^+) cells

Briefly, as described in Section 2.5, single-cell leukocyte suspensions were harvested and washed with PBS after stimulation. Mouse anti-CD19 APC-conjugated monoclonal Abs (mAbs; 1D3; eBioscience) or mouse anti-CD4 PerCP-Cyanine 5.5-conjugated mAbs (RM4-5; eBioscience) were incubated with cells to detect the surface antigens for 30 min at 4 °C. Further, cells were fixed and permeabilized with a fixation/permeabilization solution (BD Biosciences) for 20 min at 4 °C. Perm/Wash buffer (BD Biosciences) was used to maintain the cells permeabilized during washing until they were incubated for 30 min at 4 °C with mouse anti-IL-10 PE-conjugated mAbs (JES5-16E3; BD Biosciences) to analyze IL-10-producing B cells, mouse anti-IFN-γ FITC-conjugated mAbs (XMG1.2; eBioscience) for Th1 cells, or mouse anti-IL-17-PE-conjugated mAbs (eBio17B7; eBioscience) for Th17 cells. Isotype mAbs were used as negative control. Finally, cells were washed, kept in 1.5% formaldehyde fixative at 4 °C, and analyzed by a BD FACS Canto II flow cytometry immediately.

2.8. Histological analysis

The heart tissues were quickly removed in containers with 4% paraformaldehyde as soon as the mice were sacrificed. Tissues were processed, embedded in paraffin, cut into in 3-μm-thick sections, and mounted on slides. Sections were then deparaffinized and stained with hematoxylin/eosin (H&E). The pathological score, considered to represent the severity of heart lesions, was calculated by Rezkalla semi quantitative analysis as previously reported [19]. The score was between 0 (no involvement noted) and 4+ (100% involvement), with 1+, 2+, and 3+ representing 25, 50, and 75% involvement of the histological section, respectively.

2.9. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from the non-fibrotic myocardium using TRIzol Reagent (Invitrogen), and then cDNA was synthesized using a cDNA reverse transcription kit (Takara, RR047A) according to the manufacturer's protocol. RT-PCR was performed using SYBR Premix Ex Taq™ II (Takara, RR820A) on the StepOnePlus Real-Time PCR System (Applied Biosystems). Primers for T-bet (Th1 transcription factor) and RORγt (Th17 transcription factor) were designed by Primer Premier 5.0. Values for specific genes were normalized to the β-actin housekeeping control. The primers were as follows: T-bet F: GTTCAACCAG CACCAGACAGAG, R: TGGTCCACCAAGACCACATC; RORγt F: CACAG AGACACCACCGGACAT, R: CGTGCAGGAGTAGGCCACATT; β-actin F: CATCCGTAAAGACCTCTATGCCAAC; R: ATGGAGCCACCGATCCACA.

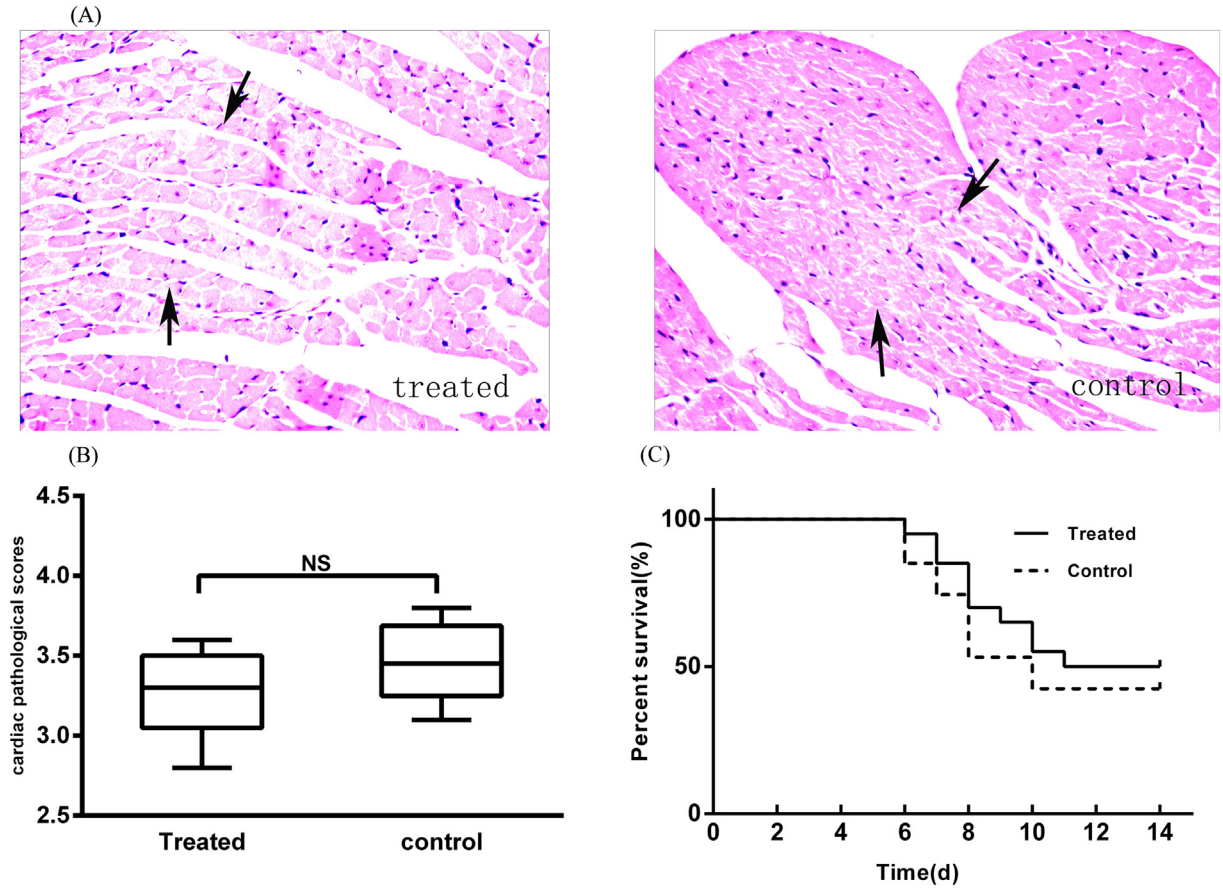

Fig. 1. IL-10-producing B cells have no effect on VMC mice without T cells. IL-10-producing B cells were transferred into SCID mice before inducing VMC (treated group). The control group received a physiological saline injection. (A) Representative pathological changes in heart tissue in SCID mice (H&E, original magnification ×400). Arrows show severe myocardial necrosis with rare inflammatory cell infiltration. (B) Statistical analysis of cardiac pathological scores. There was no difference between the treated group (n = 8) and control group (n = 8) ($P > 0.05$). (C) Survival analysis between treated group (n = 20) and control group (n = 20). The mean survival time was not different between the two groups (P > 0.05). Data are presented as P50 (P25, P75).

2.10. Statistical analysis

Statistical analysis was performed with SPSS 17.0. All data are presented as P50 (P25, P75). Rank sum testing was applied for small sample data or non-normally distributed data. A Kaplan-Meier survival curve was used to estimate the survival rate of mice and the log-rank test was used to assess the difference of the survival rate among groups. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. IL-10-producing B cells have no effect on the pathogenesis of VMC in mice without T cells

To determine whether the IL-10-producing B cells would restore VMC without the regulatory action of T cells, we adoptively transferred IL-10-producing B cells from acute VMC-BALB/c mice into SCID mice that lacked T and B cells before inducing VMC. On day 14 of VMC, H&E staining revealed extensive necrosis of the myocardium with rare inflammatory cells observed in both the treatment group and the control group (Fig. 1A). At the same time, there were no differences between the two groups in cardiac pathological scores (3.3 (3.1, 3.5) versus 3.5 (3.3, 3.7), $P > 0.05$) (Fig. 1B) and mean survival times (8.7 d ± 0.6 d versus 7.9 d ± 0.7 d, P > 0.05) (Fig. 1C). Thus, IL-10-producing B cells needed T cells to exert their effect on the pathogenesis of VMC.

3.2. IL-10-producing B cells regulate inflammatory responses of VMC via Th1 and Th17 cells

As IL-10-producing B cells were shown to be non-functional in the absence of T cells, we explored their effects on VMC in the presence of T cells. To establish a B cell-deficient VMC mouse model, $CD4^+$ T cells were transferred into SCID mice, which were simultaneously induced by CVB3. Purified IL-10-producing B cells from normal BALB/c mice (naïve group) or acute VMC-BALB/c mice (sensitized group) were transferred one day before the induction of VMC. On day 14 of VMC, H&E staining revealed mild attenuation of inflammatory cell infiltration and no change in cardiac myocyte necrosis in the naïve group and significant alleviation of both inflammatory cell infiltration and cardiac myocyte necrosis in the sensitized group compared with the control group (Fig. 2A). Compared with those of the control group, the cardiac pathological scores of the naïve group did not change (1.9 (1.0, 3.0) versus 3.2 (2.1, 3.5), $P > 0.05$), whereas those of the sensitized group decreased significantly (1.3 (0.6, 1.7) versus 3.2 (2.1, 3.5), $P < 0.01$) (Fig. 2B). Likewise, the mean survival time of the naïve group remained unchanged (12.5 d ± 0.7 d versus 11.1 d ± 0.7 d, $P > 0.05$), whereas that of the sensitized group was prolonged (13.4 d ± 0.2 d versus 11.1 d ± 0.7 d, $P < 0.05$) (Fig. 2C). Meanwhile, the spleen Th1 and Th17 cell proportions remained unchanged in the naïve group (4.4% (2.4%, 5.6%) versus 6.0% (4.0%, 7.3%); 1.9% (1.2%, 3.2%) versus 3.1% (1.6%, 4.0%); all P > 0.05), but decreased significantly in the sensitized group (2.3% (2.0%, 3.8%) versus 6.0% (4.0%, 7.3%), $P < 0.01$; 0.9% (0.3%, 2.1%) versus 3.1% (1.6%, 4.0%), $P < 0.05$)

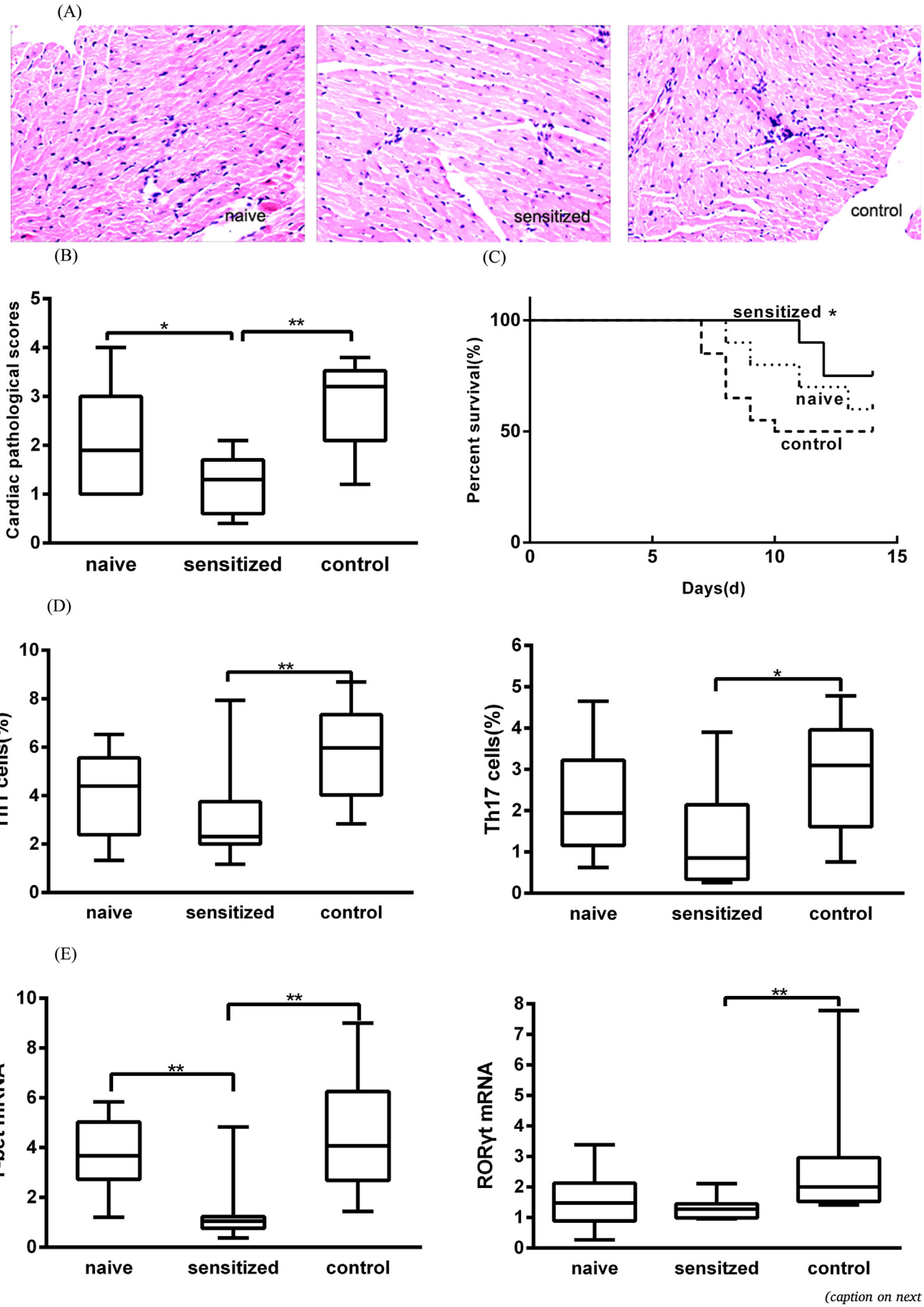

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Fig. 2. IL-10-producing B cells regulate inflammatory responses of VMC via Th1 and Th17 cells. IL-10-producing B cells from normal BALB/c mice (naïve group) or from acute VMC-BALB/c mice (sensitized group) were transferred into B cell-deficient mice one day before the induction of VMC. The control group received a physiological saline injection. (A) Representative myocardial pathological changes in naïve group, sensitized group, and control group (H&E, original magnification ×400). (B) Statistical analysis of cardiac pathological scores in the naïve group (n = 10), sensitized group (n = 10), and control group (n = 10). (D) Spleen Th1 and Th17 cell proportions among the three groups. (E) mRNA levels of Th1-specific transcription factor T-bet and Th17-specific transcription factor RORγt in myocardium among the three groups. (C) Survival analysis among the naïve group (n = 20), sensitized group (n = 20), and control group (n = 20). *P < 0.05, versus sensitized group and control group.*P < 0.05, *P < 0.01. Data are presented as P50 (P25, P75).

(Fig. 2D). The mRNA levels of T-bet (the transcription factor of Th1 cells) and RORγt (the transcription factor of Th17 cells) in the myocardium of naïve group showed no change (3.6 (2.7, 5.0) versus 4.1 (2.7, 6.3); 1.5 (0.9, 2.1) versus 2.0 (1.5, 3.0), all P > 0.05), whereas those of the sensitized group decreased (1.0 (0.8, 1.2) versus 4.1 (2.7, 6.3), P < 0.01; 1.3 (1.0, 1.4) versus 2.0 (1.5, 3.0), P < 0.01) (Fig. 2E). Thus, sensitized IL-10-producing B cells inhibited VMC responses in a Th1 and Th17 cell-dependent manner in B cell-deficient VMC mice, while naïve IL-10-producing B cells did not.

3.3. Adoptive transfer of IL-10-producing B cells before VMC induction normalizes inflammatory responses in wild-type (WT) mice

As IL-10-producing B cells had a regulatory function in B cell-deficient mice, we further confirmed whether they had the same action in WT mice before VMC induction. IL-10-producing B cells purified from VMC-sensitized mice were adoptively transferred into BALB/c mice one day before CVB3 induction. H&E staining revealed remarkable attenuation of inflammatory cell infiltration and cardiac myocyte necrosis of the myocardium in the treated group compared with the control group (Fig. 3A). Cardiac pathological scores of the treated group were lower than those of the control group (1.9 (1.8, 2.5) versus 3.0 (2.4, 3.5), P < 0.01) (Fig. 3B). Adoptive transfer of IL-10-producing B cells before VMC prolonged the mean survival time (13.8 d ± 0.1 d versus 11.8 d ± 0.6 d, P < 0.05) (Fig. 3C). The spleen Th1 and Th17 cell proportions decreased significantly (4.1% (2.9%, 6.2%) versus 9.4% (7.0%, 10.4%); 3.5% (3.2%, 3.8%) versus 5.1% (4.4%, 6.1%), all P < 0.01) (Fig. 3D). These results showed that adoption of IL-10-producing B cells into WT-VMC mice can attenuate inflammatory responses and cardiac damage by regulating Th1 and Th17 cells.

3.4. Transfer of IL-10-producing B cells on day 3 of VMC yields protection against myocardial damage, but transfer on day 7 of VMC does not

Next, we studied the effect of adoption of IL-10-producing B cells at different time points of VMC on myocardial damage and prognosis. IL-10-producing B cells from VMC-sensitized mice were adoptively transferred into BALB/c mice on day 3 or day 7 of VMC. H&E staining revealed obvious attenuation of inflammatory cell infiltration and cardiac myocyte necrosis in the myocardium following transfer of IL-10-producing B cells on day 3 of VMC (Fig. 4A). Accordingly, the cardiac pathological scores of this group were lower than those of the control group (1.5 (1.1, 2.3) versus 2.9 (2.4, 3,7), P < 0.01) (Fig. 4B). However, the mean survival time of the treated group was not longer than that of the control group (13.0 d ± 0.4 d versus 11.8 d ± 0.6 d, P > 0.05) (Fig. 4C). The treated group also showed significantly lower spleen Th1 and Th17 cell proportions than those of the control (4.7% (3.7%, 7.6%) versus 7.9% (6.5%, 10.3%); 3.3% (2.8%, 3.8%) versus 5.3% (4.0%, 5.9%), all P < 0.01) (Fig. 4D). In contrast, H&E staining revealed no difference in inflammatory cell infiltration and cardiac myocyte necrosis between the treated group and the control group following the transfer of IL-10-producing B cells on day 7 of VMC (Fig. 5A). Similar results were obtained for the cardiac pathological scores (2.3 (1.9, 3.1) versus 3.0 (2.6, 3.6), P > 0.05) (Fig. 5B) and the spleen Th1 and Th17 cell proportions (4.2% (2.9%, 6.7%) versus 6.4% (4.5%, 8.0%); 3.9% (3.6%, 4.4%) versus 4.6% (3.8%, 5.7%), all P > 0.05) (Fig. 5D). Moreover, the mean survival time of the treated group was not longer than that of the control group (12.6 d ± 0.5 d versus 12.2 d ± 0.6 d, P > 0.05) (Fig. 5C). Thus, while adoption of IL-10-producing B cells on day 3 of VMC attenuated the inflammatory responses and cardiac damage, adoption on day 7 of VMC did not have any effect in WT-VMC mice.

4. Discussion

In this study, we demonstrated for the first time that IL-10-producing B cells played a regulatory role in VMC in a T cell-dependent manner. SCID mice were used to build an acute VMC model induced by CVB3, as previous studies reported [20]. We found that severe necrosis was diffused, whereas inflammatory cells were rare in the myocardium of VMC-SCID mice due to the deficiency of adaptive immunity, which was in line with previous reports [20,21]. As a consequence, the adoptive transfer of IL-10-producing B cells could not alleviate cardiac damage nor prolong survival time. This suggested that IL-10-producing B cells had no effect on VMC in the absence of T cells. In other words, the regulation of IL-10-producing B cells is T cell-dependent, though in the presence of innate immunity. Several studies have demonstrated that IL-10-producing B cells are potent regulators of T cell-dependent autoimmune diseases and antigen-specific inflammation; in a *Listeria* model, the adoptive transfer of spleen B10 cells, a subset of IL-10-producing B cells, from MHC-II$^{-/-}$ mice into CD19$^{-/-}$ mice before infection could not alleviate the symptoms of the disease nor induce the activation and proliferation of CD4^{+} T cells to clear *Listeria* as efficiently as B10 cells from WT mice. This suggested that the effect of B10 cells on *Listeria* requires cognate interactions with CD4^{+} T cells [16]. Similarly, in a multiple sclerosis model, it is indispensable that B10 cells interact with T cells before B10 maturation to IL-10-producing B cells to functionally control the systemic immune responses under IL-21-IL-21R and CD40-CD40L conjugates. Our study supported these findings as well [10]. In summary, the adoption of IL-10-producing B cells could not alleviate cardiac lesions without the involvement of T cells in VMC.

We then determined the regulatory role of IL-10-producing B cells on T cells in VMC. According to previous reports, there are other subsets of regulatory B cells that negatively regulate autoimmunity besides IL-10-producing B cells, such as TGF-β-producing B cells [22], TIM^{+} B cells [23], and IL-35-producing B cells [24,25]. Thus, to avoid confusion between these regulatory B cells, we generated a B cell-deficient model by introducing CD4^{+} T cells into SCID mice before the induction of VMC. Remarkably, IL-10-producing B cells from VMC-sensitized mice caused a reduction in inflammatory foci and attenuation of cardiac lesions. As a consequence, the survival times of the adoption mice were longer than those of the B cell-deficient VMC mice. At the same time, the proportions of spleen Th1 and Th17 cells were reduced in the myocardium. In contrast, IL-10-producing B cells from normal mice did not reduce the cardiac lesions, although the infiltration of inflammatory cells seemed mildly reduced. Consequently, survival time was not prolonged in these mice. Hence, we demonstrated that immune adoption of sensitized IL-10-producing B cells could alleviate cardiac lesions and prolong survival time by downregulating the mRNA expression level of transcription factors T-bet and RORγt, leading to lower Th1 and Th17 cell proportions, whereas adoption of IL-10-producing B cells from normal mice could not. Regulatory B cells, mainly IL-10-producing B cells, functionally regulate both pathogenic Th1 cells and Th17 cells at the cellular level during autoimmunity and inflammation. In vitro, in

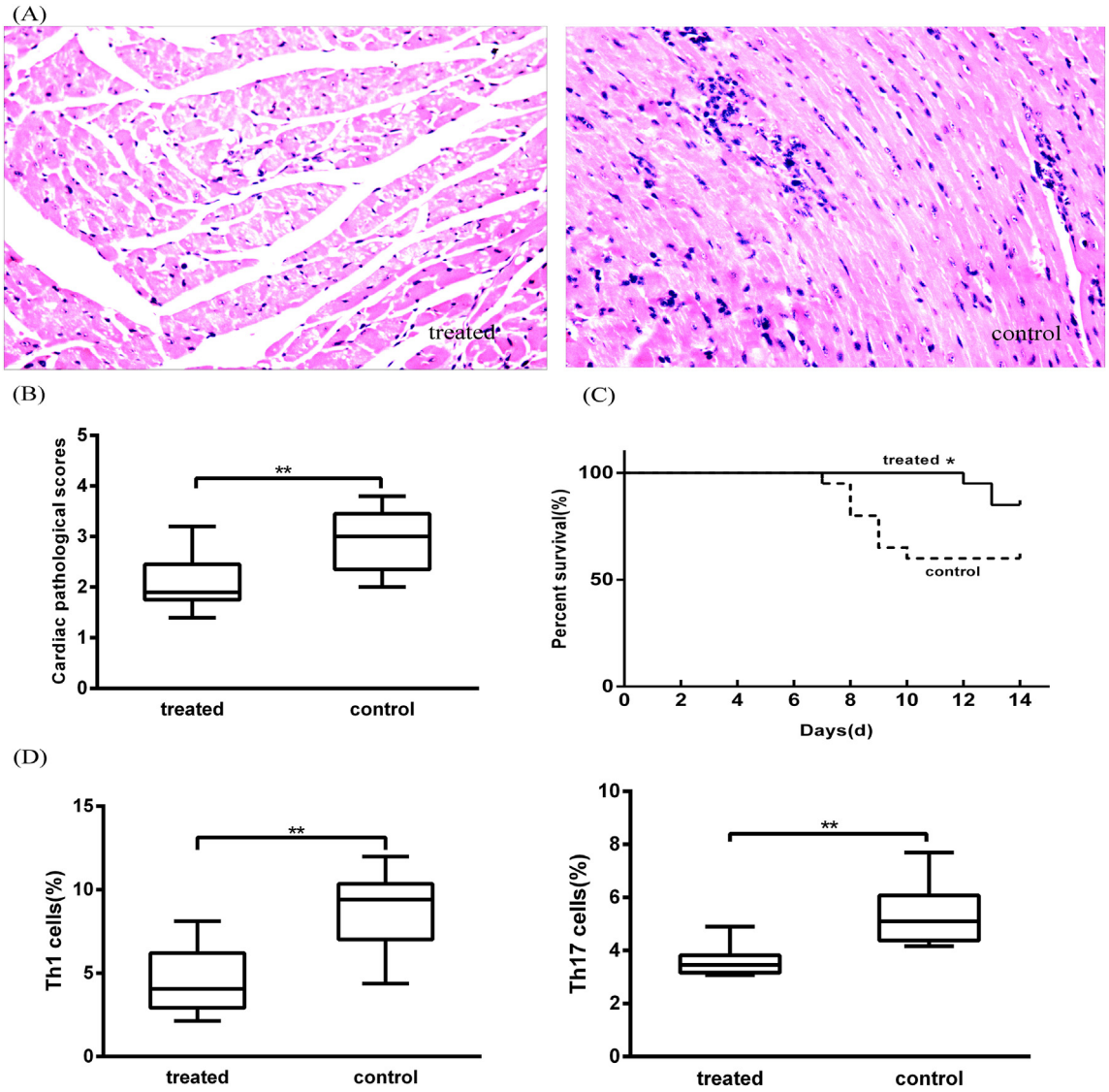

Fig. 3. Adoptive transfer of IL-10-producing B cells before VMC induction normalized inflammatory responses in WT mice. IL-10-producing B cells from acute VMC-BALB/c mice (treated group) were transferred into WT mice one day before the induction of VMC. The control group received a physiological saline injection. (A) Representative myocardial pathological changes in treated group and control group of WT-VMC mice (H&E, original magnification ×400). (B) The statistical analysis of cardiac pathological scores in the treated group (n = 10) and control group (n = 10). (D) Spleen Th1 and Th17 cell proportions in treated group and control group. (C) Survival analysis between treated group (n = 20) and control group (n = 20). *P < 0.05, *P < 0.01. Data are presented as P50 (P25, P75).

co-culture, sensitized B10 cells purified from the recovery phase of experimental autoimmune encephalomyelitis (EAE) not only directly reduce inflammatory products of $CD4^+$ T cells, but also act as antigen presenting cells (APCs) to indirectly control $CD4^+$ T cell proliferation by modulating dendritic cells [9]. In vivo, chimeric mice, specifically those with a lack of endogenous IL-10-producing B cells ($IL\text{-}10^{-/-}$ B cells), presented with more severe collagen-induced arthritis (CIA), with inflammatory Th1 and Th17 cells dramatically increased. However, transfer of sensitized T2-MZP regulatory B cells from CIA into B cell-deficient arthritic mice restored the proportions of spleen Th1 and Th17 cells and controlled the inflammatory responses [26]. Similarly, in an EAE model, B cell-deficient mice presented with EAE exacerbation due to the failure of IL-10 production and inhibition of T cell activation [27]. Indeed, our findings in this study support the above-discussed reports, suggesting that IL-10-producing B cells limit cardiac lesions in VMC mice by downregulating transcription factor T-bet and RORγt to decrease Th1 and Th17 cell proportions.

The regulation of naïve IL-10-producing B cells is discrepant between different disease models. Yanaba et al. reported that immune adoption of oxazolone sensitized $CD1d^{hi}CD5^+$ B cells (B10 cells) normalized inflammation 48 h before oxazolone induction of contact hypersensitivity, but non-$CD1d^{hi}CD5^+$ B cells or naïve $CD1d^{hi}CD5^+$ B cells did not [28]. In contrast, Yoshizaki et al. showed that transfer of naïve $CD1d^{hi}CD5^+$ B cells purified from normal mice to $CD19^{-/-}$ mice 1 day before immunization with MOG35-55 decreased $TCR^{MOG}CD4^+$ T cell proliferation, whereas sensitized $CD1d^{hi}CD5^+$ B cells presented a more powerful regulatory role towards T cells [10]. Similarly, Amu et al. suggested that $CD1d^{hi}$ regulatory B cells from *Schistosoma mansoni* infection or non-infection both prevented the occurrence of acute allergic airway inflammation (AAI) [29]. Thus, naïve IL-10-producing B cells exhibit a mild regulatory function in some studies, but not in others. What we have found suggests that the immune transfer of naïve IL-10-producing B cells could not regulate T-bet or RORγt mRNA levels, nor the proportions of Th1 and Th17 cells. Therefore, there is no effect on cardiac lesions and survival time in the B cell-deficient VMC model. The reason for the different regulatory capability of these cells needs to be elucidated in the future.

BALB/c mice share a common background with SCID mice. They

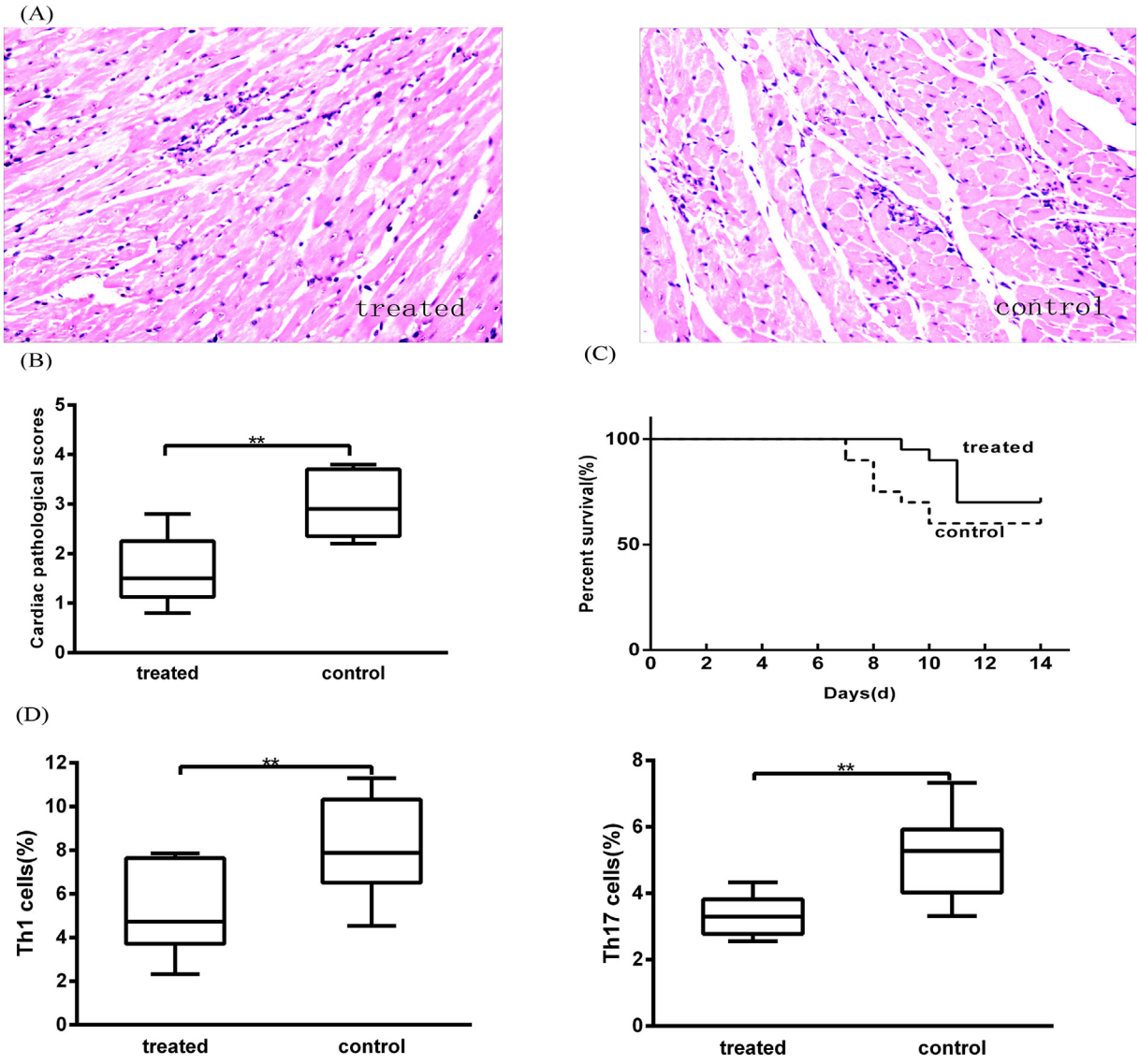

Fig. 4. Adoption of IL-10-producing B cells on day 3 of VMC reduces cardiac inflammation in VMC mice. IL-10-producing B cells from acute VMC-BALB/c mice (treated group) were transferred into WT mice on day 3 of VMC. (A) Representative myocardial pathological changes in treated group and control group of VMC mice (H&E, original magnification ×400). (B) The statistical analysis of cardiac pathological scores in the treated group (n = 10) and control group (n = 10). (D) Spleen Th1 and Th17 cells in treated group and control group. **P < 0.01. Data are presented as P50 (P25, P75). (C) Survival analysis between treated group (n = 20) and control group (n = 20). No statistical significance was found between the two groups (P > 0.05).

have normal innate and adaptive immunity. We next used BALB/c mice to build an acute VMC model that mimics human VMC more closely than the VMC-SCID mouse model. We confirmed that IL-10-producing B cells could attenuate cardiac lesions and prevent the onset of VMC. At the same time, the proportions of spleen Th1 and Th17 cells were remarkably decreased in VMC mice, suggesting that IL-10-producing B cells could downregulate the proportion of Th1 and Th17 cells to prevent VMC. To investigate the capacity of regulatory B cells, most studies utilize chimeric mice, mice with specific mAb deletion, or knock-out mice as experimental subjects, to avoid confusion with other subsets of regulatory B cells. But few studies have focused on control animals with normal immunity as subjects to induce disease and confirm the role of IL-10-producing B cells. This kind of model would more closely imitate the pathogenesis of the disease. Yoshizaki et al. showed that the transfer of activated $CD5^+$ cells to WT mice 1 day before disease induction significantly delayed onset of EAE symptoms. The result was the same as the transfer of sensitized $CD1d^{hi}CD5^+$ B to EAE B cell-deficient mice ($CD19^{-/-}$ mice or mice depleted of B cells by specific CD20 mAb) [10]. In our study, the regulatory capacity of IL-10-producing B cells in WT-VMC mice was no less than that in VMC-B cell-deficient mice, which supported the report above.

We further confirmed the negative regulatory ability of IL-10-producing B cells after the induction of VMC. While regulatory B cells show a potent function before the induction of many diseases, a few studies have also addressed their roles during the disease. The results were varied: in some studies, IL-10-producing B cells presented the same impressive regulation as before disease onset, yet in other studies showed no effect on the disease. Matsushita et al. argued that unlike the transfer of B10 cells to B cell-deficient mice, which eased EAE symptoms before the disease onset, the transfer of B10 cells on day 14 of EAE did not restrain the progression and severity of EAE. This suggested that B10 cells mainly control EAE initiation and come into full play in an early stage, while they do not participate in the progression stage [30]. Likewise, the other study carried out by Matsushita et al. also showed that in WT mice, the transfer of B10 cells remarkably prevented EAE just before the disease onset, rather than on day 7 or day 14 of EAE. Yanaba et al. also showed similar results in imiquimod-induced psoriasis-like skin inflammation [9]. However, Yoshizaki et al.

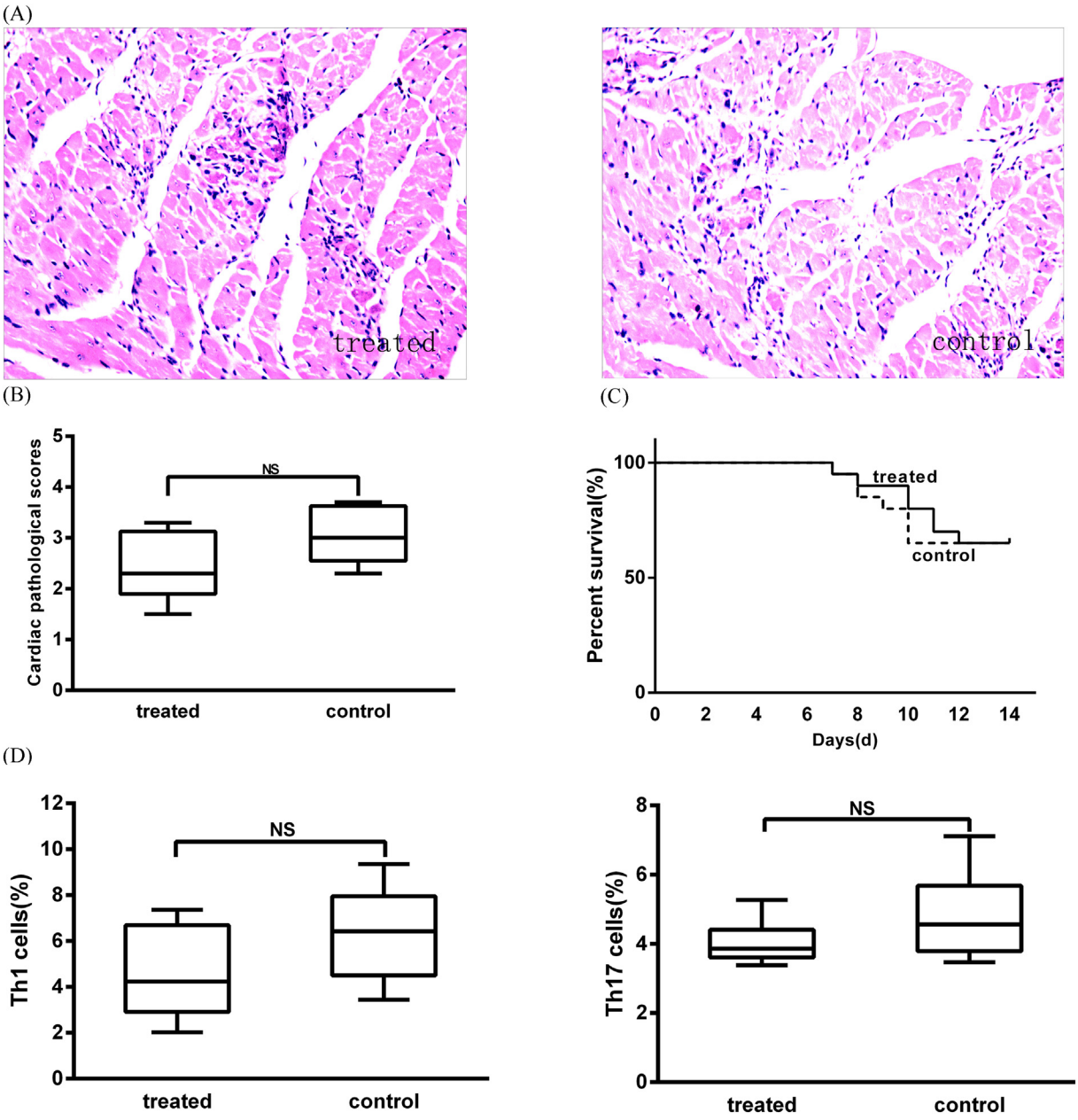

Fig. 5. Adoption of IL-10-producing B cells on day 7 of VMC does not affect cardiac inflammation in VMC mice. IL-10-producing B cells from acute VMC-BALB/c mice (treated group) were transferred into WT mice on day 7 of VMC. (A) Representative myocardial pathological changes in treated group and control group of VMC mice (H&E, original magnification ×400). (B) The statistical analysis of cardiac pathological scores in the treated group (n = 10) and control group (n = 10). (D) Spleen Th1 and Th17 cells in the treated group and control group. Data are presented as P50 (P25, P75). (C) Survival analysis between treated group (n = 20) and control group (n = 20). No statistical significance was found between the two groups ($P > 0.05$).

demonstrated that $CD5^+$ B cells sensitized ex vivo and transferred into WT mice before EAE induction or on day 7, 14, or 21 of the disease surprisingly presented potent regulation of EAE [10]. Our study found that the transfer of IL-10-producing B cells on day 3 of VMC could decrease the Th1 and Th17 cell proportions and alleviate cardiac lesions to some degree, but the regulatory function is too weak to restrain disease progression and prolong survival. Meanwhile, on day 7 of VMC when inflammation is severe and close to its peak, it is too late to adopt IL-10-producing B cells, as the cells cannot regulate Th1 and Th17 cells to alleviate inflammation, attenuate cardiac damage, or prolong the survival time. The reason for the difference in the regulatory function of IL-10-producing B cells during different stages of disease requires elucidation in the future.

5. Conclusion

We demonstrated in this work, for the first time, that IL-10-producing B cells may regulate Th1 and Th17 cells to alleviate inflammation, attenuate cardiac damage, and prolong the survival time in VMC mice, offering a promising target for the clinical treatment of VMC.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by grants from the National Natural

Science Foundation of China (no. 81450055).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116838.

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