SUPPLEMENTARY DATA

A Pro-Fibrotic Role for Interleukin-4 in Cardiac Remodeling and Dysfunction

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Supplementary Material and Methods

Animals
Eight- to twelve-week-old male wild-type (WT) Balb/c (stock number 000651) and interleukin (IL)-4[-/-] Balb/c mice (Balb/c-*Il4[tm2Nnt]*, stock number 002496) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in vented cages with a 12-hour light-dark cycle and fed *ad libitum*. WT and IL-4^{-/-} mice were infused with angiotensin II (Ang II, Bachem, Torrance, CA, 1.4 mg/kg/day) or vehicle (saline) continuously via subcutaneously implanted ALZET osmotic minipumps (Durect, Cupertino, CA). Since we previously reported that adult Balb/c mice exhibited fibrotic cardiomyopathy and developed dilated cardiomyopathy after exposure to Ang II for 8 weeks,[1] cardiac phenotype and function were assessed after 8 weeks of Ang II administration. The 4-week Ang II infusion protocol was chosen for examination of cardiac cytokines and monocyte chemoattractant protein-1 (MCP-1) because the alteration in cytokine and chemokine expression in response to Ang II precedes the development of target organ injury.[2,3] The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health and all animal protocols were approved by the Henry Ford Hospital Institutional Animal Care and Use Committee.

Measurement of systolic blood pressure (SBP) and echocardiograph
SBP was measured biweekly in conscious mice using a noninvasive computerized tail-cuff system (BP-2000, Visitech, Apex, NC) as described previously.[4]
Left ventricular diastolic dimensions (LVDd) and diastolic posterior wall thickness (PWT), as well as left ventricular ejection fraction (EF) and shortening fraction (SF), were measured in conscious mice using a Doppler echocardiograph with a 15-MHz linear transducer (Acuson c256, Mountain View, CA), as we previously described.[5,6]

Determination of interstitial collagen
Hearts were harvested as described previously.[1] The 6-µm left ventricle (LV) sections were deparaffinized, rehydrated, and stained with picrosirius red using a modification of Sweat and Puchtler's method.[7] The images of collagen morphometry were captured using a microscope (IX81; Center Valley, PA) equipped with a digital camera (DP70; Olympus American). Interstitial collagen fraction (ICF) was analyzed with Microsuite Biological Imaging software (Olympus), and expressed as percentage of interstitial collagen area of the total area of myocardium.

mRNA analysis
Total RNA from hearts or cells was isolated with TRIzol reagent and purified using an RNeasy fibrous tissue assay kit (Qiagen, Valencia, CA). Its integrity was checked on denaturing agarose gel, and it was quantified using a spectrophotometer (DU-600; Beckman, Brea, CA). The complementary DNA was obtained from 1 µg RNA using an Omniscript reverse transcription kit (Qiagen). For the detection of procollagen type-I alpha 1 (Col1α1), procollagen type-III alpha 1 (Col3α1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, quantitative real-time polymerase chain reaction (qRT-PCR) was performed with RT[2] SYBR green qPCR mastermix (Qiagen) on a Roche version 2.0 LightCycler PCR instrument (Indianapolis, IN). qRT-PCR of GAPDH was used for normalization. mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Data are presented as relative gene expression. Primers were designed by TIB MolBiol (Adelphia, NJ), as seen in Table S1.

Immunoblotting

Detection of cardiac IL-4: 60 µg LV lysate samples were prepared as we previously described.[8] The LV samples were subjected to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (2-mercaptoethanol) and electrotransferred to nitrocellulose membranes. The membranes were blocked with casein solution (Vector Laboratories, Burlingame, CA) and incubated with a rat monoclonal antibody against mouse IL-4 (11B11, 1:1000, #sc-32242, Santa Cruz Biotechnology, Santa Cruz, CA) in casein solution at 4°C overnight. Bound antibodies were visualized using a VECTASTAIN Elite ABC kit (Vector Laboratories) and enhanced chemiluminescence (ECL) reagent (Amersham Biosciences, Piscataway, NJ) according to the manufacturers' instructions. After detection of IL-4, the membrane was re-blotted with a rabbit monoclonal antibody against GAPDH (Cell Signaling Technology, Danvers, MA). Bound antibodies were visualized using a secondary antibody conjugated to horseradish peroxidase (Cell Signaling Technology) and ECL reagent. Band intensity was quantified by densitometry; IL-4 was normalized to GAPDH.

Detection of transforming growth factor-beta1 (TGF-β1): To test whether the pro-fibrotic effect of IL-4 is mediated via TGF-β1, TGF-β1 in the LV lysate was detected by Western blot under non-reducing conditions as we described previously.[9] The monoclonal antibody against TGF-β1 (1 µg/mL, #MAB240) was purchased from R&D Systems (Minneapolis, MN).

Detection of cardiac mast cells

The 6-µm LV sections were stained with toluidine blue to detect mast cell granules.[10] Mast cells stained a violet/dark blue color, and violet/dark blue-stained granule droplets were observed in some mast cells. Sections were examined by two investigators in a blinded manner. The numbers of total mast cells (intact and degranulating cells) were counted in the whole cross-sectional myocardium area and expressed as number of mast cells/10mm^2.

Immunohistochemical staining

The 6-µm LV cryosections were fixed in cold acetone and then incubated in 0.3% hydrogen peroxide to quench endogenous peroxidase activity. They were then preincubated in blocking solution (5% BSA PBS) for 30 minutes at room temperature, and finally incubated with a rat anti-mouse CD68 antibody (1:100, #MCA1957, AbD serotec, Raleigh, NC) at 4°C overnight. Sections were incubated with biotinylated rabbit anti-rat IgG antibody (Vector Laboratories), and then with a VECTASTAIN ABC reagent (Vector Laboratories). Sections were developed with 3-amino-9-ethylcarbazole and counterstained with hematoxylin. Positive cells showed red-brown staining around a blue nucleus. Sections were examined by two investigators in a blinded manner. The numbers of CD68$^+$ cells were counted and expressed as number of cells/mm^2.

Measurement of cytokines and MCP-1

About 20 mg of LV tissue was homogenized on ice in 150 µl of sample diluent (supplied with the kit) plus protease inhibitors (#P8340, Sigma-Aldrich, St. Louis, MO). Supernatants were collected after centrifugation at 14,000g for 10 minutes at 4°C, and protein contents were measured using Comassie reagent (Thermo Scientific). Protein samples were stored at -80°C until assay.

All samples were analyzed using a Bio-Plex Pro Mouse 8-plex cytokine kit (#M60-000007A, Bio-Rad, Hercules, CA) to measure IL-1β, IL-2, IL-4, IL-5, IL-10, interferon gamma (IFNγ), and

tumor necrosis factor alpha (TNFα). The assay was performed following the manufacturer's instructions with all samples diluted threefold using sample diluent supplied with the kit. Analysis of each sample was performed using a BioPlex 200 instrument (Bio-Rad). Cytokine concentrations were calculated from standard curves using Bioplex Manager 6.1 software (Bio-Rad). The amount of cytokine in the heart was corrected for the amount of protein taken for the assay and expressed as pg/mg. Values below the lowest levels of detection were reported as not detectable. The concentration ranges of detection for each cytokine were as follows: IL-1β (10.36 – 60,631 pg/ml), IL-2 (3.72 - 51,857 pg/ml), IL-4 (6.98 – 9,372 pg/ml), IL-5 (3.57 – 13,315 pg/ml), IL-10 (2.95 -12,066 pg/ml), IFNγ (1.84 – 30,164 pg/ml), TNFα (5.8 – 59,626 pg/ml). If a cytokine was detectable in some samples, but not in other samples, then concentrations below the lower limit of quantitation were set to a value half that of the lower limit of quantitation to allow for statistical analysis.[11]

MCP-1 levels were determined using a mouse/rat CCL2/JE/MCP-1 Quantikine ELISA kit (#MJE00; R&D Systems, Minneapolis, MN). The amount of MCP-1 in the heart was corrected for the amount of protein taken for the assay and expressed as pg/mg. The concentration ranges of detection are 15.6 – 1,000 pg/ml.

Cell culture
Cardiac fibroblasts were derived from ten- to twelve-week-old male WT C57BL/6 (stock number 000664), as well as STAT6^{-/-} mice [*B6.129S2(C)-Stat6^{tm1Gru}*; stock number 005977], who were mated to C57BL/6 for > 10 generations (Jackson Laboratory). Primary cultures of cardiac fibroblasts were prepared using a modified Eghbali's method,[12] with a digestion solution containing collagenase type II and trypsin (100 units/ml and 0.6 mg/ml, respectively, Worthington Biochemical Corporation, Lakewood, NJ). Five mice were used for each preparation. Fibroblasts were grown in low glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% fetal bovine serum (FBS).

To identify fibroblast population, cells grown on Nunc Lab-Tek II chamber slides (#154526) at 60% confluence were subjected to immunocytochemical staining with a mouse anti-vimentin antibody (1:200, #sc-6260, Santa Cruz Biotechnology) and a mouse anti-actin (α-smooth muscle) antibody (1:200, #A2547, Sigma) as described above for detection of CD68 in LV sections. Cells were also immunostained for cell-surface CD31 and CD68 as described below. Cultured cells at passages ≥ 3 clearly showed <15% of cells positive for α-smooth muscle actin (a marker for myofibroblasts; Figure S1A), all positive for vimentin (a marker for mesenchymal cells; Figure S1B), and all negative for both CD31 (a marker for endothelial cells) and CD68 (a marker for monocyte/macrophage lineages).

The cells at passages 3 to 5 were used in the experiments. Cells cultured in 6-well plates were made quiescent by culturing them in DMEM without FBS for 24 hours before treatment with recombinant murine IL-4 (PeproTech, Rocky Hill, NJ). IL-4 dose-dependently (0 to 50 ng/ml) induced phosphorylated STAT6 (P-STAT6) in cardiac fibroblasts, reaching peak levels at 10 ng/ml (Figure S2A). Therefore, 10 ng/ml of IL-4 was chosen for all experiments in our in vitro study.

Detection of cell-surface antigens by immunocytochemical staining
Live-cell cell-surface CD31, CD68 and IL-4 receptor alpha (IL-4Rα) in cultured cells were detected using a previously described method.[13] The following antibodies were used: a rabbit anti-IL-4Rα antibody (1:100, #sc-686, Santa Cruz Biotechnology), a rat anti-mouse CD68

antibody (1:200, #MCA1957, AbD serotec) and a rabbit anti-CD31 antibody (1:100, #ab28364, Abcam, Cambrige, MA). Briefly, cells grown on chamber slides were 60-70% confluent. Cells were incubated with an antibody (diluted with DMEM containing 10% FBS and 10 mM HEPES) on ice for 45 minutes. Each slide was washed three times in Hank's balanced salt solution with 10 mM HEPES and incubated with secondary antibody, Alexa-Fluor 488-conjugated donkey anti-rabbit IgG or Cy3-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), on ice for 30 minutes. After washing three times, cells were fixed with 1% paraformaldehyde (pH 7.4) on ice for 20 minutes. Positive cells displayed bright green (Alexa-Flour 488) or red (Cy3) fluorescent particles on the cell membrane. The images of the cells were captured using a microscope (IX81; Olympus American) equipped with a digital camera (DP70; Olympus American).

Western blot to detect IL-4Rα and phosphorylated signal transducer and activator of transcription 6 (P-STAT6)
Preparation of cell lysates and Western blot were performed as we previously described.[14] A rabbit polyclonal antibody against IL-4Rα (1:500, #sc-686) and P-STAT6 (1:500, #sc-11762) were purchased from Santa Cruz Biotechnology. The specificity of the antibody against P-STAT6 was tested in IL-4-stimulated cardiac fibroblasts from STAT6$^{-/-}$ mice (Figure S2B). Once P-STAT6 was detected, the blots were treated with a stripping buffer (Pierce, Rockford, IL) and reblotted with a rabbit polyclonal antibody against STAT6 (1:500, #sc-621, Santa Cruz Biotechnology). P-STAT6 was normalized to STAT6. GAPDH was detected to show equal protein loading for IL-4Rα.

Hydroxyproline assay
Quiescent cells on 6-well plates were cultured in 1 ml of 0.4% FBS DMEM containing 0.15 mM L-ascorbic acid in the presence or absence of IL-4 for 48 hours. Conditioned media were collected for measurement of hydroxyproline using a modified assay based on a previously described method.[15] Briefly, conditioned media were collected to a tube containing two volumes of absolute ethanol and allowed to precipitate at -20°C overnight. Cells were scraped into 100 μl of lysis buffer (Cell Signaling Technology) with cell lifter, then the protein contents of cell lysates measured using Comassie reagent (Thermo Scientific). After precipitation, medium samples were centrifuged at 14,000g for 30 minutes. The pellet was air-dried and re-suspended in 500 μl of 6N HCl in a reaction vial (Pierce), and subjected to hydrolysis at 110°C for 16 hours in the Reacti-Therm III-Heating Module (Pierce). The hydrolyzed samples were dried in a Savant (SPA131DDA; Thermo Scientific). The residue was dissolved in 100 μl of water and used for measurement of hydroxyproline as described by H. Stegemann et al.[16] The amount of hydroxyproline was determinated from a 0 - 5 μg standard curve of hydroxproline (Sigma). Collagen contents in conditioned media of fibroblast cultures were expressed as μg/mg protein, assuming collagen contains an average of 13.5% hydroxyproline.[17]

Statistical analysis
A set of pre-specified hypotheses were tested using a two-sample two-sided Wilcoxon test. The nonparametric approach was used due to the large observed differences in the variances. Each set of tests was evaluated for significance using Hochberg's method of correction for multiple testing. An overall 0.05 alpha value was set as the criteria for significance. All testing was done on SAS 9.2. Data are reported as mean plus or minus the standard error.

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Table S1. RT-PCR primers for mRNA expression (SYBR Green)

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
Col1α1	CTATCACTgCAAgAACAgCgT	TTCACCCTATTATggAgACgAT
Col3α1	AggAAACTACATTCTTCAggTCg	CAgCTACCTggTgCCTgA
GAPDH	ATTCAACggCACAgTCAAgg	TggATgCAgggATgATgTTC

RT-PCR, real-time polymerase chain reaction; Col1α1, procollagen type-I alpha 1; Col3α1, procollagen type-III alpha 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

A   α-SMA

B   Vimentin

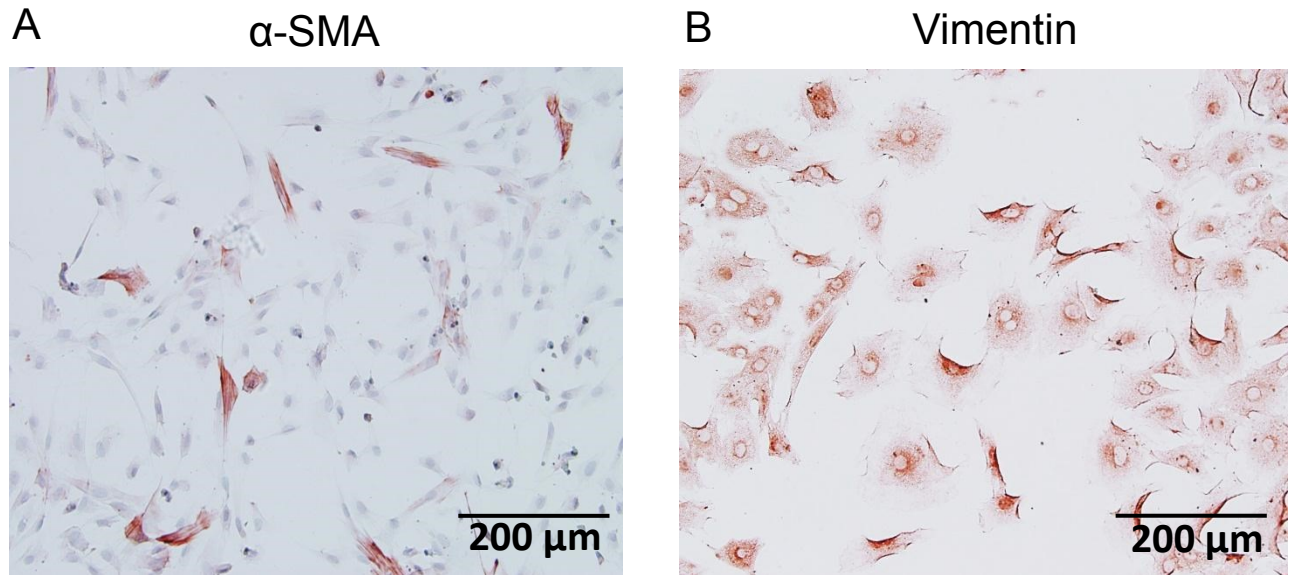Figure S1. Identification of fibroblast population in cultured adult mouse cardiac fibroblasts. Representative images of α-smooth muscle actin (α-SMA)-positive cells (**A**) and vimentin-positive cells (**B**). Positive cells show red-brown color.

A

IL-4 (ng/ml)

| | CTL | 0.4 | 2.5 | 5 | 10 | 50 |

P-STAT6

STAT6

B

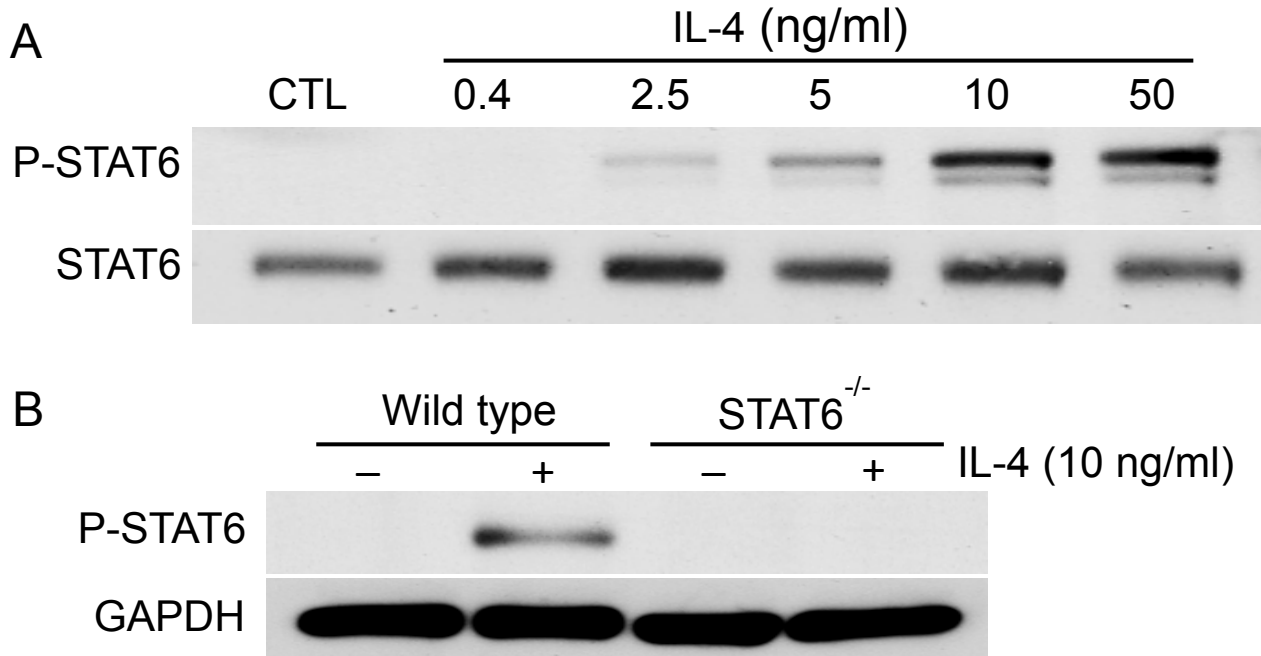| | Wild type | | STAT6$^{-/-}$ | | |
| | − | + | − | + | IL-4 (10 ng/ml) |

P-STAT6

GAPDH

Figure S2. Interleukin-4 (IL-4)-induced phosphorylation of signal transducer and activator of transcription 6 (STAT6) in cultured adult mouse cardiac fibroblasts. IL-4-induced STAT6 phosphorylation was analyzed by Western blot using 20 µg of cell lysates. Phosphorylated STAT6 (P-STAT6) increased in a dose-dependent manner after quiescent cells were stimulated with various concentrations of IL-4 for 25 minutes (**A**). Incubation of the cells from STAT6$^{-/-}$ mice with IL-4 for 25 minutes did not produce a band (**B**). The image represents results from three independent experiments.
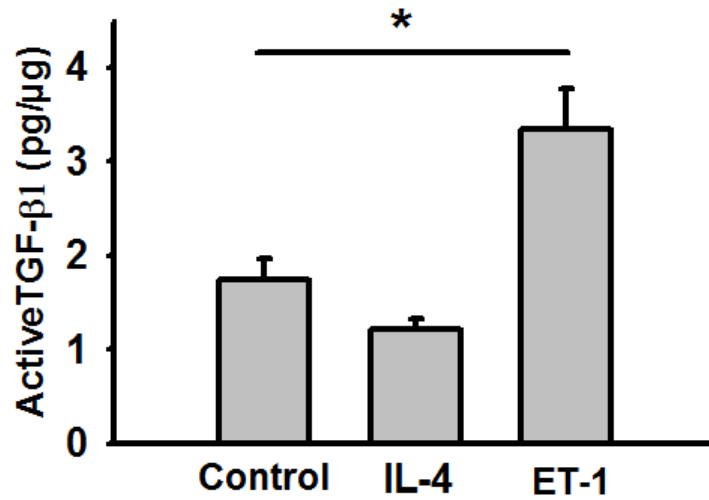
Figure S3. Interleukin (IL-4)-induced transforming growth factor-beta 1 (TGF-β1) in cultured adult rat cardiac fibroblasts. Cultured fibroblasts were treated with recombinant rat IL-4 (PeproTech, 10 ng/ml) for 48 hours. Active TGF-β1 in the conditioned media was measured using a mouse/rat/porcine/Canine TGF-β1 Quantikine ELISA kit (#MB100B, R&D Systems) with endothelin-1 (ET-1, Bachem, 10^{-8} M) serving as a positive control. The amount of TGF-β1 was corrected for the amount of protein in the cell lysate. The bars represent mean±SEM, *$p<0.05$, a two-sample two-sided Wilcoxon test with a Hochberg correction for multiple testing, n=3 per group.