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Interleukin-17 Receptor Signaling Regulates Immune Response and Slows Down Myocardial Fibrosis in Mice with Dilated Cardiomyopathy

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ABSTRACT

Immune response is a significant mechanism in dilated cardiomyopathy (DCM). The interleukin-17 receptor (IL-17R) is crucial for immune response.

A DCM model was created using doxorubicin, and IL-17R was knocked down. We assessed cardiac function, histopathological changes, fibrosis proteins, myocardial injury, and inflammation levels through echocardiography, pathological staining, immunofluorescence, and Western blot, respectively. The proportions of T cell subsets in mouse spleen tissue were identified through flow cytometry. Following these steps, we detached fibroblasts from the mouse heart and knocked down IL-17R. Angiotensin II was employed to induce cell fibrosis and co-cultured with T Helper 17 (T_H17) cells. We measured inflammation, collagen deposits, and fibrosis protein expression using Sirius red staining, immunofluorescence, and Western blot.

IL-17R exhibited significant expression in DCM mice. The systolic function of DCM mice significantly decreased. Myocardial fibrosis and collagen deposition in the left ventricle were markedly elevated. The levels of fibrosis proteins and pro-inflammatory factors were notably enhanced ($p < 0.01$). The proportion of effector CD4+ T and T_H17 cells in spleen tissue noticeably increased, while the Treg cell proportion notably decreased ($p < 0.05$). These indicators were significantly reversed after IL-17R knockdown. In the co-culture system, pro-inflammatory cytokines, collagen formation, and fibrosis-related protein levels increased significantly after fibrosis induction. However, the level of fibrosis and T_H17/Treg cell imbalance decreased significantly after IL-17R knockdown.

The knockdown of IL-17R can reduce immune reaction, which in turn improves myocardial fibrosis and alleviates DCM cardiac function.

Keywords: Cardiomyopathy; Fibrosis; Immunity; Interleukin-17; T Helper 17 cells

INTRODUCTION

Dilated cardiomyopathy (DCM) is a complex myocardial disease characterized by a poor prognosis.[1] The survival rate after 5 years stands roughly at 50%.[2] This condition is primarily marked by the enlargement

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of the cardiac chamber and the dysfunction of myocardial systolic function.[3] The main morphological indications include left ventricular or biventricular enlargement and general thinning of the ventricular wall. From a pathological standpoint, this disease is defined by extensive myocardial cell atrophy, compensatory hypertrophy of myocardial cells, and varying degrees of interstitial fibrosis.

DCM is the most common type of non-ischemic cardiomyopathy in idiopathic and familial diseases.[4] It has multiple causes, including gene mutations, alcohol consumption, infections, environmental factors, inflammation, and autoimmune diseases.[5] In its early stages, the disease can easily be overlooked by patients and progressively leads to cardiac enlargement, heart failure, and various arrhythmias. By the time most DCM patients are diagnosed, there is already cardiac damage and structural remodeling. As the disease advances, the patient's ventricles enlarge, the ventricular wall thins, and stress rises correspondingly, resulting in a decrease in remaining normal cardiomyocytes and an increase in unit cardiomyocyte load. Myocardial fibrosis (MF), particularly severe fibrosis around the capillaries, can ultimately lead to insufficient myocardial oxygen supply, energy metabolism disorder, and, in extreme cases, sudden death.[6] Moreover, there is a deficiency of targeted treatment methods in clinical practice, making the investigation of safe and effective therapeutic strategies to improve DCM particularly critical.

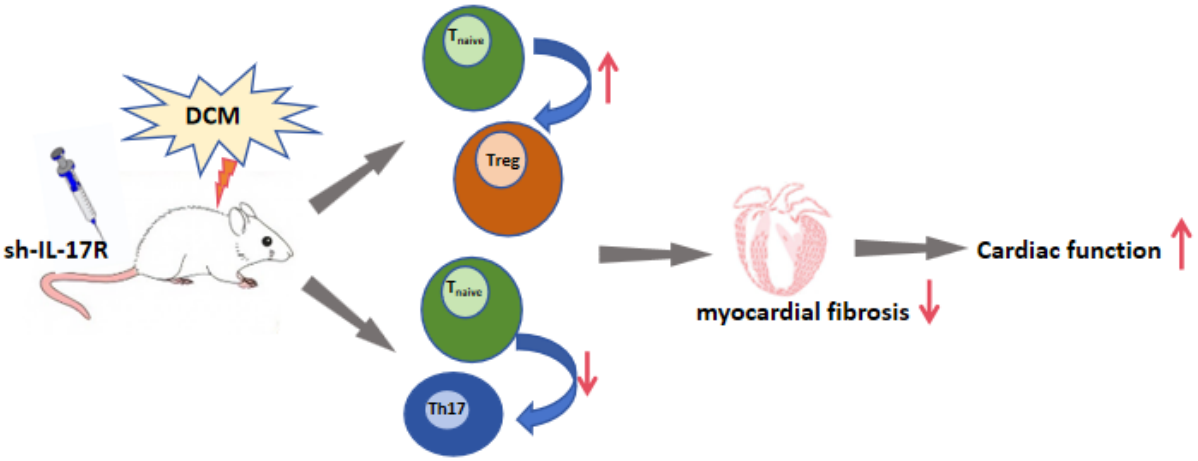
Myocardial remodeling is one of the significant pathological changes associated with DCM. This primarily involves cardiomyocyte hypertrophy and MF. MF refers to the accumulation and deposition of an extensive amount of collagen fibers in the cardiac matrix. An increase in collagen content or a change in its composition can lead to a reduction in myocardial compliance.[7,8] Furthermore, it is also a pathological process involving excessive deposition of various extracellular matrix (ECM) proteins in the myocardial interstitium, caused by various factors. Notably, cardiac fibroblasts (CFs) are the main producers of ECM proteins and play a crucial role in maintaining ECM homeostasis. This provides the cytological foundation for studying the pathogenesis of MF.[9] The pathological manifestations of MF include excessive proliferation of CFs, collagen deposition, and thickening of the ventricular wall, which leads to a decreased ejection fraction.[10]

Currently, clinical anti-MF is the principal treatment direction for DCM.[11] Hence, identifying targets that can effectively enhance MF is vitally important for DCM treatment. Reports suggest that the immune inflammatory response is a significant DCM mechanism,[12,13], and it's closely related to fibrosis progression. Oridonin mitigates MF and cardiac remodeling due to myocardial infarction in mice by inhibiting the NOD-like receptor thermal protein domain-associated protein 3 (NLRP3) inflammasome.[14] Exercise significantly reduces T-helper cell-associated cardiac inflammatory cell infiltration and stalls myocardial pyroptosis, thereby minimizing cardiac fibrosis.[15] In conclusion, enhancing myocardial immune response and MF are both DCM treatment measures; hence, our belief that DCM's MF can be eased by reducing myocardial immune damage.

The interleukin (IL)-17 signaling pathway is an impactful mediator of the inflammatory response. After activation, $CD4^+$ T lymphocytes differentiate into T Helper 17 (T_H17) cells. The primary effector factor of the T_H17 cell and the initial instigator of the inflammatory response is IL-17. Moreover, T_H17 cells can secrete additional inflammatory factors, such as IL-6 and tumor necrosis factor-α (TNF-α). These cytokines collectively stimulate, attract, and activate neutrophils. During this process, IL-17 assists in activating the mobilization of neutrophils, thereby influencing the tissue's inflammatory reaction. Research indicates that IL-17 primarily acts on fibroblasts and other mesenchymal cells, in turn inducing CFs to produce matrix metalloproteinases (MMP) and tissue inhibitor of metalloproteinases (TIMP) to promote MF.[16] IL-17 binds specifically to its receptor, interleukin-17 receptor (IL-17R), promoting the development of the inflammatory and immune response,[17] thus fulfilling its biological role.[18] Furthermore, IL-17R expression has been found on CFs.[19] To summarize, the T_H17 cell immune response plays a crucial role in improving MF. It is hypothesized that by mediating the immune response of T_H17 cells, IL-17R could influence the MF of DCM.

In conclusion, we hypothesized that IL-17R can mitigate MF in DCM mice by influencing the immune response. In light of this, the study leveraged doxorubicin (DOX) to establish a DCM mouse model,[3] identified IL-17R levels in DCM, and manipulated its expression to investigate the significance of IL-17R in DCM alongside related mechanisms. This offers fresh

perspectives for DCM treatment studies, evidencing that IL-17R is a potential treatment target for DCM. This information seeks to furnish an experimental foundation for the pursuit of safe and effective DCM treatment strategies and provide a theoretical basis for the clinical treatment of DCM.



Graphical Abstract. Knockdown of interleukin-17 receptor (IL-17R) can improve the immune response and T Helper 17 (T_H17)/Treg cell imbalance in dilated cardiomyopathy (DCM) mice, thereby reducing myocardial fibrosis damage and alleviating cardiac function damage.

MATERIALS AND METHODS

Animal Grouping and Model Preparation

Thirty-two specific pathogens free (SPF) male C57BL/6 mice, aged 7 weeks and weighing 25–30 g, were supplied by Huachuang Xinnuo Pharmaceutical Technology Co., Ltd. (Jiangsu, China). These animals were housed in an environment with a 12-hour day-night cycle, a temperature range of 20–25°C, and relative humidity between 40% and 70%. All animal experiments in this study adhered to China's Regulations on the Administration of Laboratory Animals and Guidelines for the Ethical Review of Laboratory Animal Welfare (GB/T 35892-2018). Strict adherence was given to the 3R principle during the experiment to minimize the number of animals used and ensure their welfare. The experimental protocol for this animal study was approved by the Experimental Animal Ethics Committee of the Second Hospital of Hebei Medical University (No. 2023-1258).

The mice were divided into a Control group (Saline) and a model group (DCM), each consisting of eight members. The DCM mouse model was constructed using DOX.[3,20] Mice in the DCM group received intraperitoneal injections of DOX (4 mg/kg, E2516, Selleck.cn, Shanghai, China), which was dissolved in normal saline, every other day for 2 weeks. After a three-week drug withdrawal period, mice exhibiting a left ventricular end-diastolic diameter (LVEDd) within the 95% confidence interval were deemed successful models, as confirmed by echocardiography. The Control group (Saline) received injections of an equivalent volume of normal saline, with all other procedures remaining the same.

Both the control adeno-associated virus 9 (AAV9-sh-NC) and IL-17R knockdown adeno-associated virus 9 (AAV9-sh-IL-17R) were procured from Heyuan Biotechnology Company (Shanghai, China). The mouse sh-IL-17R sequence was subsequently inserted into the adeno-associated virus 9 (AAV9)-based plasmid used for packaging the AAV9 virus. Before the DCM mouse model's establishment, mice from the DCM group received injections of either 100 µL AAV9-sh-IL-17R or AAV9-sh-NC adeno-associated virus (1.8×10^{12} vg/mL) through the tail vein. This process facilitated the creation of IL-17R knockdown mice and negative control mice.[21, 22] The DCM mouse model was established 3 weeks post-injection cessation, and the knockdown efficiency was subsequently assessed using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting (WB).

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After the modeling process, the four groups of mice were subjected to cardiac ultrasound examination and body weight measurements. Following anesthesia with Avertin, their abdominal cavities were exposed, and blood was drawn from the abdominal aorta. Following euthanasia, the heart and spleen were extracted, rinsed with PBS, dried with filter paper, and weighed. These collected samples were retained for subsequent experiments. Figure 1A illustrates the experimental process.

Echocardiographic Detection of Cardiac Function

The mice were anesthetized with Avertin and subsequently secured on an operating platform. Echocardiography was then performed using a Vevo 1100 ultrasound system (Visual Sonics, Toronto, Canada); the heart probe frequency was set to X10-23 MHz. The left ventricular end-diastolic dimension (LVDD), left ventricular end-diastolic volume (LVEDV), left ventricular ejection fraction (LVEF), and fractional shortening (FS) were measured by M-mode in the left ventricle's long-axis section. The mean values of three consecutive complete cardiac cycles were calculated for all measurements.

Pathological Staining

The left ventricular anterior wall tissue of mice was fixed in 4% paraformaldehyde (PFA), then dehydrated and embedded routinely. Paraffin sections, each of 5 μm thickness, were prepared and stained with hematoxylin-eosin (HE), Masson, and Sirius red. HE staining involved hematoxylin (C0107, Beyotime, Shanghai, China) for 15 minutes, 1% acid alcohol (containing 70% hydrochloric acid) differentiation for 30 seconds, and then 0.5% eosin (G1100, Solarbio, Beijing, China) for 3 minutes. Masson staining was performed using a Masson staining kit (G1340, Solarbio, Beijing, China). This involved performing a Masson blue stain for 3 minutes, a ponceau fuchsin stain for 8 minutes, washing with weak acid solution for 30 seconds, treatment with phosphomolybdic acid for 1 minute, and aniline blue staining for 2 minutes. The Sirius red staining employed a Sirius red staining kit (G1472, Solarbio, Beijing, China), with the stain drops left for 15 minutes. The pathology, fibrosis, and collagen deposition in the left ventricular tissue of the mice were examined under an optical microscope. Three images were collected. The proportions of blue in Masson staining and red in Sirius red staining were analyzed using the Image J image analysis system.

Flow Cytometry of Spleen Tissue

The spleen tissues from each group of mice were chopped into pieces, homogenized, and filtered twice using a 200-mesh filter. The filtrate was collected, centrifuged, and the resulting precipitate was collected. A tissue cell diluent was added to create a cell suspension, which was then added to the lymphocyte separation liquid. The lymphocyte suspension in the middle layer was centrifuged, collected, and resuspended using a phosphate buffered saline (PBS) solution, after which the lymphocyte count was performed. A volume of 2 mL lymphocyte suspension (containing 1×10^6 cells/mL) was inoculated into 6-well plates. Subsequently, ionomycin (final concentration: 1 μg/mL, II2200, Solarbio, Beijing, China), phorbol myristate acetate (final concentration: 50 μg/mL, P6741, Solarbio, Beijing, China), and monensin (final concentration: 0.1 mg/mL, 00-4505-51, eBioscience, California, USA) were added, mixed, and incubated for 6 hours. Then, either anti-Mouse CD4-FITC (100405, Biolegend, California, USA) or anti-Mouse CD25-APC (162105, Biolegend, California, USA) antibody was introduced. Following incubation and washing, IL-17-APC (506915, Biolegend, California, USA), forkhead box p3 (Foxp3)-PE (118903, Biolegend, California, USA), CD44-FITC (156007, Biolegend, California, USA), and CD62-APC (161217, Biolegend, California, USA) antibodies were added in the dark. After another dark incubation period, the material was washed with PBS and resuspended. The proportions of $CD62L^+CD44^+CD4^+$, $CD62L^+CD44\text{-}CD4^+$, $CD4^+$IL-$17A^+$ T_H17, and $CD4^+CD25^+Foxp3^+$ Treg cells were then evaluated via flow cytometry.

Isolation of Mouse CFs

The mice in the saline group were thoroughly cleaned with 75% ethanol, and their skin was gently incised to expose the thoracic cavity. The intact heart was delicately extracted using tweezers, rinsed with PBS buffer, and then sectioned into 1 mm fragments. These fragments were transferred to a 15 mL centrifuge tube and digested with trypsin (T8150, Solarbio, Beijing, China). After numerous digestions, the supernatant was centrifuged, and 3 mL of red blood cell lysis buffer (00-4333-57, eBioscience, California, USA) was added to the cell precipitate for 3 minutes. Subsequently, an equal volume of Dulbecco's Modified Eagle Medium (DMEM) medium (31600, Solarbio, Beijing, China) was added to halt lysis, centrifuged to discard the

supernatant, and the cell precipitate was resuspended and transferred to DMEM medium containing 10% fetal bovine serum (S9030, Solarbio, Beijing, China) for stable cultivation. The adherent cells, identified as CFs, were subcultured to the second generation, followed by plate preparation and experimental treatment.

Co-culture

1 mL of peripheral blood and 1 mL of PBS from mice in the saline group were added to 5 mL heparin-treated centrifuge tubes. Then, the mixture was transferred to a centrifuge tube containing 2 mL of mouse lymphocyte separation solution (P8620, Solarbio, Beijing, China) and centrifuged at 5000 r/min for 10 minutes. The lymphocyte layer was deposited into a 10 mL centrifuge tube, and the supernatant was discarded following centrifugation. Peripheral blood mononuclear cells (PBMCs) were obtained by resuspending in 1 mL of complete medium, and $CD4^+$ T cells were isolated from the PBMCs using immunomagnetic beads. The separated cells were stimulated with 20 ng/mL IL-2 and CD3/CD28 activator (212-12,11453D, Gibco, California, USA) for 2 weeks.[23] $CD4^+$ T cells were cultured in cell culture medium, which contained 50 ng/mL IL-6 (216-16, Peprotech, California, USA), 2.5 ng/mL transforming growth factor-β (TGF-β) (14-8342-80, eBioscience, California, USA), 5 μg/mL anti-Interferon-γ (IFN-γ) (16-7313-85, eBioscience, California, USA), and 5 μg/mL anti-IL-4 (16-7045-85, eBioscience, California, USA), for 3 days to induce T_H17 differentiation.

Si-IL-17R (Heyuan Biotech, Shanghai, China) was transfected into mouse CFs using the Lipofectamine™3000 transfection reagent (L3000001, Invitrogen, Austin, TX, USA), after which the CFs were treated with Angiotensin II (Ang II, 100 nM) for 24 hours. They were then co-cultured with isolated T_H17 cells at a 1:5 ratio. The cells were grouped into Control, Ang II, Ang II+T_H17, Ang II+T_H17+si-NC, and Ang II+T_H17+si-IL-17R. The Control group consisted of CFs without any treatment, while the Ang II group represented the CFs induced with Ang II fibrosis. In the Ang II+T_H17 group, fibrotic CFs were co-cultured with T_H17 cells. In the Ang II+T_H17+si-IL-17R group, IL-17R was knocked down based on the Ang II+T_H17 group. Cell specimens were collected following 2 days of co-culture. This specific grouping is illustrated in Table 1.

Table 1. The situation of each group

Group	Intervention
Control	CFs were cultured normally without any treatment.
Ang II	Ang II induced CFs fibrosis.
Ang II+T_H17	Ang II induced CFs fibrosis and was co-cultured with T_H17 cells.
Ang II+T_H17+si-NC	si-NC was transfected into CFs, and then Ang II was used to induce cell fibrosis, and co-cultured with T_H17 cells.
Ang II+T_H17+si-IL-17R	si-IL-17R was transfected into CFs to knock down IL-17R, and then Ang II was used to induce cell fibrosis, and co-cultured with T_H17 cells.

CFs: Cardiac fibroblasts; Ang II: Angiotensin II; IL-17R: Interleukin-17 receptor; T_H17: T Helper 17

qRT-PCR

Total RNA was derived from mouse myocardial tissue and CFs using TRIzol™ (15596026CN, Invitrogen, California, USA), and then reverse transcribed into cDNA with the SuperScript VILO cDNA synthesis kit (11754250, Invitrogen, California, USA). PCR detection was performed using the TransStart Top Green qPCR SuperMix (AQ131-01, TRANS, Beijing, China). The relative gene levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as the internal reference. The primer sequences were as follows:

IL-17R:

Forward: 5′-GTGGGTCTTCAAACACTTCTTCA-3′;

Reverse: 5′-CAAGGAGGCTGTGCTTAGGTT-3′.

GAPDH:

Forward: 5′-ATGGGACGATGCTGGTACTGA-3′;

Reverse: 5′-TGCTGACAACCTTGAGTGAAAT-3′.

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Western Blotting

The myocardial tissue and co-cultured CFs from mice were collected and subjected to ice-cold RIPA lysis (R0010, Solarbio, Beijing, China). Following lysis, the protein concentration was determined, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis was conducted based on a protein quantity of 60 μg per well. Subsequently, the membrane was transferred at 300 mA for 90 minutes and then blocked for 60 minutes. Primary antibodies for IL-17R (ab180904, Abcam, Cambridge, UK), alpha-smooth muscle actin (α-SMA, ab7817, Abcam, Cambridge, UK), Collagen I (ab316222, Abcam, Cambridge, UK), TIMP-1 (ab179580, Abcam, Cambridge, UK), connective tissue growth factor (CTGF, ab318148, Cambridge, UK), and GAPDH (ab8245, Abcam, Cambridge, UK) were added in a ratio of 1:1000 and incubated at 4℃ for 12 hours. Post incubation, the membrane was washed and then exposed to goat anti-rabbit IgG secondary antibody (ab205718, 1:3000, Abcam, Cambridge, UK) for 1 hour. Enhanced chemiluminescence coloration (PE0020, Solarbio, Beijing, China) was performed, followed by imaging using an automatic chemiluminescence analyzer (Tanon 5200 Multi, Tianneng Technology Co., Ltd., Shanghai, China). The results were analyzed using Image J software.

Enzyme-linked Immunosorbent Assay (ELISA)

The myocardial tissue was combined with an appropriate amount of normal saline homogenate, and the supernatants were obtained post-centrifugation. The co-cultured medium was transferred to a sterile centrifuge tube, from which the supernatants were extracted following centrifugation. The collected blood was centrifuged, and the serum from the top was gathered. The levels of IL-6 (mI098430, mlbio, Shanghai, China), IL-17 (ml037866, mlbio, Shanghai, China), IL-10 (ml037873, mlbio, Shanghai, China), TNF-α (ml002095, mlbio, Shanghai, China), lactate dehydrogenase (LDH, ml095304, mlbio, Shanghai, China), cardiac troponin I (cTnI, ml092662, mlbio, Shanghai, China), and Foxp3 (ml037859, mlbio, Shanghai, China) were evaluated by ELISA. The supernatant and antibody mixture were incubated in an ELISA plate for 1 hour. Following this, 100 μL of the substrate was added to each well, it was incubated in the dark for 10 minutes, and 100 μL of termination reaction solution was added. The optical density (OD) values of each group were evaluated with a microplate reader to determine the amounts of IL-1β, IL-6, and TNF-α in each respective sample.

CFs Sirius Red Staining

CFs were cultured on a 12-well plate positioned on a glass slide. After the co-culture, cells were fixed with 4% PFA for 15 minutes and stained with Sirius red dye for 1 hour. Subsequently, the cells were stained with hematoxylin for 5 minutes. The fiber expression was observed under the microscope. Captured images were analyzed quantitatively for the proportion of red in the staining image using the Image J image analysis system.

Immunofluorescence

The myocardial tissue sections were dewaxed; CFs cultured on 12-well plates were then co-cultured and fixed with 4% PFA for 10 minutes. Following this, tissue or cell sections were blocked with 5% bovine serum albumin for 1 hour and incubated overnight at 4℃ with primary antibodies of IL-17R (ab180904, Abcam, Cambridge, UK), α-SMA (ab7817, Abcam, Cambridge, UK), and Collagen I (ab316222, Abcam, Cambridge, UK), respectively. After this, the sections were incubated with goat anti-rabbit IgG (GB21303, Servicebio, Wuhan, China) for 1 hour, and the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes. Fluorescence microscopy (MF52-N, Guangzhou Ming-Mei Technology Co., Ltd, Guangdong, China) was utilized to observe and capture the images, and ImageJ software was employed for the quantitative analysis of the immunofluorescence intensity.

Statistical Analysis

Statistical analysis was performed using SPSS 26.0 software (SPSS Inc., Chicago, IL, USA). The data were tested for a normal distribution using the Kolmogorov-Smirnov test and were presented as the mean ± standard deviation. The student's t-test was used to analyze the difference between the two groups, while one-way analysis of variance (ANOVA) was used for comparisons across multiple groups. A p value of less than 0.05 was considered statistically significant.

RESULTS

IL-17R is Highly Expressed in DCM Mice

We used DOX to induce DCM in mice and then observed that the IL-17R gene level increased in DCM mice. This increase was statistically significant (p<0.01) (Figure 1B), suggesting that IL-17R may be associated with the development of DCM. Immunofluorescence and WB experiment results also substantiated this finding. Contrary to the Saline group, the fluorescence intensity and level of IL-17R, predominantly expressed in the membrane and cytoplasm, were elevated in the DCM group; this increase was statistically significant (p<0.01) (Figure 1C–F). Thus, IL-17R might contribute to DCM injury.

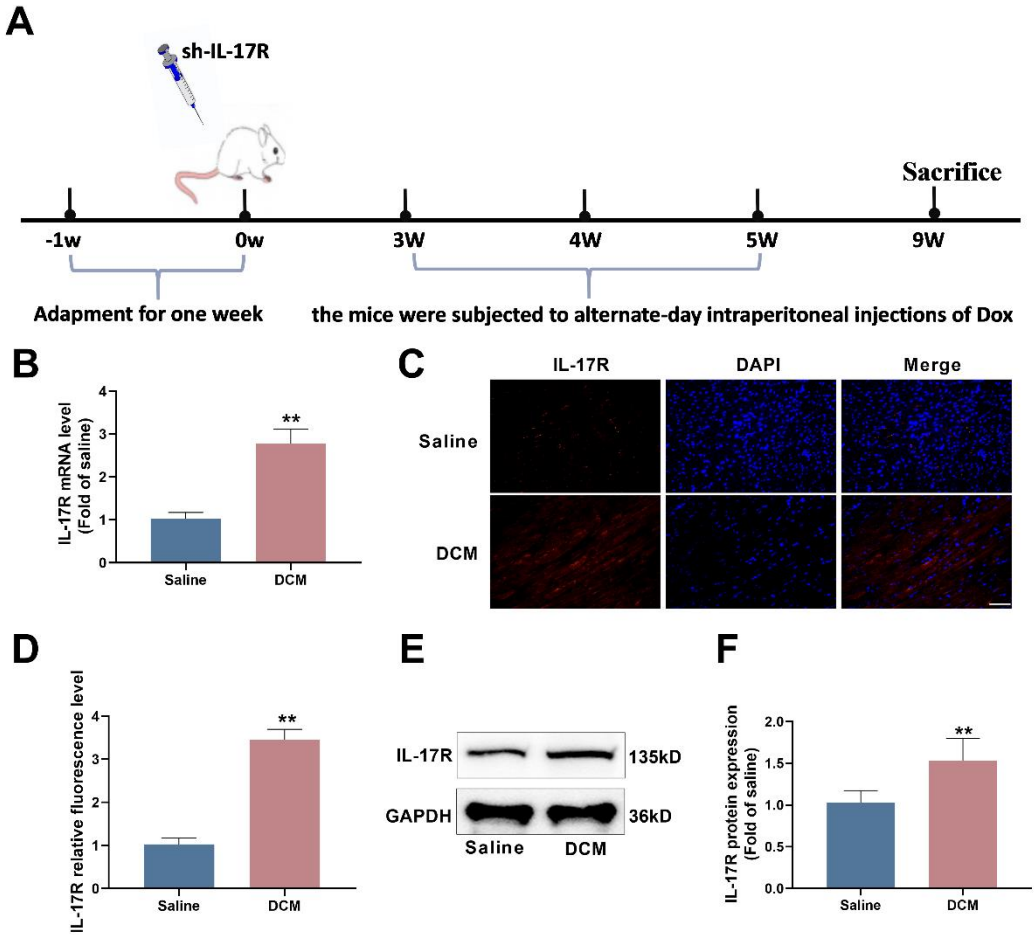

Figure 1. Interleukin-17 receptor (IL-17R) is highly expressed in dilated cardiomyopathy (DCM) mice. A: Experimental flow chart. B: Using doxorubicin (DOX) to induce DCM in mice, the level of the IL-17R gene was found using quantitative real-time polymerase chain reaction (qRT-PCR), IL-17R gene was significantly increased in DCM. C-F: IL-17R localization and level were detected through immunofluorescence and western blotting (WB). It was found that the level of IL-17R located in the membrane and cytoplasm increased significantly in DCM (×40, 50 µm). n=6, **p<0.01 vs Saline group.

Knockdown of IL-17R Can Improve Cardiac Function in DCM Mice

Increased levels of the IL-17R gene and protein were discovered in the DCM group. However, these levels decreased after the injection of AAV9-sh-IL-17R, with the differences being statistically significant (p<0.01) (Figure 2A–C). This signifies that IL-17R underwent effective downregulation, making it suitable for subsequent experiments. Cardiac ultrasound and serological tests showed significantly lower cardiac systolic function indexes, LVEF and FS values, in the DCM group (p<0.05) (Figure 2D-E). Also, diastolic

function indexes LVEDD and LVEDV values were significantly increased (p<0.01) (Figure 2F–G), and the serum myocardial injury markers LDH and cTnI levels also increased (p<0.01) (Figure 2H-I). These results suggest successful modeling of left ventricular dysfunction in the mice. After the knockdown of IL-17R, the LVEF and FS values in the mouse heart significantly decreased, while LVEDD and LVEDV values and LDH and cTnI levels notably increased. This suggests that IL-17R knockdown can significantly ameliorate cardiac function damage in DCM mice by regulating cardiac systolic and diastolic functions.

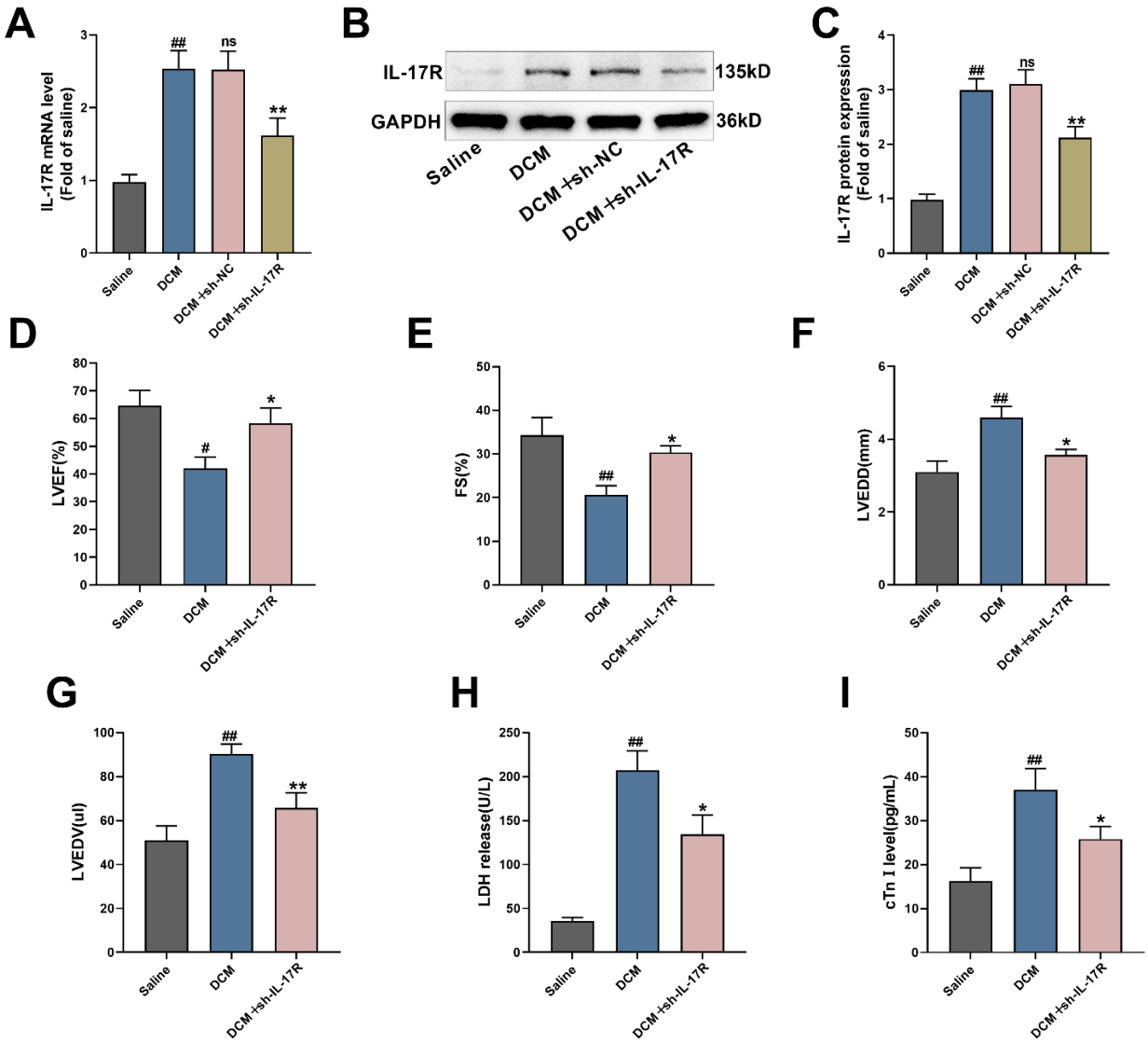

Figure 2. Knockdown of interleukin-17 receptor (IL-17R) can improve cardiac function in dilated cardiomyopathy (DCM) mice. A-C: IL-17R was knocked down, and doxorubicin (DOX) was used to induce DCM in mice. The effectiveness was detected through quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting (WB), and IL-17R was effectively knocked down (##p<0.01 vs Saline group; ns$p$>0.05 vs DCM group; **p<0.01 vs DCM+sh-NC group). D-G: The echocardiography proved that the left ventricular ejection fraction (LVEF) and fractional shortening (FS) of DCM mice were significantly decreased, and the left ventricular end diastolic dimension (LVEDD) and left ventricular end diastolic volume (LVEDV) values were raised. The above indicators were remarkably reversed after IL-17R knockdown. H-I: The levels of serum myocardial injury markers were discovered by enzyme-linked immunosorbent assay (ELISA). Lactate dehydrogenase (LDH) and cardiac troponin I (cTnI) levels increased significantly in DCM mice, and decreased significantly after IL-17R knockdown. n=6, #p<0.05, ##p<0.01 vs Saline group; *p<0.05, **p<0.01 vs DCM group.

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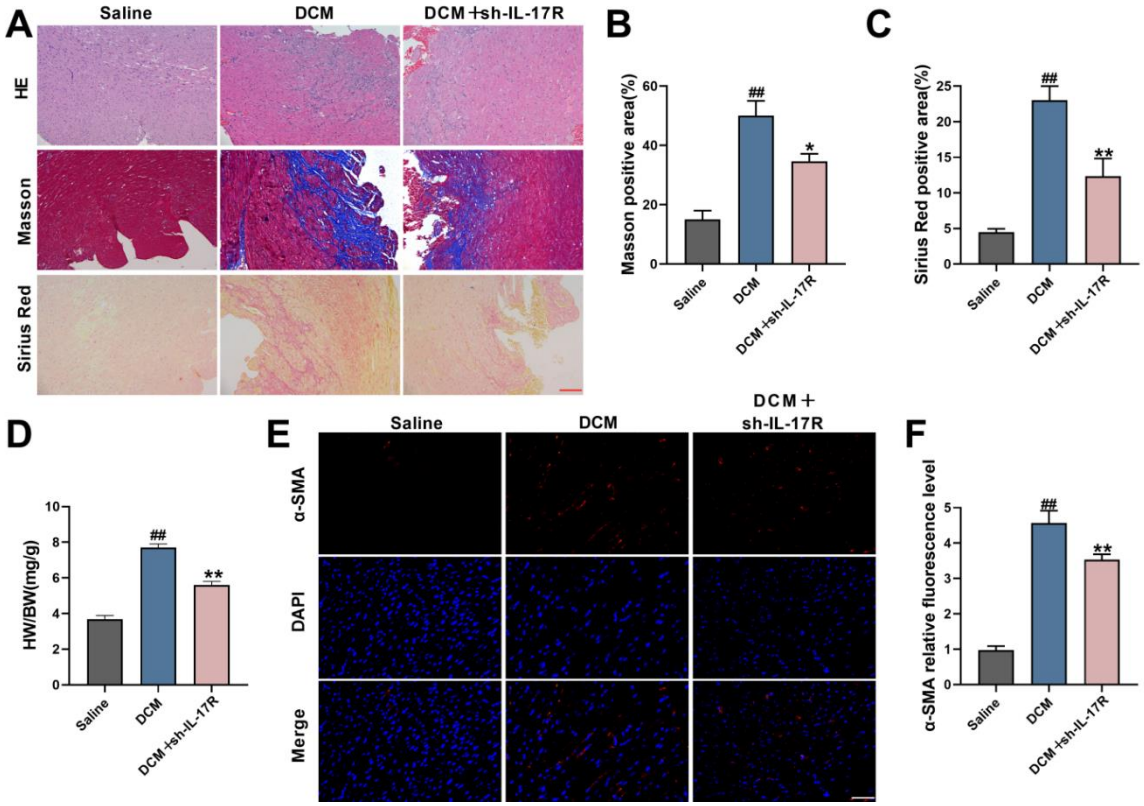
Knockdown of IL-17R Can Improve Myocardial Fibrosis in DCM Mice

The primary characteristic of DCM pathology is MF. Our research revealed that myocardial tissue in the Saline group, as observed via HE staining, presented tightly arranged central muscle fibers, distinct nuclei, uniformly colored cytoplasm, and complete, clear morphology. Blue-stained collagen fibers were sparse according to Masson staining, and Sirius red staining revealed little sizable amounts of red-stained collagen tissue. Following DOX induction, the quantity of myocardial fibers in mice decreased significantly, and the myocardial fibers' transverse diameter widened. Additionally, the myocardial cytoplasm's coloration was inconsistent, the connections between myocardial cells were slack, and the myocardial fibers' integrity and continuity were substandard, with disarranged patterns. The quantity of purple-red myocardial tissue decreased, while the amount of blue-stained and red-stained collagen fibers significantly increased. Upon IL-17R knockdown, myocardial fibers in the mice exhibited various levels of uneven staining, although the situation improved compared to the DCM group, and the prevalence of blue-stained and red-stained collagen fibers diminished (Figure 3A-C). Subsequently, the heart index (HW/BW) in DCM mice escalated but was reduced following IL-17R knockdown, a change statistically significant (p<0.01) (Figure 3D). This implies that IL-17R knockdown could enhance MF and cardiac function in DCM mice. ECM's excessive accumulation affects α-SMA and Collagen I, both critical for MF progression and onset. Also important for fibrosis formation are TIMP-1 and CTGF. The MF index had verification through immunofluorescence and WB. In DCM mice, α-SMA resided primarily in myocardial tissues' vascular walls and could be spotted in cardiomyocytes' interstitial tissue. Collagen I appeared in clusters. Both α-SMA and Collagen I fluorescence intensity were enhanced (Figure 3E-H), and the protein levels of α-SMA, Collagen I, TIMP-1, and CTGF also increased, a statistically significant finding (p<0.01) (Figure 3I–M). After IL-17R knockdown, fibrosis-related protein levels significantly decreased, further revealing that DOX can provoke significant fibrosis damage in the myocardial tissue of DCM mice. Still, fibrosis could be reduced via IL-17R knockdown by inhibiting factors linked to fibrosis and enhancing cardiac function.



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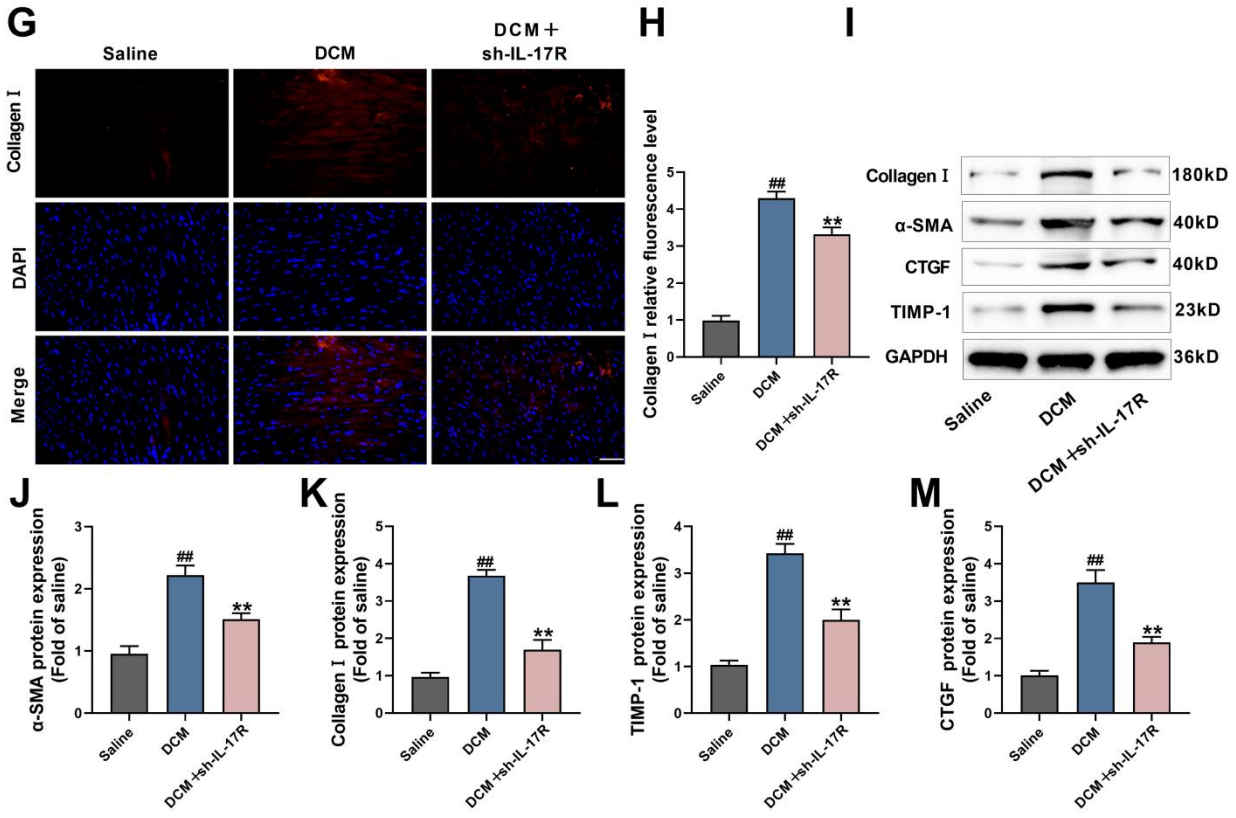


Figure 3. Knockdown of interleukin-17 receptor (IL-17R) can improve myocardial fibrosis in dilated cardiomyopathy (DCM) mice. A-C: Hematoxylin-eosin (HE) staining of myocardial tissue proved that the myocardial cytoplasm of DCM mice was unevenly stained, the color was different, the connection between myocardial cells was loose, and the integrity and continuity of myocardial fibers were poor. Masson staining found that blue-stained collagen fibers were elevated, and Sirius red staining proved that red-stained collagen fibers and tissues were elevated. After IL-17R knockdown, the myocardial fibers of mice showed different degrees of uneven staining, but the situation was alleviated, and the blue-stained collagen fibers and red-stained collagen tissues decreased (×20, 100 μm). D: The heart index heart weight (HW)/body weight (BW) of mice increased significantly in DCM and decreased significantly after IL-17R knockdown. E-H: The fibrosis proteins alpha-smooth muscle actin (α-SMA) and Collagen I were detected using immunofluorescence, which were significantly increased in DCM mice and significantly decreased after IL-17R knockdown (×40, 50 μm). I-M: Western blotting (WB) found myocardial fibrosis (MF)-related proteins α-SMA, Collagen I, tissue inhibitor of metalloproteinases-1 (TIMP-1), and connective tissue growth factor (CTGF) in DCM mice, which were significantly increased in DCM mice and significantly decreased after IL-17R knockdown. n=6, ##p<0.01 vs Saline group; *p<0.05, **p<0.01 vs DCM group.

Knockdown of IL-17R Attenuated the Immune Response in DCM Mice

It was discovered that the contents of IL-6, IL-17, and TNF-α in the DCM group had increased, and IL-10 had decreased, a statistically significant trend (p<0.01) (Figure 4A–D). This trend was significantly reversed after IL-17R knockdown, suggesting that the knockdown of IL-17R could substantially lessen the inflammatory response in DCM mice. $CD4^+$ T cells, which are situated at the interface of humoral immunity and cellular immunity, are considered a crucial component of the immune response. Consequently, we also examined the proportions of effector $CD4^+$ T cells ($CD62L^+CD44^+CD4^+$ cells) and naive $CD4^+$ T cells ($CD62L^+CD44\text{-}CD4^+$ cells) in mouse spleen tissues. Within the DCM mice, the proportion of effector $CD4^+$ T cells had increased, but after IL-17R knockdown, this increase was mitigated. We found no noticeable

difference in the proportion of naive $CD4^+$ T cells within the DCM mice when compared to the Saline group. However, in the DCM-sh-IL-17R group, there was a significant increase in the proportion of naive $CD4^+$ T cells ($p<0.05$) (Figure 4E–G). This result indicates that the knockdown of IL-17R can significantly inhibit the activation of $CD4^+$ T cells in DCM mice. In summary, the knockdown of IL-17R can ameliorate DCM by inhibiting the inflammatory response and the activation of $CD4^+$ T cells.

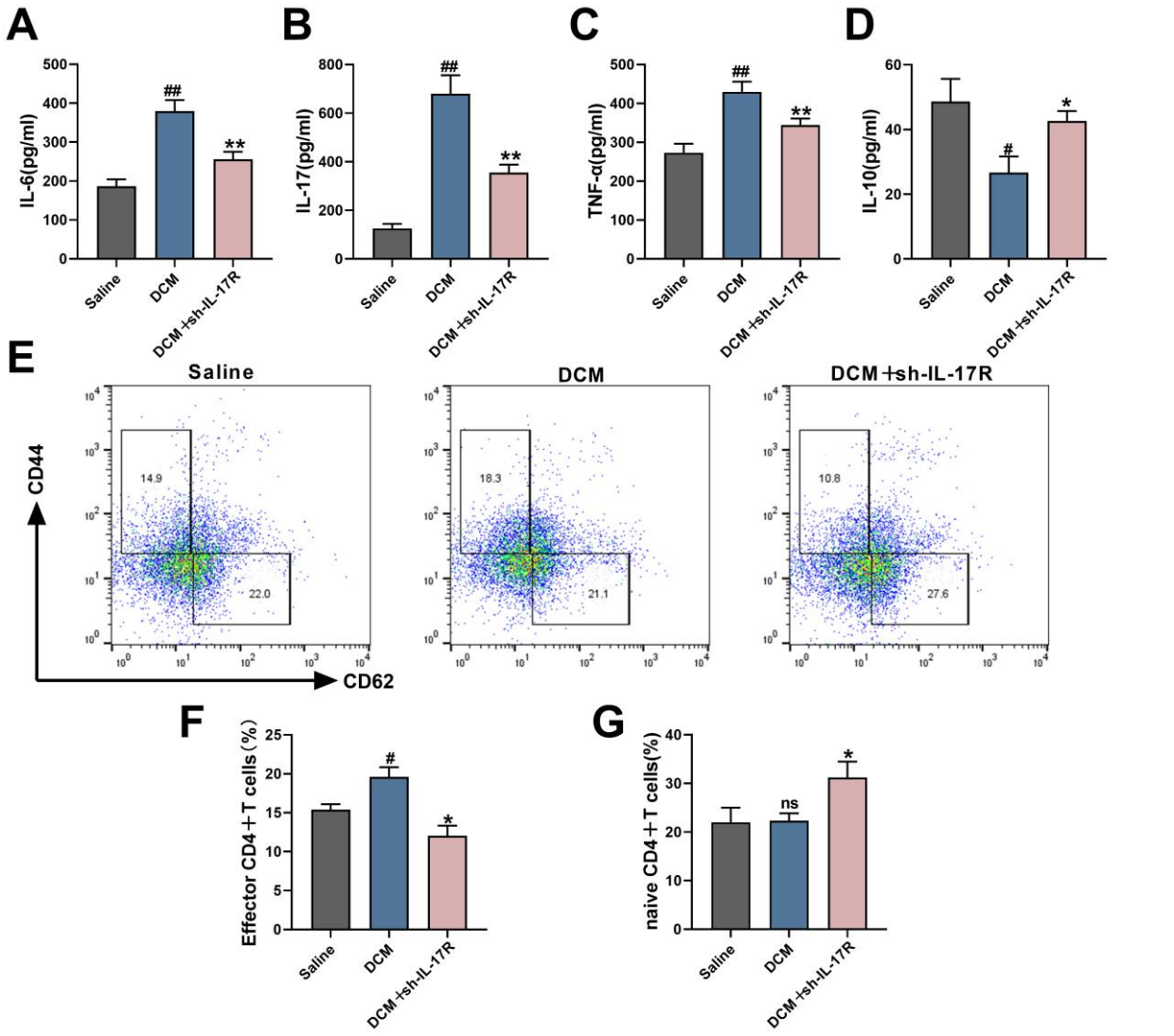


Figure 4. Knockdown of interleukin-17 receptor (IL-17R) attenuated the immune response in dilated cardiomyopathy (DCM) mice. A-D: The inflammatory factor levels were detected by an enzyme-linked immunosorbent assay (ELISA) kit. Interleukin (IL)-6, IL-17, and tumor necrosis factor-α (TNF-α) in DCM mice were notably elevated, and the level of IL-10 was significantly decreased. It was significantly reversed after IL-17R knockdown. E-G: The proportion of $CD4^+$ T cells in the spleen tissue of mice was tested by flow cytometry. The proportion of effector $CD4^+$ T cells were significantly increased in DCM mice. The proportion of effector $CD4^+$ T cells were notably decreased, and naive $CD4^+$ T cell was markedly elevated after IL-17R knockdown. n=6, #$p<0.05$, ##$p<0.01$ vs Saline group; *$p<0.05$, **$p<0.01$ vs DCM group.

Knockdown of IL-17R Improves T_H17/Treg Cell Imbalance in DCM Mice

T_H17 and Treg factor levels were detected using an ELISA kit. The level of IL-17, a marker of T_H17 cells, was found to be elevated in DCM mice, while IL-10 and Foxp3, markers of Treg cells, were reduced; these variations were statistically significant ($p<0.01$) (Figure 5A-C). Interestingly, these trends were reversed following IL-17R knockdown, suggesting it could notably elevate T_H17 and Treg levels in DCM mice. We subsequently analyzed the proportion of T_H17 ($CD4^+IL\text{-}17A^+T_H17$) and Treg ($CD4^+CD25^+Foxp3^+$Treg) cells in the spleen. An elevated percentage of T_H17 cells and a diminished Treg cell proportion were observed in the spleen of DCM mice, resulting in a pronounced increase in the T_H17/Treg ratio. This ratio decreased along with the T_H17 cell percentage, and the Treg cell percentage increased post-IL-17R knockdown; these changes were statistically significant ($p<0.01$) (Figure 6D-G). These observations indicated that the knockdown of IL-17R can notably address the T_H17/Treg cell imbalance. When taken together with the previously mentioned results, this suggests that IL-17R knockdown can ameliorate immune dysfunction in DCM and improve MF in DCM mice by inhibiting $CD4^+$ T activation and T_H17 cell differentiation.

Knockdown of IL-17R Inhibited CFs Fibrosis in Mice

To demonstrate the enhancement of IL-17R in myocardial injury in DCM mice, we initially isolated fibroblasts from mouse heart tissue. Subsequently, we knocked down IL-17R and gauged its efficiency using qRT-PCR and WB assays. It was observed that both the IL-17R gene and protein levels had declined, showing statistical significance ($p<0.01$) (Figure 6A–C). This result indicated a considerable reduction of IL-17R expression in fibroblasts.

Next, Ang II was utilized to provoke fibroblast fibrosis, and $CD4^+$ T cells were extracted from the mouse peripheral blood. These T cells were then stimulated to differentiate into T_H17 cells and co-cultured with fibroblasts. Compared with the Control group, IL-6, IL-17, and TNF-α levels in the Ang II and Ang II^+T_H17 groups increased significantly. When IL-17R was knocked down, the concentration of pro-inflammatory cells in the Ang $II^+T_H17^+$si-IL-17R group dropped considerably, reaching statistical significance ($p<0.01$) (Figure 6D–F). This finding suggests that IL-17R knockdown can notably ameliorate the inflammatory response in mouse CFs.

Sirius red staining indicated a significant increase in the area of red-stained collagen in the Ang II and Ang II^+T_H17 groups. This increase was substantially reduced post-IL-17R knockdown (Figure 6G–H). This finding proposes that IL-17R knockdown can significantly improve CF fibrosis. Concurrently, immunofluorescence and WB assays confirmed that the fluorescent intensity of α-SMA and Collagen I was elevated in the Ang II and Ang II^+T_H17 groups, decreasing significantly post-IL-17R knockdown (Figure 6I-K). The trends of α-SMA, Collagen I, TIMP-1, and CTGF protein levels corresponded with the results of the immunofluorescence assay, with statistical significance ($p<0.01$) (Figure 6L-P).

In summary, correlating with in vivo experiments, it has been shown that IL-17R knockdown can improve cardiac fibrosis damage by modulating the T_H17 cell immune response both in vivo and in vitro.

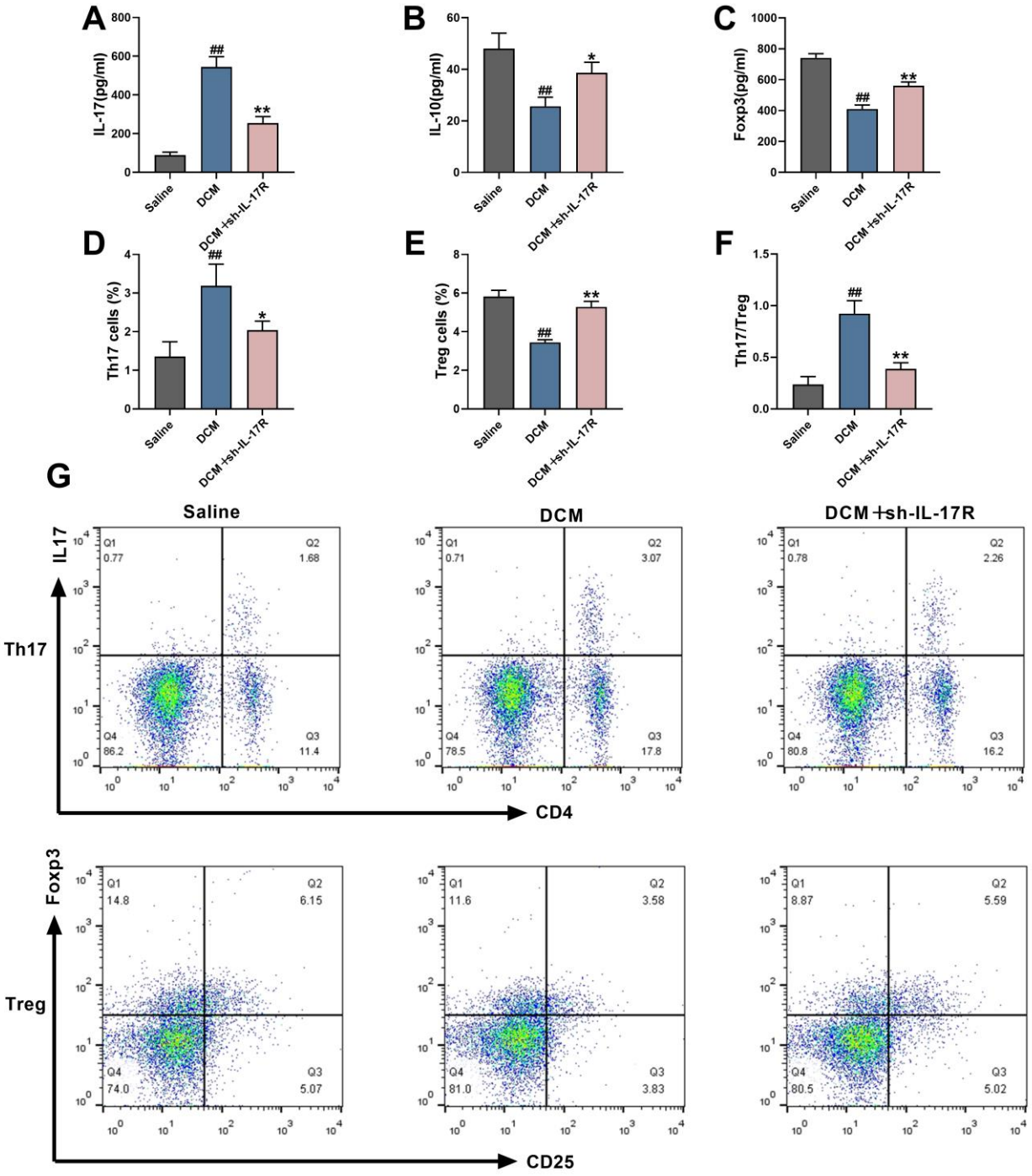
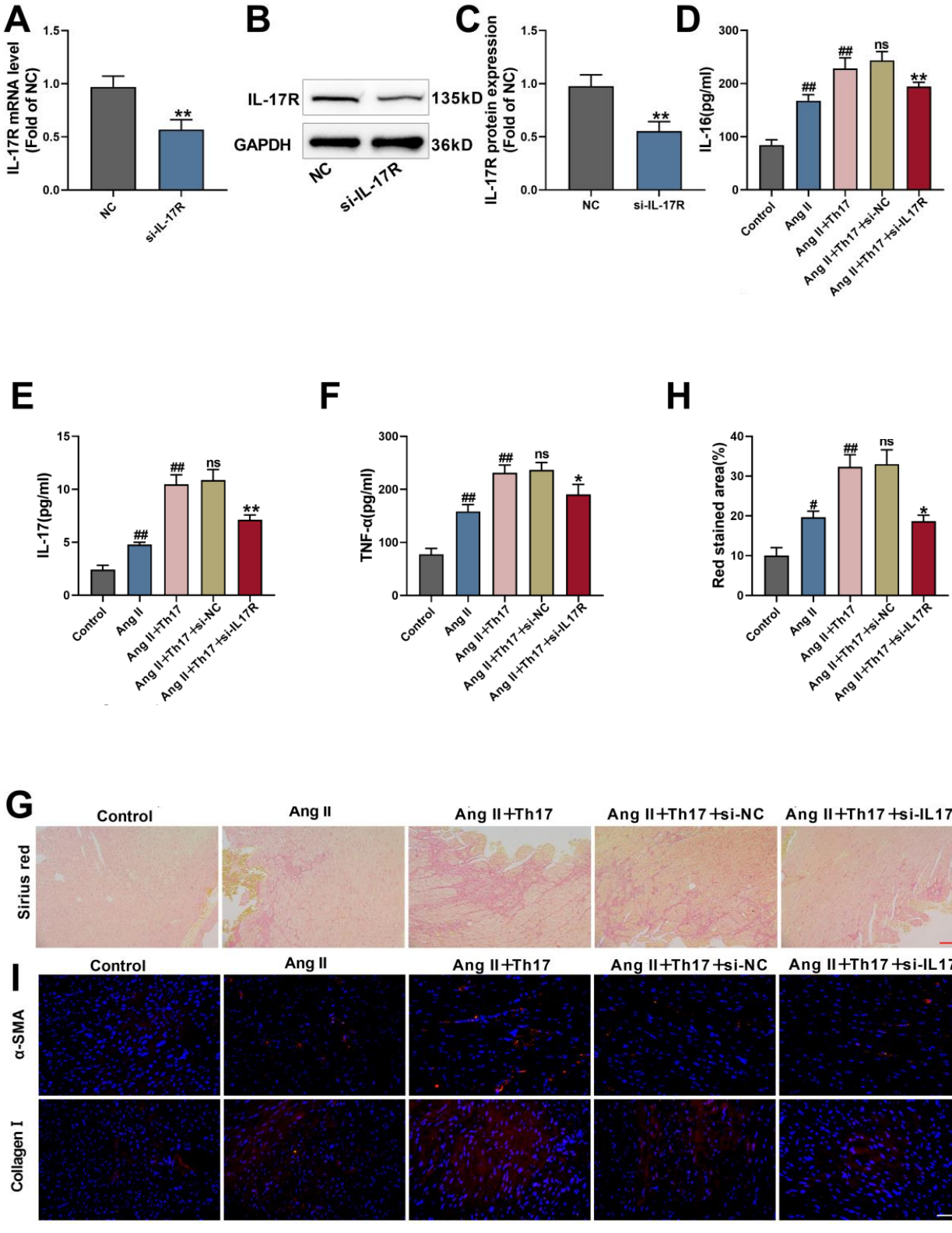


Figure 5. Knockdown of interleukin-17 receptor (IL-17R) improves T Helper 17 (T_H17)/Treg cell imbalance in dilated cardiomyopathy (DCM) mice. A-C: T_H17 and Treg cytokines were detected with an enzyme-linked immunosorbent assay (ELISA) kit. It was found that interleukin (IL-17) in the DCM group was increased, while IL-10 and forkhead box p3 (Foxp3) were considerably decreased. After IL-17R knockdown, IL-17 was decreased, and IL-10 and Foxp3 were increased. D-G: T_H17 and Treg cells in the spleen tissue were tested by flow cytometry. T_H17 cell in DCM mice was raised, Treg cell was declined, and the T_H17/Treg ratio was significantly increased. After IL-17R knockdown, T_H17 cells and the ratio of T_H17/Treg were decreased, and Treg cell was significantly increased. n=6, ##p<0.01 vs Saline group; *p<0.05, **p<0.01 vs DCM group.



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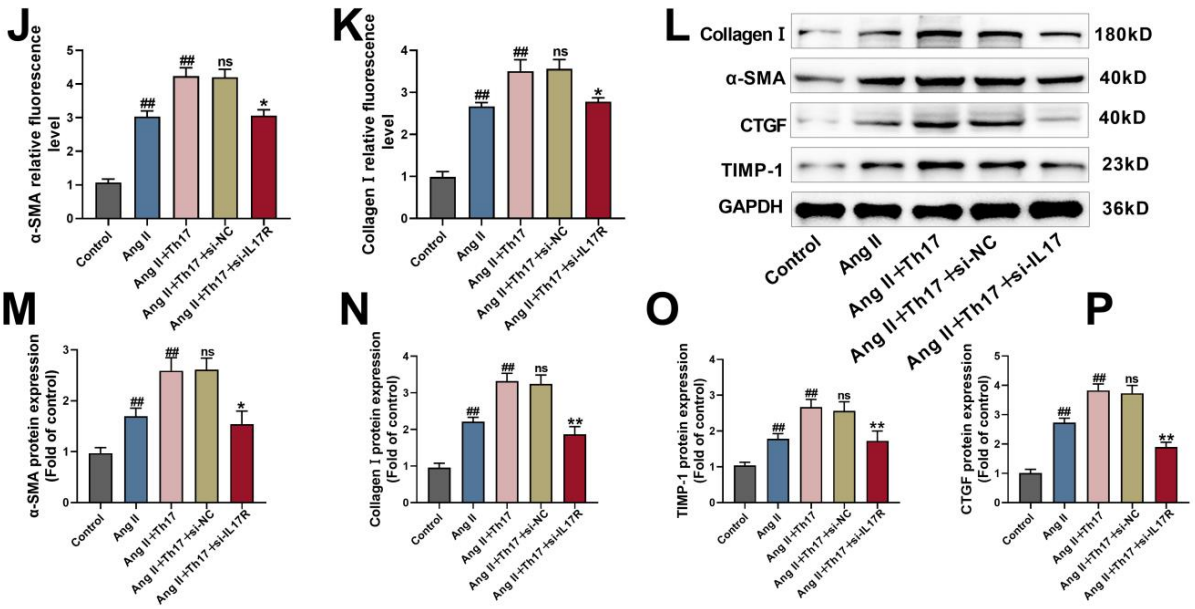

Figure 6. Knockdown of interleukin-17 receptor (IL-17R) inhibited cardiac fibroblasts (CFs) fibrosis in mice. A-C: Fibroblasts were isolated from the heart, and then IL-17R was knocked down. The efficiencies were detected with quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting (WB), and IL-17R was effectively low expressed (p<0.01 vs NC group). D-F: Angiotensin II (Ang II) was used to induce fibroblast fibrosis, and $CD4^+$ T cell was isolated from mice, and then stimulated to develop into T Helper 17 (T_H17). After that, T_H17 cell was co-cultured with fibroblasts, and the inflammatory factor in the co-culture system was detected by an enzyme-linked immunosorbent assay (ELISA) kit. It can be seen that interleukin (IL)-6, IL-17, and tumor necrosis factor-α (TNF-α) contents elevated significantly after fibroblast fibrosis, and decreased significantly after IL-17R knockdown. G-H: Sirius red staining found that the collagen fiber area was enhanced significantly after fibroblast fibrosis, and it decreased significantly after IL-17R knockdown (×20, 100 μm). I-P: alpha-smooth muscle actin (α-SMA), Collagen I, tissue inhibitor of metalloproteinases-1 (TIMP-1), and connective tissue growth factor (CTGF) were significantly increased after fibroblast fibrosis by immunofluorescence and WB, and significantly decreased after IL-17R knockdown (×40, 50 μm). n=6, ##p<0.01 vs Control group; ns$p$>0.05 vs Ang II+T_H17 group; *p<0.05, **p<0.01 vs Ang II+T_H17+si-NC group.**

DISCUSSION

DCM is a complex heart disease characterized by reduced myocardial dilation and systolic function. This condition results in a reduced ejection fraction, increased cardiac filling pressure, and decreased pump output.[24] In clinical settings, echocardiography is typically the first method used for preliminary DCM diagnosis.[25] Moreover, LDH and cTnI are crucial indicators of myocardial cell damage.

In this study, abnormalities were found in the cardiac function indexes of the DCM model mice. Echocardiography indicated a significant decrease in LVEF and FS values while showing substantial elevation in LVEDD and LVEDV values. These results suggest that the cardiac function was impaired and the cardiac ejection functionality was compromised. The elevated serum levels of LDH and cTnI pointed towards high levels of stress, heavy cardiac preload, and severe myocardial injury, confirming the successful preparation of the DCM mouse model. There was a notable increase in IL-17R in DCM mice. However, upon silencing IL-17R, an enhancement of cardiac dysfunction indexes such as LVEF and FS was seen, along with a marked reduction in LVEDD, LVEDV values, LDH, and cTnI levels. These changes imply an improvement in the mice's cardiac function, also establishing preliminary evidence of the protective effect of knocking down IL-17R on myocardial tissue damage.

Ventricular remodeling is a critical pathological alteration in the initiation and advancement of DCM.[26] This remodeling is often due to inflammation or other factors, prompting the ongoing proliferation of fibroblasts and the excessive deposition and remodeling of ECM, resulting in MF. This, in turn, triggers further ventricular remodeling. Therefore, suppressing the MF

process serves as an effective method to mitigate the progression of DCM.

Collagen deposition, particularly Collagen I, is the primary feature of MF as it is the main structural protein that forms fibrous tissue.[27] The activation and proliferation of CFs result in an increase in collagen molecules (α-SMA, Collagen I), which is a key mechanism of MF.[28] CTGF, with its mitogenic and chemotactic properties, can induce fibroblast proliferation, making it vital for the progression of MF. The expression of CTGF, to some extent, reflects the progression of MF. ECM's synthesis and degradation are regulated by vital enzymes, MMPs and TIMPs, and their interaction is intimately related to the dynamic equilibrium of ECM.[29] Simultaneously, it has been verified that tissue fibrosis can be influenced by affecting α-SMA, Collagen I, TIMP-1, and CTGF.[30,31]

The pathological staining results of myocardial tissue in the DCM model mice, constructed in this study, showed a significant reduction in the number of myocardial fibers. In contrast, there was a notable increase in collagen deposition and interstitial fibrosis. Concurrently, fibrosis factors such as α-SMA, Collagen I, TIMP-1, and CTGF were highly expressed in the myocardial tissue, mirroring results reported in previous studies.[32,33] Notably, MF can lead to cardiac dysfunction, with fibrosis factors playing a crucial role. After knocking down IL-17R, there was an effective reduction in myocardial tissue fibrosis in mice and a decrease in fibrosis factors. These findings underscore the mitigation effect of IL-17R knockdown on MF during DCM and provide partial insight into the mechanism of IL-17R knockdown as a potential therapy for DCM.

It is presumed that viral myocarditis can progress to DCM, with myocardial tissue damage resulting from inflammation commonly implicated in DCM's onset.[34] The involvement of immune-mediated inflammatory injury in the occurrence of DCM is established,[35] with a close link existing between immune dysfunction and both DCM and its progression.[24] Both T_H17 and Treg cells evolve from naive $CD4^+$ T cells that recognize antigens and subsequently transform into effector T cells.[36] $CD4^+$ T cells are the primary drivers of cardiac-specific autoimmunity in myocarditis.[37] Foxp3, a crucial transcription factor in Treg cell development, is significant in regulating immune tolerance and preserving homeostasis; it is a key marker of Treg cells.[38] However, during the progression of DCM, if T_H17 is overly activated and Treg balance is not maintained, it can lead to increased myocardial inflammation, tissue damage, and dysfunction.[39] Consequently, maintaining the T_H17/Treg dynamic equilibrium is critical for the progression of DCM.

In this study, the levels of IL-6, IL-17, and TNF-α in DCM model mice were notably increased, while the level of IL-10 significantly decreased. The proportion of Treg cells in spleen tissue markedly diminished, while the proportions of effector $CD4^+$ T cells and T_H17 cells significantly increased. The serum marker for T_H17 cells, IL-17, demonstrated a significant increase, while Treg cell markers IL-10 and Foxp3 noticeably declined. However, when IL-17R was knocked down, the proportions of effector $CD4^+$ T cells and T_H17 cells considerably decreased, while the proportions of naive $CD4^+$ T cells and Treg cells markedly increased, reversing the inflammatory cytokines significantly. The data suggest that the knockdown of IL-17R may inhibit $CD4^+$ T cell activation and T_H17 cell differentiation. Furthermore, the results indicated that an IL-17R knockdown has an inhibitory effect on the inflammatory response during the onset and progression of DCM, and it may alleviate immune dysfunction in DCM by inhibiting $CD4^+$ T cell activation and T_H17 cell differentiation.

Ang II is the principal active substance that regulates the renin-angiotensin system and is also a known important factor causing fibrosis. It has been confirmed to promote CFs activation.[28] Consequently, we used Ang II to induce CFs fibrosis and then co-cultivated it with T_H17 cells. The number of cells and collagen area significantly increased, as did the levels of pro-inflammatory factors (IL-6, IL-17, TNF-α) and fibrosis factors (α-SMA, Collagen I, TIMP-1, CTGF). However, after IL-17R was knocked down, both inflammation and fibrosis were substantially reduced. In combination with in vivo experiments, it was demonstrated that reducing IL-17R had a beneficial therapeutic effect on DCM fibrosis, with the mechanism potentially related to the regulation of the T_H17 cell immune response.

Overall, this study confirmed that the knockdown of IL-17R is vital for myocardial protection and provides a new theoretical foundation for IL-17R to become a potential therapeutic target for MF. However, this study merely conducted a preliminary examination of the improvement action of IL-17R on DCM in animal models. The potential molecular regulation mechanism of IL-17R will be elucidated through gene sequencing and cell experiments. However, this study has certain limitations. In the study, only mouse CFs were used for

the in vitro experiments, and there were no data on human cells, necessitating further in vitro studies to substantiate the results discovered in this investigation. There are also differences between the in vivo and in vitro experimental models and the true physiological and pathological environment of the human body. Therefore, further optimization of the model is required to improve the clinical correlation of the results.

STATEMENT OF ETHICS

This study was approved by the Second Hospital of Hebei Medical University Experimental Animal Ethics Committee (No. 2023-1258).

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

Not applicable

DATA AVAILABILITY

The data for this study can be accessed from the corresponding author upon a reasonable request.

AI ASSISTANCE DISCLOSURE

Not applicable.

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