TLR2 activates AP-1 to facilitate CTGF transcription and stimulate doxorubicin-induced myocardial injury

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Abstract

Objective: Our study aimed to explore the mechanism network that TLR2/AP-1 combined with SOX10 to activate the MAPK pathway via CTGF in Dox-induced myocardial injury. Methods: Rats with Dox-induced myocardial injury were treated with a TLR2 inhibitor or CTGF silencing lentiviral vector. H9c2 cells were treated with genetic vectors or MAPK pathway activators. Cardiac function was tested using echocardiography and serum markers. H&E, sirius red, and TUNEL staining were used to detect myocardial pathological changes, collagen accumulation, and apoptosis. Western blot was used to detect proteins related to cardiac hypertrophy, fibrosis, apoptosis, and MAPK pathway. H9c2 cell injury was assessed by testing cell viability, LDH release, and mitochondrial membrane potential. Results: TLR2 and CTGF were highly expressed in patients with heart failure, and Dox treatment further increased their expression. Inhibiting TLR2 or silencing CTGF improved cardiac function and reduced myocardial fibrosis and apoptosis in Dox-treated rats. Silencing TLR2 alleviated Dox-induced H9c2 cell injury, which was nullified by CTGF overexpression. TLR2 activated AP-1, which cooperated with SOX10 to promote CTGF transcription. MAPK activation aggravated H9c2 cells against Dox-induced injury. Conclusions: TLR2 activates AP-1 which cooperates with SOX10 to promote CTGF transcription and subsequently activate the MAPK pathway, thereby stimulating Dox-induced myocardial injury.

1. Introduction

Doxorubicin (Dox) is a Streptomyces-derived anthracycline antibiotic that is commonly used as a chemotherapeutic drug to treat various malignancies, such as leukemia (Tharkar-Promod et al., 2018), solid tumors (Shafei et al., 2017), and soft-tissue sarcoma (Hartmann et al., 2020). However, the dose-dependent toxicity of Dox to myocardial cells has long been a problem of great concern (Liu et al., 2020). Heart failure (HF) can be induced when the cumulative dose of Dox exceeds 400 mg/m^2 in adults and 300 mg/m^2 in children (Sallustio & Boddy, 2021). This cardiotoxicity can occur at any stage of treatment, which severely limits the clinical application of Dox. Since there is no effective therapy targeting Dox-induced cardiotoxicity, it is still an urgent need to seek effective treatment targets to alleviate Dox-induced cardiac injury.

Multiple mechanisms are involved in Dox-induced cardiotoxicity, including oxidative stress, DNA/RNA damage, autophagy, apoptosis, and fibrosis (Rawat et al., 2021). Toll-like receptors (TLRs) are well-known pattern recognition receptors expressed on a variety of cells (Pahlavanneshan et al., 2021). In recent years, TLRs have been recognized to take a key role in cardiometabolic diseases (Xu et al., 2019). Our earlier findings have shown that TLR2 mRNA and protein levels in peripheral blood mononuclear cells (PBMCs) as well as serum TLR2 expression are upregulated in patients with Dox-induced cardiac dysfunction (Shao et al., 2018)).

Activation of TLR2 is largely dependent on downstream adapter myeloid differentiation primary response protein 88 (MyD88) (Dutta et al., 2021), which subsequently activates nuclear factor-κB (NF-κB) and

activator protein-1 (AP-1) transcription factors (McKiel & Fitzpatrick, 2018). AP-1 is a collection of basic leucine zipper domain-containing transcription factors that comprise Jun, Fos, ATF, and Maf subfamilies (Wu et al., 2021). A variety of extracellular stimuli trigger c-Jun activity mostly through c-Jun N-terminal kinase (JNK) of the mitogen-activated protein kinase (MAPK) pathway, resulting in homodimerization of c-Jun or heterodimerization with c-Fos (Papavassiliou & Musti, 2020). The latest research has shown that the phosphorylation of JNK is promoted in Dox-induced cardiac injury (Li et al., 2022; Zhang et al., 2022).

TLR2 activates JNK/c-Jun to catalyze the transcription of connective tissue growth factor (CTGF) in lung fibroblasts (Lee et al., 2019). CTGF is a secreted glycoprotein produced by various cell types to interact with various regulatory modulators and thereby mediates cellular responses that are associated with fibrosis and tumorigenesis (Richeldi et al., 2020). Dox treatment increases the cardiac level of CTGF (Hullin et al., 2018). Moreover, our previous research has demonstrated that silencing CTGF suppresses myocardial fibrosis and left ventricular hypertrophy in a rat model of dilated cardiomyopathy (DCM) through blockade of the MAPK signaling pathway (Hong et al., 2018). Therefore, a hypothesis is put forward that Dox treatment induces cardiotoxicity by upregulating the expression of CTGF through activation of TLR2/AP-1.

2. Materials and methods

2.1 Bioinformatics analysis

The mouse myocardial gene expression chip GSE40289 (https://www.ncbi.nlm.nih.gov/geo/) includes 6 control samples and 6 Dox-treated samples. The R language "limma" package was used to select differentially expressed genes from the treated samples, with the screening criteria set at $|\log FC| > 1$ and p -value < 0.05. The hTFtarget database (http://bioinfo.life.hust.edu.cn/hTFtarget#!/) was used to predict target genes of AP-1, and the JASPAR database (http://jaspar.genereg.net/) to predict the AP-1-binding domain in the CTGF promoter. The STRING database (https://www.string-db.org) was utilized to analyze the interactions of the candidate genes, and a gene interaction network map was created using cytoscape software version 3.8. The GeneMANIA database (http://genemania.org/) was searched for CTGF-related genes, and the R language "clusterprofile" package was used to perform a KEGG pathway enrichment analysis of CTGF-related genes.

2.2 Clinical sample collection

The clinical tests obtained approval from the Ethics Committee of Jiangxi Provincial People's Hospital and were in keeping with the principles of the *Declaration of Helsinki* . All subjects were from this hospital and signed informed consents before tests, including 20 healthy subjects, 20 patients with chronic HF and without other diseases, and 56 patients with hematological malignancies (leukemia, lymphoma, or multiple myeloma) who had received Dox therapy. These 56 patients with tumors received three-month chemotherapy. The chemotherapy regimen and exclusion criteria were detailed in our previous study (Shao et al., 2018). After the chemotherapy, the 56 patients were divided into a non-HF group (named Dox group, n = 34) and a Dox-induced HF group (named Dox-HF group, n = 22) according to NT-pro-brain natriuretic peptide (NT-proBNP) and left ventricular ejection fraction (LVEF) values.

2.3 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Blood samples from the human subjects were mixed with Ficoll-Paque Plus reagent (Sigma-Aldrich, Darmstadt, Germany) and spun at 3000 g for 20 min to isolate PBMCs. Total RNA of PBMCs was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), followed by generation of cDNA from 400 ng of total RNA with a first strand cDNA synthesis kit (Roche Diagnostics, Basel, Switzerland). The thermal cycling parameters were: 95°C (5 min), then 40 cycles of 95°C (30 s), 56°C (20 s), and 56°C (20 s), and finally 72°C (30 s). PCR was performed using the IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), with three replicate wells for each sample. Data were analyzed by the $2^{-\Delta\Delta\hat{}\tau}$ method, with β-actin as the internal reference. The primer sequences are listed in Table 1.

2.4 Western blotting

A bicinchoninic acid kit (Beyotime, Shanghai, China) was selected to assess protein yields from lysates of PBMCs, rat myocardial tissue, and H9c2 cells. Protein samples were mixed with a loading buffer and heated for 3 min. After electrophoresis at 80 V (before bromophenol blue entered the separating gel) and then at 120 V, proteins were transferred onto a membrane (60 min, 300 mA), on which unoccupied sites were then blocked by 5% skim milk at 4°C overnight. Primary antibodies including anti-TLR2 (ab213676, 1:500), anti-CTGF (ab227180, 1:500), anti-ANP (ab209232, 1:1000), anti-BNP (ab19645, 1:500), anti-collagen I (ab260043, 1:1000), anti-c-Jun (ab40766, 1:2000), anti-SOX10 (ab227680, 1:400), anti-caspase-3 (ab184787, 1:2000), anti-Bax (ab32503, 1:2000), anti-Bcl-2 (ab196495, 1:1000), anti-ERK1/2 (ab184699, 1:10000), anti-p38 (ab170099, 1:2000), anti-JNK (ab179461, 1:1000), anti-p-ERK1/2 (ab201015, 1:1000), anti-p-p38 (ab4822, 1:1000), anti-p-JNK (ab76572, 1:5000), and anti-β-actin (ab8227, 1:2000; as an internal control) and a secondary antibody (ab205718, 1:10000) (all from Abcam, Cambridge, UK) were sequentially incubated with the membrane at ambient temperature for 60 min. Blots were treated with an ultrasensitive ECL reagent (Beyotime) and detected using a Gel Doc XR imaging device (Bio-Rad).

2.5 Animal treatment

Animals were used under the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the approval by the Institutional Animal Care and Use Committee of Jiangxi Provincial People's Hospital. Forty-eight Sprague-Dawley rats (10 weeks, 280-350 g) were reared at 20-26°C (50-60% humidity) and in 12 h:12 h light:dark cycles, with free access to food and water. Among them, 12 were randomly selected for normal control (normal group), and the left 36 were for model establishment. After one-week animal adaptation, an intraperitoneal injection of Dox (2.5 mg/kg, S1208, Selleck Chemicals, Houston, TX, USA) was administered to the 36 rats once a week for 10 consecutive weeks (Pang et al., 2022), and an equal volume of normal saline to the normal group. The 36 rats were randomly subdivided into model (n = 12), inhibitor (n = 6), shRNA (sh)-negative control (NC) (n = 6), sh-CTGF-1 (n = 6), and sh-CTGF-2 (n = 6) groups. Rats in the inhibitor group were intraperitoneally injected with 0.5 mL of 12 μg/mL TLR2 inhibitor C29 (TLR2-IN-C29) (S6597; Selleck Chemicals) after each Dox injection, also once a week for 10 consecutive weeks. Rats in the latter three groups were injected with a shRNA-mediated CTGF silencing lentiviral vector or NC vector (1×10^9PFU/mL) into the left ventricle through the apex one hour before Dox injection for 10 consecutive weeks, once a week.

2.6 Echocardiography

Seven days after the accomplishment of DCM modeling, rats were anesthetized by an intraperitoneal injection of 40 mg/kg ketamine and 10 mg/kg xylazine, and their chest was shaven. Cardiac structure and function were assessed at the long axis of the mitral valve by measuring left ventricular end-systolic diameter (LVESD), end-diastolic diameter (LVEDD), ejection fraction (LVEF), and fractional shortening (LVFS) with an HP-SONOS 5500 ultrasonic Doppler instrument (Hewlett-Packard, Andover, MA, USA).

2.7 Animal sample collection

After the cardiac function tests, the rats were weighed (body weight, BW) and then anesthetized. Blood (2 mL per rat) was collected from the retro-orbital venous plexus and centrifuged for separation of serum. The rats were then sacrificed for collection of hearts which were then weighed (heart weight, HW). Cardiac tissue was either stored at 4°C after fixation or preserved in liquid nitrogen.

2.8 Serum indices

Rat serum lactate dehydrogenase (LDH; #A020), creatine kinase-MB (CK-MB; #H197), and cardiac troponin I (cTnI; #H149) were detected using specific kits (all from Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

2.9 Hematoxylin-eosin (H&E) staining

Paraffin sections of rat heart tissue were sequentially immersed in xylene (5 min), alcohol solutions (100%, 95%, 90%, 80%, and 70%, 3 min each), and phosphate-buffered saline (PBS). The sections were then stained

with hematoxylin (5 min), differentiated with hydrochloric acid-ethanol (30 s), and stained with eosin (2 min), followed by routine dehydration, clearing, and mounting. Five random fields of view per section were photographed under an IX71 microscope (Olympus, Tokyo, Japan).

2.10 Transmission electron microscopy (TEM)

The ultrastructure of rat myocardial tissue was observed using TEM as we previously did (Hong et al., 2018). Briefly, myocardial tissue was fixed in 2.5% glutaraldehyde and 1% osmic acid, dehydrated in graded ethanol solutions and acetone, and embedded in a mixture of acetone and an embedding medium. Ultrathin sections of the tissue were stained with uranyl acetate and lead citrate and observed with a JEM-2100F microscope (JEOL Ltd., Japan).

2.11 Sirius red staining

After xylene deparaffinization and gradient ethanol rehydration, sections of rat heart tissue were dyed with sirius red (ab150681; Abcam) for 1 h and rinsed with acetic acid and absolute ethanol, followed by absolute ethanol dehydration, transparentization, mounting, and observation under the Olympus IX71.

2.12 Cardiomyocyte culture and injury induction

Rat H9c2 cardiomyocytes (ATCC, Manassas, VA, USA) were grown at 37degC in Gibco DEME (Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) in 5% CO_2. H9c2 cell injury was induced by 24-h treatment with 1 μM of Dox (Wang et al., 2022).

2.13 Cell transfection

Lipofectamine 3000 reagent (Invitrogen) was used to transfect H9c2 cells with sh-NC, sh-TLR2-1, sh-TLR2-2, sh-CTGF-1, sh-CTGF-2, pcDNA3.1 [overexpression (oe)-NC], pcDNA3.1-c-Jun (oe-c-Jun), pcDNA3.1-SOX10 (oe-SOX10), and pcDNA3.1-CTGF (oe-CTGF).

2.14 Cell viability assay

After the aforementioned cell treatments, the culture medium was replaced with a MTT solution (0.5 mg/mL) to incubate the cells for 4 h (37°C). Dimethyl sulfoxide (DMSO, 100 μL) was added to each well to dissolve formazan crystals, followed by measurement of absorbance at 570 nm.

2.15 LDH release experiment

Cells were seeded into a 96-well plate and centrifuged at 1500 rpm for 5 min. The supernatant (120 μL/well) was transferred to another 96-well plate for detection of LDH release with a LDH assay kit (Beyotime). Absorbance (optical density, OD) was detected at 490 nm. Background OD was measured using a fresh medium, and maximum OD was measured on control cells treated with 1% Triton X-100 for 1 h. LDH release rate = (sample well OD - background OD)/(maximum OD - background OD) × standard concentration.

2.16 Mitochondrial membrane potential (MMP) detection

Cells were incubated with JC-1 staining working solution for 20 min and centrifuged at 4°C. The cells were resuspended in 1 mL of JC-1 staining buffer and centrifuged at 4°C. After a repeat of the previous step, the cells were resuspended in a flow tube with 200 μL of JC-1 staining buffer, and the fluorescence intensity was detected by a flow cytometer within half an hour.

2.17 TUNEL

Cell slides were soaked in 4% paraformaldehyde for 30 min, washed once with PBS, immersed in 0.3% Triton X-100 for 5 min, and washed twice with PBS. Hydrated heart tissue sections were treated with 20 μg/mL proteinase K for half an hour, and washed thrice with PBS. Cells/tissue sections were incubated with 50 μL of prepared TUNEL detection solution (C1089; Beyotime) at 37°C for 60 min in the dark and mounted with

an anti-fluorescence quenching solution. Samples were observed under a fluorescence microscope. Nuclei were stained with 4',6-diamidino-2-phenylindole.

2.18 Co-immunoprecipitation (IP)

Cell lysate reacted with anti-c-Jun antibody (1:20, ab40766; Abcam), anti-SOX10 antibody (1:20, ab264405; Abcam), or anti-IgG antibody (a NC) overnight at 4degC on a shaker. The antigen-antibody complex was captured by overnight incubation with 50% Protein A/G agarose beads (100 μL) under rotation at 4°C and collected by a brief centrifugation (14,000 rpm, 5 s). The complex was rinsed thrice with a pre-chilled RIPA buffer (800 μL) and suspended in a 2× loading buffer (60 μL). After boiling and centrifugation, the beads were collected and the proteins were electrophoresed for western blotting.

2.19 Chromatin (Ch)-IP

A ChIP kit (Millipore, Billerica, MA, USA) was used to detect CTGF promoter-binding proteins. The crosslinks between DNA and proteins were fixed with formaldehyde for half an hour. The DNA was then isolated and sonicated into 200-1000 bp fragments which were then incubated with anti-c-Jun, anti-SOX10, or anti-IgG antibody. The pulled down DNA fragments were detected by qPCR (see primers in Table 1).

2.20 Dual-luciferase reporter assay

The JASPAR website (http://jaspar.genereg.net/) was utilized to predict the binding site of c-Jun or SOX10 in the CTGF promoter. A promoter sequence containing the wild-type or mutated binding sequence (WT-CTGF or MT-CTGF) was added to pGL3-Basic vector (Promega, Madison, WI, USA) and delivered with pRL-TK vector (Promega) and oe-c-Jun, oe-SOX10, or oe-NC into HEK293T cells. After cell transfection, a dual-luciferase reporter assay kit (Promega) was used to detect luciferase activity (relative luciferase activity = the ratio of firefly luciferase activity to Renilla luciferase activity).

2.21 Activator treatment

MAPK pathway activator (MAPK Ac, anisomycin) was dissolved in 0.1% DMSO. H9c2 cells were cultured with MAPK Ac (1 μM) for 8 h before Dox treatment (Ho et al., 2008).

2.22 Statistical analysis

Statistical analysis was performed with GraphPad Prism software version 7.0, and all data were expressed as mean ± standard deviation. One-way analysis of variance was adopted to perform multi-group comparisons of normally distributed data, followed by Tukey's test. Statistical significance was set at p -value < 0.05.

3. Results

3.1 Bioinformatics analysis

Differentially expressed genes in wild-type mouse cardiomyocytes after doxorubicin treatment were obtained through analysis of the GSE40289 dataset in GEO (Fig. 1A), among which TLR2 was significantly upregulated (Fig. 1B). TLR2 can activate NF-ϰB and AP-1. The hTFtarget database was utilized to predict target genes of AP-1. The predicted results were intersected with the upregulated genes in the GSE40289 chip (Fig. 1C). Thirty-six candidate target genes were obtained from the intersection (Fig. 1D). An analysis of the interactions of these 36 target genes further revealed that CTGF interacted with multiple genes (Fig. 1E). Moreover, the JASPAR database predicted multiple AP-1-binding domains in the CTGF promoter region. A KEGG pathway enrichment analysis of CTGF-related genes (Fig. 1F) showed that these genes were mainly enriched in signaling pathways such as MAPK (Fig. 1G). From the above, a hypothesis is put forward that highly expressed TLR2 promotes the transcription level of CTGF by activating AP-1 and subsequently activates the MAPK signaling pathway in Dox-induced myocardial injury.

3.2 High expression of TLR2 and CTGF in PBMCs from Dox-treated patients

To validate the above bioinformatics analysis results, we first detected TLR2 and CTGF expression in PBMCs from Dox-treated patients. The medical history of all participants, which showed no significant differences

between groups, is presented in Table 2. Three months after Dox treatment, the patients suffering from tumors were separated into Dox group and Dox-HF group based on their NT-proBNP and LVEF (Table 3). Before Dox therapy, the control, Dox, and Dox-HF groups showed no differences in the cardiac function indexes; the NT-proBNP of the HF group was higher than that of the control group, and the LVEF was lower. Moreover, qRT-PCR and western blotting showed that TLR2 and CTGF were highly expressed in patients with HF and that Dox treatment further increased their expression (Fig. 2A-D, $p < 0.05$).

3.3 Inhibition of TLR2 alleviates Dox-induced myocardial injury in rats

First, we performed echocardiography on the rats, and the results (Table 4) showed that the model group had larger LVESD and LVEDD and smaller LVEF and LVFS than the normal group. The LVESD and LVEDD of model rats were decreased and their LVEF and LVFS were increased by TLR2-IN-C29 treatment, suggesting that inhibition of TLR2 improved the cardiac function of Dox-treated rats. The HW/BW ratio (Fig. 3A, $p< 0.05$) and the levels of serum LDH, CK-MB, and cTnI (Fig. 3B-D, $p < 0.05$) were increased by Dox treatment; the increases were suppressed by TLR2-IN-C29. H&E staining revealed partial degeneration and dissolution, breakage of muscle fibers, and interstitial edema of cardiomyocytes in the myocardium of model rats; there were slight cardiomyocyte edema, neatly arranged muscle fibers, and fewer injuries in the myocardium of TLR2-IN-C29-treated model rats (Fig. 3E). The normal myocardium, observed using TEM, showed neatly arranged myofibrils and uniform-sized mitochondria with intact membranes. Dox treatment caused degeneration and rupture of mitochondria and breakage of myofibrils; these myocardial ultrastructural injuries were alleviated by TLR2-IN-C29 (Fig. 3F). Moreover, Dox treatment induced myocardial collagen accumulation, as revealed by sirius red staining, which was inhibited by TLR2-IN-C29 (Fig. 3G, $p< 0.05$). Increases in the expression of hypertrophy markers ANP and BNP (Wang et al., 2022) and fibrosis markers collagen I and CTGF were detected in the myocardial tissue of the model group using western blotting; however, the expression levels were reduced by inhibiting TLR2 (Fig. 3H, $p < 0.05$). Dox increased TUNEL-positive (apoptotic) myocardial cells, whereas TLR2 inhibition counteracted this impact (Fig. 3I, $p < 0.05$). To verify whether TLR2 upregulates CTGF and activates MAPK signaling by activating AP-1, we detected the expression of TLR2, c-Jun, apoptosis-related proteins, and MAPK pathway proteins by western blotting. Dox-induced increases in the expression of TLR2, c-Jun, p-ERK, p-p38, p-JNK, caspase-3, and Bax and a decrease in Bcl-2 expression were reversed by TLR2-IN-C29 treatment (Fig. 3J, $p < 0.05$). Consequently, inhibition of TLR2 improves cardiac function and reduces myocardial fibrosis and apoptosis in Dox-treated rats.

3.4 Silencing CTGF protects rats from Dox-induced myocardial injury

sh-NC treatment resulted in no changes in the cardiac parameters of model rats. The echocardiographic results (Table 5) showed that the sh-CTGF-1 and sh-CTGF-2 groups had smaller LVESD/DD and larger LVEF/FS than the sh-NC group, suggesting that silencing CTGF improved the cardiac function of Dox-treated rats. Silencing CTGF lowered the HW/BW ratio (Fig. 4A, $p < 0.05$) and the levels of serum LDH, CK-MB, and cTnI (Fig. 4B-D, $p < 0.05$) of model rats. Silencing CTGF also reduced histological injuries (manifested in slight cardiomyocyte edema and neatly arranged myocardial muscle fibers) (Fig. 4E), myocardial ultrastructural injuries (Fig. 4F), and myocardial collagen accumulation (Fig. 4G, $p < 0.05$). Myocardial expression of ANP, BNP, collagen I, and CTGF were inhibited by silencing CTGF (Fig. 4H, $p < 0.05$). Silencing CTGF decreased TUNEL-positive cells (Fig. 4I, $p < 0.05$) and p-ERK, p-p38, p-JNK, caspase-3, and Bax expression and elevated Bcl-2 expression (Fig. 4J, $p < 0.05$) in the myocardium of model rats. Taken together, these data demonstrate reductions in cardiac dysfunction, fibrosis, and apoptosis in Dox-treated rats with silenced CTGF.

3.5 Silencing TLR2 or CTGF reduces H9c2 cardiomyocyte injury

Next, the roles of TLR2 and CTGF were examined in Dox-treated rat H9c2 cardiomyocytes. The viability of H9c2 cells, tested using MTT, was decreased by Dox treatment and restored after TLR2 or CTGF silencing (Fig. 5A, $p < 0.05$). Dox treatment elevated the release rate of LDH (Fig. 5B, $p< 0.05$), increased the TUNEL-positive rate (Fig. 5C, $p< 0.05$), and decreased the MMP (Fig. 5D, $p < 0.05$) in H9c2 cells; these

pathological changes were suppressed by silencing TLR2 or CTGF. Moreover, Dox treatment promoted the expression of TLR2, c-Jun, CTGF, caspase-3, Bax, p-ERK, p-p38, and p-JNK and repressed that of Bcl-2 in H9c2 cells, which was counteracted by silencing TLR2 (Fig. 5E, $p < 0.05$). Dox-induced changes in the expression of CTGF, p-ERK, p-p38, p-JNK, caspase-3, Bax, and Bcl-2 could also be reversed by silencing CTGF (Fig. 5E, $p< 0.05$).

3.6 AP-1 cooperates with SOX10 to promote CTGF transcription

SOX10 interacts with many transcription factors, including c-Jun (Wissmuller et al., 2006). The interaction between SOX10 and c-Jun was validated by co-IP assays (Fig. 6A). The JASPAR database showed that c-Jun and SOX10 both have binding sites in the CTGF promoter (Fig. 6B), which was verified by Ch-IP experiments (Fig. 6C, $p < 0.05$), suggesting that c-Jun and SOX10 may coordinately regulate the transcription of the CTGF gene. The luciferase activity of the reporter vector inserted with WT-CTGF was enhanced by overexpression of c-Jun or SOX10 and increased further by co-overexpression of c-Jun and SOX10 (Fig. 6D, $p < 0.05$). To explore the effects of different sites in the CTGF promoter sequence on the luciferase activity, we dissected the CTGF promoter sequence into fragments F1, F2, F3, and F4 and found they all could promote the luciferase activity when c-Jun and SOX10 were co-overexpressed (Fig. 6E, $p < 0.05$).

3.7 Overexpression of CTGF offsets the protective effect of silencing TLR2 on H9c2 cells

To validate the TLR2/AP-1/CTGF axis in Dox-induced H9c2 injury, we silenced TLR2 in H9c2 cells while activating CTGF. Overexpression of CTGF inhibited TLR2 silencing-induced an increase in the viability of Dox-treated H9c2 cells (Fig. 7A, $p < 0.05$). Overexpression of CTGF elevated the release rate of LDH (Fig. 7B,$p < 0.05$), increased the TUNEL-positive rate (Fig. 7C,$p < 0.05$), and decreased the MMP (Fig. 7D, $p< 0.05$) in Dox-treated H9c2 cells with silenced TLR2. Moreover, TLR2 silencing-induced reductions in the expression of Bax, caspase-3, p-ERK, p-p38, and p-JNK and an elevation in that of Bcl-2 were reversed by overexpression of CTGF in Dox-stimulated H9c2 cells (Fig. 7E, $p < 0.05$).

3.8 Activating the MAPK pathway reverses the protective effect of CTGF silencing on H9c2 cells from Dox-induced injury

To verify the role of the MAPK pathways in Dox-induced myocardial injury, we selected sh-CTGF-2 for follow-up experiments. We treated Dox-treated H9c2 cardiomyocytes with MAPK Ac after CTGF silencing, and found that MAPK Ac treatment resulted in decreased cell viability (Fig. 8A, $p < 0.05$) and MMP (Fig. 8D, $p < 0.05$) and promoted LDH release (Fig. 8B, $p < 0.05$) and cell apoptosis (Fig. 8C, $p < 0.05$), accompanied by elevated caspase-3 and Bax levels and reduced Bcl-2 level (Fig. 8E,$p < 0.05$). These results demonstrated that activating the MAPK pathway could significantly reverse the mitigative effect of CTGF silencing on H9c2 cells from Dox-induced injury.

4. Discussion

Cardiomyopathy is a common complication in patients receiving Dox (Cheong et al., 2021). Developing anticipatory treatment targeting the signaling pathways and mechanisms involved in Dox-induced cardiotoxicity would bring about an immense improvement in oncology therapeutics (Renu et al., 2018). In this study, TLR2, CTGF, and MAPK signaling pathway were found to be contributors to Dox-induced cardiotoxicity. TLR2 promoted the expression of CTGF by activating AP-1 that coordinated with SOX10 in stimulating the transcription of CTGF. The functions of TLR2, CTGF, and MAPK signaling pathway were validated in a rat model of Dox-induced DCM and/or an H9c2 cell model of Dox-induced injury using specific inhibitors or shRNAs.

First, high expression of TLR2 and CTGF were detected in blood samples from patients with HF. The expression of TLR2 and CTGF further increased in patients with Dox-induced HF. Inhibition of TLR2 with C29 reduced histological injuries, alleviated mitochondrial damage, and reduced collagen accumulation, apoptosis, and expression of hypertrophy markers (ANP and BNP), fibrosis markers (collagen I and CTGF), c-Jun, and proteins in MAPK cascade signaling in the myocardium of Dox-treated rats. Silencing TLR2

using specific shRNAs in Dox-stimulated H9c2 cells increased their viability and MMP and reduced their apoptosis and expression of c-Jun, CTGF, and MAPK pathway proteins. A recent study by Tang et al. indicates that TLR2 and its downstream NF-ϰB are upregulated in the myocardium of rabbits with Dox-induced chronic HF (Tang et al., 2021). Crocin, a natural compound extracted from saffron, counteracts Dox-provoked cardiotoxicity by reducing oxidative stress, inflammation, apoptosis, intracellular calcium imbalance, and mitochondrial damage possibly through inactivation of the TLR2/NF-ϰB pathway (Chu et al., 2020). Antagonism of extracellular heat shock protein 70 with a neutralizing antibody ameliorates Dox-induced left ventricular dilation and dysfunction and inhibits cardiac fibrosis, which is largely attributed to the resolution of TLR2/NF-ϰB-mediated myocardial inflammation (Liu et al., 2019). Apart from NF-ϰB, TLR2 can also activate the AP-1 transcription factor family.

c-Jun is a member of the Jun subfamily of AP-1 transcription factors, which can be activated through JNK. Several researchers have indicated the participation of JNK in Dox-induced cardiotoxicity. Overexpression of secreted frizzled-related protein 1, an endogenous inhibitor of Wnt signaling, rescues H9c2 cardiomyoblasts from Dox-induced apoptosis by blocking the Wnt/planar cell polarity-JNK pathway (Hu et al., 2020). Interleukin-10 treatment sustains endoplasmic reticulum homeostasis in Dox-exposed cardiomyocytes by promoting expression of protein kinase-like endoplasmic reticulum kinase and inositol-requiring kinase 1α and also stimulates cell survival by inhibiting activation of caspase-12, phosphorylation of JNK, and expression of mitochondrial apoptotic proteins Bax and caspase-3 (Malik et al., 2022). Uric acid preconditioning reduces Dox-induced cardiotoxicity by inhibiting phosphorylated JNK as well as phosphorylated connexin 43 through activation of adenosine 5'-monophosphate-activated protein kinase/Src homology 2 domain-containing protein tyrosine phosphatase (Wang et al., 2020).

Sometimes c-Jun works in concert with other transcription factors to control gene expression (Martin & Tremblay, 2009; Roumaud et al., 2017). This study found that c-Jun and SOX10 coordinately promoted the transcription of CTGF in H9c2 cells. SOX genes are master regulators of cell fate during development, which are fundamental to sex determination in a multitude of organisms (Sreenivasan et al., 2022). There is synergy between c-Jun and SOX8/SOX9 in the regulation of mouse Gja1 transcription in Sertoli cells (Ghouili et al., 2018). Our previous research has demonstrated that overexpression of SOX8 promotes hypoxia/reoxygenation-induced cardiomyocyte injury by increasing the expression of metallopeptidase 2/9 (Cai et al., 2020). The role of SOX10 in Dox-induced cardiotoxicity can be explained by its downstream CTGF.

CTGF expression tends to increase in the pathogenesis of malignancies and tissue fibrosis (Ramazani et al., 2018). Under the stimulation of the fibrosis inducer transforming growth factor-β, CTGF is overexpressed in human lung fibroblasts through the MEKK1/JNK pathway (Lin et al., 2018). The expression of CTGF can also be induced by a disintegrin and metalloproteinase 17 through the ERK pathway in human lung fibroblasts as well as epithelial cells (Chen et al., 2018; Ou et al., 2020). These findings indicate a close relationship between CTGF and MAPK pathways in fibrotic processes. Our study discovered that silencing CTGF reduced Dox-induced myocardial fibrosis and apoptosis and also inhibited the expression of MAPK pathway proteins. Overexpression of CTGF nullified the protective effects of TLR2 silencing on Dox-treated H9c2 cells, which confirmed the regulation of TLR2 on CTGF in Dox-elicited cardiotoxicity. Previous findings have also demonstrated upregulated expression of CTGF in Dox-induced cardiac injury (Qian et al., 2022). An agonist monoclonal antibody for activation of MET receptors inhibits Dox-induced increases in the expression of CTGF and other fibrosis markers (Gallo et al., 2020). These data suggest that fibrosis is a vital mechanism underlying Dox-induced cardiac injury.

In summary, TLR2 promotes CTGF transcription by activating AP-1 and activates the MAPK signaling pathways, thereby stimulating Dox-induced myocardial injury. The understanding of the mechanism of Dox-induced cardiac dysfunction may provide new targets for mitigating the side effects of Dox therapy and therefore broaden the prospects of this promising anti-cancer drug.

Author contribution

CXY, HL and SL conceived the ideas. CXY, HL, SL and ZYL designed the experiments. CXY, HL, ZYL, LST and ZPT performed the experiments. CXY, HL, SL, ZYL and CYM analyzed the data. CXY, HL and SL provided critical materials. CXY, HL, ZYL and LST wrote the manuscript. SL supervised the study. All the authors have read and approved the final version for publication.

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Conflicts of interests

The authors report no relationships that could be construed as a conflict of interest.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The clinical tests obtained approval from the Ethics Committee of Jiangxi Provincial People's Hospital and were in keeping with the principles of the *Declaration of Helsinki* . All subjects were signed informed consents before tests.

Consent for publication

All participants were informed of the specific details of the study and signed the informed consents before enrollment.

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Figure legends

Figure 1. Bioinformatics analysis. A: A volcano plot of differentially expressed genes in mouse cardiomyocytes after Dox treatment in the GSE40289 chip (the red and green dots respectively represent significantly upregulated and downregulated genes in the Dox-treated samples). B: Differential expression of TLR2 in GSE40289. C: A heat map of partial upregulated genes in GSE40289 (the abscissa and ordinate list the sample numbers and gene names, respectively). D: The intersection of upregulated genes in GSE40289 and predicted target genes of AP1. E: An interaction network of the candidate target genes. F: CTGF-related genes (the circles represent the genes, and the lines connecting the circles indicate correlations between the genes). G: A KEGG pathway enrichment analysis of CTGF-related genes (the abscissa and ordinate show the GeneRatio and KEGG pathways, respectively).

Figure 2. High expression of TLR2 and CTGF in PBMCs from Dox-treated patients. A: qRT-PCR detection of TLR2 mRNA. B: Western blotting detection of TLR2 protein. C: qRT-PCR detection of CTGF mRNA. D: Western blotting detection of CTGF protein. n = 20 in the control group, n = 20 in the HF group, n = 34 in the Dox group, and n= 22 in the Dox-HF group. One-way analysis of variance was used for comparisons between multiple groups, followed by Tukey's multiple comparisons test. *, p -value < 0.05.

Figure 3. Inhibition of TLR2 alleviates Dox-induced myocardial injury in rats. A: Heart/body weight ratio. B-D: Levels of serum LDH, CK-MB, and cTnI. E: H&E staining was used to reveal pathological changes of myocardial tissue. F: Ultrastructure of myocardial cells was observed using transmission electron

microscopy. G: Sirius red staining was used to detect myocardial fibrosis. H: Western blot was used to detect hypertrophic markers ANP and BNP and fibrosis markers collagen I and CTGF. I: TUNEL staining was used to detect myocardial apoptosis. J: Western blot was used to detect TLR2, c-Jun, apoptosis-related proteins (caspase-3, Bax, and Bcl-2), and MAPK pathway proteins (p-ERK, p-p38, and p-JNK). n = 6 in each group. One-way analysis of variance was used for comparisons between multiple groups, followed by Tukey's multiple comparisons test. *, p -value < 0.05.

Figure 4. Silencing CTGF protects rats from Dox-induced myocardial injury. A: Heart/body weight ratio. B-D: Levels of serum LDH, CK-MB, and cTnI. E: H&E staining was used to reveal pathological changes of myocardial tissue. F: Ultrastructure of myocardial cells was observed using transmission electron microscopy. G: Sirius red staining was used to detect myocardial fibrosis. H: Western blot was used to detect ANP, BNP, collagen I, and CTGF. I: TUNEL staining was used to detect myocardial apoptosis. J: Western blot was used to detect caspase-3, Bax, Bcl-2, p-ERK, p-p38, and p-JNK. n = 6 in each group. One-way analysis of variance was used for comparisons between multiple groups, followed by Tukey's multiple comparisons test. *, p -value < 0.05.

Figure 5. Silencing TLR2 or CTGF reduces H9c2 cardiomyocyte injury. A: MTT viability assay on H9c2 cells. B: LDH release assay to assess H9c2 cytotoxicity. C: TUNEL detection of H9c2 cell apoptosis. D: JC-1 staining to detect mitochondrial membrane potential of H9c2 cells. E: Western blot detection of TLR2, c-Jun, CTGF, caspase-3, Bax, Bcl-2, p-ERK, p-p38, and p-JNK. The cell experiments were repeated thrice. One-way analysis of variance was used for comparisons between multiple groups, followed by Tukey's multiple comparisons test. *, p -value < 0.05.

Figure 6. AP-1 cooperates with SOX10 to promote CTGF transcription. A: Co-IP was used to detect the binding of c-Jun protein and SOX10 protein in H9c2 cells. B: JASPAR database predicted the binding sites of c-Jun and SOX10 in the CTGF promoter. C: Ch-IP was used to detect the binding of c-Jun and SOX10 to the CTGF promoter. D: Dual-luciferase reporter assay was used to assess the effects of c-Jun, SOX10, and c-Jun + SOX10 on the luciferase activity of CTGF promoter-inserted vectors. E: Dual-luciferase reporter assay was used to detect the luciferase activity of vectors inserted with different CTGF promoter fragments. The cell experiments were repeated thrice. One-way analysis of variance was used for comparisons between multiple groups, followed by Tukey's multiple comparisons test. *, p -value < 0.05.

Figure 7. Overexpression of CTGF offsets the protective effect of silencing TLR2 on H9c2 cells. A: MTT viability assay on H9c2 cells. B: LDH release assay to assess H9c2 cytotoxicity. C: TUNEL detection of H9c2 cell apoptosis. D: JC-1 staining to detect mitochondrial membrane potential of H9c2 cells. E: Western blot detection of TLR2, c-Jun, CTGF, caspase-3, Bax, Bcl-2, p-ERK, p-p38, and p-JNK. The cell experiments were repeated thrice. One-way analysis of variance was used for comparisons between multiple groups, followed by Tukey's multiple comparisons test. *, p -value < 0.05.

Figure 8. Activating the MAPK pathway exacerbates H9c2 cells from Dox-induced injury. A: MTT viability assay on H9c2 cells. B: LDH release assay to assess H9c2 cytotoxicity. C: TUNEL detection of H9c2 cell apoptosis. D: JC-1 staining to detect mitochondrial membrane potential of H9c2 cells. E: Western blot detection of caspase-3, Bax, and Bcl-2. The cell experiments were repeated thrice. One-way analysis of variance was used for comparisons between multiple groups, followed by Tukey's multiple comparisons test. *, p -value < 0.05.

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