

Original Article

The different levels of IL-10-producing B cells, paralleling Th1 cells, between chronic myocarditis in cardiomyopathy induced by coxsackie virus B3

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Received December 22, 2015; Accepted March 20, 2016; Epub April 1, 2016; Published April 15, 2016

Abstract: Background: IL-10-producing B cells, mostly within CD1d^{hi}CD5⁺B cells, have recently been identified playing a regulatory role in autoimmunity. They mainly suppress CD4⁺ T cells to affect autoimmune diseases. However, whether they are involved in the pathogenesis of late phase of myocarditis and cardiomyopathy remain unclear. Methods: Male BALB/c mice were intraperitoneally infected coxsackievirus B3 (CVB3) to establish viral myocarditis (VMC) and cardiomyopathy (DCM) models. Frequencies of spleen IL-10-producing B cells, CD1d^{hi}CD5⁺B cells and Th1 cells (IFN- γ CD4⁺ T cells) were examined by flow cytometry on 1 week, 1, 3, 6 month post injection. Numbers of immune cell infiltrations loci in heart tissue were evaluated as severity of cardiac inflammation. Results: IL-10-producing B cells were increased in chronic VMC, whereas were not in DCM. They paralleled Th1 cells, presenting expansion in acute VMC and a descent tendency in chronic VMC and DCM, whereas did not parallel with severity of cardiac inflammation. CD1d^{hi}CD5⁺B cells also expanded mainly in acute VMC and early stage of chronic VMC, which were similar to IL-10-producing B cells. Conclusions: These results suggest IL-10-producing B cells, paralleling Th1 cells, possibly have an important role mainly in acute VMC, even in chronic VMC.

Keywords: IL-10-producing B cells, Th1 cells, viral myocarditis, dilated cardiomyopathy

Introduction

Viral myocarditis (VMC) is a potentially fatal inflammatory heart disease that frequently progresses to dilated cardiomyopathy (DCM) [1, 2]. The main causes of cardiac injury in VMC are involved in the immune-related responses, which include a number of immunocompetent cells [3-7]. It is generally agreed that B cells participate in immune responses by secreting antigen-specific antibodies [8]. However, a small subset of B cells which has negative regulatory activities recently attract our more concentrations.

IL-10-producing B cells, a subset of regulatory B cells, are recently found to have an important role in autoimmunity [9-12] and in inflammation [13-17]. They nevertheless present low frequencies of B cells following 5 h of in vitro stimulation with different stimulus groups [11, 18, 19]. IL-10-producing B cells produce IL-10, a

potent regulatory cytokine, to control CD4⁺ T cells function and downregulate proinflammatory cytokines, such as IFN- γ [20-22] and IL-17 [23-25]. In contrast, deletion or deficiency of IL-10-producing B cells result in a disbalance of Th1/Th17 and exacerbate diseases [23, 24]. CD1d^{hi}CD5⁺B cells are a unique phenotypic subset of IL-10-producing B cells, which enrich 15-20% of IL-10-producing B cells. Adoptive transfers of spleen CD1d^{high}CD5⁺B cells also ameliorate inflammation in B cell-depleted mice model [18, 26].

We previously reported that IL-10-producing B cells were elevated both in acute VMC in mice model [27] and in primary dilated cardiomyopathy in human [28]. Further, in this study we paid our attention on the role of IL-10-producing B cells in chronic myocarditis and dilated cardiomyopathy induced by coxsackie B3 virus (CVB3) and the correlation of IL-10-producing B cells and Th1 cells (IFN- γ CD4⁺ T cells) in mice.

IL-10-producing B cells in chronic myocarditis, paralleling Th1 cells

Materials and methods

Animal model

All animal studies were performed in accordance with the Guangxi Medical University Ethics Committee. Male 4-wk-old BALB/c mice were purchased from laboratory animal center of Foshan, Guangdong Province, China and were bred in a specific pathogen-free barrier facility at the Laboratory Animal Center of the Guangxi Medical University. CVB3 were kindly provided by the Institute of Immunology of Guangxi Medical University. To induce VMC and DCM, male BALB/c mice were inoculated intraperitoneally (i.p.) with 0.1 ml of phosphate-buffered saline (PBS) containing 5×10^5 plaque-forming units of CVB3 per month until the fifth month. Controls received the same dose of PBS. BALB/c mice were randomly divided into 8 groups, and each group were included treated subgroup (VMC group or DCM group, $n=10$) and control subgroup ($n=10$). The day of first injection was as week 0. Mice were sacrificed on 1 week (acute VMC), 1 month (early stage of chronic VMC), 3 month (late stage of chronic VMC) or on 6 month (DCM phase) respectively post i.p. injection.

Histology

Heart tissues were quickly removed in container with 4% paraformaldehyde as soon as mice were sacrificed. Tissues were processed, blocked in paraffin wax, cut into in 5- μ m-thick sections and mounted on slides. Sections then were deparaffined and stained with hematoxylin/eosin (H&E). The severity of heart inflammation was graded as numbers of immune cell infiltrations loci by two independent investigators. Every immune cell infiltrations loci contained at least 20 immune cells.

Cell preparation

Spleen tissues were removed and dissected in a gentle way from mice. Single-cell leukocyte suspensions from spleens tissues were prepared, lysed red blood cells, washed with PBS, and then were cultured at 1×10^6 cells/ml in complete medium at 37°C with 5% CO₂ atmosphere for 5 h. Complete medium was added with PMA (100 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich), LPS (10 μ g/ml; Sigma-Aldrich), and Brefeldin A (10 μ g/ml;

Sigma-Aldrich) to stimulate B cells, or was added with PMA (50 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich), and Brefeldin A (10 μ g/ml; Sigma-Aldrich) to stimulate T cells.

IL-10-producing B cells and Th1 (IFN- γ ⁺CD4⁺ T cells) cells analysis

Briefly, as described previously [18], single-cell leukocyte suspensions were harvested and washed with PBS after stimulation. Before staining, Fc receptors were blocked with Fc receptor mAbs (2.4G2; BD Biosciences). Mouse anti-CD19 APC-conjugated 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 minutes at 4°C. Cells, then were fixed and permeabilized with a fixation/permeabilization solution (BD Biosciences) for 20 minutes at 4°C. After that, Perm/Wash buffer (BD Biosciences) was used to maintain cells permeabilized during washing until cells were incubated for 30 minutes at 4°C with mouse anti-IL-10 PE-conjugated mAbs (JES5-16E3; BD Biosciences) to analyze IL-10-producing B cells, or mouse anti-IFN- γ FITC-conjugated mAbs (XMG1.2; eBioscience) for Th1 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 as soon as possible. For CD1d^{hi}CD5⁺B cells analysis, also as described above, briefly, Mouse anti-CD19 APC-conjugated mAbs (1D3; eBioscience), mouse anti-CD5 PerCP-Cyanine5.5 mAbs (53-7.3; eBioscience) and mouse anti-CD1d PE-conjugated (1B1; eBioscience) were incubated with cells for 30 minutes at 4°C, then were washed and analyzed by flow cytometry as soon as possible.

Statistical analysis

Statistical analysis was performed by SPSS Statistics17.0. Data were presented as mean \pm SD. Student t test was applied in two groups' comparison and one-way ANOVA followed by the LSD significant difference test were applied in multiple groups' comparison. Analysis of covariance was applied in two linear regression equations. $P < 0.05$ was considered to be statistically significant.

IL-10-producing B cells in chronic myocarditis, paralleling Th1 cells

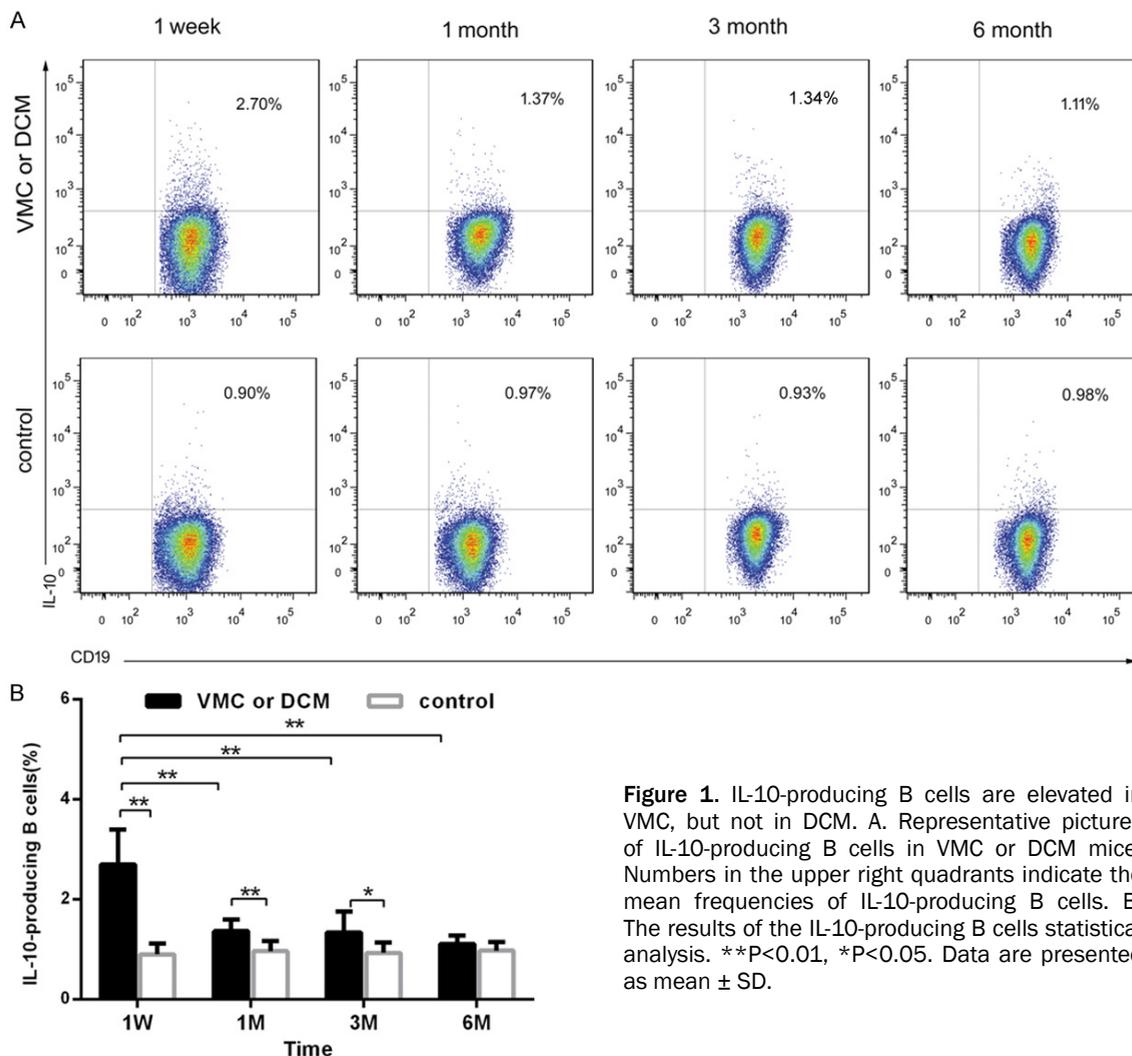


Figure 1. IL-10-producing B cells are elevated in VMC, but not in DCM. A. Representative pictures of IL-10-producing B cells in VMC or DCM mice. Numbers in the upper right quadrants indicate the mean frequencies of IL-10-producing B cells. B. The results of the IL-10-producing B cells statistical analysis. ** $P < 0.01$, * $P < 0.05$. Data are presented as mean \pm SD.

Results

IL-10-producing B cells were elevated on 1 week, 1 month and 3 month after CVB3 infection

As IL-10-producing B cells are significantly increased in autoimmune or inflammatory mice, IL-10-producing B cells were observed whether they expanded in the course of CVB3-induced VMC and DCM as well. Murine IL-10-producing B cells frequencies were measured by flow cytometry after stimulation in vitro and stained for cytoplasmic IL-10 expression (Figure 1A). After CVB3 inflammation, IL-10-producing B cells were significantly elevated on 1 week, 1 month and 3 month ($2.70\% \pm 0.69\%$ versus $0.90\% \pm 0.22\%$, $1.37\% \pm 0.23\%$ versus $0.97\% \pm 0.21\%$, $1.34\% \pm 0.42\%$ versus

$0.93\% \pm 0.21\%$, respectively, $P < 0.05$) during VMC, however they were not found increased on 6 month ($1.11\% \pm 0.17\%$ versus $0.98\% \pm 0.17\%$, $P = 0.09$) in the time of DCM in contrast to control mice (Figures 1, 2). During the period of VMC, IL-10-producing B cells significantly expanded on 1 week, in comparison with that on 1 month and 3 month, which were just slightly increased ($P < 0.01$) (Figure 1B).

Th1 cells paralleled IL-10-producing B cells

As mice IL-10-producing B cells functionally regulate T cells differentiation in autoimmune diseases, Th1 cells here were also observed in CVB3-induced VMC and DCM. After CVB3 inflammation, spleen Th1 cells were stained with cytoplasmic IFN- γ antibodies and quantified the frequencies in vitro (Figure 2A). Similar

IL-10-producing B cells in chronic myocarditis, paralleling Th1 cells

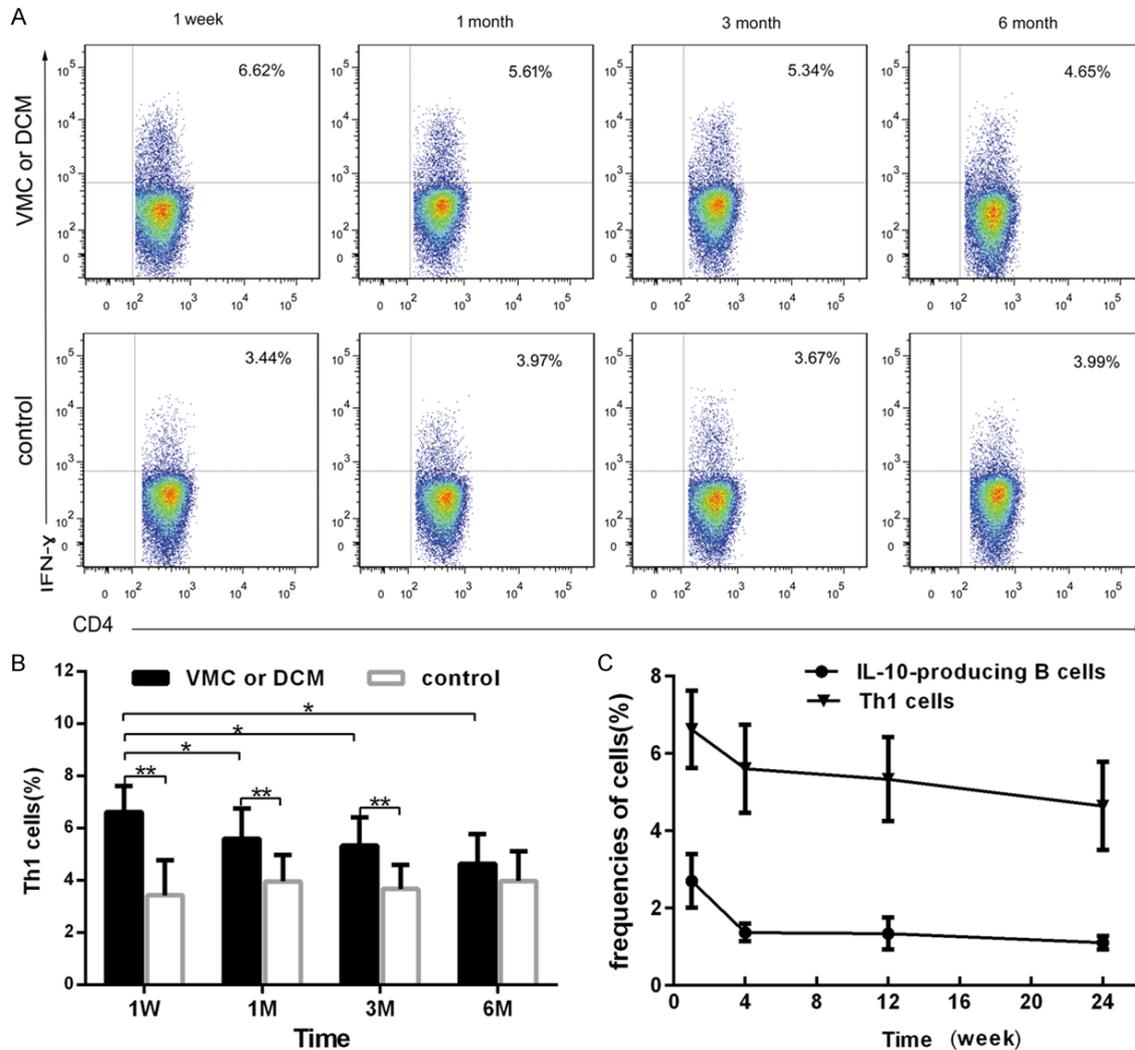


Figure 2. Th1 cells, paralleling IL-10-producing B cells, are elevated in VMC, but not in DCM. **A.** Representative pictures of Th1 cells in VMC or DCM mice. Numbers in the upper right quadrants indicate the mean frequencies of Th1 cells. **B.** The results of the Th1 cells statistical analysis. **C.** Th1 cells parallel IL-10-producing B cells. No statistic significance is shown between the slopes of the two lines. ** $P < 0.01$, * $P < 0.05$. Data are presented as mean \pm SD.

to IL-10-producing B cells, Th1 cells were significantly increased on 1 week, 1 month and 3 months ($6.62\% \pm 1.00\%$ versus $3.44\% \pm 1.34\%$, $5.61\% \pm 1.14\%$ versus $3.97\% \pm 1.01\%$, $5.34\% \pm 1.09\%$ versus $3.67\% \pm 0.93\%$, respectively, all $P < 0.01$) in VMC, but were not in DCM ($4.65\% \pm 1.14\%$ versus $3.99\% \pm 1.14\%$, $P > 0.05$) in contrast to control mice (**Figure 2B**). During VMC, Th1 cells paralleled IL-10-producing B cells, which both had a change of mono-peak curve presenting the maximum of frequencies on 1 week and a descent on 1 month and on 3 months (6.62% versus 5.61% , 6.62% versus 5.34% , all $P < 0.05$). No statistic significance

was shown between these two frequencies curves in the whole phase of VMC and DCM ($P > 0.05$) (**Figure 2C**).

CD1d^{hi}CD5⁺B cells were increased on 1 week and on 1 month, similar to IL-10-producing B cells

As the spleen CD1d^{hi}CD5⁺B cells, one of the IL-10-producing B cells subsets, acquired the ability to function like IL-10-producing B cells in other autoimmunity and inflammation, they were at the same time stained with the surface antibodies and were identified whether they expanded during VMC and DCM (**Figure 3A**).

IL-10-producing B cells in chronic myocarditis, paralleling Th1 cells

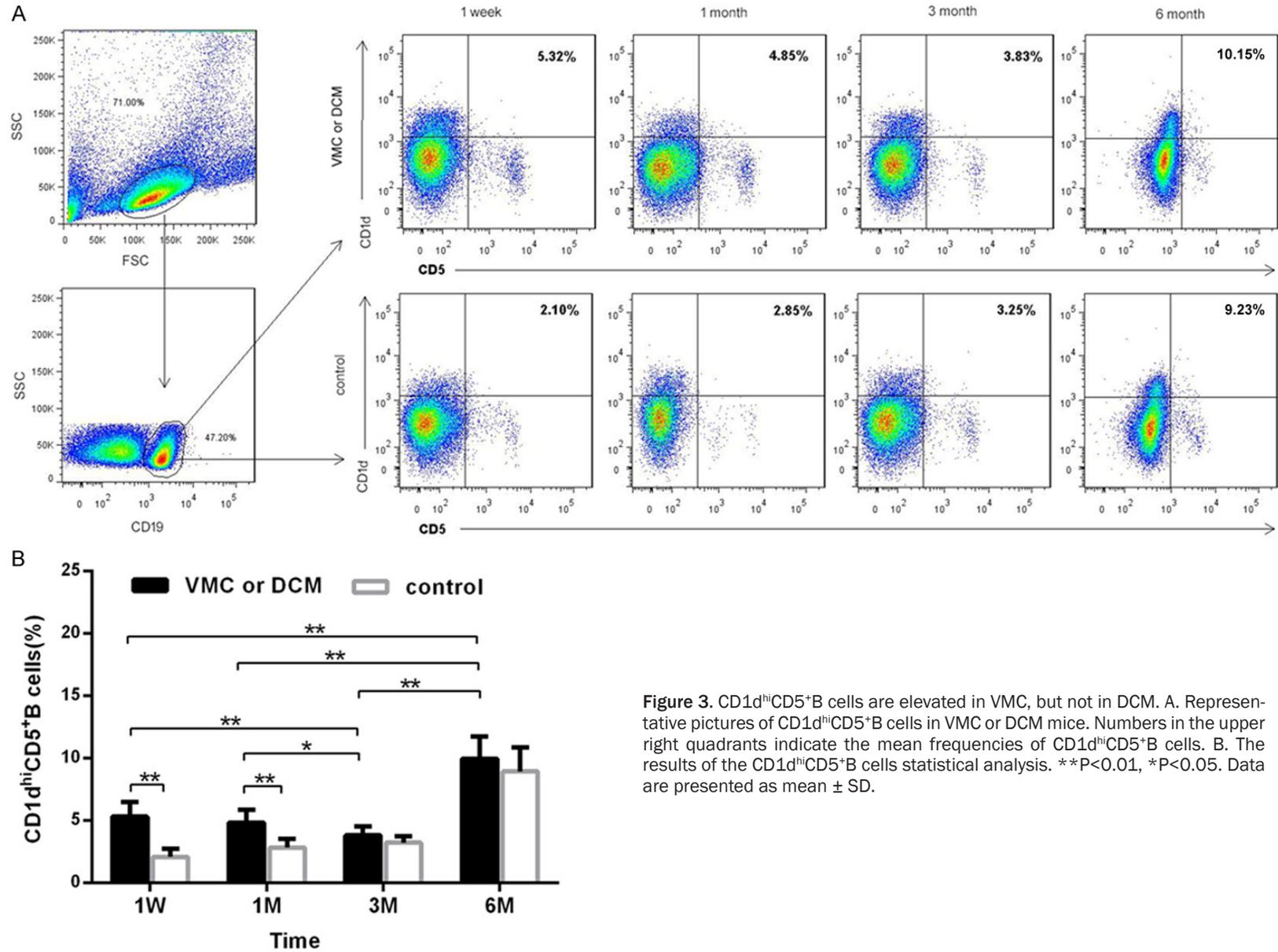


Figure 3. CD1d^{hi}CD5⁺B cells are elevated in VMC, but not in DCM. A. Representative pictures of CD1d^{hi}CD5⁺B cells in VMC or DCM mice. Numbers in the upper right quadrant indicate the mean frequencies of CD1d^{hi}CD5⁺B cells. B. The results of the CD1d^{hi}CD5⁺B cells statistical analysis. **P<0.01, *P<0.05. Data are presented as mean ± SD.

IL-10-producing B cells in chronic myocarditis, paralleling Th1 cells

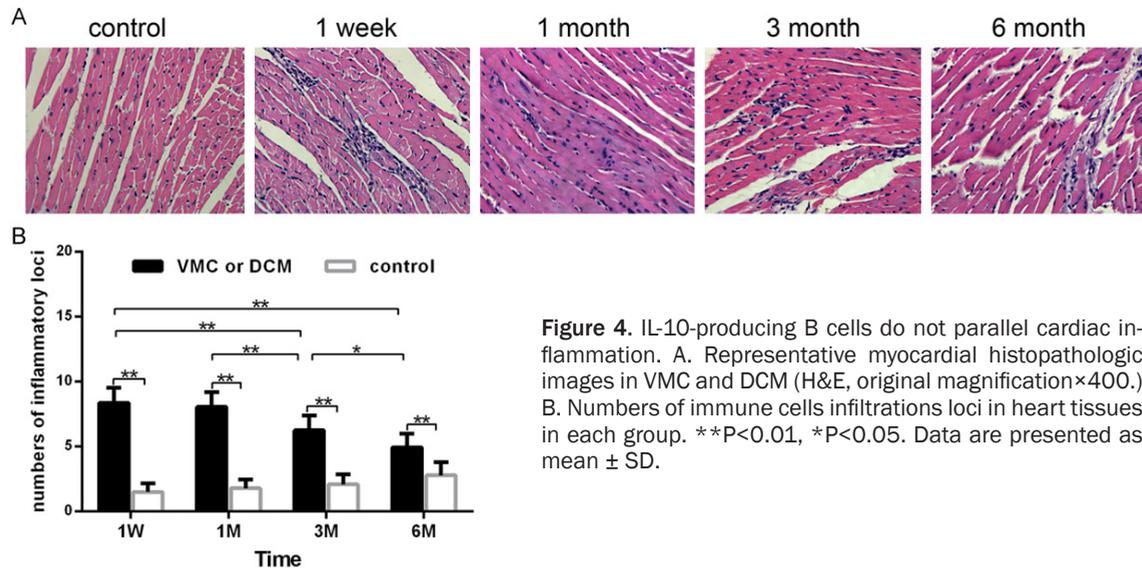


Figure 4. IL-10-producing B cells do not parallel cardiac inflammation. A. Representative myocardial histopathologic images in VMC and DCM (H&E, original magnification×400.) B. Numbers of immune cells infiltrations loci in heart tissues in each group. **P<0.01, *P<0.05. Data are presented as mean ± SD.

CD1^{hi}CD5⁺B cells frequencies were 2.5- and 1.7-fold higher than in control group on 1 week (5.32%±1.20% versus 2.10%±0.66%, P<0.01) and on 1 month (4.85%±1.02% versus 2.85%±0.69%, P<0.01), respectively. However they were not found increased on 3 month (3.83%±0.70% versus 3.25%±0.52%, P=0.05). During VMC, CD1^{hi}CD5⁺B cells on 1 week were higher than on 3 month (5.32% versus 3.83%, P<0.01), presenting a steady declining tendency. In addition CD1^{hi}CD5⁺B cells on 6 month were not elevated in contrast to control mice (10.15%±2.00% versus 9.23%±2.14%, P>0.05) in the time of DCM, even though they were doubled that of VMC (**Figure 3B**).

IL-10-producing B cells did not parallel cardiac inflammation

As quantitative differences in IL-10-producing B cells influence inflammation in autoimmune disease, the severity of heart inflammation then was evaluated. The numbers of immune cell infiltrations loci in heart tissues were counted as grade of heart inflammation (**Figure 4A**). During the whole VMC and DCM after CVB3 induced, the numbers of inflammatory loci were all significantly increased on 1 week, 1 month, 3 month and 6 month (8.35±1.18 versus 1.50±0.67, 8.05±1.17 versus 1.80±0.68, 6.25±1.14 versus 2.10±0.77 and 4.95±1.07 versus 2.80±1.00, respectively, all P<0.01) when compared with control mice. Meanwhile, the severity of heart inflammation maintained a

high level from 1 week to 1 month, nevertheless significantly decreased on 3 month (8.35 versus 6.25, 8.05 versus 6.25, respectively, P<0.01) and 6 month (8.35 versus 4.95, 8.05 versus 4.95, respectively, all P<0.01) (**Figure 4B**).

Discussion

In this study, we have shown for the first time that IL-10-producing B cells were increased in chronic VMC, whereas they were not in DCM. Meanwhile we have observed that IL-10-producing B cells expanded in acute VMC by two times, but remarkably decreased and maintained at a low level during VMC. It is reported that IL-10-producing B cells predominantly controlled disease initiation and the number of endogenous regulatory B cells directly influenced autoimmune disease pathogenesis [20]. Thus what we were found here suggest that IL-10-producing B cells possibly have an important role mainly in acute VMC, but mild in chronic VMC and DCM. Furthermore, it is verified that lacking or deletion of IL-10-producing B cells aggravated disease, while immune adoption of IL-10-producing B cells alleviate disease [9, 23]. Combining with our findings here, we will find out next whether IL-10-producing B cells functionally contribute to regulate acute VMC, even affect chronic VMC in the future. In contrast to previous report that IL-10-producing B cells were elevated in DCM patients [28], our data showed that little differ-

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ence of IL-10-producing B cells was observed in DCM. This difference might relate to the object of study: we used a murine model of DCM, whereas previous report studied of DCM patients.

Our data have shown spleen Th1 cells were similar to IL-10-producing B cells, elevated in acute VMC and in chronic VMC, whereas were not in DCM comparing to control mice. Well paralleling the changes of IL-10-producing B cells in different stages, Th1 cells were predominantly high in acute VMC, then presented a declining trend during VMC and DCM. It is confirmed that *in vitro*, IL-10-producing B cells are potent to inhibit Th1 cells activation and reduce IFN- γ production of Th1 cells [21]. *In vivo*, deficiencies of IL-10-producing B cells lead to an increased Th1 frequencies, IFN- γ production as well and exacerbate immune disease, whereas adoptive transfer of IL-10-producing B cells could reset misbalance of Th1 and ameliorate disease [20-22]. Thus, taking into account of our data and those mentioned above, we suggest that elevating IL-10-producing B cells likely endeavor to normalize Th1 cells and functionally regulate VMC, but do not affect DCM.

CD1d^{hi}CD5⁺B cells, a phenotypically unique subset in spleen, predominantly contain IL-10-producing B cells and appear to only produce IL-10 [18]. In this study, we found that CD1d^{hi}CD5⁺B cells were significantly increased in acute VMC and mildly in early stage of chronic VMC, while were no changes in late stage of chronic VMC and DCM. Meanwhile CD1d^{hi}CD5⁺B cells were shown a steady descent during the whole VMC, which was similar to IL-10-producing B cells. Previous reports have confirmed that adoptive transferring CD1d^{hi}CD5⁺B cells into B cell deficient mice could ameliorate immune diseases. By contrast, transferring CD1d^{lo}CD5⁺B cells could not affect disease severity [18, 21, 26]. Taking together what we had shown in this study, we suggest that CD1d^{hi}CD5⁺B cells likely have a potent regulatory ability when adoptively transferred into CVB3-induced mice recipient in acute VMC, even in early stage of chronic VMC. We will confirm about this further in the future. Although the frequencies of CD1d^{hi}CD5⁺B cells in DCM doubled that of VMC, they yet did not have statistic significance in contrast to control mice. By contrast, CD1d^{hi}CD5⁺B cells are found increased in peripheral blood of

DCM patients [28]. The discrepancy between the two results may also due to the objective for study, which is similar to IL-10-producing B cells.

After CVB3 induced, the numbers of inflammatory loci, as severity of heart inflammation, were significantly increased when compared with control mice in the whole VMC and DCM. They maintained a high level from acute VMC to early stage of chronic VMC, just then slightly decreased in late stage of chronic VMC and DCM. These findings do not parallel IL-10-producing B cells. We suggest there are two possible explanation of the discrepancy between cardiac inflammations and IL-10-producing B cells. For one thing, not only IL-10-producing B cells, but also a large numbers of other immune cells are involved in inflammatory responses. Each type of cells plays an important role on heart inflammation in different stage of VMC and DCM [7]. For another thing, previous reports have confirmed that IL-10-producing B cells predominantly control immune disease initiation or expand in acute phase of inflammation [20], which is further supported by our findings. Thus, IL-10-producing B cells although functionally regulate cellular immune responses in many inflammatory diseases [13-17], we do not find a direct correlation between IL-10-producing B cells and heart inflammation in CVB3-induced VMC and DCM.

Acknowledgements

This work was supported by grants from the Nature Science Foundation of China (81450-055 to Weifeng Wu).

Disclosure of conflict of interest

None.

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