Treg/Th17 Ratio Regulation May Play an Important Role in Epigallocatechin-3-Gallate–Mediated Attenuation of Increased Afterload-Induced Cardiac Hypertrophy

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Abstract: The aim of this study was to investigate whether Treg/Th17 ratio regulation plays an important role in epigallocatechin-3-gallate (EGCG) in attenuating increased afterload-induced cardiac hypertrophy. Three-month-old male C57BL/6 mice were divided into sham + vehicle, abdominal aortic constriction (AAC) + vehicle, and AAC + EGCG groups. Intraperitoneal EGCG (50 mg/kg/d) administration was conducted. Cardiac structure and function were examined by ultrasonography. Pathology was examined by hematoxylin and eosin staining, wheat germ agglutinin staining, and Masson's trichome staining. T-lymphocyte subtypes were analyzed using immunofluorescence and flow cytometry assays. Ultrasonography showed that the ventricular wall in the AAC + vehicle group was thicker than that in the sham + vehicle group ($P < 0.05$). Hematoxylin and eosin staining revealed cardiomyocyte hypertrophy accompanied by a small amount of inflammatory cell infiltration in the AAC + vehicle group. The results of wheat germ agglutinin staining demonstrated the presence of hypertrophic cardiomyocytes in the AAC + vehicle group ($P < 0.01$). Masson's trichome staining showed cardiac fibrosis in the AAC + vehicle group, and the immunofluorescence assay revealed infiltration of CD4+ cells in both AAC + vehicle and AAC + EGCG groups. Splenic flow cytometry showed a significant increase in the proportion of Treg cells in the AAC + EGCG group ($P < 0.05$). The proportion of Th17 cells in the AAC + vehicle group was significantly higher than that in the sham + vehicle group ($P < 0.05$). In conclusion, changes in the Treg/Th17 ratio are associated with the occurrence of myocardial hypertrophy caused by increased afterload. Moreover, regulation of the Treg/Th17 ratio by EGCG may play an important role in the attenuation of myocardial hypertrophy.

Key Words: cardiac hypertrophy, epigallocatechin-3-gallate, increased afterload, Treg, Th17

INTRODUCTION

Hypertension is a globally prevalent chronic disease and a significant contributing factor in heart diseases (hypertrophy and heart failure), kidney diseases, and other systemic diseases.[1] Long-term hypertension increases cardiac afterload and directly leads to heart remodeling through hemodynamics. Moreover, heart remodeling because of the immune response caused by afterload has received increased attention.[2]

Hypertension stimulates T lymphocytes and macrophages.[3] Th17 cells produce interleukin (IL)-17, IL-6, and tumor necrosis factor-α (TNF-α) and are responsible for several inflammatory diseases. IL-17 is a powerful proinflammatory factor.[4] Treg cells are a subset of T cells that suppress excessive immune responses by releasing anti-inflammatory cytokines.[5] Normally, Th17 and Treg cells are balanced in organisms. Studies have shown that Th17 and Treg cell levels increase simultaneously with the increase in the level of angiotensin II (Ang II), suggesting that the increase in Treg levels balances that in Th17 levels induced by Ang II.[6] Adoptive transfer of Treg cells reduces the damage caused by hypertension-induced immune response in target organs, but its potential side effects affect the application of such intervention measures.[7] Therefore, it is of great significance to find safer drugs to regulate the immune response and reduce hypertension-related cardiac remodeling.

Epigallocatechin-3-gallate (EGCG) is the most bioactive compound among catechins because of the 6 o-phenolic hydroxyl groups in its unique stereochemical structure. It affects multiple physiological functions, including immune regulation.[8] EGCG plays an immunoregulatory role by inhibiting the production and activity of nuclear factor-κB (NF-κB), which regulates the expression of various inflammatory

factors, such as TNF-α, IL-1, IL-6, and IL-8.[9] In addition, EGCG can reduce the immune response caused by nitric oxide overproduction by reducing the expression of inducible nitric oxide synthases[10] and regulate immune function through Treg cells.[11,12] Treg and Th17 cells play an important role in hypertension-induced target organ injury. Therefore, the aim of this study was to investigate whether the regulation of the Treg/Th17 ratio plays a role in EGCG-mediated alleviation of myocardial hypertrophy caused by increased afterload and provide favorable evidence for clinical translation of EGCG in the treatment of this disease.

MATERIALS AND METHODS

Experimental Animals

This study was approved by the Ethical Committee on Animal Research of Chongqing Medical University (20201118001). The procedures in this study were performed based on the standard animal care guidelines established by the ethical committee. C57BL/6 mice (male, 12 weeks, 25 ± 3 g) were purchased from the Animal Department of Chongqing Medical University (Chongqing, China). All mice were housed under standard laboratory conditions at 24 ± 1°C. The lighting duration in the breeding room ranged from 7:00 AM to 7:00 PM.

Mouse Model

The model of increased afterload was constructed in C57BL/6 mice by abdominal aortic constriction (AAC) according to a previous study.[13] The mice fasted without food and water for 8 hours and 4 hours, respectively. Then, they were anesthetized with 10% of chloral hydrate (3 mL/kg, intraperitoneal injection) and fixed in the supine position. Their abdominal hair was removed with depilation cream, and the area was disinfected with povidone–iodine. The abdominal skin and peritoneum were cut open, 0.9% of stroke-physiological saline solution was spread on the area with a gauze, and the stomach and spleen were gently turned out with cotton swabs to expose the abdominal aorta. A 5–0 silk suture was snared and pulled back around the aorta between the superior mesenteric artery and celiac trunk. A 27G blunt acupuncture needle was then placed next to the aorta. The suture was tied snugly around the needle and the aorta, and the needle was removed immediately after ligation. Then, the area was sprinkled with penicillin powder, and the stomach and spleen were replaced, followed by the suturing of the peritoneum and skin. The mice were placed on a heating mat and, on awakening, returned to the cage. The sham group underwent the same operation as the operation group, except for ligation.

Study Protocol

Before surgery, the mice were randomly divided into 2 groups (30 mice in the AAC group and 15 mice in the sham group). After surgery, the AAC group was randomly divided into 2 groups (15 mice in the AAC + EGCG group and 15 mice in the AAC + vehicle group), and the sham group was classified as sham + vehicle group. On the fifth day postsurgery, 200 mg of EGCG (purity 99%, Selleck) was weighed and dissolved in

1 mL of dimethyl sulfoxide; this was diluted with 0.9% of stroke-physiological saline solution at a ratio of 1:20 and administered intraperitoneally at a rate of 50 mg/kg/d per mouse for 6 weeks. The sham + vehicle and AAC + vehicle groups were treated with the same amount of dimethyl sulfoxide diluted with 0.9% of stroke-physiological saline solution at a 1:20 ratio.

Echocardiography

After 6 weeks of EGCG or vehicle administration, cardiac function and the left ventricular wall of the mice were evaluated using echocardiography.[14] The mice were anesthetized with isoflurane and fixed on a hard plate in the supine position. Anesthesia was maintained with isoflurane through a mask, the precardiac hair of the mice was removed, and the ultrasonic coupling agent was applied. The cardiac function and ventricular wall of the mice were evaluated in the short-axis section of the heart using an ultrasound machine (VEVO3100, probe frequency 40 Hz).

Body Weight, Heart Weight, and Heart Weight/Body Weight

After completing the echocardiographic analysis, the mice were weighed using an electronic scale, and blood was collected from their eyeballs. Then, the mice were euthanized by cervical dislocation, their sternum was opened to expose the heart, and the right atrium was cut. Perfusion was performed from the apex with phosphate-buffered saline (PBS) until all organs turned white. The heart was then removed, rinsed in a petri dish containing PBS, dried with absorbent paper, and weighed and photographed under natural light. Body weight (BW) and heart weight (HW) were measured, and the HW/BW ratio was calculated to evaluate the hypertrophic response to pressure overload.

Histopathology

The removed heart tissue was fixed with 4% of paraformaldehyde for 48 hours, dehydrated with gradient alcohol, and embedded in paraffin. The paraffin block was cut into 5 μm sections and then subjected to hematoxylin and eosin and Masson's trichome staining.[15] Microscopic inspection was completed by a board-certified pathologist in a blinded manner.

Fluorescein isothiocyanate–conjugated wheat germ agglutinin (WGA) (Sigma L4895, 1:500) was used to further analyze the cell size. The paraffin sections were dewaxed, and antigen retrieval was performed by placing the sections in a box filled with EDTA antigen repair buffer (pH 8.0), followed by boiling for 8 minutes at medium power and then for 7 minutes at medium low power in a microwave. The WGA working solution was then added and placed in the incubator for 30 minutes at room temperature. Finally, the nuclei were dyed with 4′,6-diamidino-2-phenylindole (DAPI), and the sections were sealed, examined, and photographed under a fluorescence microscope at 400× magnification; the results were analyzed using NIS-Elements Viewer.

Immunofluorescence Staining

Mouse hearts were fixed with 4% of paraformaldehyde for 24 hours; dehydrated with a 10%, 20%, and 30% sucrose

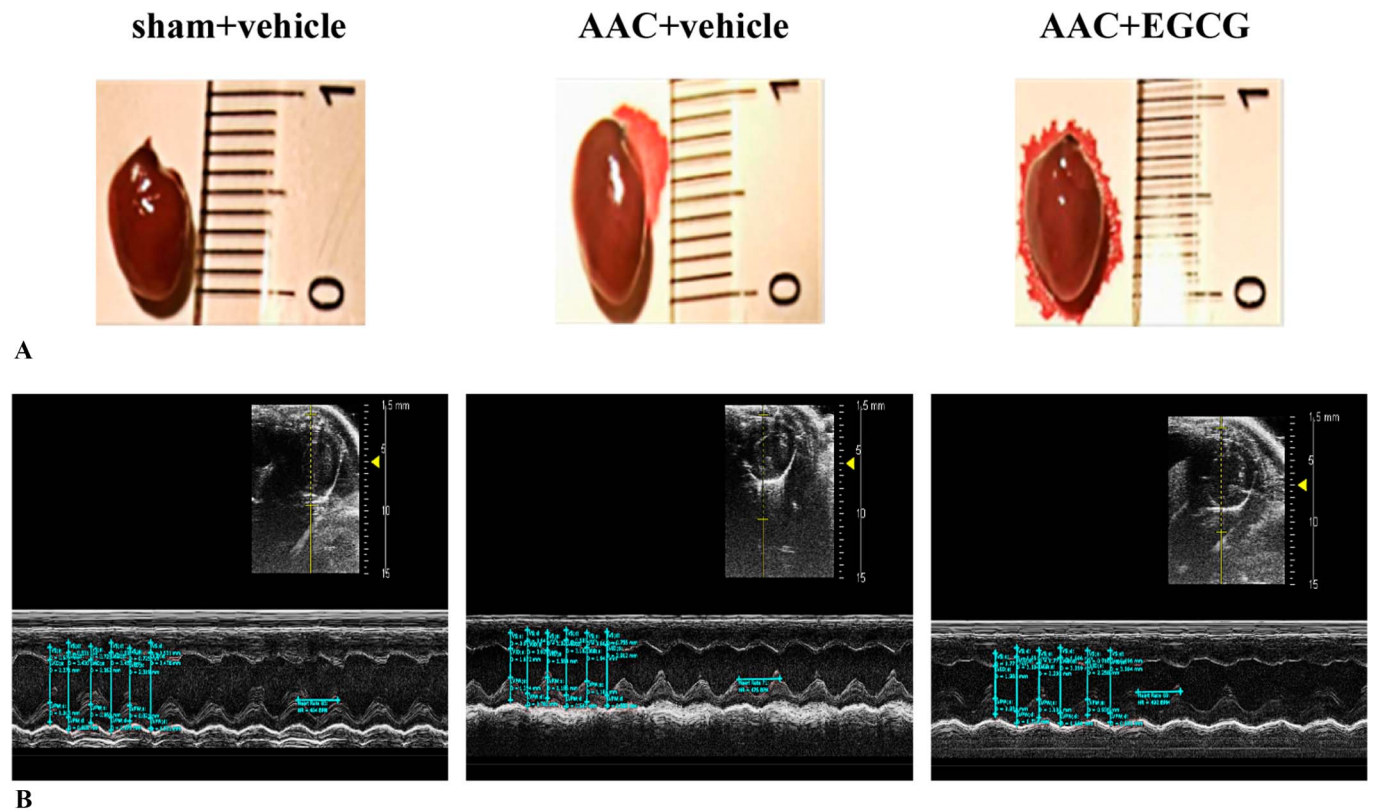**sham+vehicle**   **AAC+vehicle**   **AAC+EGCG**

A

B

FIGURE 1. EGCG improved cardiac hypertrophy. (A) Gross hearts under natural light; (B) short-axis view of cardiac ultrasound.

gradient; embedded with optimal cutting temperature compound; and prepared into frozen 8 μm sections. The sections were sealed with 5% of bovine serum albumin in PBS and then incubated overnight with anti-CD4 McAb (Wanleibio, 1:100) and Coralite 488-conjugated CD8 monoclonal antibody (1 G2B10, ProteinTech, 1:100); the former was labeled with Cy3-labeled goat anti-mouse IgG (H + L) (Beyotime, 1:300) fluorescent secondary antibody. The nucleus was stained with DAPI. The sections were observed under a laser confocal microscope.

Flow Cytometric Analysis of Treg and Th17 in the Spleen

The spleens of mice were removed, and the blood was washed off with PBS solution. They were cut into small pieces, ground with a 2 mL syringe head, filtered through a 200-mesh sieve, centrifuged at 400*g* at 4°C for 5 minutes, and resuspended to obtain a splenic single lymphocyte suspension. Treg and Th17 cells were labeled using the Mouse Regulatory T-cell Staining Kit #1 (eBioscience) and IL-17A monoclonal antibody (eBio17B7), PerCP-Cyanine5.5, according to the manufacturer's instructions. The results were detected by using BD FACSaria II flow cytometer and analyzed using FlowJo v10.

Statistics

All results are presented as mean ± SD. Multiple-group comparisons were performed using 1-way ANOVA followed by the Tukey post hoc test. The Kruskal–Wallis test followed by the Dunn test comparison of pairs was used to analyze data that were not normally distributed. Statistical significance was set at $P < 0.05$. All statistical analyses were performed using GraphPad Prism (version 8.3.0).

RESULTS

EGCG Improved Cardiac Hypertrophy

Gross heart tissue showed enlargement in the AAC + vehicle group compared with the other 2 groups; there was no significant difference between the AAC + EGCG and sham + vehicle groups (Fig. 1A). In general, increased HW and HW/BW ratio are the main indicators of cardiac hypertrophy.[13] There was no significant difference in BW among the 3 groups (Table 1). The HW in the sham + vehicle group was lower than that in the AAC + vehicle and AAC + EGCG

TABLE 1. Heart Weight Related Indicators

	Sham + Vehicle	AAC + Vehicle	AAC + EGCG
BW (g)	27.00 ± 0.52	27.03 ± 1.85	27.03 ± 1.45
HW (mg)	133.00 ± 12.17	163.33 ± 21.08	145.00 ± 10.15
HW/BW (mg/g)	4.92 ± 0.40	6.02 ± 0.38*	5.36 ± 0.11

*Represents $P < 0.05$ versus the sham + vehicle group.
BW, body weight; HW, Heart Weight. n = 3.

groups, whereas the HW in the AAC + EGCG group was lower than that in the AAC + vehicle group. The HW/BW ratio was significantly higher in the AAC + vehicle group than that in the sham + vehicle group ($P < 0.05$), whereas the AAC + EGCG group showed no significant difference compared with the sham + vehicle group.

Ventricular wall and cardiac function were evaluated using echocardiography after 6 weeks of EGCG or vehicle administration. Echocardiographic short-axis sections of the heart (Fig. 1B) showed that compared with those in the sham + vehicle group, the left ventricular thickness increased and the left ventricular diameter decreased in the AAC + vehicle group; however, there was no significant difference in left ventricular thickness and left ventricular diameter between the sham + vehicle and AAC + EGCG groups (Table 2). However, there were no significant differences in ejection fraction and fractional shortening among the 3 groups, suggesting that structural changes but not functional changes occurred in the heart at this time.

EGCG Improved the Heart Histopathology

Microscopic observation of hematoxylin and eosin–stained sections under 200× magnification revealed little infiltration of inflammatory cells and an orderly arrangement of myocardial cells in the AAC + vehicle and AAC + EGCG groups compared with the sham + vehicle group; under 400× magnification, hypertrophy of cardiomyocytes was observed in the AAC + vehicle group, whereas no hypertrophy was observed in the AAC + EGCG and sham + vehicle groups (Fig. 2A). Furthermore, WGA staining indicated that the cross-sectional area of cardiomyocytes in the AAC + vehicle group was significantly greater than that in the sham + vehicle group, whereas no significant difference was observed in the AAC + EGCG group compared with the sham + vehicle group (Fig. 2B).

Masson's trichome staining showed fibrosis around the myocardial cells in the AAC + vehicle group but not in the AAC + EGCG group (Fig. 2C).

Infiltrating Lymphocyte Subtypes in the Heart

Anti-CD4 and anti-CD8 antibodies were used to perform immunofluorescence detection on frozen sections of the heart to show the lymphocyte subtypes infiltrating the heart. CD4-positive and CD8-positive fluorescent markers were not found in the sham + vehicle group, whereas a small amount of CD4-positive red fluorescence and no CD8-positive fluorescent markers were found in the AAC + vehicle and AAC + EGCG groups (Fig. 3).

EGCG Changed the Proportion of Treg and Th17 Cells in the Spleen

Flow cytometry was used to detect the subtypes of splenic lymphocytes, mainly Treg (CD4$^+$CD25+Foxp3+) and Th17 (CD4+IL-17+) cells. Compared with that in the sham + vehicle group, there was no significant difference in the proportion of Treg cells and a significant increase in that of Th17 cells in the AAC + vehicle group, whereas there was a significant increase in that of Treg cells but no significant difference in that of Th17 cells in the AAC + EGCG group (Figs. 4A–D).

DISCUSSION

An increased afterload model was constructed using AAC to simulate hypertension-induced cardiac hypertrophy, and EGCG was used as an intervention in the early stages of the disease. The results suggested that there is an immune response disorder in this model, and the alteration of the Treg/Th17 cell ratio plays an important role in the attenuation of cardiac hypertrophy by EGCG in this model.

Increased afterload results in the chronic stimulation of the heart, leading it slowly to hypertrophy, followed by heart failure, providing a good model for studying hypertrophy. Cardiac hypertrophy begins 4 weeks after AAC, and heart failure is more severe at 8 weeks.[13] In this study, 6 weeks after the administration of EGCG was also 6–7 weeks after the AAC; it was found that the ventricular wall thickened and the ventricular cavity

TABLE 2. Echocardiographic Data

	Sham + Vehicle	AAC + Vehicle	AAC + EGCG
Heart Rate (beats/min)	487 ± 23	488 ± 17	477 ± 20
LV			
IVS; s (mm)	0.83 ± 0.087	0.971 ± 0.072*	0.755 ± 0.069###
LVID; s (mm)	2.496 ± 0.382	1.886 ± 0.199*	2.049 ± 0.182
LVPW; s (mm)	0.982 ± 0.144	1.321 ± 0.228*	0.915 ± 0.102##
IVS; d (mm)	0.527 ± 0.062	0.567 ± 0.092	0.510 ± 0.065
LVID; d (mm)	3.972 ± 0.222	3.442 ± 0.402*	3.411 ± 0.234*
LVPW; d (mm)	0.680 ± 0.083	0.712 ± 0.157	0.570 ± 0.050
EF (%)	67.151 ± 9.689	76.948 ± 5.898	71.085 ± 6.795
FS (%)	37.303 ± 7.918	44.941 ± 5.610	39.766 ± 5.841

*Represents $P < 0.05$ versus the sham + vehicle group.
and ### represents $P < 0.01$ versus the AAC + vehicle group.
LV, left ventricle; IVS; s, interventricular septal thickness at end-systolic; LVID; s, left ventricular internal dimension at end-systole; LVPW; s, left ventricular posterior wall thickness at end-systole; IVS; d, interventricular septum thickness at end-diastole; LVID; d, left ventricular internal dimension at end-diastole; LVPW; d, left ventricular posterior wall thickness at end-diastole; EF, ejection fraction; FS, fractional shortening. Sham + vehicle: n = 6; AAC + vehicle and AAC + EGCG: n = 7.
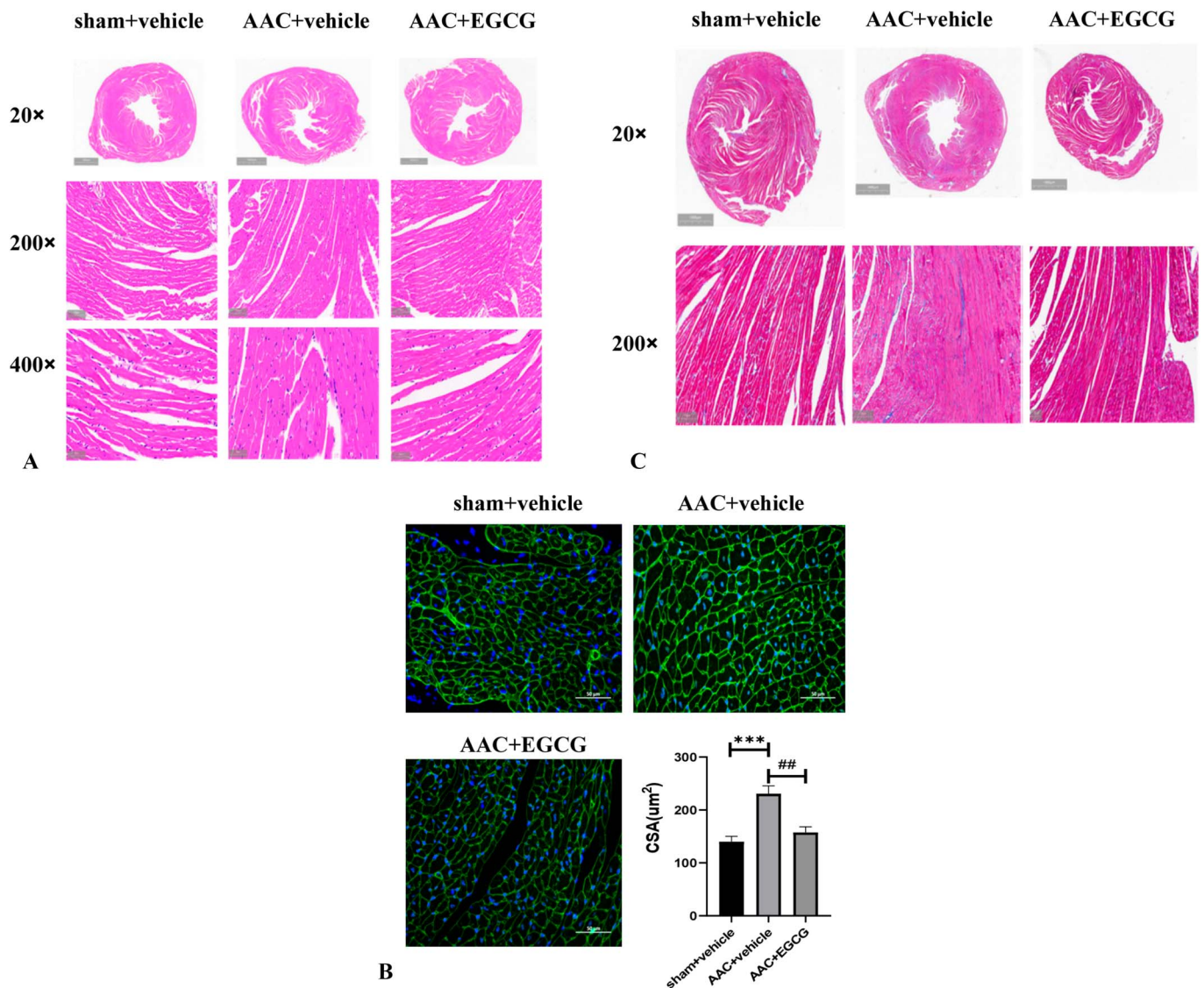
FIGURE 2. EGCG improved heart histopathology. A, Hematoxylin and eosin staining (20×, 200×, 400×). (B) WGA staining (400×, n = 4). C, Masson's trichome staining (20×, 200×). *** represents $P < 0.01$ comparison with the sham + vehicle group; ## represents $P < 0.01$ comparison with the AAC + vehicle group.

shrank during systole, and ejection fraction and fractional shortening increased, indicating that the heart was in the compensatory period of hypertrophy at this time.

Hypertension causes cardiac afterload to increase, leading to pathological changes in the heart. Initially, cardiac lesions manifest as left ventricular hypertrophy, which is an independent risk factor for cardiovascular death in this disease.[16] In a coarctation model of the abdominal aorta, cardiac damage because of increased afterload pressure manifests as centripetal hypertrophy and later as heart failure.[17] The results of our experiment are consistent with these observations. Cardiac conditions were detected by echocardiography 6–7 weeks after AAC, and it was found that the left ventricular inner diameter in systolic and diastolic periods was smaller than that in the sham group, and the wall of the left ventricle was thickened, showing centripetal hyperplasia.

The best-known mechanism of cardiac injury is remodeling of the heart in response to changes in hemodynamics caused by increased afterload.[18] With an increase in afterload, the left ventricular ejection pressure increases, and then, the compensatory hypertrophy of the left ventricle enhances the myocardial contractility to maintain sufficient cardiac displacement. However, this causes cardiomyocyte hypertrophy, muscle fiber thickening, and chronic degenerative changes. Long-term increased pressure leads to an excessive increase in afterload, resulting in vascular wall thickness, cardiac centripetal hypertrophy, diastolic relaxation impairment, and finally a decrease in myocardial contractility, resulting in hypertensive heart disease and chronic left heart failure.

The immune system is associated with high blood pressure, and recent data have identified the role of T cells and various T-cell–derived cytokines in several experimental hypertension models.[19] T lymphocytes can be divided into CD4+ and
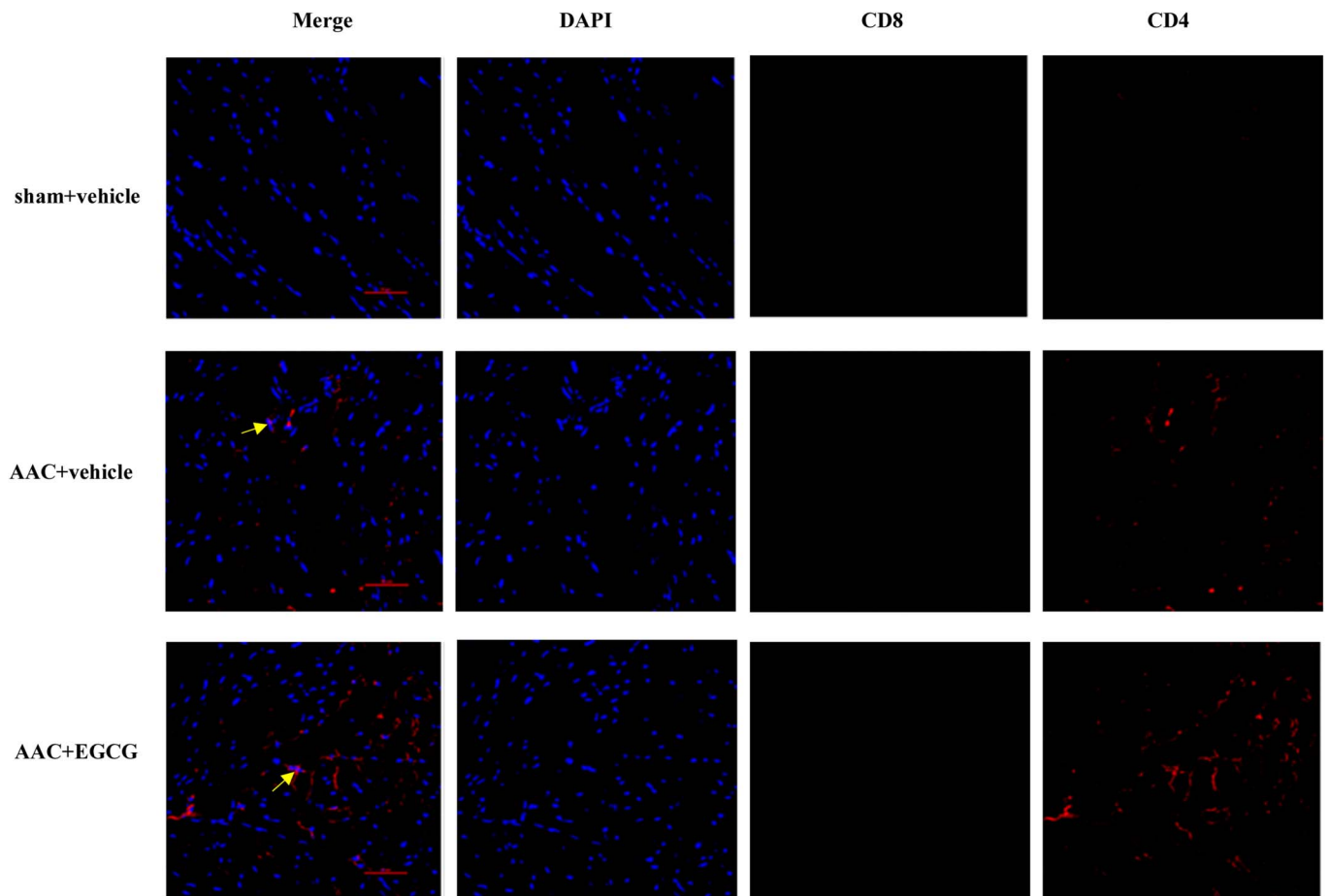
FIGURE 3. EGCG improves lymphocyte infiltration in the heart. CD8 is green, CD4 is red, and the nucleus is blue. Photograph was taken at 400×. The yellow arrows are CD4$^+$ cells.

CD8$^+$ subtypes according to their cell surface antigens; CD4$^+$ T lymphocytes promote the development of compensatory hypertrophy, such as heart failure, in the presence of chronic stress overload.[20] In this study, the results of immunofluorescence staining of frozen sections of the heart also showed that under the stimulation of increased afterload, the main infiltration of the heart was by CD4$^+$ T lymphocytes, whereas CD8$^+$ T lymphocytes did not show significant infiltration.

CD4$^+$ T lymphocytes also contain the following subtypes: Th1, Th2, Th17, Treg, and follicular helper T cells. In Ang II-induced hypertension, IL-17 is critical to maintain hypertension and vascular dysfunction.[21] Plasma levels of the anti-inflammatory cytokine IL-10, which is mainly secreted by Treg cells, are increased in mice injected with Ang II.[22] Flow cytometry was used to directly analyze the CD4$^+$ T-lymphocyte subtypes in the spleen tissue, and the results showed that in the AAC + vehicle group, the proportion of proinflammatory Th17 cells increased, whereas that of anti-inflammatory Treg cells was not significantly different, compared with the sham + vehicle group; this finding suggested that the anti-inflammatory cell count was relatively insufficient in the AAC + vehicle group, leading to immune disorders and promoting the occurrence of inflammation.

Epidemiological findings indicate that green tea consumption is associated with a reduced risk of all-cause death and death from the 3 major causes: heart disease, cerebrovascular disease, and respiratory disease.[23] Green tea extract is primarily a series of catechins, including EGCG, epicatechin gallate, epigallocatechin, and epicatechin, which have many beneficial properties that can help prevent atherosclerotic diseases by regulating obesity,[24] hypertension,[25] diabetes,[26] and oxidative stress.[27] Among these components, EGCG is the most abundant tea polyphenol, possesses the most biological activity,[28] and has previously been found to improve cardiac hypertrophy due to increased afterload.[29] In our experiment, the results of ultrasound and WGA staining showed that the ventricular wall thickness and myocardial area in the AAC + EGCG group were significantly improved compared with those in the AAC + vehicle group, but no significant difference was found compared with the sham + vehicle group, indicating that EGCG improves cardiac hypertrophy caused by increased afterload.

The main active ingredient in green tea extract, EGCG, provides antioxidant, anticancer, antiseptic, anticollagenase, and antifibrotic effects.[8] It plays an anti-inflammatory role in many ways, among which it can regulate the immune
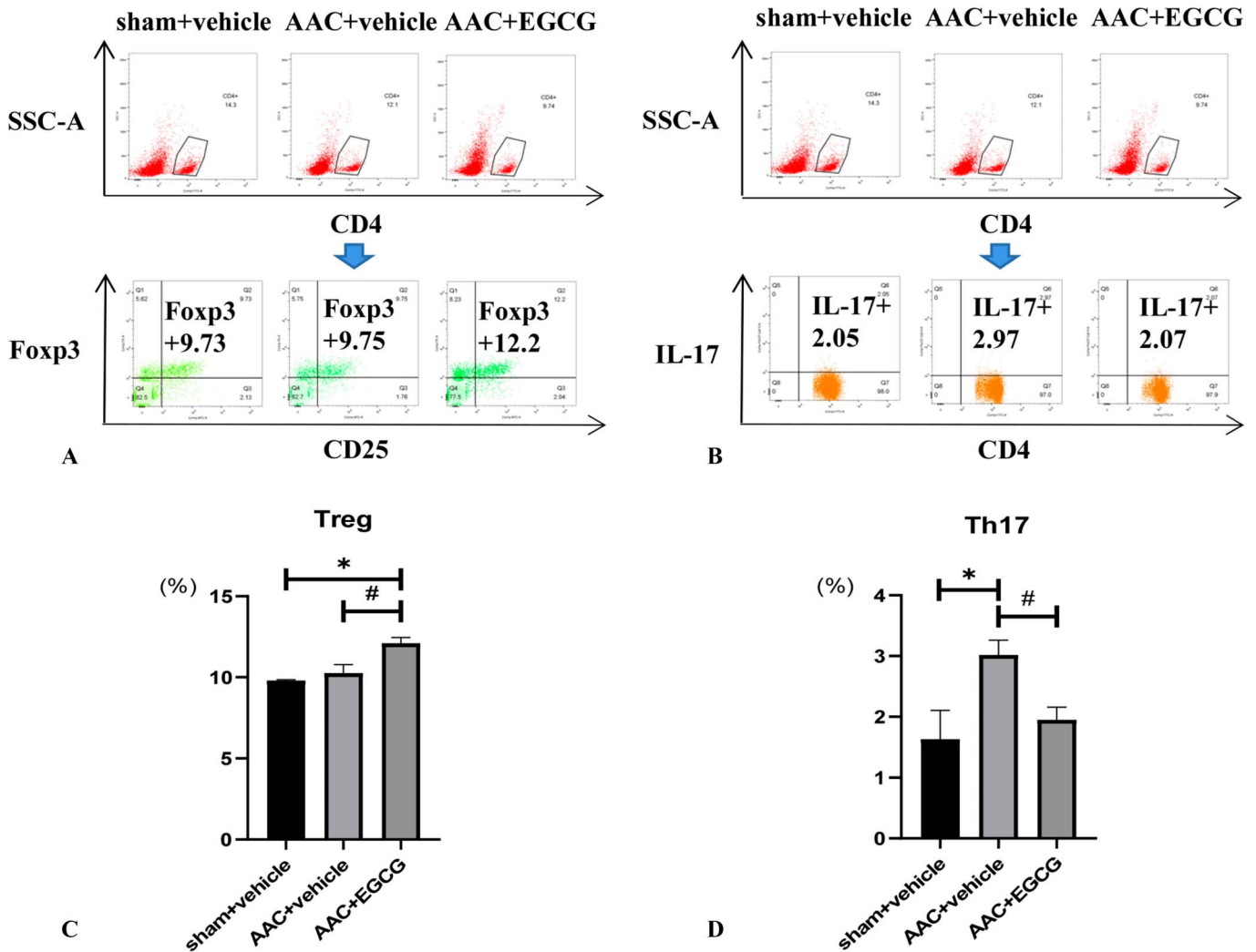
FIGURE 4. EGCG changed the proportion of Treg and Th17 cells in the spleen. (A) CD4+CD25+Foxp3+ cells in the spleen; (B) CD4+IL-17+ cells in the spleen; (C) the percentage of CD4+CD25+Foxp3+ cells in the spleen; (D) the percentage of CD4+IL-17+ cells in the spleen (n = 3). * represents $P < 0.05$ versus the sham + vehicle group, # represents $P < 0.05$ versus the AAC + vehicle group.

response by regulating Treg levels, thereby affecting the inflammatory response of the body.[11,30] EGCG can improve cardiac function in rats with chronic heart failure by regulating the balance between Treg and Th17 cells.[31] In our study, the splenic lymphocyte subtypes detected by flow cytometry showed that the proportion of Treg cells was significantly increased in the AAC + EGCG group, whereas that of Th17 cells was significantly decreased, suggesting that EGCG altered the balance of Treg and Th17 cells in AAC mice.

CONCLUSIONS

This study has demonstrated that cardiac hypertrophy caused by increased afterload stimulation is related to immune response disorder, especially the change in the Treg/Th17 balance. Moreover, the regulation of the Treg/ Th17 ratio may play an important role in the attenuation of cardiac hypertrophy by EGCG in this disease model.

Prospects

This study showed that cardiac hypertrophy induced by increased afterload stimulation is related to immune response disorder and demonstrated that the change in the Treg/Th17 ratio may play an important role in the improvement of increased afterload-induced cardiac hypertrophy by EGCG. It provides a possible mechanism of EGCG action in this disease and further provides strong support for the clinical translation of EGCG treatment. Following our previous study demonstrating successful application of EGCG to treat children with diastolic dysfunction,[32] we will conduct further clinical translational studies to explore the clinical efficacy of EGCG on cardiac hypertrophy caused by increased afterload and the accompanying immune changes.

Declarations

Ethics Approval

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