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Original Article

IL-10-producing B cells involved in the pathogenesis of Coxsackie virus B3-induced acute viral myocarditis

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Abstract: Background: Interleukin-10 (IL-10)-producing B cells, a subset of regulatory B cells, play critical roles in autoimmune and infectious diseases. However, the role of IL-10-producing B cells in acute viral myocarditis (AVMC) remains unknown. Methods: BALB/c mice were intraperitoneally (i. p.) infected with coxsackievirus B3 (CVB3) to establish AVMC models (AVMC group), while control mice (control group) were treated with phosphate-buffered saline (PBS) i. p. According to the time after injection, the AVMC group mice or control group mice were randomly separated into 1 week and 2 week subgroup. Myocardial histopathological changes were observed by hematoxylin and eosin staining and the frequency of splenic IL-10-producing B cells was measured by flow cytometry. Results: Histopathologic examination of heart tissues showed that mice infected with CVB3 developed AVMC. Compared with control group, the frequency of splenic IL-10-producing B cells was increased significantly in the AVMC group, with the 1 week AVMC subgroup (3.58 ± 0.47%) higher than the 2 week AVMC subgroup (2.50 ± 0.42%) (all $P < 0.05$). Conclusions: IL-10-producing B cells are increased in CVB3-induced AVMC, indicating that IL-10-producing B cells may play an important role in the pathogenesis of CVB3-induced AVMC.

Keywords: IL-10-producing B cells, regulatory B cells, viral myocarditis

Introduction

Myocarditis is an inflammatory disease of the myocardium. Approximately 30% of cases, biopsy-proven myocarditis, can progress to dilated cardiomyoparthy, a terminal condition leaving heart transplantation as the only treatment [1]. Viruses are the infectious pathogens that most frequently implicated in acute myocarditis. Coxsakievirus B3 (CVB3), a member of the positive-stranded RNA virus family Picornaviridae, is one of the major viruses identified to cause acute viral myocarditis (AVMC) [2]. Importantly, the availability of murine model of myocarditis has facilitated much of our understanding of the disorder. A growing number of studies suggest that not only can viral proliferation in cardiac myocytes cause direct tissue injury, but also most tissue damage in myocarditis results from the T cell-mediated inflammation and autoimmune responses, triggered by virus [3, 4]. Besides Th1 and Th22 cells, our previous reports have demonstrated that Th17 cells also involve in the pathogenesis of CVB3-induced AVMC [5]. Nevertheless, the fundamental mechanisms responsible for AVMC are not completely clarified.

B cells have been traditionally considered to play roles during the immune responses, such as antibody production, antigen presentation and T cells activation. In contrast to those conventional B cells, IL-10-producing B cells, a novel subset of regulatory B cells, have been recently functionally identified in mice and humans [6-8]. The immunoregulatory function of these rare IL-10-producing B cells is mediated by anti-inflammatory cytokine IL-10, and thus distinguish them from other regulatory B cell subsets [9, 10]. Mechanisms of IL-10-producing B cells development and function have not been fully elucidated yet, but various factors, including B-cell antigen receptor (BCR)-related signals, toll-like receptor (TLR) signals as well as CD40 ligation, major histocompatibility complex class II (MHC-II) and IL-21, are shown to be involved [8, 11]. Though there is no known cell surface or transcription factor signa-

ture unique to IL-10-producing B cells and, currently, the only way to identify these specific subset of regulatory B cells is functionally by intracellular IL-10 staining [9]. Developing literature has demonstrated that IL-10-producing B cells could suppress T-cell-mediated inflammation in autoimmune and viral diseases, including experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), systemic lupus erythematosus (SLE) and human immunodeficiency virus (HIV) infection, chronic hepatitis B virus (CHB) infection [12-15]. Thus, it seems IL-10-producing B cells provide unique immune regulatory functions to modulate tissue inflammation and immune responses.

The immunomodulatory roles of IL-10-producing B cells have been studied in numerous autoimmune and infectious diseases, but little is known in CVB3-induced AVMC. Our present study was therefore designed to examine the change of IL-10-producing B cells in CVB3-induced AVMC, and explore whether or not IL-10-producing B cells involved in the pathogenesis of CVB3-induced AVMC.

Materials and methods

Mice

Specific pathogen-free male BALB/c mice (4 weeks old) were purchased from Guangdong Medical Laboratory Animal Centre, Foshan, China (Certificate No. SCXK (Yue) 2008-0002). All animals were housed under standard conditions in the experimental animal center (Experimental Animal Center of Guangxi Medical University, Nanning, China), and the animal handling and experimental procedures were implemented in accordance with protocols approved by Guangxi Medical University Animal Ethics Committee.

CVB3 virus

A Nancy variant of CVB3, which was bountifully provided by the Institute of Immunology of Guangxi Medical University, was propagated in Hep-2 cell monolayers, and then stored at -80°C. The supernatant from infected cell cultures was collected, and viral titers were determined in 96-well plates by the end-point dilution method. Briefly, tenfold serial dilutions (1:10 to $1:10^{-10}$) of PBS (Solarbio Science & Technology, Beijing, China) were prepared, and the 50% tissue culture infectious dose ($TCID_{50}$) titer was determined by the cytopathic effects visible after 72 h. The $TCID_{50}$ value for Hep-2 cells was 1×10^{-7}.

Induction of AVMC

A total of 30 male BALB/c mice were included in the study. CVB3 was diluted in PBS, BALB/c mice infected by an i. p. injection of 0.1 ml of PBS containing approximately 100 $TCID_{50}$ of the CVB3 were taken as AVMC group (n = 20) and according to the time point post injection, the AVMC group mice were separated into 2 subgroups: 1 week (1 w) AVMC subgroup (n = 10) and 2 week (2 w) AVMC subgroup (n = 10); Mice administered i. p. with 0.1 ml PBS were taken as control group (n = 10) and according to the time point post injection, the control group mice were separated into 2 subgroups as well: 1 week (1 w) control subgroup (n = 5) and 2 week (2 w) control subgroup (n = 5). The day when mice were injected i. p. was defined as 0 week, all surviving animals were sacrificed at 1 week and 2 weeks after i. p. injection, and spleens as well as myocardial tissues were aseptically collected.

Histopathology

Transverse midsections of hearts were fixed in 10% phosphate-buffered formalin, embedded in paraffin, cut into 5 μm sections, and processed for H & E (hematoxylin and eosin) staining to determine the level of inflammation. Stained sections were observed by using a Nikon Eclipse E800 light microscopy (Nikon, Kawasaki, Japan).

Flow cytometry

Spleens from surviving mice were harvested aseptically. Single cell suspensions of splenic cells were prepared by dispersing the organs through nylon mesh and lysed red cells with Lysing buffer. For intracellular cytokine IL-10 staining, lymphocytes (2×10^6 cells/ml) were resuspended in RPMI 1640 medium (Gibco, USA) with 10% fetal bovine serum (FBS, Gibco, USA), stimulated with ultrapure lipopolysaccharide (LPS, 10 μg/ml, Sigma-Aldrich, USA), phorbol myristate acetate (PMA, 50 ng/ml, Sigma-Aldrich) and ionomycin (1 μg/ml, Sigma-Aldrich) in the presence of GolgiPlug (1 μl/10^6 cells, BD Biosciences) at 37°C, 5% CO_2 of a 24-well culture plate. After 5 hours, the cells were surface-stained with PE-Cy7-anti-CD19 (eBioscience,

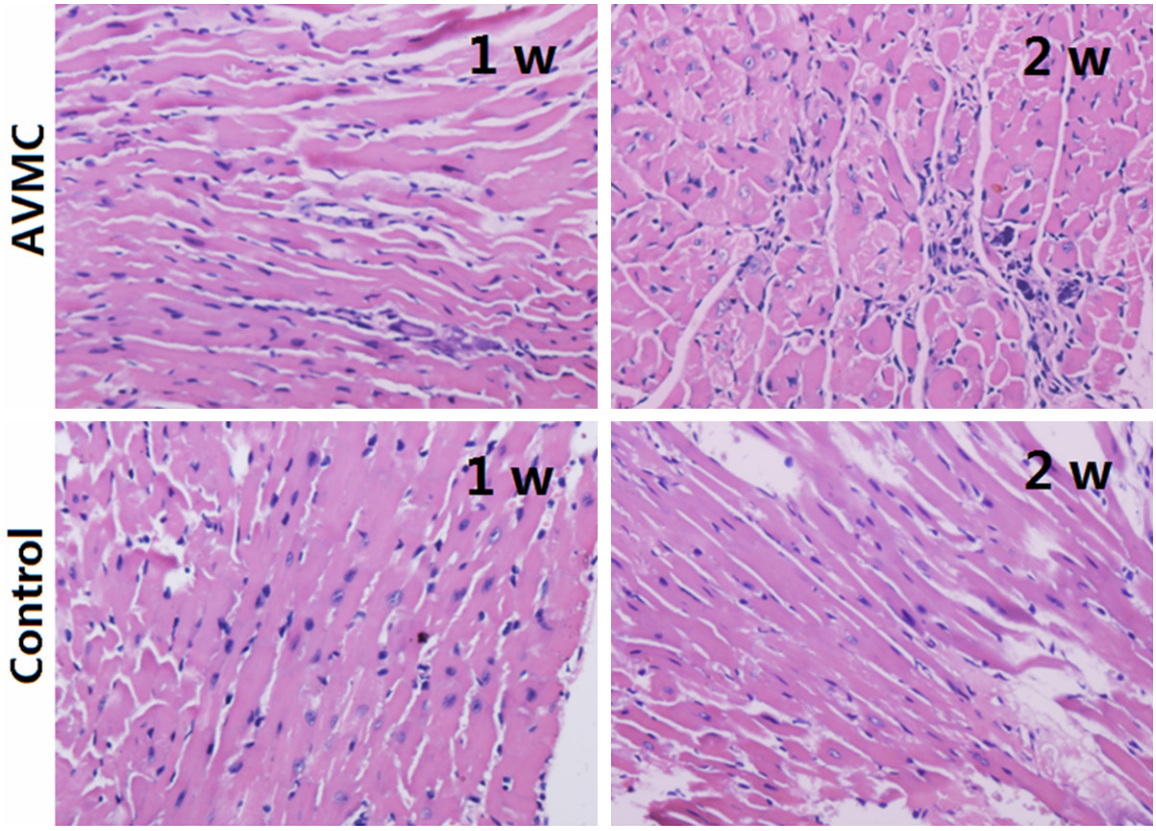

Figure 1. Histopathologic changes of heart tissue in each groups. Representative images of inflammation in heart tissue (H & E, original magnification × 400).

USA), fixed and permeabilized with Cytofix/Cytoperm kit (BD Bioscience) according to the manufacturer's instructions. After washing, then intracellular cytokine was stained with anti-IL10 mAb conjugated with APC (eBioscience, USA), APC-anti-IgG2 (eBioscience, USA) acted as the isotype control. Detected using a FACS-Calibur flow cytometer (BD Bioscience). FlowJo (v7.6.1) software was used for data acquisition.

Statistical analysis

Data were expressed as mean ± standard derivation (SD). Statistical analyses of data were performed with Student's t-test. All data were analyzed with SPSS 16.0. A value of $P < 0.05$ was considered to be statistically significant.

Results

Severity of AVMC

Mice in AVMC group developed myocarditis one week after CVB3-infection, and the symptoms of AVMC were apparent, such as weakness, coat ruffling, weight loss, lethargy and even death. In contrast, the mice in control group kept in good healthy throughout the study. There were 9 and 8 mice remaining alive in 1 w AVMC subgroup and 2 w AVMC subgroup, respectively. No mice died in control group. The typical histopathologic changes of AVMC, such as massive inflammatory cellular infiltration were seen in the AVMC group mice, but there were no changes in the control group mice. (**Figure 1**).

Increased frequency of IL-10-producing B cells in AVMC

To explore whether IL-10-producing B cells were present in each groups mice, the frequency of $CD19^+IL\text{-}10^+$ B cells in splenic $CD19^+$B cells was measured by flow cytometry (**Figure 2A**, **2B**). The results showed that the frequency of $CD19^+IL\text{-}10^+$ B cells in AVMC group was significantly increased than those in control group at different time points (all $P < 0.01$). In addition, compared with those in 2 w AVMC subgroup, the frequency of $CD19^+IL\text{-}10^+$ B cells was higher in 1 w AVMC subgroup ($P < 0.01$). The frequen-

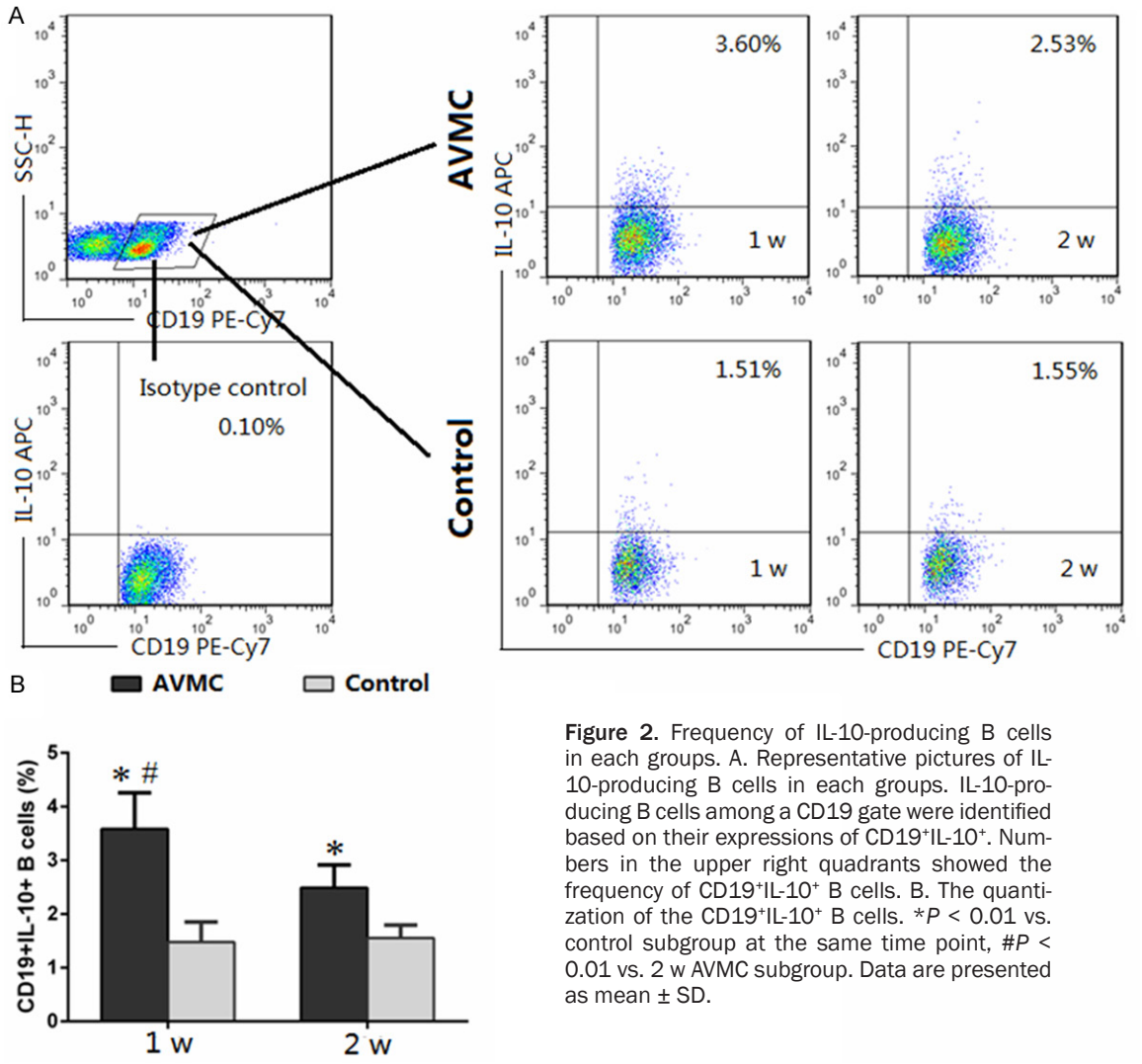

Figure 2. Frequency of IL-10-producing B cells in each groups. A. Representative pictures of IL-10-producing B cells in each groups. IL-10-producing B cells among a CD19 gate were identified based on their expressions of $CD19^{+}IL\text{-}10^{+}$. Numbers in the upper right quadrants showed the frequency of $CD19^{+}IL\text{-}10^{+}$ B cells. B. The quantization of the $CD19^{+}IL\text{-}10^{+}$ B cells. $*P < 0.01$ vs. control subgroup at the same time point, $\#P < 0.01$ vs. 2 w AVMC subgroup. Data are presented as mean ± SD.

cy of $CD19^{+}IL\text{-}10^{+}$ B cells in 1 w AVMC subgroup and 2 w AVMC subgroup was 3.58 ± 0.47%, 2.50 ± 0.42% respectively, and in 1 w control subgroup and 2 w control subgroup was 1.48 ± 0.37%, 1.55 ± 0.24% respectively.

Discussion

IL-10-producing B cells, as a novel subset of regulatory B cells, are recently identified with capacity of regulating immune responses, playing an important role in maintaining immune homeostasis [6, 7, 10]. However, there are no reports regarding the frequency of IL-10-producing B cells in the progression of CVB3-induced AVMC. In current study, we firstly observed that the frequency of IL-10-producing splenic B cells was dramatically increased in CVB3-induced AVMC mice. Our findings indicated that IL-10-producing B cells may play an important role in the pathogenesis of AVMC.

Recent researches have evidenced that the IL-10-producing B cells are increased and regulate immune responses in viral infection and T-cell-mediated autoimmune diseases, such as HIV infection, CHB infection, SLE, EAE and rheumatoid arthritis [6, 12, 14-16]. IL-10-producing B cell frequency and number were increased during the course of EAE, further more, transferring of wild-type $CD1d^{hi}CD5^{+}B$ cells, which contain IL-10-producing B cells, into $CD19^{-/-}$ mice before EAE induction significantly reduced EAE severity, while the adoptive transfer of $CD1d^{low}CD5^{-}B$ cells did not affect EAE severity [12]. These findings reveal that the loss of IL-10-producing B cells may exacerbate inflammation. Here, we firstly showed that the

frequency of IL-10-producing splenic B cells was significantly elevated in CVB3-induced AVMC mice in both 1 w and 2 w subgroup. In addition, we found that the frequency of IL-10-producing B cells in 1 w AVMC subgroup was higher than that in 2 w AVMC subgroup. Thus, our results suggest that the dynamic changes of IL-10-producing B cells may participate in the pathogenesis of CVB3-induced AVMC. However, a recent study reported that the levels of IL-10-secreting B cells were elevated in dilated cardialmyopathy (DCM) patients, but not significantly higher than those in healthy controls [17]. The virus infection condition as well as stage of the disease may contribute to the differences, and whether or not the IL-10-producing B cells participate in the progression of viral myocarditis into DMC warrants further investigation.

Yanaba et al. have demonstrated that BCR and TLR signals involved in the development of IL-10-producing B cells [7, 8]. In addition, a recent Nature published study showed that supply of CD40L (CD154) and IL-21, activating CD40 and IL-21 receptor signals respectively, not only can drive IL-10-producing B cells development and expansion ex vivo, but also generate these cells secreting IL-10 that dramatically suppress disease when transferred into EAE mice [11]. A similar result was found in lupus-prone MRL/lpr mice, referred to that IL-21 induced IL-10-producing B cells to secrete IL-10 via activation of phosphorylated signal transduction and activator of transcription 3 (p-STAT3) [16]. Therefore, there are several possible mechanisms for the elevation of IL-10-producing B cells in AVMC: Firstly, it has been shown that the frequency of IL-10-producing B cells is correlated with virus infection [14, 15]. In addition, virus infectivity and viral RNA in the myocardium and blood collected from CVB3-infected mice at the first week were higher than that at the second week [18], which results were parallel with our findings that the frequency of IL-10-producing B cells was greater in 1 w AVMC subgroup than that in 2 w AVMC subgroup. So we speculate that CVB3 and/or CVB3-derived products may promote the differentiation of IL-10-producing B cells. Secondly, it has been demonstrated that the T cells, particularly $CD4^+$ T cells, participate in the course of AVMC [3-5]. The activated $CD4^+$ T cells, including Th17 cells, express CD40L (CD154) and secrete IL-21, which are potent stimulators of IL-10-producing B cells maturation and proliferation. Of course, micro-environmental cytokines or other various complex regulation factors should not be excluded in the development of IL-10-producing B cells during CVB3-induced AVMC. Further study is need.

In conclusion, the results of this study shown for the first time that the IL-10-producing-B cells are significantly increased in CVB3-induced AVMC, indicating that IL-10-producing B cells may play an important role in the pathogenesis of CVB3-induced AVMC mice. However, the differentiation and function of IL-10-producing B cells, especially in human systems, remain to be further investigated in AVMC.

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Disclosure of conflict of interest

None.

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