Progressive cardiac fibrosis is a major maladaptive response to hemodynamic stress and various profibrotic stimuli. It leads to a reduction in myocardial compliance and eventually to cardiac failure. Cytokine interleukin (IL)-4 levels are associated with cardiac fibrosis in hypertension and heart failure in both patients and experimental animals. We hypothesized that chronically elevated IL-4 induces cardiac fibrosis, resulting in a predisposition of the heart to angiotensin II–induced damage. Wild-type Balb/c (WT, high circulating IL-4) and IL-4–deficient Balb/c mice (IL-4−/−) were used. WT mice exhibited cardiac fibrosis (evidenced by an increase in expression of procollagen genes/interstitial collagen fraction), enlarged left ventricle chamber, and declined cardiac function associated with a greater number of mast cells and macrophages in the heart compared with IL-4−/−. In contrast, IL-4−/− mice had normal cardiac architecture/function while showing a 57.9% reduction in heart interstitial collagen compared with WT, despite elevated proinflammatory cytokines in heart tissue. In response to angiotensin II administration, IL-4−/− had reduced interstitial myocardial fibrosis and were protected from developing dilated cardiomyopathy, which was seen in WT mice. This was associated with increased macrophage infiltration into the hearts of WT mice, despite a similar degree of hypertension and increased cardiac transforming growth factor-β1 in both groups. In vitro data demonstrated that IL-4 upregulates procollagen genes and stimulates collagen production in mouse cardiac fibroblasts. This process is mediated by signal transducer and activator of transcription 6 signaling pathway via IL-4 receptor alpha. This study not only establishes a causal relationship between IL-4 and cardiac fibrosis/dysfunction, but also reveals a critical role for IL-4 in angiotensin II–induced cardiac damage. IL-4 could serve as an additional target for the treatment of cardiac fibrosis.

Key Words: angiotensin II • balb/c mice • fibrosis • heart • interleukin-4

Abstract—Elevated interleukin-4 (IL-4) levels are associated with cardiac fibrosis in hypertension and heart failure in both patients and experimental animals. We hypothesized that chronically elevated IL-4 induces cardiac fibrosis, resulting in a predisposition of the heart to angiotensin II–induced damage. Wild-type Balb/c (WT, high circulating IL-4) and IL-4–deficient Balb/c mice (IL-4−/−) were used. WT mice exhibited cardiac fibrosis (evidenced by an increase in expression of procollagen genes/interstitial collagen fraction), enlarged left ventricle chamber, and declined cardiac function associated with a greater number of mast cells and macrophages in the heart compared with IL-4−/−. In contrast, IL-4−/− mice had normal cardiac architecture/function while showing a 57.9% reduction in heart interstitial collagen compared with WT, despite elevated proinflammatory cytokines in heart tissue. In response to angiotensin II administration, IL-4−/− had reduced interstitial myocardial fibrosis and were protected from developing dilated cardiomyopathy, which was seen in WT mice. This was associated with increased macrophage infiltration into the hearts of WT mice, despite a similar degree of hypertension and increased cardiac transforming growth factor-β1 in both groups. In vitro data demonstrated that IL-4 upregulates procollagen genes and stimulates collagen production in mouse cardiac fibroblasts. This process is mediated by signal transducer and activator of transcription 6 signaling pathway via IL-4 receptor alpha. This study not only establishes a causal relationship between IL-4 and cardiac fibrosis/dysfunction, but also reveals a critical role for IL-4 in angiotensin II–induced cardiac damage. IL-4 could serve as an additional target for the treatment of cardiac fibrosis. (Hypertension. 2015;66:582-589. DOI: 10.1161/HYPERVISIONAHA.115.05627.) • Online Data Supplement

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fibrosis, dilated left ventricular chamber, and declined cardiac function) in Ang II–induced hypertension. Furthermore, we examined the effects of IL-4 on cardiac fibroblasts, the primary cellular source of collagens in the heart, and found that IL-4 not only significantly increased procollagen mRNA but also promoted collagen production through the signal transducer and activator of transcription 6 (STAT6) signaling pathway via IL-4 receptor alpha (IL-4Rα).

**Methods**

Detailed description of methods is available in the online-only Data Supplement.

**Results**

**Systolic Blood Pressure, Cardiac Remodeling, and Function**

Basal systolic blood pressure was similar between WT and IL-4−/− mice (Figure 1A). Ang II administration resulted in a significant increase in systolic blood pressure within 2 weeks of exposure to Ang II and stayed elevated until the end of the experiment (8 weeks). There were no differences in the degree of induced hypertension between the groups at any time point measured (Figure 1A). Ang II also induced comparable cardiac hypertrophy in WT and IL-4−/− mice as indicated by significantly increased left ventricle (LV) weights after 8 weeks of Ang II infusion (Figure 1B).

Cardiac geometry and function are shown in Figures 1C–1F. At 16 to 20 weeks of age, WT mice showed significantly enlarged LV chamber and declined cardiac function compared with IL-4−/−. WT mice exhibited increased LV weight, severe LV chamber dilatation, wall thinning, and dramatically decreased ejection fraction and shortening fraction after 8 weeks of Ang II administration. In contrast, IL-4−/− mice had significantly increased LV weight to tibia length ratio and diastolic posterior wall thickness in response to Ang II and increased systolic blood pressure. In Ang II–treated IL-4−/− mice, ejection fraction and shortening fraction were decreased compared with vehicle-treated mice.

**Cardiac Fibrotic Remodeling**

As detected by immunoblotting, IL-4 was expressed in the hearts of WT but was absent in IL-4−/− mice (Figure 2A). The fibrosis shown in WT mice with Ang II–induced hypertension was characterized as a dense crisscrossing meshwork of collagen fibers encircling muscle fibers and accumulated collagen extending outward from perivascular/pericapillary space (Figure 2B). Cardiac fibrosis seen in WT mice showed a significantly higher interstitial collagen fraction in the LV myocardium when compared with mice with genetic IL-4 deletion (Figure 2C). IL-4 deletion attenuated cardiac fibrosis by 57.9% (3.85%±0.53% in WT versus 1.62%±0.22% in IL-4−/−, \( P < 0.005 \)). Ang II infusion for 8 weeks resulted in a significant increase in interstitial collagen fraction in both WT and IL-4−/− mice with significantly higher interstitial collagen fraction in WT compared with IL-4−/− mice (Figure 2C). In addition, significantly higher mRNA levels of procollagen type-I alpha 1 (Col1α1) and procollagen

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**Figure 1.** Systolic blood pressure (SBP) and cardiac remodeling in wild-type (WT) and interleukin-4 (IL-4)−/− mice at the steady-state condition and angiotensin (Ang) II–induced hypertension. **A**, SBP data. Results represent mean±SEM. \( *P < 0.005 \) vs WT+vehicle; \( *P < 0.005 \) vs IL-4−/−+vehicle, a Student’s \( t \) test with a Hochberg correction for multiple testing. Cardiac hypertrophy/remodeling and function assessed by left ventricle weight (LVW) to tibia length (TL) ratio (**B**) or echocardiography as the sum of diastolic posterior wall thickness (PWT; **C**), left ventricular diastolic dimension (LVDd; **D**), ejection fraction (**E**), and shortening fraction (**F**) after 8 weeks of Ang II treatment in WT and IL-4−/− mice. The bars represent mean±SEM. \( *P < 0.05 \), \( **P < 0.005 \), a 2-sample 2-sided Wilcoxon test with a Hochberg correction for multiple testing, \( n = 6 \) to 14 per group.
type-III alpha 1 (Col3α1) were found in the hearts of WT mice compared with that in IL-4−/− (Figure 2E). Ang II induced significant upregulation of these 2 procollagen genes in the hearts of both WT and IL-4−/− mice with significantly higher mRNA level of Col3α1 in WT+Ang II than in the IL-4−/− mice+Ang II group (Figure 2D and 2E).

**Mast Cells and CD68+ Macrophages in the Heart**

Mast cells were primarily observed surrounding the vessels and the pericardium (Figure 3A). A more than 2-fold increase in total mast cells was seen in the hearts of WT mice compared with IL-4−/−; however, Ang II infusion for 8 weeks did not change the numbers of mast cells in the hearts (Figure 3B). A similar pattern was observed with CD68+ macrophages in the hearts of vehicle-treated WT and IL-4−/− mice. Chronic Ang II administration significantly increased the numbers of macrophages in the hearts of WT but not in IL-4−/− mice (Figure 3C and 3D). Noticeable accumulation of macrophages was also seen around the vessels.

**Cardiac Cytokines and Monocyte Chemoattractant Protein-1 in the Heart**

There were no significant differences in cardiac transforming growth factor-β1 (TGF-β1) levels between WT and IL-4−/− mice. Ang II administration for 8 weeks significantly increased TGF-β1 levels in the hearts of both groups (Figure 4A). Values of IL-4 and IL-5 in the mouse hearts were below the lowest levels of detection. Interferon gamma (IFNγ) in the hearts of WT mice was too low to detect with the used kit; however, IL-4 deletion induced robust IFNγ production (Figure 4B). Significant increases in IL-10, IL-1β, IL-2, and tumor necrosis factor-α were observed in the hearts of IL-4−/− mice compared with WT mice (Figure 4C–4F). Interestingly, chronic Ang II administration for 4 weeks resulted in a significant reduction in cardiac IL-1β, IL-2, and tumor necrosis factor-α only in IL-4−/-deficient mice. Cardiac monocyte chemoattractant protein-1 (MCP1) levels in WT mice were >2× greater than those in IL-4−/− mice. Ang II infusion for 4 weeks induced increases in MCP-1 in the hearts, which was significant in WT mice (Figure 4G).

**IL-4 Signaling and Collagen Production in Cardiac Fibroblasts**

Immunocytochemistry results showed constitutive expression of functional IL-4Rα on the cell-surface of mouse cardiac fibroblasts (Figure 5A). Western blot analysis revealed that IL-4Rα expression was doubled after cells were incubated with IL-4 for 24 hours in a STAT6-dependent fashion (Figure 5B). IL-4 rapidly induced phosphorylation of STAT6.
(within minutes), with the phosphorylation lasting >24 hours (Figure 5C). Importantly, IL-4 markedly increased collagen production (Figure 5D) and significantly induced Col1α1 and Col3α1 mRNA expression by cardiac fibroblasts (Figure 5E and 5F); these effects were STAT6-dependent.

**Discussion**

This is the first study to establish a causal relationship between chronically elevated IL-4 and cardiac fibrotic remodeling and dysfunction. Not only does high IL-4 induce LV fibrosis and dysfunction, but its deletion markedly attenuates this effect as demonstrated in Balb/c hearts and IL-4−/− Balb/c hearts, respectively. The present study also showed that in contrast to WT, IL-4−/− mice were protected from fibrotic lesions and did not develop DCM in Ang II–induced hypertension.

The role for IL-4 in the regulation of blood pressure has not been adequately addressed previously. We did not observe significant differences in systolic blood pressure between WT and IL-4−/− mice at both basal condition and Ang II–induced hypertension. This finding is in line with a previous study showing that systemic administration of an anti-IL-4 neutralizing antibody did not affect the increased blood pressure in C57BL/6 mice with aortic coarctation. Vascular endothelial cells express IL-4Rα and respond to IL-4. In the lungs, IL-4 activates pulmonary endothelial cells and induces endothelin-1 production, resulting in the development of pulmonary hypertension. Therefore, we cannot exclude the possibility that IL-4 may play a role in the development of hypertension over time, especially under the conditions combined with other activated signaling pathways, such as Ang II signaling.

An increase in content or a transformed structure of cardiac fibrillar collagen can impede muscular contraction and relaxation. Cardiac fibrosis may cause systolic dysfunction through several distinct mechanisms, including impaired force generation by myocytes, disrupted normal coordination of myocardial excitation–contraction coupling, and an asynchronous contraction of the myocardium. These proposed mechanisms may be involved in cardiac fibrosis–induced decline in the cardiac function observed in Balb/c mice. The fact that this decline in cardiac function disappeared when cardiac fibrosis was absent in IL-4−/− further supports the notion of cardiac fibrosis–induced systolic dysfunction.

In the present study, we found positive correlation between IL-4 and Col1α1/Col3α1 gene expression/collagen production in the hearts of mice, as well as in cultured cardiac fibroblasts. In cultured cells, upregulation of Col1α1/Col3α1 gene was mediated through STAT6 signaling via IL-4Rα. These findings are consistent with the results of in vivo and in vitro studies using noncardiac tissue and fibroblasts, respectively. The important finding of our study was an increase in IL-4Rα expression on cardiac fibroblasts in response to IL-4, which was mediated via STAT6 signaling. This phenomenon was also observed in T and B cells from Balb/c mice. Increased IL-4 receptor expression by IL-4 provides an important mechanism for amplification of IL-4–dependent activation of STAT6 signaling in cardiac fibroblasts. Therefore, IL-4–induced collagen production in fibroblasts significantly contributes to cardiac fibrosis and dysfunction.

We observed that IL-4 was associated with an increase in the number of mast cells in the heart. This finding could have
been because of IL-4–induced mast cell proliferation because mast cells express IL-4Rα in vivo.25 In disease states, mast cells can be activated by cytokines, such as IL-4, leading to either secretion of mediators by degranulation or release of distinct mediators without overt degranulation.13,26 The positive association between total mast cells and cardiac fibrosis shown in our study is consistent with reported results from clinical studies27 and in experimental animal models. 28 Notably, a study using mast cell–deficient mice29 confirms a profibrotic role for mast cells in the evolution of congestive heart failure. Here, we provide evidence for the first time that mast cells, which are known to secrete IL-4,30 might participate in mediating IL-4–induced fibrotic cardiomyopathy. Thus, IL-4 produced by mast cell may function in an autocrine manner, leading to further mast cell proliferation and IL-4 production. We propose that mast cells could be an important cellular source of cardiac IL-4, which is significantly elevated in Balb/c mice.

Our data indicate that IL-4 contributes to an increased number of macrophages in the fibrotic heart. This finding may be as a result of IL-4–induced upregulation of MCP-1 expression because deletion of IL-4 was associated with a markedly decreased number of macrophages and MCP-1 in the myocardium. The mechanisms underlying IL-4–induced accumulation of macrophages in the heart could be because of (1) IL-4–induced recruitment of the cells via upregulation of expressions of adhesion molecules and MCP-1 in endothelial cells31–33 and (2) IL-4–stimulated proliferation of resident and recruited macrophages.34 In an IL-4 rich environment, macrophages polarize toward an M2 phenotype (alternative activation),35 which has been shown to significantly contribute to cardiac fibrosis.36

Although it has been reported that IL-4 might mediate fibrosis in part by increasing the expression of the TGF-β mRNA in fibroblasts,37 we observed neither a significant difference in TGF-β1 levels between WT and IL-4–deficient mouse hearts nor an induction of TGF-β1 production in cultured rat cardiac fibroblasts stimulated with IL-4 (Figure S3 in the online-only Data Supplement). Our data are consistent with reported findings showing that IL-4–induced cardiac fibrosis in hypertension was independent of TGF-β1.13 IL-4

Figure 4. Cytokine and monocyte chemoattractant protein-1 (MCP-1) expression in the hearts of wild-type (WT) and interleukin (IL)-4−/− mice. Left ventricle lysates from WT and IL-4−/− mice treated with either vehicle or angiotensin (Ang) II were processed by Western blot for transforming growth factor-β1 (TGF-β1; A) by a cytokine bead array for interferon gamma (IFNγ), IL-10, IL-2, IL-1β, and tumor necrosis factor-α (TNFα; B-F) and by ELISA for MCP-1 (G). The bars represent mean±SEM, *P<0.05, **P<0.005, a 2-sample 2-sided Wilcoxon test with a Hochberg correction for multiple testing, n=4 to 5 per group.
not only promotes Th2 differentiation, but at the same time suppresses Th1 differentiation. 38 Not surprisingly, genetic deletion of IL-4 in Balb/c mice results in the skew from Th2 differentiation to Th1 as evidenced by markedly increased IFN\(\gamma\) in the myocardium of IL-4\(−/−\) mice. Our finding that cardiac fibrosis was abrogated along with a marked induction of IFN\(\gamma\) and IL-10 in IL-4–deficient Balb/c mice is consistent with reports indicating antifibrotic role of IFN\(\gamma\) in the heart39 and IL-10’s efficacy in the treatment of fibrosis in disease models.40,41

Enhanced Th1 responses, as a result of a striking induction of IFN\(\gamma\), were observed in the hearts of IL-4\(−/−\) mice as evidenced by markedly increased IFN\(\gamma\) in the myocardium of IL-4\(−/−\) mice. Our finding that cardiac fibrosis was abrogated along with a marked induction of IFN\(\gamma\) and IL-10 in IL-4–deficient Balb/c mice is consistent with reports indicating antifibrotic role of IFN\(\gamma\) in the heart39 and IL-10’s efficacy in the treatment of fibrosis in disease models.40,41

Figure 5. Effects of interleukin-4 (IL-4) on cultured mouse cardiac fibroblasts. A, Representative immunofluorescent image of cell-surface IL-4 receptor alpha (IL-4R\(\alpha\)) on mouse cardiac fibroblasts (green). Primary cardiac fibroblasts were prepared from wild-type (WT) or signal transducer and activator of transcription 6 (STAT6)\(−/−\) mice, IL-4R\(\alpha\) expression (B) and IL-4–induced phosphorylated STAT6 (P-STAT6; C) were analyzed by Western blot. A and C, Results from 3 independent experiments. B, Collagen contents in conditioned media of cardiac fibroblasts treated with IL-4 (10 ng/mL) for 48 hours. mRNA abundance of procollagen type-I \(\alpha_1\) (Col1\(\alpha_1\); E) and procollagen type-III \(\alpha_1\) (Col3\(\alpha_1\); F) after cells were incubated with IL-4 (10 ng/mL) for 6 hours. The bars represent mean±SEM, *P<0.05, **P<0.005, a 2-sample 2-sided Wilcoxon test with a Hochberg correction for multiple testing, n=4 per group.

Figure 5. Effects of interleukin-4 (IL-4) on cultured mouse cardiac fibroblasts. A, Representative immunofluorescent image of cell-surface IL-4 receptor alpha (IL-4R\(\alpha\)) on mouse cardiac fibroblasts (green). Primary cardiac fibroblasts were prepared from wild-type (WT) or signal transducer and activator of transcription 6 (STAT6)\(−/−\) mice, IL-4R\(\alpha\) expression (B) and IL-4–induced phosphorylated STAT6 (P-STAT6; C) were analyzed by Western blot. A and C, Results from 3 independent experiments. B, Collagen contents in conditioned media of cardiac fibroblasts treated with IL-4 (10 ng/mL) for 48 hours. mRNA abundance of procollagen type-I \(\alpha_1\) (Col1\(\alpha_1\); E) and procollagen type-III \(\alpha_1\) (Col3\(\alpha_1\); F) after cells were incubated with IL-4 (10 ng/mL) for 6 hours. The bars represent mean±SEM, *P<0.05, **P<0.005, a 2-sample 2-sided Wilcoxon test with a Hochberg correction for multiple testing, n=4 per group.

Ang II significantly increased TGF-\(\beta\) levels in the hearts of both WT and IL-4\(−/−\) mice. In fibroblasts, TGF-\(\beta\) induces a sustained increase in Col1\(\alpha_1\) mRNA expression and collagen type-I secretion, but does not do the same with Col3 mRNA and collagen type-III.44,45 This phenomenon may explain why only Col3\(\alpha_1\) mRNA levels, but not Col1\(\alpha_1\), were significantly different between Ang II–treated WT and IL-4\(−/−\) mice in our study. This finding stems from the fact that significant increases in Col1\(\alpha_1\) mRNA in both WT and IL-4\(−/−\) mice diminish the difference in Col1\(\alpha_1\) mRNA expression induced by IL-4. TGF-\(\beta\) also induces other profibrotic factors, including connective tissue growth factor,44 which might upregulate both Col1\(\alpha_1\) and Col3\(\alpha_1\) mRNAs in both WT and IL-4\(−/−\) mice treated with Ang II. This results in significantly higher Col1\(\alpha_1\)/Col3\(\alpha_1\) mRNA levels compared at least 16 to 20 weeks of age. However, it is possible that cardiac function of IL-4\(−/−\) mice could gradually decline with age.
with its vehicle-treated group. An interesting finding of the current study was the reduction of IL-1β, IL-2, and tumor necrosis factor-α in the hearts of IL-4−/− mice treated with Ang II. The mechanisms by which these cytokines were reduced in mice with Ang II exposure are unknown. Recent studies have provided evidence of an important role for MCP-1 in the recruitment of bone marrow–derived fibroblast precursors in Ang II–induced cardiac fibrosis. In addition, it has been shown that IL-4 induces MCP-1 expression in endothelial cells. The cardiac MCP-1 levels in our WT mice were significantly higher than that in IL-4−/−. Therefore, robust MCP-1 induction by IL-4 and Ang II results in a strong recruitment of monocytes (then M2 macrophage differentiation) and bone marrow–derived fibroblast precursors. This may explain the dense collagen extending outward from the perivascular/pericapillary space to the myocardium in our Ang II–treated WT mice. The pathophysiologic effects of Ang II administration over imbalanced Th1/Th2 cytokine expression on the heart are complex and involve many variables. Our data suggest that in the presence of high levels of IL-4 and Ang II–induced high systolic afterload and stimulation, cardiac fibrosis is a major cause for impaired cardiac function in mice. This could be the outcomes of synergic effects of IL-4 and Ang II on cardiac resident cells and infiltrated immune cells.

Perspectives

The present study establishes the causal relationship between chronically elevated IL-4 and fibrotic cardiomyopathy by using WT and IL-4−/− Balb/c mice. In addition, by infusing Ang II, we develop a hypertension-induced DCM mouse model in Balb/c mice. This is likely the result of IL-4 accumulation over time because IL-4–deficient mice were protected from exacerbated fibrotic cardiac remodeling and DCM. Our in vitro study proposes a mechanism by which IL-4–enhanced collagen production in cardiac fibroblasts is mediated through the STAT6 signaling pathway. Of note, fibrogenesis has been recognized as a major cause of morbidity and mortality in many chronic diseases, but for which no specific therapies are available yet. The discovery of IL-4 upregulating fibrogenesis would be of great clinical interest and would allow IL-4 to serve as a potential target for future therapeutic intervention.

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Disclosures

None.

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K Shi


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What Is Relevant?
• Cardiac fibrosis represents a critical component of chronic heart deseases and hypertension-related end-organ damage.

What Is New?
• This study shows that chronically elevated interleukin-4, a cytokine produced by immune cells, is sufficient to induce cardiac fibrosis/dysfunc-
tion, resulting in the heart being susceptible to angiotensin II-induced cardiac damage.

What Is Significant?
• Fibrogenesis represents a common pathophysiological change in many chronic diseases, becoming a major cause of morbidity and mortality, but no specific therapies are currently available to halt or reverse fibrosis.

Summary
Our findings reveal that interleukin-4 is a key profibrotic factor in the heart, which implicates interleukin-4 as an additional diagnos-
tic test for the diseases and a promising new therapeutic target.
Profibrotic Role for Interleukin-4 in Cardiac Remodeling and Dysfunction
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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−/−, 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 RT2 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−ΔΔCt 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.

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)-Stat6tm1Gru; 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, 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. 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-Flour 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. 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 rebotted 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. 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. The amount of hydroxyproline was determined from a 0 - 5 µg standard curve of hydroxyproline (Sigma). Collagen contents in conditioned media of fibroblast cultures were expressed as µg/mg protein, assuming collagen contains an average of 13.5% hydroxyproline.

**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.
References:


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