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SHORT COMMUNICATION

High fat diet downregulates regulatory T cells in the myocardium of spontaneous hypertensive rats

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Abstract *Background and aim:* Regulatory T cells (Tregs) play an important role in cardiovascular complications with the immune response. However, the role of Tregs in high fat diet (HFD)-induced myocardial fibrosis has not been fully elucidated to date. Therefore, we investigated whether HFD suppresses Tregs activation in the myocardium of spontaneously hypertensive rats (SHRs), which aggregates myocardial fibrosis.
Methods and results: Eight-week-old male SHRs were fed to either HFD or control diet (CHO) groups for 12 weeks. We measured Tregs (CD4+FoxP3+) in the heart and mediastinal lymph nodes (LNs). The flow cytometry analysis confirmed that SHR-HFD exhibited a decreased Tregs compared to that of SHR-CHO in the heart and mediastinal LNs. Furthermore, the CD4 and FoxP3 antigens were used in the immunofluorescence microscopy of Tregs in the heart tissues. In the heart, dual staining for the Treg population was increased more in SHR-CHO than it was in SHR-HFD rats. In line with these findings, SHR-HFD significantly exacerbated myocardial fibrosis.
Conclusion: We found that diet-induced obesity typically showed an exacerbated myocardial fibrosis and down-regulation of Tregs pathway in the heart and mediastinal LNs. Therefore, we suggest that the up-regulation of Tregs may be a promising therapeutic approach to preventing obesity induced heart failure.

Introduction

Obesity plays a central role in the development of insulin resistance, type 2 diabetes mellitus, hypertension, and dyslipidemia, which are collectively termed metabolic syndrome [1]. In general, the development of obesity is associated with the cardiovascular disease including left ventricular (LV) hypertrophy, vascular remodeling, and heart failure [2]. Obesity induced myocardial fibrosis may lead to diastolic dysfunction and ultimately cause heart failure [3,4]. Significantly, obesity is also strongly associated with congestive heart failure [5]. Obesity contributes to the development of target organ damage by the interaction of dietary, genetic, epigenetic and environmental factors [2]. The immune and inflammatory response systems play a pivotal role in the pathogenesis of obesity-induced cardiac remodeling [2]. Cardiac fibrosis is the result of chronic inflammation and repair, and many

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immune cells contribute to the process [6]. Regulatory T cells (Tregs), anti-inflammatory T cells, modulate the innate and adaptive immune responses [7]. Cardiac inflammation may play a pivotal role in the pathogenesis of obesity-induced myocardial fibrosis.

Recently, several studies have revealed the relevance of Tregs in the pathophysiology of target organ damage with obesity [2,8]. Tregs modulate Ang II-induced blood pressure elevation, vascular oxidative stress, and endothelial dysfunction through an anti-inflammatory action [9]. Amador et al. also recently reported that the treatment of deoxycorticosterone acetate (DOCA)-salt hypertensive rats with spironolactone increased cluster of differentiation 4 (CD4)+/forkfead box protein P3 (FoxP3)+ cells and induced high levels of FoxP3 mRNA in the heart and kidneys [10].

However, the role of Tregs and its associated anti-inflammation in obesity-induced myocardial fibrosis has not been elucidated to date. Therefore, we investigated whether high fat diet (HFD) suppresses Tregs activation in the myocardium of spontaneously hypertensive rats (SHRs), which aggregates myocardial fibrosis.

Methods

Animals and experimental design

Eight-week-old male SHRs and non-hypertensive Wistar-Kyoto (WKY) control rats were purchased from the Central Animal Laboratory of our institution. The animals were divided into three groups (n = 6 each) and fed to either HFD or control diet (CHO) for 12 weeks.: (1) normal controls, WKY; (2) non-obese controls, SHRs fed a CHO (10% fat, 20% protein, 70% carbohydrate by calories; Orient, Seongnam, Korea; SHR-CHO); (3) SHRs fed an HFD (60% fat, 20% protein, 20% carbohydrate by calories; SHR-HFD). We chose 60% fat diet formula based on the previous studies [11]. All procedures were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee in the School of Medicine, The Catholic University of Korea, Seoul, Republic of Korea. (Approval number: CUMS-2014-0179-01).

Tissue preparation

The perivascular and interstitial areas were observed using a light microscope (ZEISS, Jena, Germany) at × 100 magnification. The collagen volume fraction was quantified as percentage Sirius red-stained fibrosis area per total myocardial tissue area.

Flow cytometry analysis

Fluorescence-conjugated monoclonal antibodies were used to examine the phenotype of the Tregs. Briefly, single cell suspensions were obtained from the heart and mediastinal lymph nodes (LNs), which were perfused through the LV with 10 mL ice-cold PBS. And flow cytometry analysis was done according to the protocol.

Immunofluorescence staining

The frozen cryosections incubated with rabbit anti-rat CD4 antibody (NBP1-19371, Novus Biologicals Inc., Littleton, CO, USA) or mouse anti-rat FoxP3 (N-12clone, Santa Cruz Biotechnology, Santa Cruz, CA, USA). All the sections were subsequently observed using an LSM 510 META laser confocal microscopy (Carl Zeiss, Jena, Germany) and the images were captured using a ZEN 2009 Light software program.

Statistical analysis

The data are expressed as means ± standard deviation (SD). The differences between two or multiple groups were analyzed using the Student *t*-test or analysis of variance (ANOVA) with a Bonferroni post hoc test, where appropriate. If the data were not normally distributed or the sample size in one of the experimental groups was <10, a nonparametric test (Mann-Whitney) followed by the Bonferroni post hoc correction was performed. All comparisons were two-sided and a p-value < 0.05 was considered statistically significant.

Detailed description of procedures is available in Methods in the Supplementary material online.

Results

High fat diet increased myocardial fibrosis in the hearts of SHR

The heart tissue sections from the HFD-fed SHRs were stained using Masson Trichrome staining. The myocardial fibrosis was significantly higher in the SHR-HFD group than it was in the WKY or SHR-CHO group (Fig. 1). In addition, the collagen fibrosis area demonstrated that HFD increased the amount of fibrosis in the perivascular and interstitial heart tissue (Fig. 1).

High fat diet decreased activated FoxP3$^+$ Tregs in the hearts and mediastinal L/Ns of SHR

The flow cytometry analysis confirmed that the administration of HFD reduced the Treg populations in the heart and mediastinal LNs of SHR (Fig. 2A, B). The SHR-HFD group exhibited a decreased Treg activation compared with that of the WKY group (Fig. 2A, B). In contrast, the SHR-CHO exhibited an increased Tregs compared to that of the SHR-HFD in the heart. Furthermore, SHR-CHO slightly increased the Foxp3+ compared with that of the SHR-HFD in the mediastinal LNs (Fig. 2A, B).

The CD4 and FoxP3 antigens were used in the immunofluorescence microscopy of Tregs in the heart tissues of SHR. In the heart, dual staining for the Treg population was increased more in the SHR-CHO rats than it was in the SHR-CHO rats (Fig. 2C).

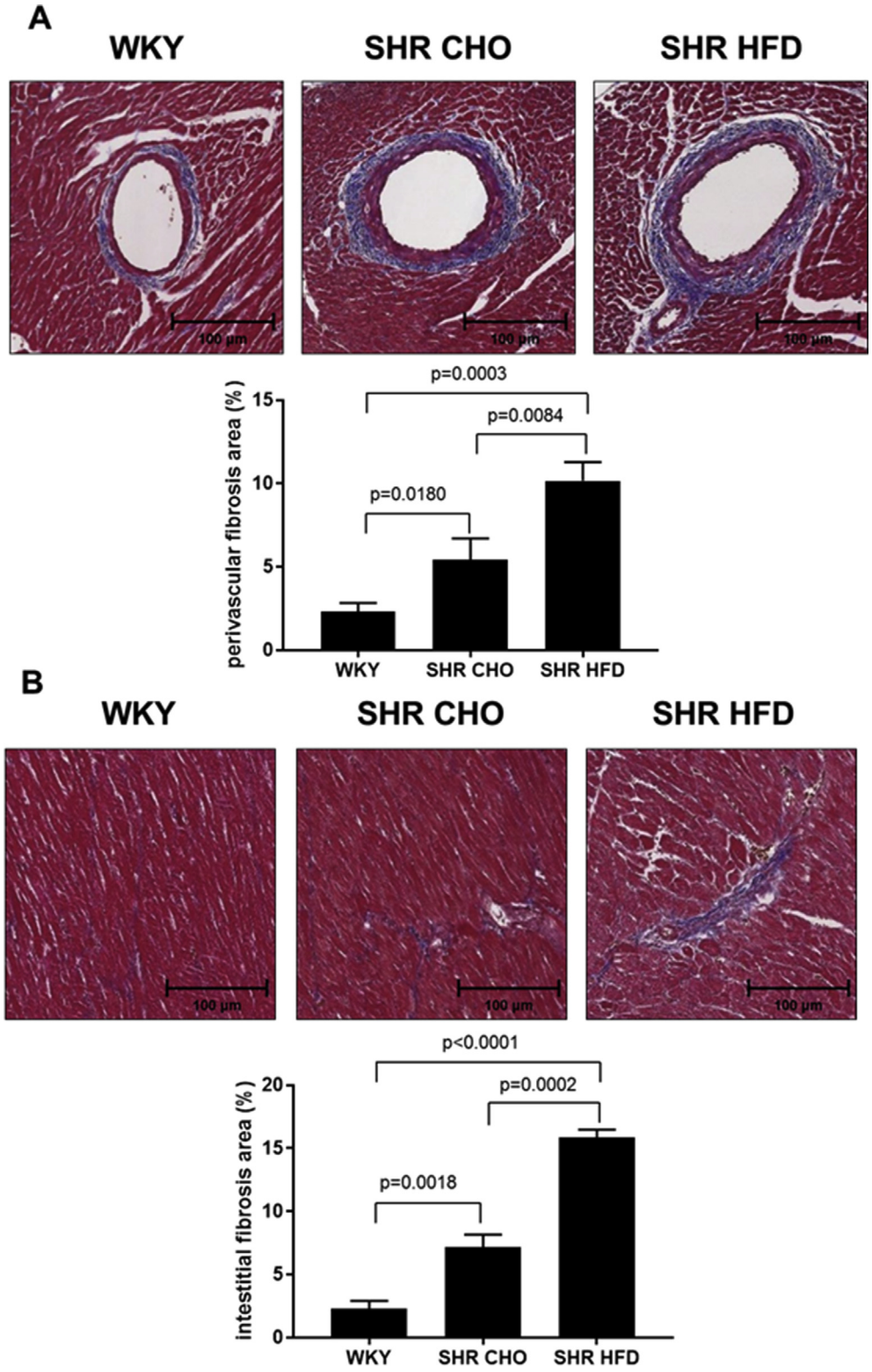

Figure 1 Fibrosis of perivascular area and interstitial area was increased in spontaneously hypertensive rats fed high-fat diet (SHR-HFD) compared with that of SHR fed chow diet (SHR-CHO). (A), SHR-HFD rats significantly increased perivascular fibrotic areas. Quantitative measurement using collagen volume fraction shows HFD significantly increased fibrosis in perivascular areas. (B), SHR-HFD rats significantly increased interstitial fibrotic areas. Quantitative measurement using collagen volume fraction shows HFD significantly increased fibrosis in interstitial areas. Data are mean ± standard deviation (SD, n = 6).

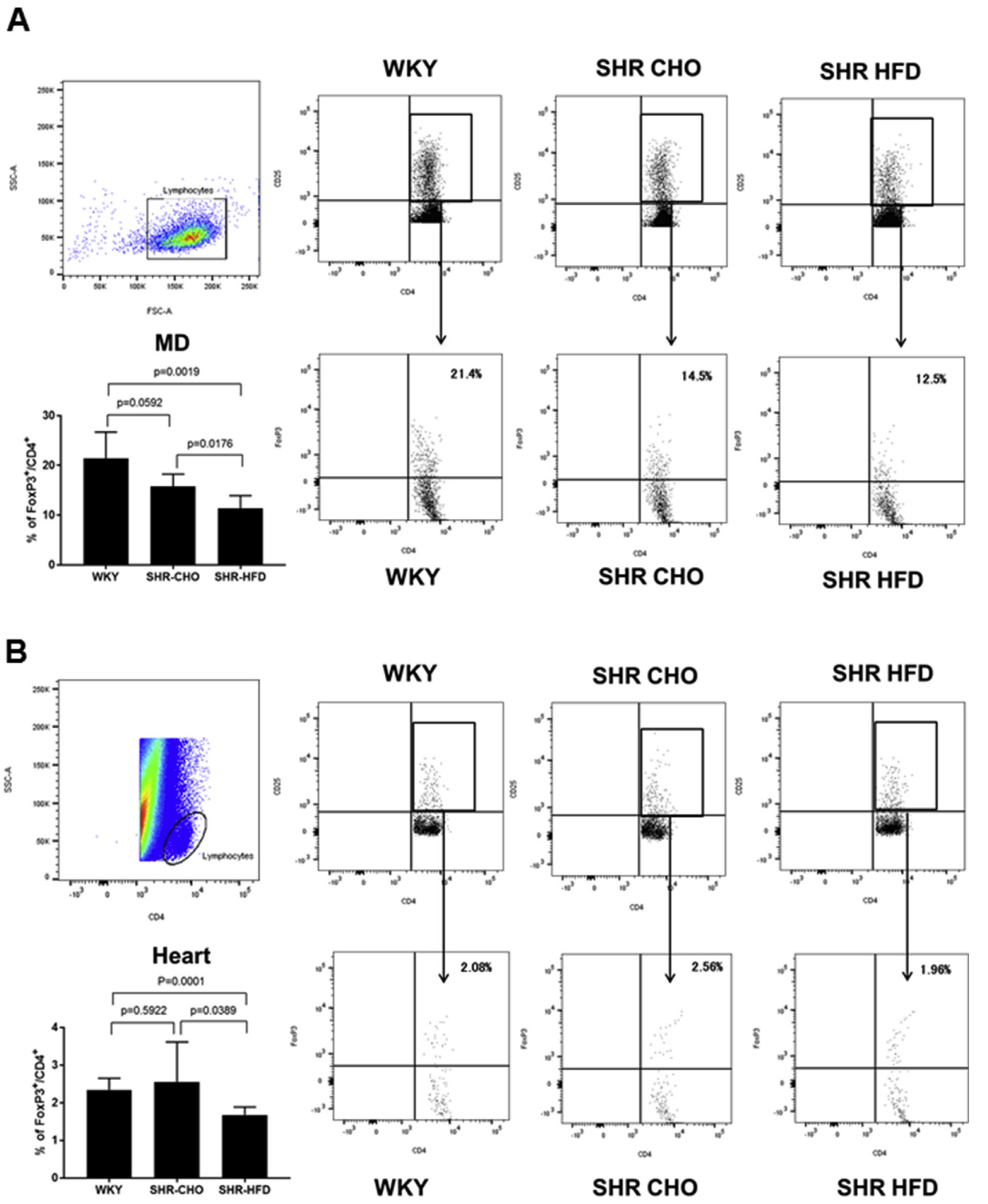

Figure 2 (A) and (B), Flow cytometry analysis of regulatory T cells (Tregs) in hearts and mediastinal lymph nodes (LNs). Lymphocytes were gated according to $CD4^+$ of heart single cells and mediastinal LNs. Representative dot plots of proportions of $CD4^+$ forkhead box protein P3 $(FoxP3)^+$. Comparison of proportions of $CD4^+FoxP3^+$ Tregs of $CD4^+$ T cells between WKY control groups, spontaneously hypertensive rats fed high-fat diet (SHR-HFD) and SHR fed chow diet (SHR-CHO) groups. (C), Immunofluorescence staining of Tregs in hearts. Regulatory T cells (Tregs) stained with anti-cluster of differentiation 4 (CD4) and forkhead box protein P3 (FoxP3) antibody in rat hearts. Representative images of Tregs are shown in each panel (arrows) using immunofluorescence staining using confocal microscopy. CD4 (green), FoxP3 (red), and DAPI (blue). Merged fluorescent and phase images are indicated. Data are mean ± standard deviation (SD, n = 6).

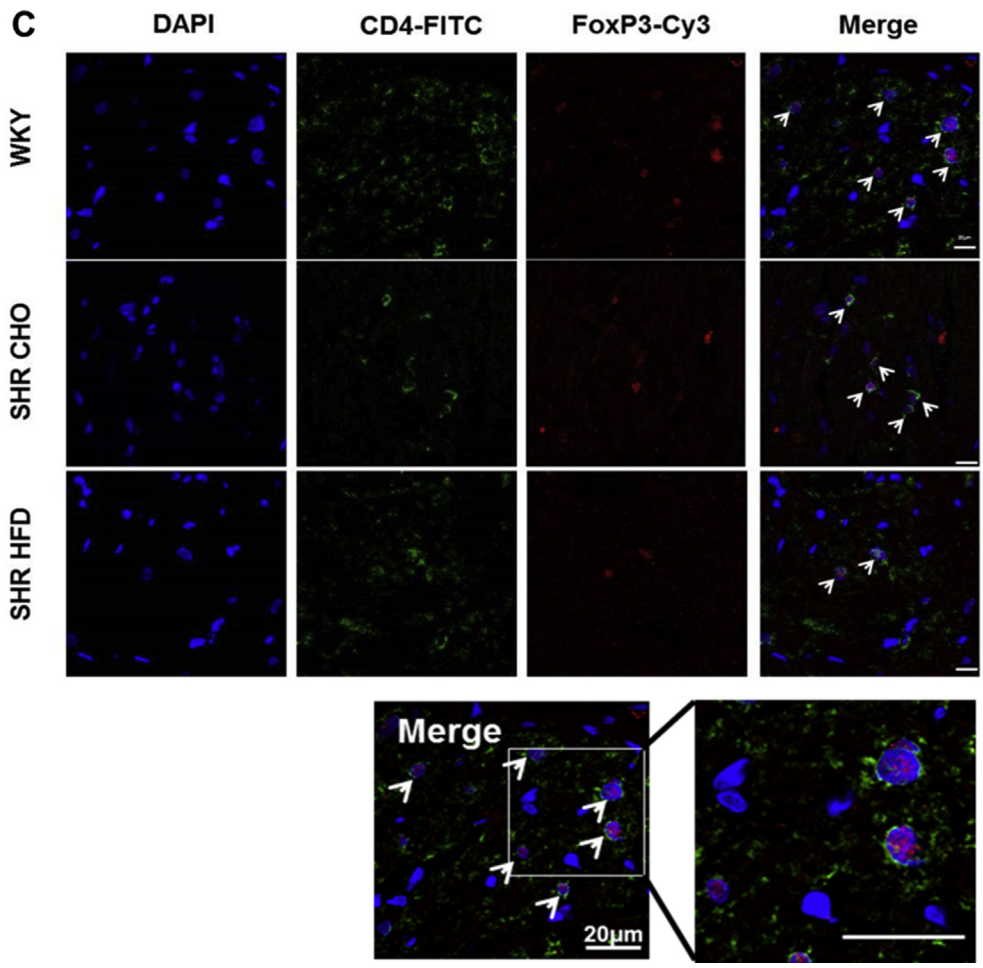

Figure 2 (*continued*).

Discussion

In this study, we discovered that HFD downregulated the Tregs pathway activity in the heart and mediastinal L/Ns of SHR and significantly increased myocardial fibrosis in the heart of SHR.

Dietary factors are important in modulating the development of obesity and metabolic dysfunction. There were several diet-induced obesity animal models, but studies comparing HFD with other diets are lacking. We used SHR-HFD as the diet-induced obesity (DIO) rat model in this study. Because SHR are already hypertensive and insulin resistant, the SHR has been used as a model for metabolic syndrome [12]. We already demonstrated that DIO activates the TGF-β1 and Smad2/3 pathways in the myocardium, which in turn leads to myocardial fibrosis and LV diastolic dysfunction [13].

The immune and inflammatory response systems play a pivotal role in the pathogenesis of obesity-induced cardiac remodeling [2]. Tregs are anti-inflammatory T cells that modulate the innate and adaptive immune responses and play an important role in cardiovascular complications and inflammatory action of the immune response and obesity-induced target organ damage [7,8,14]. Some studies reported that Tregs dysfunction was involved in the hypertension and vascular inflammation in hypertensive rodent models [9,15], but spironolactone improved the Treg dysfunction in the heart and kidney of DOCA-salt hypertensive rats [10]. Recently, some studies reported a relationship between Tregs and myocardial remodeling in pressure overload and hypertensive animal models [16,17]. Especially, Kvakan et al. showed that the immunosuppressive effects of transferred Tregs ameliorated the cardiac damage [17]. Previous studies showed the relationships between Tregs dysfunction and cardiovascular complications in the hypertensive animal model. However, to our knowledge, there has been no study of the role of Tregs in the myocardial fibrosis of diet induced obesity animal model. We demonstrated that CD4+FoxP3+T cells are decreased in the heart and mediastinal LNs by HFD feeding when compared to control diet in the SHR. Wang B et al. reported the suppression of Tregs in the HFD induced obesity mice but they showed only the expression of Tregs in the peripheral blood and spleen [18]. Furthermore, in our study, HFD decreased Tregs in the mediastinal LNs as well as in the heart of SHR. The

mediastinal LNs are located in the thoracic cavity and surround the pericardium and the major vessels of the heart. In particular, myocardial infarction or chronic heart failure induces the activation and proliferation of Tregs in the LNs and some researchers have identified heart-draining mediastinal LNs [19]. However, this study did not show the cause-and-effects relation between the changes in Treg and those in LV wall. In addition, recent studies suggested that obesity may lead to immunological alterations in the epicardial adipose tissue, which may have important implications for the myocardial fibrosis under metabolic stress or pathologic conditions. Therefore, immunological alterations within the epicardial adipose tissue may be also another possible mechanism of obesity-induced myocardial fibrosis [20,21]. Further study is needed in the future. Eventually, we found that diet-induced obesity typically showed an exacerbated myocardial fibrosis and down-regulation of Tregs pathway in the heart and mediastinal LNs. Therefore, we suggest that the up-regulation of Tregs may be a promising therapeutic approach to preventing obesity induced heart failure.

Conclusion

In this study, we discovered that the SHR-HFD increased myocardial fibrosis, and down-regulation of the Tregs pathway activity. The immune and inflammatory systems play a pivotal role in the pathogenesis of obesity-induced myocardial remodeling.

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Disclosures

The authors have no conflicts of interest to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.numecd.2019.08.004.

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