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Research article

Cell autonomous TLR4 signaling modulates TGF-β induced activation of human cardiac fibroblasts

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A B S T R A C T

Fibrosis is one of the major outcomes following injury in the heart. Immune response in the injury niche modulates fibrosis, yet little is known about how cell-autonomous immune signaling in adult cardiac fibroblasts regulates fibrosis. Using FACS, single-cell sequencing of cardiac fibroblasts from Collagen1-α1GFP mice and human heart failure patients, we demonstrate that TLR4 is the major immune sensor expressed in cardiac fibroblasts. Inhibition of TLR4 signaling reduces TGF-β induced fibrotic changes such as contractibility and migration of adult human cardiac fibroblasts in TGF-β treated fibrotic conditions. TGF-β treated cardiac fibroblastss show enhanced cytokine expression, and inhibition of TLR4 signaling reduces the expression of cytokines, thereby reducing TGF-β targets such as extracellular matrix genes. Thus, our data demonstrate that TLR4 and other signaling molecules downstream of TLR4 are expressed in cardiac fibroblast, and inhibition of TLR4 modulates fibrotic changes in vitro.

1. Introduction

Cardiac fibroblasts (CF), the principal fibrotic cells in the adult heart, exist in a quiescent state in an uninjured heart [1]. In response to an injury, CFs get activated to myofibroblasts with dynamic deposition of extracellular matrix (ECM) proteins, forming fibrosis [2,3]. Excessive fibrosis is pathological and interferes with cardiac tissue's normal architecture and function.Transforming growth factor-β (TGF-β) is the primary mediator of cardiac fibroblast activation, enhancing the expression of different pro-fibrotic genes [4]. The activation of cardiac fibroblasts is associated with profound changes in their transcriptional network, causing them to proliferate, migrate, and secrete fibrotic extracellular matrix [5]. In addition, the regulatory networks governing fibrosis are tissue specific [6]. Understanding the molecular mechanism that mediates the pathological fibrotic process is essential to develop novel therapeutic approaches to treat fibrosis and prevent it from contributing to cardiac dysfunction following injury.

During tissue injury, endogenous stress signals generated from dying and damaged myocytes function as molecular signals, enabling immune cells and fibroblasts to sense and respond to the damage [7–9]. Toll-like receptors (TLR) are the primary pattern recognition sensors expressed in various cells that mediate inflammation. Among different TLRs, Toll-like receptor 4, although initially identified as the receptor for lipopolysaccharide (LPS) during microbial infections, is emerging as the major receptor that senses endogenous damage signals, triggering a downstream signaling network that modulates the tissue repair process during non-sterile injury [10,11]. Moreover, TLR4 knockout mice are protected from experimentally induced fibrosis in different organs [12–14]. Suppressing TLR4 signaling using metformin reduces left ventricular dysfunction after myocardial infarction [15].

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Although TLR4 signaling in macrophages and other immune cells and its contribution to cardiac fibrosis is well described [16,17], the role of TLR4 in cardiac fibroblasts remains unclear. In this study, we investigated the expression of TLR4 receptors and downstream signaling molecules of TLR4 in adult cardiac fibroblasts in steady state and during injury. We observed that adult cardiac fibroblasts express TLR4 receptors in mice and in humans during homeostatic conditions and increase the expression of TLR4 and downstream cytokines in response to injury in mice and in heart failure patients. Inhibiting TLR4 signaling modulates the transcriptional programs in human cardiac fibroblasts, inhibiting the activation of adult cardiac fibroblasts in vitro by modulating cytokine expression. Thus, modulation of TLR4 signaling or its targets in cardiac fibroblasts may be a potential treatment modality to inhibit non-functional fibrosis following cardiac injury.

2. Methodology

2.1. Single cell sequencing data analysis

To determine the TLR and cytokine expression in cardiac fibroblasts, publicly available mouse (GSE132144 [18]) and human single-cell data (GSE 121893 and GSE109816 [19]) from control conditions were used. Single-cell data of Collagen1α1-GFP mice, in which Collagen1α1 expressing fibroblasts were genetically labelled with GFP, were used. Cardiac injury in Collagen1α1-GFP mice was made by permanent ligation of the left anterior descending (LAD) coronary artery. For cardiac injury, 8 to 10-week-old mice, under anesthesia, left thoracotomy was performed between the fourth and fifth intercostal space. After dissection of the muscles, the LAD coronary artery was permanently ligated using a 7/0 non-absorbable ethylene suture. The successful occlusion of the coronary artery was visually confirmed by observing signs of anemia and akinesis in the apex and anterior-lateral walls of the heart. Subsequently, the thorax was closed in layers, and the mice were allowed to recover. The animals were later sacrificed, and heart samples were taken 7 days, 14 days, and 30 days following injury, along with healthy controls.

Seurat package in R was used to analyze the single-cell data. The mouse data from healthy myocardium were filtered for cells with mitochondrial DNA of less than 10 % and minimum features of 200. The data was normalized using 'NormalizeData' with the 'LogNormalize' method. Further, highly variable features were identified using the 'FindVariableFeatures' function using the 'vst' method. The data was scaled using the 'Scaledata' function, and linear dimensional reduction was performed by the 'RunPCA' function. Based on their PCA scores, the dimensionality of the data was determined by JackStraw plotting, where significant PCs with a strong enrichment of low p-value features were plotted. Again, the heuristic method that generates an 'Elbow plot' where the ranking of principal components based on the percentage of variance was also used to find the dimensionality. Then, a KNN graph based on the Euclidean distance in PCA space was calculated for the data using the 'FindNeighbors' function. Further, the cells were clustered using 'FindClusters' with a resolution of 0.5. The expression of TLRs visualized in mouse and human healthy controls were determined using the 'Findallmarkers' function with a minimum pct 0.2.

To determine the gene expression in injured Vs healthy mice, we integrated the data of control (healthy) and data at 7 days, 14 days, and 30 days following injury (GSE132144). A Seurat object was created for both control and injury samples, and both were filtered for cells with mitochondrial DNA less than 10 % for control, 20 % for 7 days, and 10 % for 14 days and 30 days, with gene counts of more than 200. A merged Seurat object was created and further split by sample identities. Each sample was normalized using 'NormalizeData' and using the function 'FindVariableFeatures,' variable genes were found, and these variable features were then used for iterative pairwise integration [20]. Further, the 'SelectIntegrationFeatures' was used to determine the features for integrating the datasets together. Next, with 'FindIntegrationAnchors,' anchor points were identified, and data was integrated using the 'IntegrateData' function. Finally, the data was scaled, and dimensionality reduction was done by 'RunPCA' and 'RunUMAP' functions using the default assay as 'Integrated.' Further, to find the differentially expressed genes, the assay was changed to RNA, normalized the features using the 'NormalizeData' function, and scaled by the 'ScaleData' function. Markers in clusters were identified using the 'FindAllMarkers' function, comparing each cluster to all other cells. Genes that are expressed in at least 20 % of cells with a p-value <0.05 is plotted further.

For human single-cell data (GSE121893 and GSE109816), integrated analysis of healthy and heart failure samples was done. Samples were filtered for cells with mitochondrial percentages of less than 72 % and less than 200 genes. Like mouse data, a merged Seurat object was created with all samples. Then, samples were split by sample identities and further normalized. Variable features were found, and the data set was integrated using the 'FindIntegrationAnchors' function. Then, the data was scaled and visualized by running 'RunUMAP' functions using the default assay as 'Integrated.' Genes that are expressed in at least 20 % of cells with a p-value <0.05 is plotted further. The clusters were identified in human data based on the gene expression of cell type markers. The expression of genes in the fibroblast group comprising the clusters (10 and 11) was calculated. Gene expression in cells was compared and visualized using the DotPlot and Featureplot functions.

2.2. Human cardiac fibroblasts culture

Primary human cardiac fibroblasts (Promo Cell, Germany) were used for bulk RNA sequencing, and immortalized human cardiac fibroblasts (ABM, Canada) were utilized for all other functional assays and confirmatory in vitro experiments. Cardiac fibroblasts were cultivated as described before [21]. Briefly, the cells were grown in Ham's F-12K (Kaighn's) medium (GIBCO), supplemented with 10 % FBS, 10 ng/ml bFGF (Sigma), and 1X penicillin/streptomycin (GIBCO). For cardiac fibroblast activation, cells were treated with 10 ng/ml of TGF-β (Thermo) for 48 h in F-12K media with 2 % FBS.

2.3. Modulation of TLR4 signaling

To inhibit TLR4 signaling, the cells were pretreated with TAK-242 (4uM, Sigma) for 4 h, followed by a 60 h treatment with TGF-β (10 ng/ml) plus TAK-242 (4uM) in F-12K media with 2 % FBS. Cardiac fibroblasts treated with TGF-β in F-12K with 2 % FBS medium and cells in 2 % FBS medium with vehicle served as controls. The concentration of TAK-242 is based on previous literature [22,23]. Another TLR4 inhibitor, TLR4-IN-C34 (IN-C34, Sigma) at 50μM [24,25], was also pretreated for 4 h and then treated with TGF-β for 60 h. Cardiac fibroblasts treated with TGF-β in F-12K media with 2 % FBS served as control. Lipopolysaccharide (LPS) from E. coli (Sigma) was used as the TLR4 signaling agonist [26–28]. LPS at 1μg/ml was treated along with TGF-β, and samples were collected after 60 h of incubation. In this experiment, TGF-β alone and cells in 2 % FBS medium were also used as controls. To confirm the role of IL6 in TLR4-mediated modulation of cardiac fibroblast activation in Fig. 5F–H, we treated cardiac fibroblasts with exogenous IL6 (Pepro-Tech; Cat# 200-06) at 50 ng/ml along with TAK-242(4μM) and TGF-β (10 ng/ml). The cells were pretreated with TAK-242 for 4 h, followed by treatment with TGF-β only, TGF-β plus TAK-242, and TGF-β plus TAK-242 plus IL6 for 60 h in growth medium with 2 % FBS. Cardiac fibroblasts in 2 % FBS-containing growth medium served as control. We normalized the fold change in gene expression of the fibrotic genes ∝-SMA, Collagen1A1, and Periostin for the TGF-β condition. We plotted the difference in the fold expression of fibrotic genes in TGF-β+TAK-242 and TGF-β+TAK-242 supplemented with exogenous IL6 condition compared to TGF-β alone in cardiac fibroblasts.

2.4. Flow cytometry to determine TLR4 expression

To evaluate the TLR4 expression in cardiac fibroblasts using flow cytometry, cells were detached using accutase and then incubated with an anti-TLR4-APC antibody (eBioscience, Catalog No. 17-9917-42) for 30 min on ice. After washing, the cells were subjected to flow cytometric analysis in a BD (Becton, Dickinson) flow cytometer (FACSCalibur). The flow cytometric data were analyzed and visualized using Flowjo software (version 4.1).

2.5. Western blot assay to quantitate TLR4 expression

To determine the expression of TLR4 using Western blot, whole-cell lysates were prepared from 10^5 cells in lysis Buffer (25 mM Tris, 2 % β-mercaptoethanol, 1 % SDS, 0.1 % bromophenol blue, 5 % glycerol, pH 6.8) and further heated at 95 °C for 10 min. Samples were separated using a 7.5 % SDS gel and transferred to a PVDF membrane. The membranes were blocked with 5 % skimmed milk in Tris Buffered Saline with Tween-20 (TBS-T) and incubated overnight with primary antibodies, anti-TLR4 monoclonal antibody (Santa Cruz Biotechnology), and anti-GAPDH antibody (Origin Biosciences). Proteins were detected with chemiluminescence kit (Super-signal, Thermofisher Scientific). The images were captured using the GelDoc MP Imaging System (Bio-Rad). Bands from the Western blot were quantified with ImageLab software version 5.2 (Biorad). The band density was calculated using "Volume Tools" with background subtraction, and the density of the TLR4 bands was normalized to GAPDH and plotted in Fig. 1B.

2.6. Cytokine measurement using cytometric bead array

The Human TH1/TH2/TH17 Cytokine kit (Becton Dickinson Biosciences Pharmingen, San Diego, CA, USA) was used for quantitative analysis of IL-2, IL-4, IL-6, IL-10, TNF, IFN-g and IL-17A. Cytokine quantification was carried out according to the manufacturer's instructions. Briefly, 50 μl of the culture supernatant (cardiac fibroblasts under different treatment conditions) was mixed with an equal amount of capture beads and visualization reagent, then incubated for 3 h at room temperature in the dark. After washing, the beads were analyzed using the flow cytometer (FACSCalibur, BD) alongside serially diluted standards. The data was analyzed, and the concentration of cytokines was calculated based on the standard values in FCAP™ Array Software (BD® CBA Analysis Software).

2.7. Bulk RNA sequencing

For bulk RNA-seq, control cardiac fibroblasts and cells treated with TAK-242 alone, TGF-β alone, and TGF-β with TAK-242 for 48 h were harvested, and total RNA was extracted using RNeasy Mini kit (Qiagen). Libraries were prepared using standard Illumina RNA-seq library construction protocols, followed by sequencing using Illumina HiSeq X. Reads were aligned to the hg19 human genome using HISAT2 (2.1.0), and the raw reads were normalized using DESeq2. Differentially expressed genes (DEGs) in cardiac fibroblasts treated with either TGF-β alone, compared to untreated controls, and TGF-β Vs TGF-β plus TAK-242 were calculated. The identified DEGs with an adjusted p value less than 0.05 with a log2 fold change more than 1 or less than −1 were analyzed further. Gene Ontology and KEGG pathway analysis was performed to identify functional modules modulated with TAK-242 treatment. The top KEGG pathways significantly altered in each analysis were plotted with -log P-values. Heatmaps were visualized using the 'pheatmap' function in R.

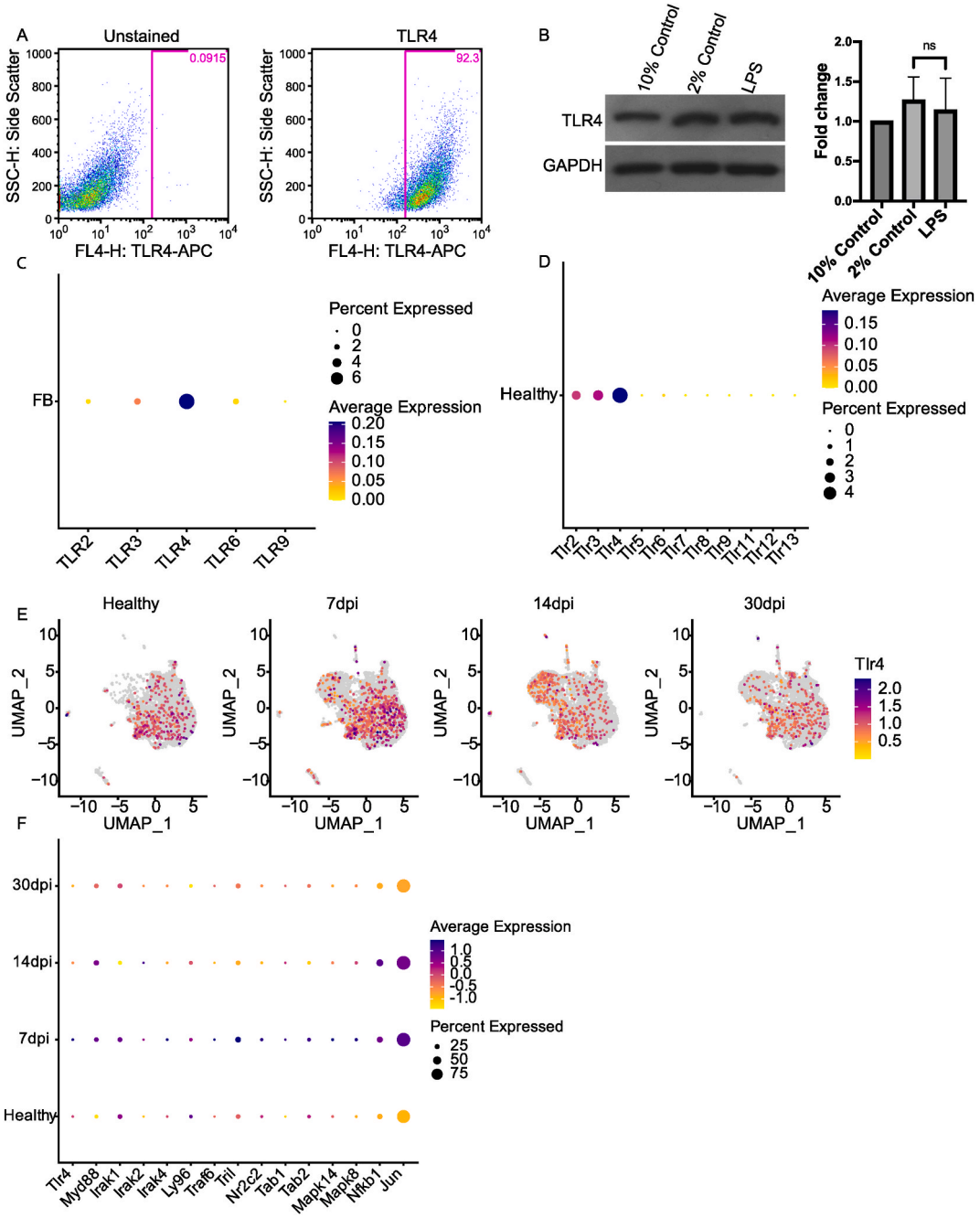

Fig. 1. The expression of TLRs in adult cardiac fibroblasts: (A) FACS analysis for TLR4 in human cardiac fibroblasts with unstained control and cardiac fibroblasts stained with anti-TLR4 APC antibody. (B) Western blot and quantitative analysis showing the expression of TLR4 in CF in growth medium with 10 % serum, 2 % serum and with LPS stimulation.(C) Dot plot representing the expression of different TLRs in human cardiac fibroblast clusters. (D) Dot plot representing the average expression and percentage cells expressing different Tlr genes in healthy mouse adult Col1-GFP labelled cardiac fibroblasts by single-cell RNA seq data. (E) Temporal changes in gene expression of Tlr4 following cardiac injury: UMAP demonstrating the expression of Tlr4 in Col1-GFP single-cell RNA seq data of healthy and at different time points at 7 days, 14 days, and 30 days following injury. (F) Dot plot demonstrating the expression of Tlr4, Myd88, Tril, Traf6, Tab2, Mapk14, Nfkb1, and Jun in healthy control and injured Col1-GFP labelled cardiac fibroblasts at 7 days, 14 days and 30 days post-injury by single-cell sequencing (n = 3).

2.8. Quantitative RT-PCR

RNA was isolated from cardiac fibroblasts cultured in the presence or absence of TAK-242 (F12K, 1X Penicillin/Streptomycin, 2 % FBS, ± TGF-β), grown for 48 h using the Total RNA Isolation Kit (Qiagen), and the cDNA was synthesized using superscript RT III

(Invitrogen). qPCR was performed using SYBR (Takara) on a Quantstudio5 thermocycler (Thermofisher Scientific). The quantitative real-time PCR primers used in the study are listed in Table 1.

2.9. Cell migration assay

The migration of cardiac fibroblasts was measured using a scratch-wound assay where the cells migrate from a confluent area to a wound area made by mechanically striping cells. Confluent monolayer of human cardiac fibroblasts grown in the growth medium was serum-deprived overnight, and then a scratch was made using a p1000 pipette tip. The plates were then rinsed with PBS to remove the suspended cells and incubated with F-12K with 2 % FBS supplemented with TGF-β in the presence and absence of TAK-242. Cells with 10 % FBS and 2 % FBS were included as controls. Wound closure was monitored, and images of the well area were captured at 0 h, 16 h, and 24 h following injury. The scratch area at different time points was measured using ImageJ, and the percentages of the final scratch area to the initial area were calculated for each sample. These experiments were repeated more than four times with triplicates each time.

2.10. Gel contraction assay

For gel contraction assay, $2x10^5$ cells were resuspended in type I collagen solution (3 mg/ml), 1M NaOH and F12K medium supplemented with 10 % FBS to get a final concentration of 1.2 mg/ml collagen in a total volume of 500μl. The mixture of collagen and cells was added to 24 well plates and polymerized for 20 min at room temperature. The gels were carefully detached from the well, and 500μl of F12K medium containing 2 % FBS with TGF-β was added to each well, with or without TAK-242. Gels with 10 % FBS and 2 % FBS with vehicle were included as controls. The cells were serum-deprived for 8 h and pretreated with TAK-242 for 4 h. The gels were photographed at 0 and 16 h, and the area of each gel was measured using ImageJ. Percentage reduction in gel area compared to the initial gel area in each well was calculated at all time points.

2.11. MTT assay to determine the viability of cardiac fibroblasts with drug treatment

In order to determine the viability with TLR4 inhibitors and agonist, human cardiac fibroblasts were seeded in 96-well microplates at a density of 5000 cells/well. The cells were treated with different concentrations of TAK-242, TLR4-IN-C34 and LPS for 24 and 48 h. MTT assay was done using EZcount MTT Cell Assay Kit (HiMedia) following the manufacturer's protocols. Briefly, the treatment media was replaced with media containing 10 % MTT reagent (v/v), and the cells were incubated for 2.5 h at 37 °C in a CO2 incubator. After incubation, 100ul of solubilization solution was added to each well. The plates were wrapped in aluminum foil and kept in a shaker to enhance the dissolution of the formazan crystals for 30 min. The absorbance was recorded at 570 nm with a reference wavelength of 630 nm.

2.12. Protein–protein interaction (PPI) network construction and visualization

The PPI network analysis was done using Search Tool for the Retrieval of Interacting Genes (STRING, https://string-db.org), a database of protein–protein interactions. To construct the network, we have used differentially expressed genes that were common in CF Vs TGF-β and TGF-β Vs TGF-β-TAK 242. Protein interaction networks with a score >0.4 was considered statistically significant and visualized using Cytoscape (version 3.8.2).

The most significant module in the network was identified using the Cytoscape plug-in Molecular Complex Detection (MCODE) (version 2.0), by clustering based on its topology. The hub genes were identified using the CytoHubba pluggin in the Cytoscape. Various topological features of the PPI network such as degree, centrality, density of maximum neighborhood component, ecCentricity, closeness, maximum neighborhood component, maximal clique, edge percolated component, bottleneck, radiality, and betweenness were analyzed to calculate the hub genes. Based on the degree method, the top 20 hub genes were selected that have the highest degree of connectivity. The nodes common to all topological features were considered as the hub genes in the network.

2.13. Statistical analysis

Data represents the mean ± SEM. Statistical significance between groups was calculated using a 2-tailed Student's t-test. A p-value of less than 0.05 was considered significant. qRT-PCR, migration, and gel contraction data were analyzed using GraphPad Prism10.

3. Results

3.1. TLR4 is the major immune sensor expressed in adult cardiac fibroblasts

To investigate how cardiac fibroblasts sense cellular damage, we screened the expression of different TLR receptors and observed that TLR4 is abundantly expressed in adult human cardiac fibroblasts (Supplementary Fig. 1A). Flow cytometric analysis using an anti-TLR4 antibody demonstrates that more than 90 % of the cardiac fibroblasts express TLR4 (Fig. 1A). The TLR4 expression in cardiac fibroblasts is further confirmed by Western blot analysis (Fig. 1B). By analysing the single-cell data of healthy human cardiac cells, we observed that TLR4 is the major TLR in cardiac fibroblast clusters (Fig. 1C). Moreover, using single-cell data from Collagen1α1-GFP

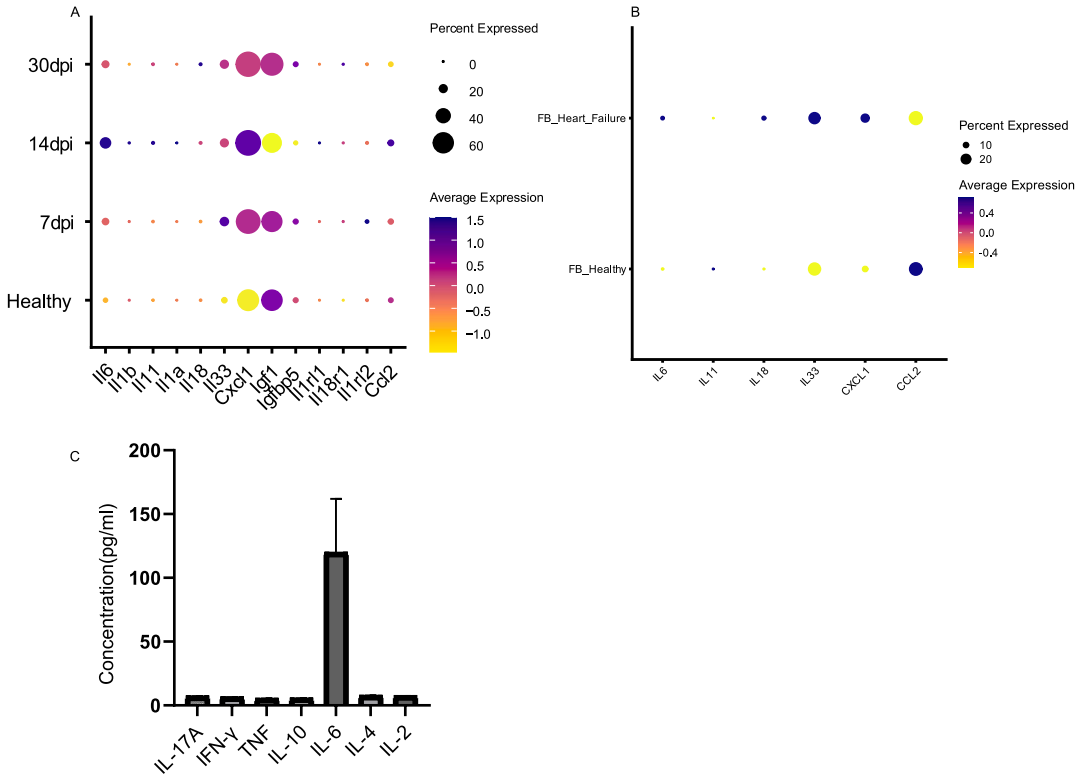

Fig. 2. Expression of genes encoding cytokines: (A) Expression of cytokines and related genes in mouse Col1-GFP cardiac fibroblasts in healthy control and on different days following injury. (B) Expression of cytokines and related genes in human cardiac fibroblast clusters of healthy and heart failure patients' sample. (C) Expression of cytokines in human cardiac fibroblasts in basal conditions in vitro.

mouse, having genetically labelled cardiac fibroblasts, we determined Tlr4 expression. We observed that Tlr4 is highly expressed in fibroblasts in the uninjured Collagen1α1-GFP mouse heart (Fig. 1D). Following cardiac injury in Collagen1α1-GFP mice by ligation of the left anterior descending (LAD) coronary artery, the expression of Tlr4 increased within 7 days of acute injury and remained persistently elevated even at 14 days and declined by 30 days (Fig. 1E). In addition, we found that the molecules associated with the Tlr4 signaling pathway, such as Myd88, Irak4, Cd4, Irf7, Hif1a, Tbk1, Traf6, Tab1, and Tab2, are significantly increased in cardiac fibroblasts after injury, suggesting active Tlr4 signaling in cardiac fibroblasts (Fig. 1F). Thus, TLR4 is expressed in basal conditions and increases with injury in vivo. However, in vitro, TLR4 is highly expressed under basal conditions, and the expression did not increase significantly when stimulated with lipopolysaccharide (LPS), the known TLR4 ligand (Fig. 1B). Based on these observations, we hypothesize that TLR4 acts as a significant sensor in adult cardiac fibroblasts and modulates fibroblast activation in response to changes in the extracellular niche.

Since Tlr4 pathway genes are induced in cardiac fibroblasts upon injury, we next examined the expression of downstream effectors of Tlr4 signaling, such as cytokines and other immune signaling molecules, in the single cell data from mouse. We found that several genes encoding cytokine and immune-related molecules were significantly induced in mouse cardiac fibroblasts in-vivo after injury with a peak expression by 7–14 days, with a few genes persistently elevated even by 30 days after injury, although at lower levels (Fig. 2A). The cytokine expression profile in cardiac fibroblasts closely correlates with the temporal pattern of Tlr4 pathway gene expression following injury. Moreover, cytokines and chemokines such as IL6, IL18, IL33 and CXCL1 are induced in the cardiac fibroblast clusters of heart failure patients (Fig. 2B), corroborating the single-cell sequencing results in Collagen1α1-GFP cells. Next, we analyzed the cytokine profile of adult human cardiac fibroblast secretome in vitro using a TH1/TH2/TH17 cytokine bead array. We found that IL6 is one of the major cytokines expressed in cardiac fibroblasts under baseline conditions, among those cytokines checked (Fig. 2C).

3.2. *Inhibiting TLR4 signaling reduces TGF-β mediated activation of cardiac fibroblasts*

Next, to understand the functional role of TLR4 signaling in cardiac fibroblasts in the presence of TGF-β, we inhibited TLR4 signaling using TAK-242 and analyzed how TLR4 signaling modulates the fibrotic gene expression, the contractile and migratory properties of cardiac fibroblasts. For that, we treated cardiac fibroblasts with TGF-β (10 ng/ml) for 48 h and examined the conventional fibrotic genes Collagen1A1 and α-Sma. We observed a 2.81 ± 0.31-fold increase in Collagen1, and a 1.83 ±0.18-fold increase in α-Sma expression in TGF-β treated cells compared to control, confirming the activation of cardiac fibroblasts (Fig. 3A, mean ± SE, n = 8).

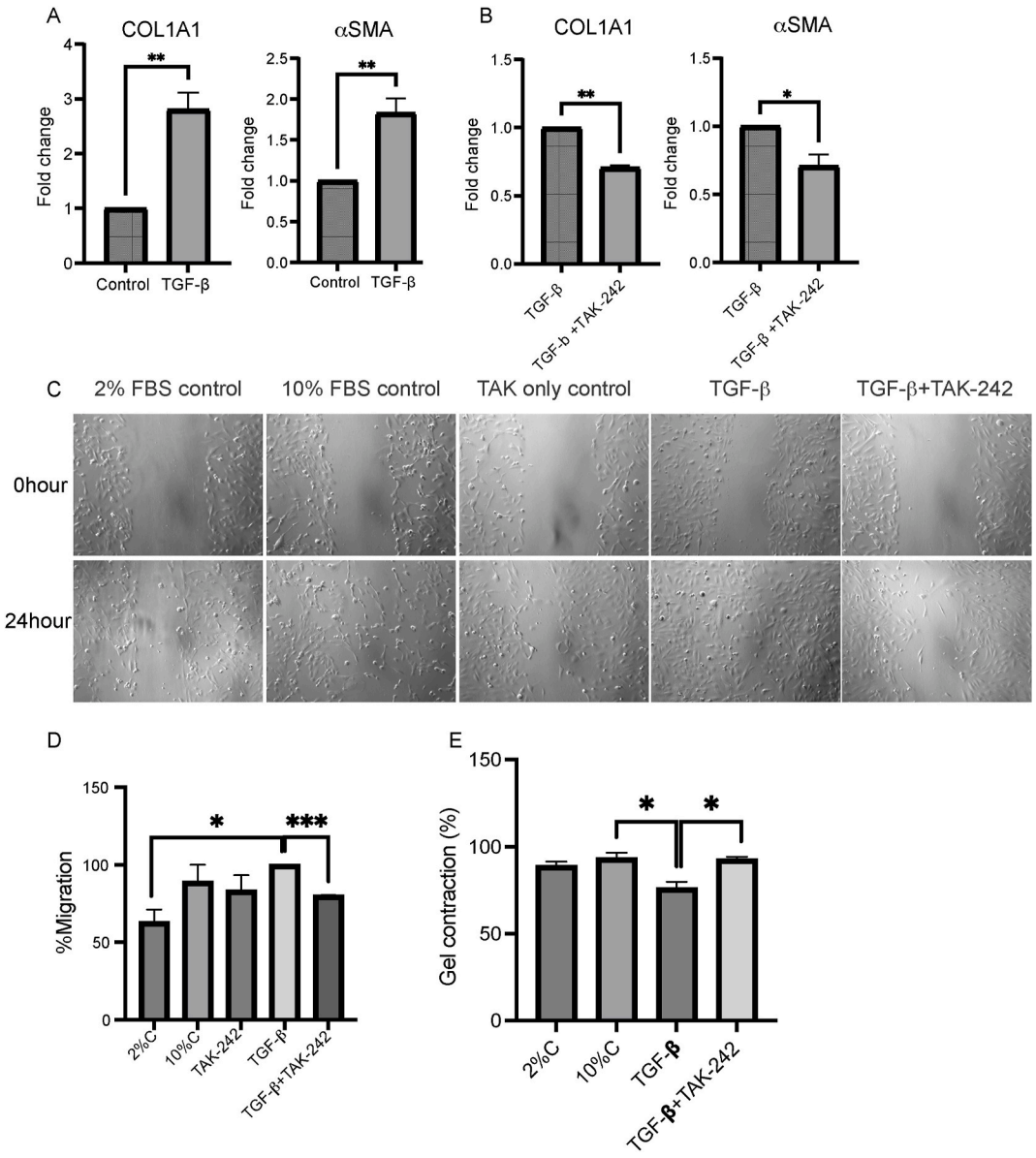

Fig. 3. Inhibiting TLR4 signaling reduces differentiation and migration of myofibroblasts: (A) qPCR showing the expression of fibrotic genes, Collagen1A1 and α-Sma in cardiac fibroblasts treated with TGF-β, n = 8. (B) qPCR showing the expression of fibrotic markers Collagen1A1 and α-Sma in TGF-β plus TAK-242 treated cells compared to TGF-β with vehicle alone control,n = 5.(C) Scratch wound assay to determine the migration of cardiac fibroblasts in the presence of TGF−β plus TAK-242, TGF-β alone and controls cells in medium with 10 % FBS and 2 % FBS. (D) Percentage area of cardiac fibroblast migration in TGF-β condition versus TGF-β plus TAK-242 treatment and controls,n = 4. (E) Cardiac fibroblasts seeded on contractable collagen gel matrices were assayed for gel contraction after treatment with TGF-β and TGF-β plus TAK-242 for 24 h, n = 4. Data are represented as the mean ± SE, *p < 0.05, **p < 0.01, ***p < 0.0001, ns = p > 0.05.

Interestingly, inhibition of TLR4 signaling reduced the α-Sma expression to 0.7± 0.86 ($p < 0.05$) fold and Collagen1A1 to 0.78±0.02($p < 0.05$) fold (Fig. 3B, mean ± SE, n = 5). Using an MTT assay, we confirmed that TAK-242 is not toxic to cardiac fibroblasts even at a concentration of 8μM (Supplementary Fig. 2A).

Next to determine whether the migration of myofibroblasts was modulated with TLR4 inhibition, we performed the scratch wound assay where the confluent cardiac fibroblasts treated with TGF-β in the absence and presence of TAK-242 in 2 % FBS medium for 48 h, were mechanically disrupted using a pipette tip and allowed to close the pseudo wound. We conducted the studies after serum deprivation for 8 h and at a low serum concentration of 2 % FBS to eliminate serum-induced modulation of cell migration. The cells in the medium containing 10 % FBS without TGF-β and TAK-242 act as the positive control, and cells in the medium containing 2 % FBS without TGF-β and TAK-242 act as negative control. The migration of myofibroblasts was visualized at 0 h, 16 h, and 24 h after wounding. We quantified the uncovered area from images taken at different points using the ImageJ. Data shows that under 10 % FBS

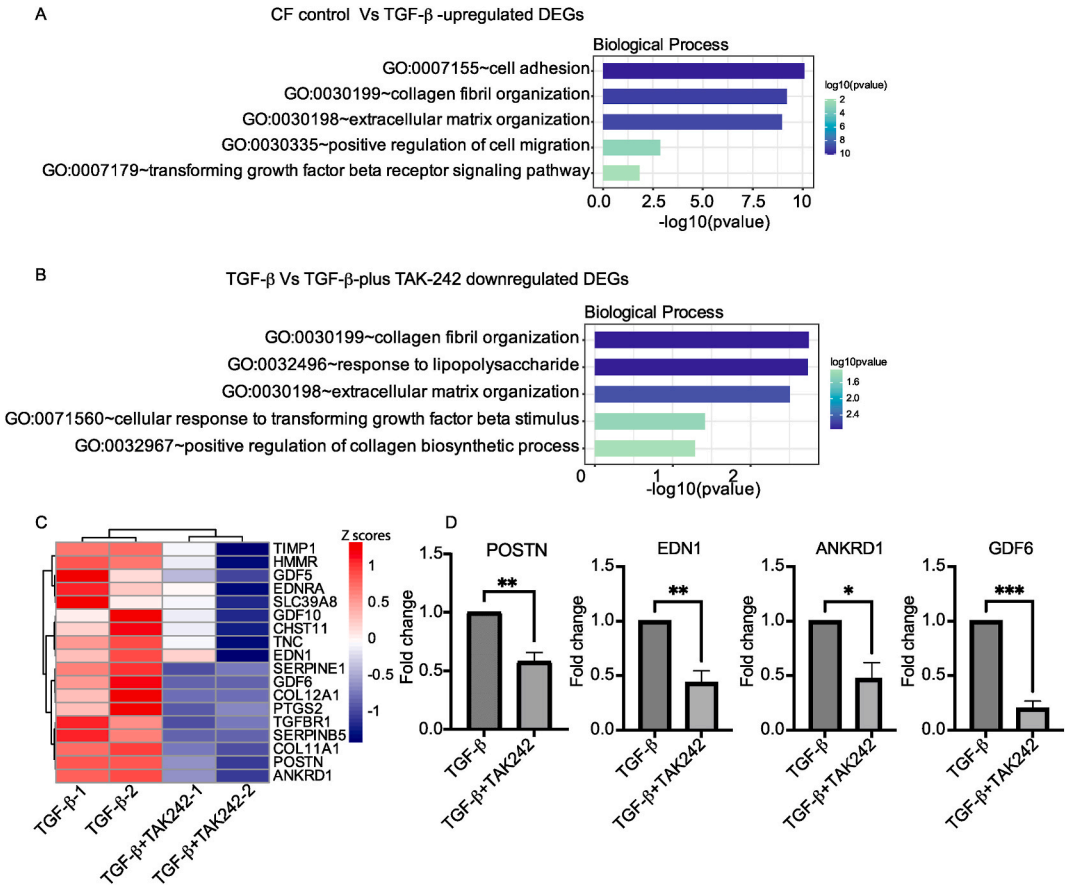

Fig. 4. Inhibiting TLR4 signaling reduces the expression of extracellular matrix genes: (A) GO enrichment analysis to determine the biological processess that are significantly altered with TGF-β treatment (for 48 h) in cardiac fibroblasts compared to control cells without TGF-β. (B) GO enrichment analysis to determine the biological processess significantly inhibited by TAK-242 in TGF-β treated cardiac fibroblasts. (C) Heatmap showing the scaled expression of extracellular matrix genes with TAK-242 treatment. (D) Expression of genes encoding profibrotic factors and extracellular matrix by qPCR in TGF-β+TAK-242 compared to TGF-β only, n = 4. Data are represented as the mean ± SE, *p < 0.05, **p < 0.01, ***p < 0.0001.

and in TGF-β conditions, cardiac fibroblasts have repopulated the wound area by almost 100 % within 24 h, in contrast, the TLR4 inhibition in the TGF-β condition inhibited cell migration to 80 ± 0.4 % (mean ± SE, n = 4, p < 0.001, Fig. 3C and D). Thus, cell migration determined by the percentage area of migration shows that TAK-242 inhibited the TGF-β-induced migration of human cardiac fibroblasts.

Further, to investigate whether TLR4 signaling modulates the gel contraction ability of cardiac fibroblasts, we stimulated cardiac fibroblasts in a 3D collagen gel with TGF-β, with and without TAK-242. Cells with TGF-β alone act as the control. The contraction of cells in 2 % FBS with TGF-β alone shows significantly increased contraction compared to control cells in 2 % FBS (p < 0.05). Inhibition of TLR4 signaling significantly reduced the TGF-β mediated gel contraction of cardiac fibroblasts with an increase in percentage gel area from 76.18 ± 3.68 to 92.82 ± 1.39 % (n = 4, mean ± SE, p < 0.05) (Fig. 3E). Thus, taken together, inhibition of TLR4 signaling significantly reduces fibrotic gene expression, migratory properties and gel contraction ability of cardiac fibroblasts.

3.3. TLR4 downregulation inhibits the pro-fibrotic transcriptional signature in cardiac fibroblasts

Next, to determine the molecular mediators inhibiting cardiac fibroblast activation, we conducted a transcriptome analysis by RNA sequencing of cardiac fibroblasts stimulated with TGF-β for 48 h in the presence and absence of TAK-242. Stimulation of cardiac fibroblasts with TGF-β significantly induced the upregulation of 988 genes and downregulation of 1709 genes using a filtering criterion of log2FC ≥ 1 and <=-1, respectively, with p-value- <0.05. Moreover, inhibition of TLR4 signaling in TGF-β treated CFs significantly downregulated 143 genes and upregulated 124 genes. Functional analysis of differentially expressed genes from TGF-β stimulated cells shows strong enrichment for biological processes related to extracellular matrix organization, cell adhesion, collagen fibril organization, cell migration, and TGF-β signaling pathway (Fig. 4A). This confirms the activation of cardiac fibroblasts with TGF-β treatment. Whereas inhibition of TLR4 signaling with TAK-242 significantly blocks some of those significant pathways and processes upregulated

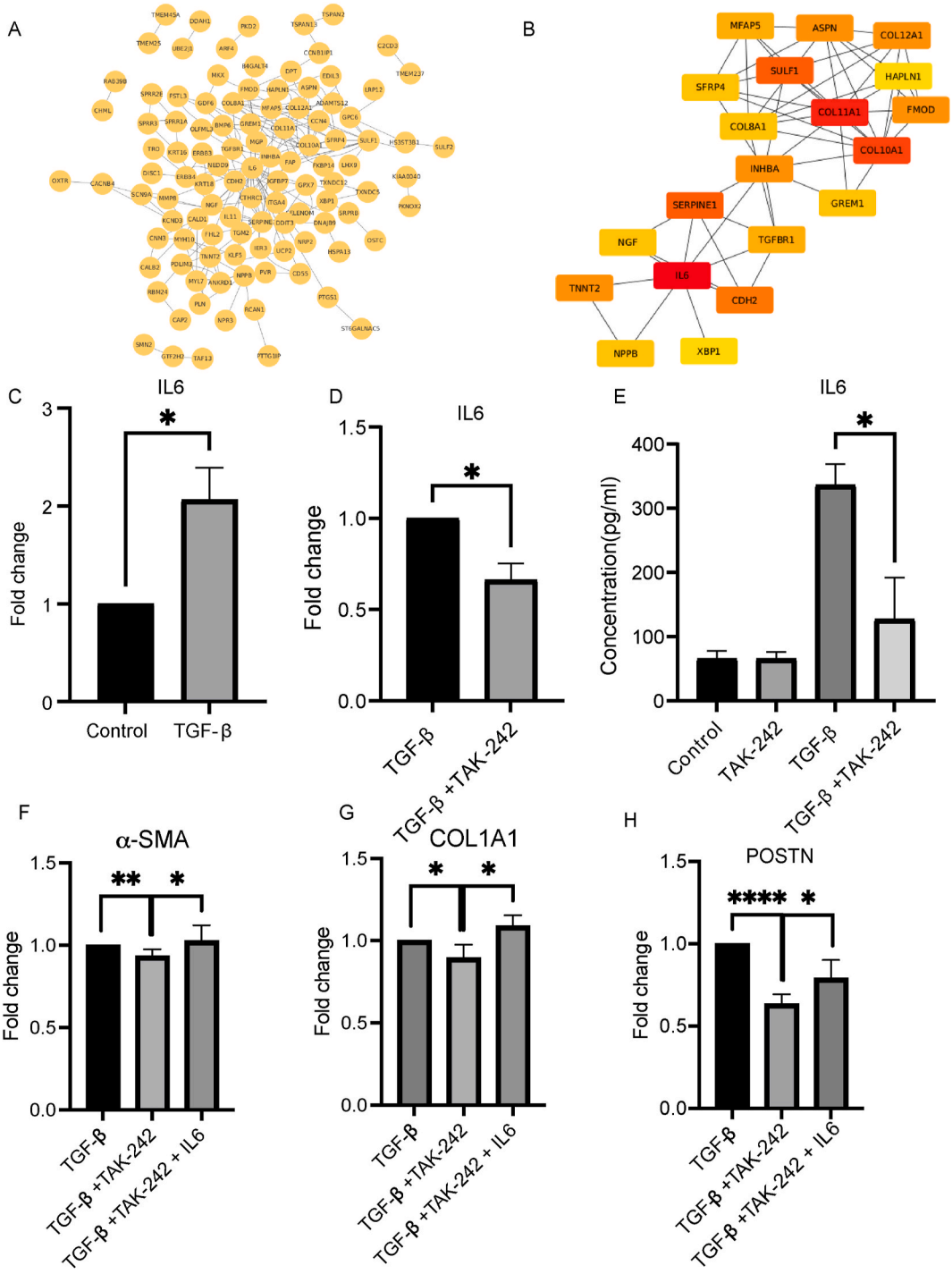

Fig. 5. Protein-protein interaction network analysis: (A) Protein-protein interaction network of cells treated with TAK-242 with TGFβ compared to TGFβ alone. (B) Top 20 hub genes identified using cytohubba in Cytoscape. (C) qPCR showing the fold increase in IL6 expression with TGFβ treatment in cardiac fibroblasts in vitro,n = 6,p < 0.05.

with TGF-β treatment (Fig. 4B).

Extracellular matrix genes are most differentially expressed genes 7–14 days following injury in-vivo in cardiac fibroblast (Supplementary Fig. 3A). Further, human heart failure data analysis also demonstrated that a diverse set of extracellular matrix-related genes, including several that are minimally expressed in the healthy heart, are robustly increased in the diseased cardiac fibroblasts (Supplementary Fig. 3B). We observed that inhibition of TLR4 signaling in cardiac fibroblasts inhibited a spectrum of extracellular

matrix genes (Fig. 4C).

Moreover, qPCR also confirms that TGF-β treatment increases the expression of extracellular matrix genes, Periostin by 11.76 ±2.88-fold, EDN1 by 2.96 ± 0.18, GDF6 by 14.23±3.01 and ANKRD1 by 1.61 ±0.06-fold (mean ± SE) compared to control (Supplementary Fig. 3C). Inhibition of TLR4 signaling in TGF-β treated cells reduced Periostin expression to 0.53± 0.09-fold ($p < 0.01$), EDN1 to 0.43± 0.11-fold ($p < 0.01$), GDF6 to 0.2±0.06-fold ($p < 0.001$) and ANKRD1 to 0.47 ±0.15-fold($p < 0.05$) (Fig. 4D, $n = 4$).

To further confirm the inhibition of fibroblast activation, we treated CF with another TLR4 signaling inhibitor, TLR4-IN-C34, along with TGF-β and analyzed the expression of Collagen1A1, Periostin, and α-Sma. We observed a slight reduction in α-SMA expression, but it was not significant. Expression of Collagen reduced significantly to 0.73 ±0.09-fold (mean ± SE, $p < 0.05$, $n = 4$) and Periostin to 0.69 ± 0.09-fold (mean ± SE, $p < 0.05$, $n = 4$), compared to cells treated with TGF-β alone. This data confirms the inhibition of extracellular matrix gene expression with the inhibition of TLR4 signaling (Supplementary Fig. 4). On the other hand, when we treated cardiac fibroblasts (CF) with LPS (1μg/ml) along with TGF-β, we observed a 1.82 ± 0.23-fold (mean ± SE, $p < 0.05$, $n = 4$) increase in Periostin compared to TGF-β alone. However, with LPS treatment, we did not observe a significant change in α-Sma and Collagen (Supplementary Fig. 5). In addition, cardiac fibroblasts in control conditions without TGF-β did not show any significant change in fibrotic genes when TLR4 signaling was modulated, except for LPS treatment. When cardiac fibroblasts were treated with LPS for 48 h, the expression of α-Sma significantly dropped to 0.72± 0.034-fold (Supplementary Fig. 6, mean ± SE, $p < 0.01$, $n = 4$). The data warrant further investigation to understand the role of TLR4 in inducing myofibroblast differentiation of cardiac fibroblast in control conditions. We further confirmed using an MTT assay that neither TLR4-IN-C-34 nor LPS has any cytotoxic effect in cardiac fibroblasts (Supplementary Fig. 2 B &C).Thus, these data collectively demonstrate that modulation of TLR4 signaling in adult cardiac fibroblasts influences TGF-β mediated synthesis of extracellular matrix proteins.

3.4. Protein-protein interaction network analysis reveals IL6 as the major hub gene reduced with TLR4 inhibition

In order to understand the regulatory molecules that induce the changes with TLR4 inhibition, we analyzed the protein-protein interaction (PPI) network of genes that are upregulated with TGF-β treatment and downregulated with TGF-β plus TAK-242 treatment in STRING. Using the MCODE algorithm in the Cytoscape, we identified two functional modules in the network. Module 1 has a cluster score of 4.5 with 5 nodes and nine edges. Module 2 has 7 nodes with 11 edges and a 3.667 cluster score. Subsequently, by hub gene analysis using the cytohubba plugin, we found that IL6 is one of the top-ranked hub genes (Fig. 5A–B). The hub gene analysis revealed that IL6 is connected to several key genes involved in cardiac fibrosis, such as COL11A1, COL10A1, SERPINE1, SULF1, CDH2, ASPN, FMOD, COL12A1, INHBA, MFAP, TNNT2, TGFBR1, COL8A1, SFRP4, GREM1, NGF, NPPB, HAPLN1 and XBP1.

Furthermore, qPCR showed that IL6 exhibited a 2.07±0.33-fold (mean ± SE, $p < 0.05$, $n = 6$) increase in TGF-β treated cells compared to control cells (Fig. 5C). Moreover, inhibition of TLR4 signaling in TGF-β treated cells exhibited a significant reduction to 0.66± 0.13-fold (Fig. 5D, $p < 0.05$, $n = 5$). Additionally, we confirmed that IL6 secretion in the medium was significantly reduced from 336.3 ±32.4 pg/ml in cardiac fibroblasts treated with TGF-β alone to 127.48± 64.85 pg/ml (mean ± SE) in TGF-β plus TAK-242 treated cells (Fig. 5E, $p < 0.05$, $n = 4$).

To confirm the role of IL-6 in the TLR4-mediated fibrotic response, we treated cardiac fibroblasts with IL-6 (5 ng/ml) in the presence of TGF-β and the TLR4 inhibitor TAK-242. We observed a significant reduction in the expression of fibrotic genes: COL1A1 to 0.89 ±0.03, α-SMA to 0.9 ±0.01, POSTN to 0.64 ±0.02-fold with TAK-242 treatment (mean ± SE, $p < 0.05$, $n = 6$, Fig. 5F–H). Further, adding IL6 to the TLR4-inhibited condition (TGF-β plus TAK-242 plus IL-6) significantly restored the expression of fibrotic genes.

Table 1
q-PCR primer sequences.

Primer list:	
Primer name	Primer Sequence (5′-3′)
TLR4 F	CTGCAATGGATCAAGGACCA
TLR4 R	TTATCTGAAGGTGTTGCACATTCC
POSTN F	GCGCTTTAGCACCTTCCT
POSTN R	GCACAAATAATGTCCAGTCTCC
α-SMA F	ACTGAGCGTGGCTATTCCTCCGTT
α-SMA R	GCAGTGGCCATCTCATTTTCA
COL1A1 F	GAACGCGTGTCAATCCCTTGT
COL1A1 R	GAACGAGGTAGTCTTTCAGCAACA
EDN1 F	GCTCGTCCCTGATGGATAAA
EDN1 R	TTCCTGCTTGGCAAAAATTC
ANKRD1 F	AGACTCCTTCAGCCAACATGATG
ANKRD1 R	CTCTCCATCTCTGAAATCCTCAGG
GDF6 F	TGCACGTGAACTTCAAGGAG
GDF6 R	CCCGCGTCGATGTATAGAAT
IL6 F	AGTCCTGATCCAGTTCCTGC
IL6 R	CTGGCATTTGTGGTTGGGTC
GAPDH F	GGTGTGAACCATGAGAAGTATGA
GAPDH R	GAGTCCTTCCACGATACCAAAG

Specifically, COL1A1 to 1.1 ± 0.02, α-SMA to 1.03 ± 0.03, Periostin to 0.8 ± 0.05-fold compared to the TGF-β condition (mean ± SE, $p < 0.05$, $n = 6$, Fig. 5F–H).

In conclusion, we found that in addition to immune cells in the heart, cardiac fibroblasts express TLR4 and the pro-inflammatory cytokines in response to injury. Inhibition of TLR4 signaling during TGF-β treatment significantly downregulated genes associated with the synthesis of cytokines, and extracellular matrix genes, thereby modulating the fibrotic response in cardiac fibroblasts in vitro. Thus, taken together, these observations demonstrate that the cardiac fibroblasts in the adult heart express TLR4 and respond to TLR4, suggesting a role of TLR4-dependent regulation of cardiac fibroblast activation.

4. Discussion

Immune cells such as macrophages, neutrophils, and T lymphocytes are long known to modulate the fibrotic/regenerative response in an injury niche [29–31]. However, how the immune response in cardiac fibroblasts, irrespective of the presence of other immune cells, regulates fibrotic changes is not clear. Our study shows that TLR4 signaling is active in adult cardiac fibroblasts, and inhibiting TLR4 signaling significantly reduces the fibrotic changes induced by TGF-β.

Our data demonstrates that TLR4 is the primary immune sensor in adult human and mouse healthy cardiac fibroblast. Furthermore, single-cell data of mouse CF at different time points following injury demonstrates that expression of TLR4 and components of the TLR4 signaling pathway increases following injury, implying active TLR4 signaling in CF during pathological stress conditions. The results are consistent with previous studies, which show that with TLRs and RLRs stimulation, human cardiac fibroblasts produce pro-inflammatory cytokines [32].

To investigate the functional role, we inhibited TLR4 in human cardiac fibroblasts in vitro using a known TLR4 signaling inhibitors, TAK-242, a small molecule [33] and TLR4-IN-C34. The inhibition reduced the expression of known fibrotic genes. Further, inhibition of TLR4 signaling has reduced the migration by 83 % and gel contraction of cardiac fibroblasts by 80 %. These results agree with the reports that silencing TLR4 using shRNA in spontaneously hypertensive rats inhibits atrial fibrosis and susceptibility to atrial fibrillation by downregulating NLRP3-TGF-β [34]. The results are also in agreement with reports of TLR4 mediated activation of organ fibrosis in skin and lung [35,36].

Furthermore, TLR4 activation in CF with LPS, protects against apoptosis induced by ischemia reperfusion injury through the activation of survival signaling pathways, Akt and ERK1/2 [37]. Transcriptome analysis shows that TLR4-inhibited cardiac fibroblasts downregulates several pro-fibrotic factors and ECM-related genes that are upregulated with TGF-β treatment. For example, Periostin is significantly inhibited with TLR4 downregulation and upregulated with TLR4 activation. Periostin expression is vital in fibrosis, and ablation of periostin-positive cells significantly inhibited fibrosis and improved heart functions [38].Periostin interacts with ECM and promotes myofibroblast differentiation, ECM production, and scar formation [39,40].

CF shows enhanced expression of cytokines, chemokines, and other pro-fibrotic growth factors upon injury in vivo and with TGF-β treatment in vitro. This confirms previous studies, which show that TLR4 inhibition with TAK-242 reduced LPS-mediated increased expression of cytokines, VCAM-1, and ICAM-1, which enhances the recruitment of neutrophils [41].

Our data by single cell analysis shows that IL6 is the major cytokine increased in cardiac fibroblasts after injury in mice and in heart failure patients. TGF-β treated cardiac fibroblasts in vitro also show enhanced expression of IL6 and inhibition of TLR4 signaling significantly reduces IL6 expression. The data again confirms that TLR4 signaling is active in cardiac fibroblasts and results in the expression of cytokines such as IL6.

IL6 augments TGF-β signaling during fibrosis [42] in different anatomical locations including heart [43–45]. For example, IL6 enhances fibrosis in skin and lung [44,46]. However, the molecular mechanisms by which IL6 modulates TGF-β signaling and induces fibrotic changes in cardiac fibroblast needs further investigation. In addition, treatment of TLR4 agonist in cardiac fibroblast in control conditions without TGF-β showed significant reduction in fibrotic gene expression, indicating a context dependant role of TLR4 in modulating the intrinsic properties of cardiac fibroblast.

So, the data from the paper demonstrates that cardiac fibroblasts are active participants in the immune environment following injury, with TLR4 being one of the significant sensors. Our findings support the hypothesis that TLR4 plays a role in modulating the intrinsic properties of cardiac fibroblasts independently of non-cardiac fibroblast cells such as immune cells present in vivo. In addition to the functional role in accelerating fibrosis, cell-autonomous TLR4 signaling may mediate the recruitment of other immune cells to the injury niche, modulating the repair process, which needs further investigation. TLR4 is a primary receptor in immune cells, and TLR4 signaling is required for a normal immune response; thus the inhibition of TLR4 may not be a viable strategy for treating fibrosis. However, downstream targets like reducing Periostin or IL6 would be considered a therapeutic option to avoid pathological fibrosis in the heart.

CRediT authorship contribution statement

Gayathri Vijayakumar: Writing – review & editing, Writing – original draft, Validation, Methodology, Conceptualization. **Anisha Latha:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Aiswaria P. Anil:** Methodology, Investigation. **Yogini Surve:** Visualization, Methodology, Investigation. **Aiswarya R:** Visualization, Methodology, Investigation. **Bipin G. Nair:** Resources, Project administration. **Indulekha CL. Pillai:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Data availability statement

Data generated from this manuscript will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

[1] J.G. Travers, F.A. Kamal, J. Robbins, K.E. Yutzey, B.C. Blaxall, Cardiac fibrosis: the fibroblast awakens, Circ. Res. 118 (2016), https://doi.org/10.1161/CIRCRESAHA.115.306565. Preprint at.
[2] P. Camelliti, T.K. Borg, P. Kohl, Structural and functional characterisation of cardiac fibroblasts, Cardiovasc. Res. 65 (2005), https://doi.org/10.1016/j.cardiores.2004.08.020. Preprint at.
[3] E.M. Zeisberg, R. Kalluri, Origins of cardiac fibroblasts, Circ. Res. 107 (2010).
[4] M. Bujak, N.G. Frangogiannis, The role of TGF-β signaling in myocardial infarction and cardiac remodeling, Cardiovasc. Res. 74 (2007), https://doi.org/10.1016/j.cardiores.2006.10.002. Preprint at.
[5] M.S. Stratton, et al., Dynamic chromatin targeting of BRD4 stimulates cardiac fibroblast activation, Circ. Res. 125 (2019).
[6] M. Zeisberg, R. Kalluri, Cellular mechanisms of tissue fibrosis. 1. Common and organ-specific mechanisms associated with tissue fibrosis, Am. J. Physiol. Cell Physiol. 304 (2013).
[7] N.A. Turner, Inflammatory and fibrotic responses of cardiac fibroblasts to myocardial damage associated molecular patterns (DAMPs), J. Mol. Cell. Cardiol. 94 (2016), https://doi.org/10.1016/j.yjmcc.2015.11.002. Preprint at.
[8] L. Timmers, et al., Toll-like receptor 4 mediates maladaptive left ventricular remodeling and impairs cardiac function after myocardial infarction, Circ. Res. 102 (2008).
[9] J.G. Rurik, H. Aghajanian, J.A. Epstein, Immune cells and immunotherapy for cardiac injury and repair, Circ. Res. 128 (2021), https://doi.org/10.1161/CIRCRESAHA.121.318005. Preprint at.
[10] C. García-Rodriguez, Toll-like receptor 4-dependent pathways as sensors of endogenous 'danger' signals. New evidences and potential therapeutic targets, Inmunologia 26 (2007), https://doi.org/10.1016/S0213-9626(07)70090-2. Preprint at.
[11] S. Bhattacharyya, J. Varga, Endogenous ligands of TLR4 promote unresolving tissue fibrosis: implications for systemic sclerosis and its targeted therapy, Immunol. Lett. 195 (2018), https://doi.org/10.1016/j.imlet.2017.09.011. Preprint at.
[12] R.Q. Dong, et al., Toll-like receptor 4 knockout protects against isoproterenol-induced cardiac fibrosis: the role of autophagy, J. Cardiovasc. Pharmacol. Therapeut. 20 (2015).
[13] P.B. Katare, P.K. Bagul, A.K. Dinda, S.K. Banerjee, Toll-like receptor 4 inhibition improves oxidative stress and mitochondrial health in isoproterenol-induced cardiac hypertrophy in rats, Front. Immunol. 8 (2017).
[14] H. Wu, et al., TLR4 activation mediates kidney ischemia/reperfusion injury, J. Clin. Investig. 117 (2007).
[15] H. Soraya, et al., Chronic treatment with metformin suppresses toll-like receptor 4 signaling and attenuates left ventricular dysfunction following myocardial infarction, Eur. J. Pharmacol. 737 (2014).
[16] Y. Yang, et al., The emerging role of toll-like receptor 4 in myocardial inflammation, Cell Death Dis. 7 (2016), https://doi.org/10.1038/cddis.2016.140. Preprint at.
[17] D. Kaczorowski, A. Nakao, K. McCurry, T. Billiar, Toll-like receptors and myocardial ischemia/reperfusion, inflammation, and injury, Curr. Cardiol. Rev. 5 (2009).
[18] A. Ruiz-Villalba, et al., Single-cell RNA sequencing analysis reveals a crucial role for CTHRC1 (collagen triple helix repeat containing 1) cardiac fibroblasts after myocardial infarction, Circulation 142 (2020).
[19] L. Wang, et al., Single-cell reconstruction of the adult human heart during heart failure and recovery reveals the cellular landscape underlying cardiac function, Nat. Cell Biol. 22 (2020).
[20] Y. Hao, et al., Integrated analysis of multimodal single-cell data, Cell 184 (2021).
[21] I.C.L. Pillai, et al., Cardiac fibroblasts adopt osteogenic fates and can Be targeted to attenuate pathological heart calcification, Cell Stem Cell 20 (2017).
[22] C. Muñoz-Rodríguez, et al., Expression and function of TLR4- induced B1R bradykinin receptor on cardiac fibroblasts, Toxicol. Appl. Pharmacol. 351 (2018).
[23] F. Olivares-Silva, et al., Heparan sulfate potentiates leukocyte adhesion on cardiac fibroblast by enhancing Vcam-1 and Icam-1 expression, Biochim. Biophys. Acta, Mol. Basis Dis. 1864 (2018).
[24] Y. Chen, D. Li, L. Sun, K. Qi, L. Shi, Pharmacological inhibition of toll-like receptor 4 with TLR4-IN-C34 modulates the intestinal flora homeostasis and the MyD88/NF-κB axis in ulcerative colitis, Eur. J. Pharmacol. 934 (2022).
[25] S.S. Zhang, et al., TLR4-IN-C34 inhibits lipopolysaccharide-stimulated inflammatory responses via downregulating TLR4/MyD88/NF-κB/NLRP3 signaling pathway and reducing ROS generation in BV2 cells, Inflammation 45 (2022).
[26] J. Sandstedt, et al., Human cardiac fibroblasts isolated from patients with severe heart failure are immune-competent cells mediating an inflammatory response, Cytokine 113 (2019).
[27] X. Wu, Y. Lv, Z. Li, Z. Yang, Serelaxin inhibits lipopolysaccharide-induced inflammatory response in cardiac fibroblasts by activating peroxisome proliferator-activated receptor-γ and suppressing the nuclear factor-kappa B signaling pathway, J. Cardiovasc. Pharmacol. 82 (2023).

[28] J. Ji, et al., Protective effects of rolipram on endotoxic cardiac dysfunction via inhibition of the inflammatory response in cardiac fibroblasts, BMC Cardiovasc. Disord. 20 (2020).
[29] A. Moskalik, J. Niderla-Bielińska, A. Ratajska, Multiple roles of cardiac macrophages in heart homeostasis and failure, Heart Fail. Rev. 27 (2022).
[30] T.A. Wynn, K.M. Vannella, Macrophages in tissue repair, regeneration, and fibrosis, Immunity 44 (2016), https://doi.org/10.1016/j.immuni.2016.02.015. Preprint at.
[31] Y. Zaidi, E.G. Aguilar, M. Troncoso, D.V. Ilatovskaya, K.Y. DeLeon-Pennell, Immune regulation of cardiac fibrosis post myocardial infarction, Cell. Signal. 77 (2021), https://doi.org/10.1016/j.cellsig.2020.109837. Preprint at.
[32] Z. Li, T.T. Nguyen, A. Valaperti, Human cardiac fibroblasts produce pro-inflammatory cytokines upon TLRs and RLRs stimulation, Mol. Cell. Biochem. 476 (2021).
[33] N. Matsunaga, N. Tsuchimori, T. Matsumoto, M. Ii, TAK-242 (resatorvid), a small-molecule inhibitor of Toll-like receptor (TLR) 4 signaling, binds selectively to TLR4 and interferes with interactions between TLR4 and its adaptor molecules, Mol. Pharmacol. 79 (2011).
[34] C. Ge, Y. Zhao, Y. Liang, Y. He, Silencing of TLR4 inhibits atrial fibrosis and susceptibility to atrial fibrillation via downregulation of NLRP3-TGF- β in spontaneously hypertensive rats, Dis. Markers 2022 (2022).
[35] S. Bhattacharyya, et al., TLR4-dependent fibroblast activation drives persistent organ fibrosis in skin and lung, JCI Insight 3 (2018).
[36] S., B, et al., Blockade of TLR4 signaling by TAK242 ameliorates experimental organ fibrosis, Arthritis Rheumatol. 66 (2014) S430.
[37] P. Parra-Flores, et al., Toll-like receptor 4 activation prevents rat cardiac fibroblast death induced by simulated ischemia/reperfusion, Front. Cardiovasc. Med. 8 (2021).
[38] H. Kaur, et al., Targeted ablation of periostin-expressing activated fibroblasts prevents adverse cardiac remodeling in mice, Circ. Res. 118 (2016).
[39] S. Zhao, et al., Periostin expression is upregulated and associated with myocardial fibrosis in human failing hearts, J. Cardiol. 63 (2014).
[40] P. Snider, et al., Origin of cardiac fibroblasts and the role of periostin, Circ. Res. 105 (2009), https://doi.org/10.1161/CIRCRESAHA.109.201400. Preprint at.
[41] R. Anfossi, et al., Interferon-β decreases LPS-induced neutrophil recruitment to cardiac fibroblasts, Front. Cell Dev. Biol. 11 (2023).
[42] J.H. Wang, et al., Hypoxia-stimulated Cardiac Fibroblast Production of IL-6 Promotes Myocardial Fibrosis via the TGF-B1 Signaling Pathway, 96, Laboratory Investigation, 2016.
[43] L.Z. Xiao, N. Topley, T. Ito, A. Phillips, Interleukin-6 regulation of transforming growth factor (TGF)-β receptor compartmentalization and turnover enhances TGF-β1 signaling, J. Biol. Chem. 280 (2005).
[44] S.A. Papiris, et al., High levels of IL-6 and IL-8 characterize early-on idiopathic pulmonary fibrosis acute exacerbations, Cytokine 102 (2018).
[45] C.P. Denton, et al., Therapeutic interleukin-6 blockade reverses transforming growth factor-beta pathway activation in dermal fibroblasts: insights from the faSScinate clinical trial in systemic sclerosis, Ann. Rheum. Dis. 77 (2018).
[46] C.A. Fielding, et al., Interleukin-6 signaling drives fibrosis in unresolved inflammation, Immunity 40 (2014).