IL-17 contributes to cardiac fibrosis following experimental autoimmune myocarditis by a PKCβ/Erk1/2/NF-κB-dependent signaling pathway

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Abstract
Myocarditis is a common clinical cardiovascular disease, and some patients progress to dilated cardiomyopathy (DCM) with chronic heart failure. Common viral infections are the most frequent cause of myocarditis, but other pathogens and autoimmune diseases have also been implicated. T_{h17} cells are novel IL-17-producing effector T helper cells that play an important role in the development of autoimmune myocarditis. Furthermore, IL-17 is also important in post-myocarditis cardiac remodeling and progression to DCM. However, the mechanisms whereby IL-17 and IL-17-producing cells promote the progression of cardiac fibrosis remain unclear. We therefore investigated whether IL-17 directly induced cardiac fibrosis in experimental autoimmune myocarditis (EAM) and explored the possible molecular mechanisms. The EAM model was induced and serum IL-17 level was detected by ELISA; western blot, immunofluorescence and sirius red staining were used to analyze the collagen expression. PCR was used to assay the IL-17RA and IL-17RC. The results indicated that IL-17 induced cardiac fibrosis both in vitro and in vivo. The protein kinase C (PKC)δ/Erk1/2/NF-κB (Nuclear Factor κappa B) pathway was involved in the development of myocardial fibrosis and IL-17 contributed to cardiac fibrosis following EAM via this pathway. These results provide the first direct evidence for the involvement of the PKCδ/Erk1/2/NF-κB signaling pathway in IL-17-induced myocardial fibrosis.

Keywords: cardiac fibrosis, experimental autoimmune myocarditis, IL-17, T_{h17}

Introduction
Myocarditis presents with a spectrum of symptoms ranging from mild dyspnea or chest pain that spontaneously resolves without treatment to cardiogenic shock and sudden death. The major long-term consequence is dilated cardiomyopathy (DCM) with chronic heart failure. Common viral infections are the most frequent cause of myocarditis, but other pathogens, hypersensitivity reactions and systemic and autoimmune diseases have also been implicated.

CD4⁺ T cells play an important role in the development of cardiovascular diseases (1–3). Historically, two major CD4⁺ T-cell subsets have been defined according to their cytokine-production patterns: T_{h1} and T_{h2} cells, which produce IFN-γ and IL-4, respectively. A third subset of IL-17-producing effector T helper cells, T_{h17} cells, has recently been discovered and characterized. T_{h17} cells produce IL-17, IL-17F and IL-22, thereby inducing a massive tissue response because of the broad distributions of IL-17 and IL-22 receptors (4). The importance of T_{h17} cells in clearing pathogens during the host defense response and in inducing tissue inflammation in autoimmune disease has been recognized. The results of some studies have indicated that IL-17 and IL-17-producing cells are also important in the formation of liver fibrosis (5) and pulmonary fibrosis (6) and that IL-17 plays a critical role in post-myocarditis cardiac remodeling and the progression to DCM (7). However, the significance of IL-17 and IL-17-producing cells in promoting the progression of DCM remains unclear.

Our previous studies and other reports suggest that T_{h17} cells contribute to the development of autoimmune myocarditis (2, 8, 9). Experimental autoimmune myocarditis (EAM) is a mouse model of post-infectious cardiomyopathy that can be induced by inoculation with the Coxsackie B virus or cardiac myosin (10, 11). Both these replicate CD4⁺ T-cell mediated autoimmune diseases in BALB/c
mice, which are characterized by infiltration of inflammatory cells into the myocardium (12), followed by myocyte fibrosis, edema and necrosis, leading to ventricular wall dysfunction and heart failure (13).

Autoimmune myocarditis can progress to DCM. T_h17 cells contribute to the pathogenesis of autoimmune myocarditis, and IL-17 is involved in the formation of liver and pulmonary fibrosis. The present study therefore aimed to clarify if EAM could progress to cardiac fibrosis, to verify whether cardiac fibrosis can be directly induced by IL-17, and to identify the signaling pathway involved in the formation of cardiac fibrosis.

Materials and methods

Materials

Recombinant murine IL-17A (catalog no. 210-17) was purchased from PeproTech. Anti-col3A1 (catalog no. sc-28888), anti-col1A1 (catalog no. sc-8784), anti-β-actin (catalog no. sc-47778) antibodies and protein kinase C (PKC)β inhibitor (catalog no. sc-204199) and anti-PKCβ (catalog no.sc-210) were all obtained from Santa Cruz. Anti-phosphorylated PKCβII (catalog no. 2252-1) and anti-phosphorylated p65 (catalog no. 1546-1) were purchased from Epitomics. Anti-phosphorylated Erk1/2 (catalog no. 9101S) was purchased from Cell Signaling Technology. Hoechst 33342 was purchased from Sigma–Aldrich. Type II collagenase and TRIzol reagent were purchased from Invitrogen Life Technologies.

Mice

Male BALB/c mice, 5–6 weeks old, were purchased from the Animal Center of Yangzhou University and maintained in the animal center of Jiangsu University. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Jiangsu University.

Induction of EAM and treatment

Cardiac myosin peptide MyHC-Induction of EAM and treatment (Ac-SLKLMATLF-STYASAD-OH) was synthesized by TASH Biotechnology Company and dissolved in a solution of NH_3H_2O at a concentration of 10 mg ml\(^{-1}\). To produce EAM, each mouse was immunized with 100 μg/0.2 ml of an emulsion containing cardiac myosin with an equal volume of CFA by subcutaneous injection on days 0 and 7. Control mice were immunized with PBS/CFA (14).

A cell-permeable pharmacologic inhibitor, sc-204199, was used to assess the roles of PKCβ in EAM model. The EAM mice were injected with 10 μg inhibitor (intravenous) or same volume dimethyl sulfoxide (DMSO) every other day from day 15 to 35.

Enzyme-linked immunosorbent assay

IL-17 levels in serum were measured by ELISA (eBioscience) according to the manufacturer’s guidelines. Recombinant cytokine standards were used to assess quantities using a standard curve, with optical densities acquired at 450 nm using an ELISA reader.

Culture of cardiac fibroblasts and treatment

Cardiac fibroblasts were isolated from 2- to 3-week-old BALB/c mice. Hearts were removed under sterile conditions and cut into pieces and digested with 0.2% type II collagenase/0.1% trypsin at 37°C for 1 h, until the tissue blocks disappeared. Dissociated cells were then centrifuged at 350 \( \times \) g, resuspended in DMEM supplemented with 10% newborn calf serum (NBCS), 2 mM glutamine, 100 U ml\(^{-1}\) penicillin and 100 μg ml\(^{-1}\) streptomycin and plated in cell culture dishes. After 1 h in culture, non-adherent cells were removed and adherent cells were cultivated in DMEM supplemented with 10% NBCS. The cultures were maintained at 37°C in 5% CO\(_2\), and the medium was replaced every 2 days. When the cells approached confluence, they were passaged after 1:3 dilution with fresh medium. Fibroblasts from passages 3 to 5 were used in each experiment. The cells were morphologically homogeneous with typical bipolar configuration observed by inverse microscopy.

RNA extraction and reverse transcription–PCR amplification

To examine the expression of IL-17RA and IL-17RC in cardiac fibroblasts, total RNA was isolated using TRIzol reagent and cDNA was synthesized from 2 μg of total RNA with oligo dT and murine Moloney leukemia virus reverse transcriptase. The mouse primers were IL-17RA forward, 5′-AGTGTTCCTCTGAGCTTAC-3′; reverse 5′-GAAAACCCTGACCCCTTAC-3′ and IL-17RC forward, 5′-AACAGCCACTGCTCTGGGAGC-3′; reverse, 5′-TCGGCCAGTGCAGAAGCAGCA-3′.

Tissue preparation and staining

Mice were sacrificed on days 21, 35 and 54, respectively, after the first injection. Hearts were fixed in 4% PFA and embedded in paraffin. The blocks were cut into 4-μm slices, heated for 3 h at 37°C in an incubator and then dewaxed and stained with hematoxylin and eosin. One slice from each mouse was analyzed under a microscope.

Immunofluorescence staining

Immunofluorescence staining of cardiac fibroblasts plated in 24-well cell culture flasks was performed as follows: after stimulation with rIL-17 for different lengths of time, medium was aspirated from the plates and cells were washed twice with PBS. Fibroblasts were fixed with 4% PFA solubilized in PBS/0.1% Triton-X100 for 30 min at room temperature and then blocked for 1 h with PBS/1% BSA. The first antibody was applied for 2 h at room temperature, followed by addition of the PE-labeled second antibody for 90 min. Finally, the cells were stained with Hoechst 33342 for 10 min.

All the sections were cover slipped with vectashield mounting medium, viewed under an Olympus fluorescence microscope and analyzed using Image J software.

Sirius red staining

Paraffin sections of hearts were dewaxed and put into ethanol with different concentration, followed by double-distilled H\(_2\)O for three washes. Samples were then put into saturated picric acid sirius red liquid for 60 min and dehydrated with
ethanol. One sample from each mouse was analyzed under a microscope.

Western blot analysis
Cardiac fibroblast lysates were electrophoresed on SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Perkin Elmer, USA). Membranes were blocked with 5% (w/v) BSA in TBST for 1 h at room temperature and incubated overnight with primary antibodies at 4°C, followed by HRP-conjugated second antibodies. The immunoreactive bands were detected by chemiluminescence (ECL Plus, Millipore) and relevant blots were quantified by densitometry using the accompanying computerized image analysis program (Amercontrol Biosciences, USA).
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Statistical analysis

Statistical analysis was performed using Prism5 (GraphPad Software). All experiments were performed at least in triplicate. Statistical differences between groups were compared by one-way analysis of variance, followed by Dunnett’s multiple comparison test to identify significantly different results. Values of $P < 0.05$ were considered to be statistically significant.

Fig. 2. IL-17 promoted collagen types I and III expression in vitro. (A) IL-17RA and IL-17RC express in mouse cardiac fibroblasts. M: DL2000 Marker, lane 1: IL-17RC, lane 2: IL-17RA and lane 3: NTC (no-template control, i.e. blank). (B) Immunofluorescence staining of collagen types I and III in cardiac fibroblasts treated with IL-17 (100 ng ml$^{-1}$) for 24, 48 or 72 h. Images were analyzed using Image J software and all values were tested by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant. (C) Western blot analysis of collagen types I and III levels in cardiac fibroblasts treated with IL-17 (100 ng ml$^{-1}$) for 24, 48 or 72 h. $\beta$-Actin served as a loading control. Densitometric analysis blots. Data are given as means ± SD from three independent experiments. All values were tested by one-way ANOVA. $P < 0.05$ was considered statistically significant.
Results

**Myocarditis progressed to myocardial fibrosis in EAM model**

EAM was induced by MyHC-α614-629. Serum IL-17 levels on day 21 were 623.37 ± 53.08 pg ml⁻¹ in EAM mice versus 97.34 ± 19.37 pg ml⁻¹ in control mice (P < 0.05, Fig. 1A). Lymphocyte infiltration was significantly increased in EAM mice in accordance with the results of previous studies (9, 15). Fibroblast proliferation occurred and collagen was deposited in myocardium on day 54 (Fig. 1B). Sirius red staining showed that cardiac fibrosis was significantly increased in EAM mice compared with the control group (P < 0.001, Fig. 1C).

**IL-17RA and IL-17RC expression in cardiac fibroblasts and IL-17 treatment in vitro**

Cardiac fibroblasts were isolated from BALB/c mice. PCR confirmed IL-17RA and IL-17RC expression in the cells (Fig. 2A). To address the effect of IL-17 on cardiac fibrosis, cardiac fibroblasts were treated with 100 ng ml⁻¹ rIL-17 for 24, 48 or 72 h in vitro. Immunofluorescence staining showed that the expression levels of collagen types I and III were significantly increased at 24 h compared with controls (P < 0.05, Fig. 2B). These results were confirmed by western blotting, which demonstrated increased expression of collagen types I and III at 24 h compared with control cells (P < 0.05, Fig. 2C).

**IL-17 contributed to cardiac fibrosis via the PKCβ/Erk1/2/NF-κB-dependent pathway**

High glucose has been reported to promote the production of extracellular matrix via PKC (16), which may play an important role as a modulator of fibroblast proliferation in patients with diabetes (17). In the current study, we assessed if IL-17 contributed to cardiac fibrosis via the PKCβ/Erk1/2/Nuclear Factor κappa B (NF-κB)-dependent pathway. As shown in Fig. 4A, IL-17 increased PKCβ phosphorylation (upper panel, Fig. 3A) in fibroblasts and altered the phosphorylation status of Erk1/2 (upper panel, Fig. 3B). In addition, IL-17 also activated NF-κB in fibroblasts, as indicated by increased expression of p65 (a subunit of NF-κB) (upper panel, Fig. 3C).

**Inhibition of PKCβ ameliorated IL-17-induced cardiac fibrosis**

We demonstrated that IL-17 induced cardiac fibrosis via a PKCβ/Erk1/2/NF-κB-dependent pathway. To confirm if targeting PKCβ activation inhibited IL-17-induced cardiac fibrosis, cardiac fibroblasts were exposed to the PKCβ inhibitor sc-204199 10 μM for 2 h, followed by treatment with IL-17 for 24 h. We found PKCβ inhibitor sc-204199 notably attenuated PKCβ expression via western blotting (Fig. 4A). Then immunofluorescence staining showed that IL-17-induced collagen types I and III expression levels were significantly attenuated by PKCβ inhibition (Fig. 4B) compared with the untreated group (P < 0.05). These results were confirmed by the results of western blotting (Fig. 4C).

In order to confirm the above data in vivo, the mice were injected with 10 μg of inhibitor (intravenous) or DMSO on alternate days from day 15 to 35. As shown in
some reports have indicated that IL-6 and transforming cells secrete IL-17, conflicting results have been published regarding the role of this cytokine in various models of autoimmune disease. In our model, however, Th17 cells were shown to promote the development of myocardial fibrosis and cardiac remodeling leading to pulmonary and liver fibrosis (6, 26). The results of the current study demonstrate a critical role for IL-17 in the development of myocardial fibrosis and cardiac remodeling leading to end-stage DCM, in accordance with the results of Balde et al. (6). The PKCβ1 isoform in the heart, aorta and retina and of growth factor-β, has been reported in pathological conditions, including in diseased hearts removed from cardiac transplant recipients (28). Preferential activation of the PKCβ pathway in IL-17-induced myocardial fibrosis.

Fig. 4. Inhibition of PKCβ ameliorated collagen deposition in the myocardium. (A) PKCβ was inactivated by inhibitor sc-204199 (10 μM in DMSO) for 2 h and then t-PKCβ expression was detected by western blotting. (B) Cardiac fibroblasts were treated with inhibitor for 2 h followed by IL-17 and DMSO solvent control from days 15 through 35 by intravenous injection and sacrificed. (A) Hematoxylin and eosin staining showed fibroblast proliferation in treatment group significantly decreased compared with the untreated (Fig. 5A). Sirius red staining also was determined as the percent area fraction (%AF) of sirius red staining. Six mice were included in each group.
cells secrete IL-17, conflicting results have been published regarding the role of this cytokine in various models of autoimmune inflammation (4, 19–21). We and others have previously identified an important role for IL-17 in the pathogenesis of autoimmune myocarditis (22). However, although some reports have indicated that IL-6 and transforming growth factor-β, both important proinflammatory factors in autoimmune myocarditis, are involved in myocardial fibrosis (23–25), the direct involvement of IL-17 remains unclear. Published data suggest that IL-17 contributes to pulmonary and liver fibrosis (6, 26). The results of the current study demonstrate a critical role for IL-17 in the development of myocardial fibrosis and cardiac remodeling leading to end-stage DCM, in accordance with the results of Baldeviano et al. (7).

We also investigated the mechanism whereby IL-17 leads to myocardial fibrosis. Previous studies have indicated that activation of PKC is an important intracellular signaling pathway for modulating cardiac myocyte development, inotropic functions and cellular growth (27). PKC is composed of a family of serine–threonine kinases whose isoform-specific functional roles have not been clearly identified in vivo. PKC activation, in particular PKCβ, has been reported in pathological conditions, including in diseased hearts removed from cardiac transplant recipients (28). Preferential activation of the PKCβ2 isoform in the heart, aorta and retina and of the PKCβ isoform in the renal glomeruli has also been observed in diabetic animals (29, 30). Some reports have indicated that the IL-17 receptor is constitutively expressed on cardiac fibroblasts. As there is evidence that a functional response to IL-17 requires expression of a heteromeric complex of both IL-17RA and IL-17RC (31–33), we established that both IL-17RA and IL-17RC were expressed by isolated cardiac fibroblasts. We furthermore provided, by using a pharmacological block, evidence of a pivotal role for PKCβ in IL-17-mediated cardiac fibrosis. Erk1/2 and NF-κB were also shown to be involved in myocardial fibrosis. We therefore suggest that myocardial fibrosis induced by IL-17 is dependent on the PKCβ/Erk1/2/NF-κB signaling pathway. To the best of our knowledge, these results provide the first direct evidence for the involvement of the PKCβ/Erk1/2/NF-κB signaling pathway in IL-17-induced myocardial fibrosis. However, other signaling molecules may also be involved in IL-17-mediated cardiac fibrosis. Using an isoproterenol-induced heart failure model, Feng et al. (34) showed that IL-17 depended on the receptor activator of NF-κB ligand/osteoprotegerin system to induce matrix metalloproteinase (MMP)-1 production, which is another important index of fibrosis. IL-17 stimulated MMP-1 expression also via p38 MAPK- and ERK1/2-dependent C/EBPβ, NF-κB and AP-1 activation.

In addition, although Th17 cells are characterized by the production of IL-17, recent studies have shown that T cells expressing the γδTCR also produce IL-17. Wilson et al. (6) suggested that bleomycin-mediated pulmonary fibrosis was highly dependent on IL-17 and that IL-17-producing T cells included CD4+IL-17A+ T cells and γδTCR+IL-17A+ T cells. In our model, however, Th17 cells were shown to promote the

![Fig. 5](image.png)

Fig. 5. The PKCβ inhibitor ameliorated cardiac fibrosis in mice with established myocarditis. WT BALB/c mice (EAM, day 14) were treated with PKCβ inhibitor sc-204199 and DMSO solvent control from days 15 through 35 by intravenous injection and sacrificed. (A) Hematoxylin and eosin-stained sections. (B) Sirius red staining was used to detect collagen deposition in the myocardium. Collagen deposition was determined as the percent area fraction (%AF) of sirius red staining. Six mice were included in each group. *P < 0.05 was considered to be statistically significant by one-way analysis of variance with Bonferroni correction.

Discussion

Th17 cells are implicated in the pathogenesis of autoimmune diseases in mice, as well as humans (4, 18). Although Th17 cells secrete IL-17, conflicting results have been published regarding the role of this cytokine in various models of autoimmune inflammation (4, 19–21). We and others have previously identified an important role for IL-17 in the pathogenesis of autoimmune myocarditis (22). However, although some reports have indicated that IL-6 and transforming growth factor-β, both important proinflammatory factors in autoimmune myocarditis, are involved in myocardial fibrosis (23–25), the direct involvement of IL-17 remains unclear. Published data suggest that IL-17 contributes to pulmonary and liver fibrosis (6, 26). The results of the current study demonstrate a critical role for IL-17 in the development of myocardial fibrosis and cardiac remodeling leading to end-stage DCM, in accordance with the results of Baldeviano et al. (7).

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development of autoimmune myocarditis, and the IL-17 production in myocarditis and fibrosis appears to originate mainly from T<sub>h</sub>17 cells.

In contrast to other autoimmune diseases such as rheumatoid arthritis, there are currently no immunomodulatory therapies for autoimmune myocarditis. Myocarditis in humans is highly heterogeneous in etiology (2, 35); however, recent reports indicate that a subgroup of myocarditis patients may benefit from immune-targeted therapies (36). The results of the current study also suggest that treatment with IL-17-antagonists or PKC<sub>i</sub> inhibitors might be able to prevent or slow the progression to DCM in patients with established myocarditis.

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