Supplementary information

Cytokine receptor-like factor 1 (CRLF1) promotes cardiac fibrosis via ERK1/2 signaling pathway

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Materials and methods

Animals

Healthy C57BL/6 mice (8 weeks old, male, 20–25 g) were bought from Gempharmatech (Nanjing, China) and housed with enough animal care. The procedures for myocardial infarction (MI) establishment were the same as described previously (Sun et al., 2019). The cardiac fibrosis models were obtained by ligating the left anterior descending coronary artery for 4 weeks, while the sham operated mice were carried out the same experimental procedures without ligation.

Masson's trichrome

Mice were anesthetized and sacrificed, then the hearts were taken out from the chest. Hearts were fixed for 24 h by using 4% paraformaldehyde. After excess paraformaldehyde was flushed, tissues were embedded with paraffin, then cut into cross-sectional slices with 5 μm thickness. According to the protocol of the commercial Masson's Trichrome Staining Kit (Solarbio, Beijing, China), the slices were stained. Blue color represented fibrotic area while red color suggested that myocardium was stained. The degree of cardiac fibrosis was calculated and analyzed with Image-Pro Plus (Media Cybernetics, Bethesda, USA).

Cell culture

Neonatal mice cardiac fibroblasts (NMCFs) were separated, extracted, and cultured as previously reported (Li et al., 2019; Luo et al., 2021). To be brief, healthy neonatal (within 3 d post-birth) C57BL/6 mice were purchased from the animal center of southern medical university. After sterilization, the hearts were taken out and the remaining blood in the heart cavity was washed with phosphate-buffered solution (PBS; Gibco, Waltham, MA, USA). Hearts were further cut into chunks and digested with trypsin-EDTA solution (Gibco) and terminated with Dulbecco's modified Eagle's medium (DMEM; Gibco) with 10% (v/v) fetal bovine serum (FBS; ExCell, Shanghai, China). After filtration and centrifugation, cells were resuspended and plated in culture plates with DMEM containing 10% (v/v) FBS and penicillin (100 U/mL)/streptomycin (0.1 mg/mL) (Gibco). Because of the preferential attachment of cardiac fibroblasts (Zhou et al., 2017; Zhang et al., 2018; Li et al., 2020), the cell resuspension was planked onto a culture flask for 90 min, which allows NMCFs to plank on the bottom. The weakly attached cells, mainly NMCMs, were removed and seeded into another plank. In addition, human cardiac fibroblasts (HCFs), endothelial cells (ECs), and adult mouse cardiac fibroblasts (AMCFs) were purchased from Bena Biology (Beijing, China), these cells were also cultured in the same conditions with NMCFs and NMCMs.

Plasmids and small interfering RNAs (siRNAs)

The plasmids of cytokine receptor like f actor 1 (CRLF1) overexpression were constructed by Generay (Shanghai, China) by using pcDNA3.1(+) carrying CRLF1 sequence. The empty pcDNA3.1(+) vector was used as a negative control. The siRNAs and scramble sequences (negative control, NC) were designed and synthesized by RiboBio (Guangzhou, China). The sequences of siRNAs were listed as follows: siRNA-1 sequences, GGCTCAAGAAGCACGCAT; siRNA-2 sequences, GATCTGATGTCCTCACACT; siRNA-3 sequences, TCCTCTTCCAAGCC AAGTA. The scramble sequences (used as negative control) were also purchased from RiboBio.

In vitro experiments

NMCFs were transfected with siRNAs (100 nmol/L) or overexpression plasmids (100 ng/mL) separately using X-treme Gene siRNA transfection reagent (Roche, Basel, Switzerland) and Advanced transfection reagent (Zeta Life, Menlo Park, CA, USA) with serum-free DMEM while the medium was replaced with DMEM containing 10% (v/v) FBS at 6 h after transfection. For TGF-β1 treatment, NMCFs and HCFs were treated with mouse TGF-β1 (20 ng/mL; MCE, New Jersey, USA) and human TGF-β1 (20 ng/mL; MCE) respectively. For TGF-β1 and plasmids or siRNAs co-treatment, TGF-β1 was used at 6 h after transfection when the medium with DMEM containing 10% (v/v) FBS was changed. For the interventions with different inhibitors, SIS3 (1 μmol/L; Topscience, Shanghai, China), Temuterkib (1 μmol/L; MCE), SP600125 (25 μmol/L; MCE), SB203580 (10 μmol/L; MCE), Y-27632 (10 μmol/L; MCE), and LY294002 (10 μmol/L; MCE) were respectively for SMAD3 (Tu et al., 2011; Sundararaj et al., 2016; Meng et al., 2020; Shen et al., 2020), ERK1/2 (Wang et al., 2019; Wang et al., 2021), JNK (Singh et al., 2021), p38

MAPK (Stratton et al., 2019), ROCK (Childers et al., 2019), and PI3K (Lei et al., 2015; Yu et al., 2020) inhibition in NMCFs. Cell samples were collected in 48 h (for proteins) or 24 h (for RNAs). All procedures were in accordance with the manufacturer's instructions.

Cell viability assay

NMCFs were cultured in 96-well plate followed by the corresponding treatments. In the end, cell viability was measured through cell counting kit-8 (CCK-8; Beyotime, Shanghai, China). The absorbance value of each well was measured by SpectraMax i3x (Molecular Devices, San Jose, CA, USA) at 450 nm.

Measurement of collagen content

Soluble collagen contents were determined by Sircol Soluble Collagen Assay (Biocolor, Carrickfergus, the United Kingdom) through using the same procedures as previously reported (Chu et al., 2012; Qu et al., 2017). The total protein contents were determined by Pierce™ BCA Protein Assay Kit (Thermofisher Scientific, Carlsbad, USA) according to the protocol. Then, soluble collagen contents were standardized by total protein contents. The absorbance at 540 nm (collagen) or 562 nm (protein) were measured by SpectraMax i3x (Molecular Devices).

5-ethynyl-2'-deoxyuridine (EdU) staining

The proliferation ability of neonatal mice cardiac fibroblasts was detected by the Cell-Light EdU Apollo488 In Vitro Kit (RiboBio). Briefly, the proliferating cardiac fibroblasts were labeled with EdU staining. Total number of neonatal mice cardiac fibroblasts in the field of vision was determined with Hoechst 33342. The samples were observed under IX73 inverted fluorescence microscope (Olympus, Tokyo, Japan). The proliferation ability of neonatal mice cardiac fibroblasts was determined by the ratio of EdU positive cells to Hoechst 33342 positive cells.

Enzyme-linked immunosorbent assay (ELISA)

For detecting CRLF1 level in cell supernatant, Mouse CRLF1 ELISA Kit was bought from Signalway Antibody (SAB; Maryland, USA). For detecting intracellular phospho-ERK1/2 (p-ERK1/2) level, Mouse pERK1/2 ELISA Kit (mlbio, Shanghai, China) was bought. NMCFs were cultured in plates followed by the corresponding treatments. According to the manufacturer's instructions, CRLF1 and p-ERK1/2 level were measured. The absorbance was detected by SpectraMax i3x (Molecular Devices) at 450 nm.

Amplified luminescent proximity homogenous assay-linked immunosorbent assay (AlphaLISA)

AlphaLISA SureFire Ultra p-ERK1/2 (Thr202/Tyr204) Assay Kit (PerkinElmer, Waltham, Massachusetts, USA) and AlphaLISA SureFire Ultra Total ERK1/2 Assay Kit (PerkinElmer) were separately applied to detect p-ERK1/2 and total-ERK1/2 (t-ERK1/2) proteins in cytoplasm. According to its respective groups, NMCFs were seeded to 96-well plate and treated. For surefire

assay, experiments were performed according to the manufacturer's instructions. In brief, culture medium was removed from wells, and freshly prepared 50 μL lysis buffer was added to wells. Then, the plate was shaken for 10 min at room temperature. A total of 10 μL cell lysates were transferred to the new plate (PerkinElmer) and incubated with acceptor mix for 1 h at room temperature, followed by addition of donor mix under subdued light. After incubation for 1 h at room temperature in the dark, the alpha signal was measured using plate reader with standard AlphaLISA settings.

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qRT-PCR)

Total RNA samples were collected from NMCFs, NMCMs, AMCFs, ECs, HCFs, and murine hearts using AG RNAex Pro Reagent (Accurate Biology, Changsha, China). By the way, total RNA samples of hearts were extracted from left ventricular peri-infarct region (1–2 mm area between the infarct region and normal tissue) and corresponding regions of sham-operated hearts. Total RNA was converted to cDNA through Evo M-MLV RT Premix for qPCR (Accurate Biology). The mRNAs expression levels of were detected by Hieff® qPCR SYBR Green Master Mix (YEASEN, Shanghai, China) on QuantStudio™ 6 Flex instrument (Applied Biosystems, Waltham, MA, USA). By using the $2^{-\Delta\Delta CT}$ method, the relative quantitative expression was determined. β-actin was used as an internal control. The specific primers were designed and synthesized by Generay as follows (Table S1).

Table S1 Primer sequences designed for qRT-PCR

Primer	Sequences (5'–3')	Species
CRLF1-F	CCACCCTTCTCATCGGCTC	Mouse
CRLF1-R	TGCCTGGACCCATTAAGGTTAG	Mouse
CRLF1-F	CCCAGAGAAACCCGTCAACA	Human
CRLF1-R	TGTGTTGTCCTGGCCATACC	Human
Col-1A1-F	AAGAAGACATCCCTGAAGTCA	Mouse
Col-1A1-R	TTGTGGCAGATACAGATCAAG	Mouse
Col-1A2-F	GCCCTTCTGGTCCTATTGG	Mouse
Col-1A2-R	CTACCAGTGTTGCCAGTGTC	Mouse
Col-3A1-F	CCCCTGGTTCTTCTGGACAT	Mouse
Col-3A1-R	CCTGACTCTCCATCCTTTCCA	Mouse
α-SMA-F	GTCCCAGACATCAGGGAGTAA	Mouse
α-SMA-R	TCGGATACTTCAGCGTCAGGA	Mouse
β-actin-F	ACTGCCGCATCCTCTTCCT	Mouse
β-actin-R	TCAACGTCACACACTTCATGATGGA	Mouse
β-actin-F	CTCGCCTTTGCCGATCC	Human
β-actin-R	GGGGTACTTCAGGGTGAGGA	Human

Western blotting

Total proteins were extracted from NMCFs, HCFs, and murine hearts with RIPA lysis buffer (Beyotime). Same as extraction method of total RNA samples, total proteins of hearts were also extracted from left ventricular peri-infarct region (1–2 mm area between the infarct region and normal tissue) and corresponding regions of sham-operated hearts. SDS-PAGE (Epizyme, Shanghai, China) was used to separate proteins at 110 V, and proteins were subsequently transferred onto the Amersham™ Protran™ 0.45 nitrocellulose membrane (Cytiva, New York, USA). Then, membranes were blocked with 5% (w/v) skim milk for 1 hour, incubated with the primary antibodies overnight at 4 °C, and conjugated with a Goat anti-rabbit or anti-mouse antibody (LI-COR, Lincoln, USA). Protein levels were detected by the Odyssey infrared scanning system (LI-COR) and quantified with β-actin. The primary antibodies were used as follows: β-actin mAb (Zsbio, Beijing, China), Col-1 antibody (Proteintech, Wuhan, China), Col-3 antibody (Proteintech), α-SMA antibody (Proteintech), CRLF1 antibody (Bioss, Beijing, China), p-ERK1/2 antibody (Proteintech), and t-ERK1/2 antibody (Proteintech). All antibodies were diluted and used according to the instructions in the manual.

Statistical analysis

All data were displayed as the mean±SD. After passing normality tests, the student *t*-test was used for comparisons between two groups and the analysis of variance (ANOVA) was employed for comparisons of multiple groups by GraphPad Prism software (GraphPad Inc., San Diego, CA, USA). A value with $P<0.05$ was considered statistically significant.

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