Interleukin-12p35 Deletion Promotes CD4 T-Cell–Dependent Macrophage Differentiation and Enhances Angiotensin II–Induced Cardiac Fibrosis

Yulin Li, Congcong Zhang, Yina Wu, Yalei Han, Wei Cui, Lixin Jia, Lun Cai, Jizhong Cheng, Huihua Li, Jie Du

Objective—Interleukin-12 is essential for the differentiation of naïve T cells into interferon-γ–producing T cells, which regulate inflammatory responses. It is a key regulator of cardiac remodeling and development of fibrosis. Macrophages, as master regulators of inflammation, are highly plastic and can differentiate into M1 (classically activated) or M2 (alternatively activated) macrophages. M1 macrophages, induced by lipopolysaccharide (LPS) or T-helper (Th) 1–produced interferon-γ (IFN-γ), are associated with inflammation and tissue destruction, whereas M2 macrophages, induced by Th2-produced interleukin-4 (IL-4) and IL-13, show an anti-inflammatory phenotype associated with tissue repair and angiogenesis. Therefore, Th1/2 cell changes may be the key regulators that control macrophage differentiation and the inflammatory process.

IL-12 is a heterodimeric cytokine composed of 2 covalently linked p40 and p35 subunits, produced principally by antigen-presenting cells such as macrophages and dendritic cells (DCs). Heart, spleen, and lung show low IL-12 expression. The primary effect of IL-12 is to stimulate naïve T cells (TCs) to differentiate into Th1 cells, but IL-12 may also be a key controller of the macrophage phenotype in certain infected conditions. For example, IL-12 pretreatment can program macrophages to release more tumor necrosis factor-α and NO in the presence of LPS or Trypanosoma cruzi infection. Moreover, IL-12-deficient DCs show increased levels of M2-associated genes and reduced levels of M1-associated genes in response to LPS. Furthermore, IL-12 is involved in the development of pulmonary inflammation and fibrosis. Deficiency of IL-12p35 suppresses the infiltration of macrophages and neutrophils in coxsackievirus B3–induced myocarditis but presents robust silica-induced pulmonary inflammation and fibrosis. In contrast, administration of IL-12 inhibits pulmonary fibrosis induced by schistosomiasis or bleomycin. Interestingly, IL-12p40 deficiency aggregates bleomycin-induced fibrosis but protects against silica-induced fibrosis, which suggests

Methods and Results—Mice infused with angiotensin II showed a marked increase in interleukin-12p35 expression in cardiac macrophages. The degree of cardiac fibrosis was significantly enhanced in interleukin-12p35 knockout (p35-KO) mice compared with wild-type (WT) littermates in response to angiotensin II. Fibrotic hearts of p35-KO mice showed increased accumulation of alternatively activated (M2) macrophages and expression of M2 genes such as Arg-1 andFizz1. Bone marrow–derived macrophages from WT or p35-KO mice did not differ in differentiation in response to angiotensin II treatment; however, in the presence of CD4+ T cells, macrophages from p35-KO mice differentiated into M2 macrophages and showed elevated expression of transforming growth factor-β. Moreover, CD4+ T-cell–treated p35-KO macrophages could stimulate cardiac fibroblasts to differentiate into α-smooth muscle actin–positive and collagen I–positive myofibroblasts in 3-dimensional nanofiber gels. Neutralizing antibodies against transforming growth factor-β inhibited myofibroblast formation induced by M2 macrophages.


Key Words: interleukin-12 ■ fibrosis ■ macrophages ■ CD4-positive T-lymphocytes ■ transforming growth factor beta

Cardiac fibrosis is a major complication of hypertensive heart disease and is characterized by excessive deposition of extracellular matrix protein, which leads to stiffening of ventricles and heart failure. Increasing evidence has demonstrated that infiltrating inflammatory cells such as macrophages and lymphocytes play critical roles during cardiac remodeling and development of fibrosis. Macrophages, as master regulators of inflammation, are highly plastic and can differentiate into M1 (classically activated) or M2 (alternatively activated) macrophages. M1 macrophages, induced by lipopolysaccharide (LPS) or T-helper (Th) 1–produced interferon-γ (IFN-γ), are associated with inflammation and tissue destruction, whereas M2 macrophages, induced by Th2-produced interleukin-4 (IL-4) and IL-13, show an anti-inflammatory phenotype associated with tissue repair and angiogenesis. Therefore, Th1/2 cell changes may be the key regulators that control macrophage differentiation and the inflammatory process.

IL-12 is a heterodimeric cytokine composed of 2 covalently linked p40 and p35 subunits, produced principally by antigen-presenting cells such as macrophages and dendritic cells (DCs). Heart, spleen, and lung show low IL-12 expression. The primary effect of IL-12 is to stimulate naïve T cells (TCs) to differentiate into Th1 cells, but IL-12 may also be a key controller of the macrophage phenotype in certain infected conditions. For example, IL-12 pretreatment can program macrophages to release more tumor necrosis factor-α and NO in the presence of LPS or Trypanosoma cruzi infection. Moreover, IL-12-deficient DCs show increased levels of M2-associated genes and reduced levels of M1-associated genes in response to LPS. Furthermore, IL-12 is involved in the development of tissue inflammation and fibrosis. Deficiency of IL-12p35 suppresses the infiltration of macrophages and neutrophils in coxsackievirus B3–induced myocarditis but presents robust silica-induced pulmonary inflammation and fibrosis. In contrast, administration of IL-12 inhibits pulmonary fibrosis induced by schistosomiasis or bleomycin. Interestingly, IL-12p40 deficiency aggregates bleomycin-induced fibrosis but protects against silica-induced fibrosis, which suggests...
a conflicting role of IL-12 in fibrosis. Moreover, the myofibroblast differentiation of isolated fibroblasts was not affected by treatment with exogenous recombinant IL-12.11 Therefore, the functional roles and mechanism of IL-12 in fibrosis remain to be explored. Given the role of IL-12 in the differentiation of macrophages in infection and the plasticity of macrophages in the development of fibrosis, we aimed to test whether IL-12 may affect cardiac fibrosis by regulating the inflammatory microenvironment in angiotensin II (Ang II)–induced hypertension, an inflammatory but not infectious process. Recent studies clarified the divergent function of IL-12 and IL-23 in autoimmune and cardiac fibrosis. Deficiency of IL-12p35 markedly promoted cardiac fibrosis. Overexpression of IL-23p19, on the other hand, significantly decreased cardiac fibrosis.12 Therefore, the functional roles and mechanism of IL-12 in fibrosis remain to be explored.

Materials and Methods

Reagents and Antibodies
Ang II, collagenase II, dispase II, phorbol-12-myristate-13-acetate, and ionomycin were from Sigma-Aldrich (St. Louis, MO). The following antibodies were used: anti-Mac2, anti-inducible NO synthase, anti-Ang-1, anti-TGF-β1, anti-IL12p35, anti-CD206 (all from Santa Cruz Biotechnology, Santa Cruz, CA), anti-α-smooth muscle actin (α-SMA) (Abcam, Cambridge, MA), anti-IL-10 (Abbo-tec, San Diego, CA), anti-total or phospho-signal transduction and activator of transcription (STAT) 3, STAT4, nuclear factor (NF)-κB p65, anti-glycerolaldehyde 3-phosphate dehydrogenase (all from Cell Signaling Technology, Beverly, MA), anti-IL-23p19, anti-IL-12p40, anti-IL-12p70, anti-mouse IL-12p35–fluorescent (from all R&D Systems, Minneapolis, MN), Percp-Cy5.5–conjugated anti-CD45.2, fluorescein isothiocyanate (FITC)–conjugated anti-CD206, phycocerythrin-conjugated anti-F4/80, FITC-conjugated anti-IFN-γ, FITC-conjugated anti-IL-13, phycocerythrin-conjugated CD4, FITC-conjugated anti-CD3, anti-CD28, isotype antibodies (all from Biologend, San Diego, CA), and anti-mouse CD16/32 (eBioscience, San Diego, CA). Macrophage colony-stimulating factor was from PeproTech (Rocky Hill, NJ). Recombinant mouse IL-12, neutralizing antibodies against IL-23p19, and mouse IL-12p70 ELISA kit were from R&D Systems. GolgiPlug transport inhibitor was from BD Bioscience (Boston, MA).

Animal Model
We used 10- to 12-week-old male p35 and p40-KO mice, littermate WT mice, and enhanced green fluorescent protein (EGFP)-transgenic mice (all in a C57BL/6 background). The p35 and p40-KO mice were from The Jackson Laboratory (Bar Harbor, ME) and had been backcrossed with C57BL/6 mice for 10 generations. The genotype of p35 and p40-KO mice was confirmed by polymerase chain reaction as described.19 Mice were maintained in specific pathogen-free conditions described.19 Mice were maintained in specific pathogen-free conditions (National Institutes of Health publication No. 85-23, 1996) and were approved to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).20

Histopathology
After 7 days of saline or Ang II infusion, mice were euthanized by an overdose of pentobarbital (100 mg/kg), and heart tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned (5 μm). Hematoxylin and eosin, and Masson trichrome staining were as described.20 Cardiac fibrosis was calculated by the proportion of collagen-stained areas to total myocardial area. Heart sections were incubated with antibodies against IL-12p35 (1:200), green fluorescent protein (GFP) (1:200), α-SMA (1:400), inducible NO synthase (1:200), Arg-1 (1:200), CD206 (1:200), TGF-β1 (1:300), or IL-10 (1:200) at 4°C overnight, then with secondary antibodies at 4°C for 1 hour and detected with 3,3′-diaminobenzidine for immunohistochemistry. For immunofluorescence, frozen heart sections or cells were labeled with primary antibody, and then incubated with FITC- and tetramethylrhodamine isothiocyanate–conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Images were captured by the use of a Nikon Eclipse TE2000-S microscope (Nikon, Tokyo, Japan) and analyzed by a person blinded to treatment with use of Image Pro Plus 3.0 (Nikon).

Flow Cytometry
Mouse hearts were perfused with cold PBS for 4 minutes, quickly minced into small pieces, and then digested with 0.1% collagenase II and 2.4 U/mL dispase II in PBS at 37°C for 30 minutes.21 The cell suspension was filtered, centrifuged, and resuspended. Cells were blocked with CD16/32 antibody, and then incubated with antibodies for FITC-conjugated CD206, phycocerythrin-conjugated F4/80, and Percp-C5.5–conjugated CD45.2 diluted in PBS with 1% fetal bovine serum for 30 minutes at cytometer 4°C. To assess cell-specific expression of IL-12p35, IFN-γ, and IL-13 production, isolated cells from digested hearts were stimulated with phorbol-12-myristate-13-acetate (5 ng/mL), ionomycin (500 ng/mL), and 1 μL GolgiPlug in 1640 medium containing 5% fetal bovine serum at 37°C for 4 hours. Cells were stained with antibodies for surface markers for 30 minutes. For intracellular protein staining, cells were fixed and permeabilized with BD Perm/Wash buffer (Cytosift/Cytoperm, BD) and stained with antibody against IL-12p35, IFN-γ, or IL-13. The expression of surface molecules and intracellular cytokines was analyzed by Beckman Coulter Epics XL flow cytometer (Beckman Coulter, Miami, FL).

Real-Time Quantitative PCR for RNA analysis
RNA was extracted by the Trizol reagent method (Invitrogen). Aliquots of 2 μg total RNA were used for first-strand cdNA synthesis with moloney murine leukemia virus reverse transcriptase (Promega, Southampton, UK). Aliquots of 2 μL of reaction mixture were amplified with 10 μL SYBR Green PCR Master Mix and 1 μmol/L primers. The table shows the primers used. Amplification was at 95°C for 5 minutes, 95°C for 45 seconds, and 60°C for 1 minute for each step for 45 cycles. Gene levels were normalized to that of glyceraldehyde-3-phosphate dehydrogenase. All samples were run in duplicate.

Cell Isolation and Culture
Bone marrow–derived macrophages were prepared as described previously.22 Briefly, the bone marrow cells were obtained by flushing
Table. Primers Used for qRT-PCR

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*IL indicates interleukin; iNOS, inducible NO synthase; IFN-γ, interferon-γ; TGF-β1, transforming growth factor-β1; α-SMA, α-smooth muscle actin; and GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Adaptive Transfer of EGFP+ Macrophages Into Mice

Bone marrow–derived macrophages were isolated from EGFP transgenic mice and used as donor cells for transfer into p35-KO mice. As described previously,26 macrophages from EGFP transgenic mice were isolated and cultured, and then 2×106 macrophages in 100 μL DMEM were adoptively transferred into p35-KO mice by tail-vein injection, which were infused with Ang II or saline the next day.

Statistical Analysis

Data are presented as mean±SEM. Statistical analysis involved 1-way ANOVA followed by Bonferroni test for selected pairs with use of GraphPad Prism 4.01 (GraphPad Software Inc., San Diego, CA). P<0.05 was considered statistically significant.

Results

Ang II Infusion Stimulates IL-12p35 Expression in Cardiac Macrophages of Mice

To assess the regulatory role of IL-12 in response to Ang II, we first examined whether Ang II infusion stimulated IL-12 expression in the WT mice heart. qRT-PCR revealed that IL-12p35 mRNA level increased time-dependently (Figure 1A). Immunohistochemistry also demonstrated higher IL-12p35 protein expression in Ang II–infused than saline control hearts (Figure 1B). To identify the cellular source of IL-12p35, we used flow cytometry to analyze the types of infiltrated inflammatory cells in Ang II–treated mice hearts. Ang II infusion stimulated the infiltration of circulating inflammatory cells (CD45+ cells) into hearts (Figure 1C). CD45+ leukocytes in hearts were mainly composed of F4/80+ macrophages and CD3+ T cells. The number of infiltrated inflammatory cells was higher in Ang II–infused than saline control hearts: CD45+ cells: 4.30±0.82% versus 1.5±0.45%, P<0.05; CD45+F4/80+ cells: 2.37±0.85% versus 0.82±0.18%, P<0.05; CD45+CD3+ cells: 1.03±0.30% versus 0.35±0.15%, P<0.05. In isolated bone marrow–derived macrophages

For macrophage-CD4TCs coculture, 5×106 macrophages (MΦ) from WT or p35-KO mice were cocultured with or without 2.5×106 CD4+TCs from WT or p35-KO mice for 48 hours for the following study groups: (1) WT MΦ; (2) KO MΦ; (3) WT MΦ+Ang II; (4) KO MΦ+Ang II; (5) WT MΦ+WT CD4+TCs+Ang II; (6) WT MΦ+KO CD4+TCs+Ang II; (7) KO MΦ+WT CD4+TCs+Ang II; and (8) KO MΦ+KO CD4+TCs+Ang II. After 48-hour culture, CD206 expression in macrophages was detected by flow cytometry and immunofluorescence, and CD4+ TCS were used for qRT-PCR.

For macrophage-fibroblast coculture, 5×106 CFs were cultured with or without 1×106 macrophages from WT or p35-KO mice. After 72-hour culture, RNA and proteins were extracted from cells for qRT-PCR and Western blot analysis. To examine fibroblast differentiation into myofibroblasts, macrophage-fibroblasts were cocultured in a 3-dimensional coculture system as described with modification.24,25

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The TCs were used for detection of α-SMA expression by Western blot and immunofluorescence analysis.

Coculture Experiments

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In brief, macrophages from WT or p35-KO mice were pretreated with CD4+ TCS and separately with fibroblasts in 3-dimensional nanofiber gels made of the peptides K2QK6K2 (k-lysine; QL-glutamine-leucine). The lyophilized K2QK6K2 peptides at 20 mg/mL were dissolved in deionized water, and the pH was adjusted to 7.4. The peptide solution was gelated by supplementation with phosphate buffer, because the lysine-containing peptides were cross-linked in the presence of negatively charged phosphate ions. The 50-μL mixture of 4×106 macrophages and 2×106 fibroblast suspension was added with the 50-μL peptide solution (20 mg/mL) to create 100-μL gels. The cells encapsulated in gels were seeded in 48-well plates, and 200 μL of full DMEM was added 30 minutes later. Culture medium was changed every other day. After 72 hours, 3-dimensional nanofiber gels with cells were washed with PBS, fixed in 4% paraformaldehyde for 20 minutes, frozen with optimum cutting temperature, and then sectioned into 7-μm-thick sections for α-SMA immunofluorescence staining. The macrophage-fibroblast coculture system was supplemented with concentrations of TGF-β (10, 20, 40 μg/mL) or IgG control for 72 hours, and then the cells were used for detection of α-SMA expression by Western blot and immunofluorescence analysis.

The TCs were stimulated with Ang II (100 nmol/L) or saline for 24 hours to extract cell RNA for mRNA analysis. Cardiac fibroblast (CF) isolation was performed using an enzymatic digestion as described previously.21 Isolated bone marrow–derived macrophages, CD4+ TCS, and CFs were prepared for coculture experiments.

The femurs and tibias of 8-week-old WT or p35-KO mice. Cells were plated in DMEM complete medium (10% FCS, 50 U/mL penicillin, 50 μg/mL streptomycin) supplemented with murine macrophage colony-stimulating factor (50 ng/mL) cultured for 5 days to allow macrophage differentiation. For purified TCs separation, the spleens from WT or p35-KO mice were mashed through the cell strainer into the culture dish. The TCs were purified from the splenocytes using CD3+ or CD4+ MΦ activated in complete RPMI 1640 medium and activated in the presence of negatively charged phosphate ions. The 50-μL mixture of full DMEM was added 30 minutes later. Culture medium was changed every other day. After 72 hours, 3-dimensional nanofiber gels with cells were washed with PBS, fixed in 4% paraformaldehyde for 20 minutes, frozen with optimum cutting temperature, and then sectioned into 7-μm-thick sections for α-SMA immunofluorescence staining. The macrophage-fibroblast coculture system was supplemented with concentrations of TGF-β (10, 20, 40 μg/mL) or IgG control for 72 hours, and then the cells were used for detection of α-SMA expression by Western blot and immunofluorescence analysis.
and spleen-derived CD3+ TCs treated with Ang II for 24 hours, IL-12p35 mRNA expression was enhanced 6-fold in macrophages but only 1.2-fold in CD3+ T cells (Figure 1D). IL-12p35 expression in infiltrated macrophages and TCs of mouse heart after Ang II infusion was further confirmed by flow cytometry. IL-12p35 macrophages were in the spleen and liver of p35-KO mice as detected by anti-green fluorescent protein antibody staining. EGFP+ macrophages in the heart of p35-KO mice restored IL-12p35 expression detected by immunohistochemistry (G) and immunofluorescence staining (H).}

**Figure 1.** Angiotensin II (Ang II) induces IL-12p35 expression in the heart tissue and macrophages. Wild-type (WT) mice received saline or Ang II (1500 ng/kg per min) for 7 days. A, Quantitative RT-PCR (qRT-PCR) analysis of IL-12p35 mRNA expression after 0, 3, and 7 days of Ang II infusion (n=5 per group). *P<0.05 vs Ang II infusion on 0 day. B, Immunohistochemistry of IL-12p35 protein expression in myocardial tissues after 7 days of Ang II infusion. Bars=50 μm. C, Flow cytometry of infiltrated leukocytes gated on CD45+ cells, revealing F4/80+ macrophages and CD3+ T cells (n=5 per group). D, qRT-PCR of IL-12p35 mRNA expression in bone marrow–derived macrophages (BMDMs) and CD3+ T cells with saline or Ang II for 24 hours (*P<0.05 vs saline. E, Flow cytometry of IL-12p35 expression gated on CD3+ or F4/80+ cells in Ang II–infused mouse hearts (n=3). F, Adoptive transfer of macrophages from EGFP transgenic mice into IL-12p35-KO (p35-KO) mice, and then saline or Ang II infusion. EGFP+ macrophages were detected in the spleen and liver of p35-KO mice as detected by anti-green fluorescent protein antibody staining, EGFP+ macrophages in the heart of p35-KO mice restored IL-12p35 expression detected by immunohistochemistry (G) and immunofluorescence staining (H). Bars=50 μm. IL-12 indicates interleukin-12; qRT-PCR, quantitative real-time polymerase chain reaction; and KO, knockout.

**IL-12p35 Deficiency Aggravates Ang II–Induced Cardiac Fibrosis**

IL-12 can attenuate or enhance pulmonary fibrosis induced by different stimuli. To determine whether IL-12 is involved in Ang II–induced cardiac fibrosis, WT or p35-KO mice received Ang II (750 or 1500 ng/kg per min) for 7 days. Ang II infusion induced a similar increase in blood pressure in WT and p35-KO mice (Figure 2A), and the degree of collagen disposition identified by Masson trichrome staining was significantly higher in the hearts of p35-KO than WT mice with both low- and high-dose Ang II (Figure 2B). Moreover, immunohistochemical staining of heart sections
with α-SMA, a marker of myofibroblast activation, showed an increased number of α-SMA–positive cells in p35-KO than WT mice (Figure 2C). These results were confirmed by Western blot analysis (Figure 2D), which indicates that IL-12p35 deficiency promotes cardiac fibrosis by activation of myofibroblasts.

IL-12 and IL-23 share the p40 subunit, and studies have indicated the divergent function of IL-12 and IL-23 in inflammation and tumor.17,18 IL-12p35-KO mice may leave more IL-12p40 to combine with p19 subunit, which leads to an increased IL-23 level; thus, we measured p40 and p19 protein levels in WT and p35-KO mice. p40 and p19 levels were increased in both WT and p35-KO mouse hearts with Ang II infusion, with no difference between the 2 groups (Figure IIA in the online-only Data Supplement). p70 level was higher in WT than p35-KO hearts after Ang II infusion.

To determine whether p40-KO affects Ang II–induced cardiac fibrosis, p40-KO mice received Ang II (1500 ng/kg per min) for 7 days. The degree of cardiac fibrosis did not differ between WT and p40-KO mice, and α-SMA expression did not differ on immunohistochemical staining and Western blot analysis (Figure 2E–2G).

Moreover, the role of IL-23 in cardiac fibrosis was evaluated by the use of a neutralizing antibody of p19 subunit of IL-23. C57B/L6 mice were administered 100 μg/mouse neutralizing antibody of p19 or IgG control via tail vein and infused with Ang II for 0, 2, 4, and 6 days. The efficacy of IL-23 neutralization by p19 antibody was determined by the expression of IL-17 (downstream of IL-23) after Ang II infusion. IL-17...
mRNA expression was decreased 54.7% in p19-antibody–treated hearts compared with control hearts. Interestingly, cardiac fibrosis and myofibroblast formation were significantly decreased with p19-antibody treatment (Figure IIB–IID in the online-only Data Supplement). However, p19 level was similar in p35-KO and WT hearts before and after Ang II treatment (Figure IIA in the online-only Data Supplement), so IL-12 rather than IL-23 activation was involved in increased fibrosis in Ang II–infused p35-KO mouse hearts.

To explore the antifibrotic role of IL-12, we administered p35-KO mice with recombinant murine IL-12 (rIL-12) or control. ELISA showed higher blood levels of IL-12p70 with recombinant murine IL-12 than control treatment (Figure IIIA in the online-only Data Supplement). p35-KO mice showed lower collagen deposition (Figure IIIB and IIIC in the online-only Data Supplement) and α-SMA expression (Figure IIB and IIIID in the online-only Data Supplement) with recombinant murine IL-12 than control treatment, which suggests that supplementation with recombinant murine IL-12 in vivo could suppress the increased fibrotic response in Ang II–infused p35-KO mice.

**IL-12p35 Deficiency Stimulates Macrophages**

**Toward a M2 Phenotype Through NF-κB Signaling**

Because macrophage phenotypic change plays a critical role in tissue repair and fibrosis,27,28 we characterized the effect of IL-12 deficiency on macrophage differentiation by examining type–specific surface marker and gene expression. We used mannose receptor (CD206), a typical selective M2 macrophage surface marker, to distinguish macrophage subpopulations. Flow cytometry revealed a higher ratio of M2 macrophages (CD45+ F4/80+ CD206+ cells) in p35-KO than WT hearts after Ang II infusion (Figure 3A). The expression of CD206 was further confirmed in heart sections by immunohistochemistry (Figure 3B). To provide additional evidence for the change in macrophage phenotype, qRT-PCR analysis revealed a marked decrease in mRNA level of inducible NO synthase (a marker of M1 macrophages) and an increase in that of Fizz1 and Arg-1 (markers of M2 macrophages) in p35-KO than WT hearts (Figure 3C), with further confirmation by immunohistochemistry (Figure 3D). Therefore, IL-12 deficiency promoted macrophage differentiation into M2 macrophages.

The NF-κB or STAT3 signaling pathway is essential for the polarization of macrophages.29 We therefore examined the effect of deficiency in IL-12 on NF-κB or STAT3 signaling in mouse hearts by Western blot analysis. The phosphorylation of p65 induced by Ang II infusion was significantly lower in p35-KO than WT hearts, with no change in STAT3 phosphorylation (Figure 3E). Upon binding of IL-12 to the IL-12R complex, activation of Jak kinases (Tyk-2 and Jak-2) occurs, thus leading to phosphorylation of STAT4.30 We investigated STAT4 phosphorylation and found that Ang II infusion activated STAT4, with no difference between WT and p35-KO mice (Figure 3E). IL-23R has a cytoplasmic STAT4 binding domain for signal transduction,30 so endogenous p40 and p19 might compensate for p35 deficiency in activating STAT4 in vivo.

To determine NF-κB activation, we performed double immunostaining of F4/80 and p-p65 and found NF-κB p65 activation in WT macrophages but not p35-KO macrophages (Figure 3F). NF-κB is important in M1 polarization,30 and because it is not activated in p35-KO hearts, M2 is a predominant phenotype in p35-KO mice.

**IL-12p35 Deficiency–Induced M2 Polarization Requires CD4+ TCs**

Previous studies demonstrated that IL-12 can prime CD4+ TCs differentiation into Th1 cells and stimulate expression of Th1 cytokines such as IFN-γ.6,7 We thus determined whether IL-12 regulates Th1/2 cytokine expression in CD4+ TCs and found the mRNA level of the Th1 cytokine IFN-γ to be lower and that of the Th2 cytokine IL-13 to be higher in p35-KO than WT CD4+ TCs after coculture with macrophages (Figure 4A). The alteration in IFN-γ or IL-13 mRNA expression in WT and p35-KO hearts was confirmed by qRT-PCR (Figure 4B). Similar patterns of IFN-γ and IL-13 levels in CD4+ TCs from hearts were detected by flow cytometry (Figure 4C). Thus, IL-12 deficiency promotes CD4+ TCs differentiation into Th2 cells.

To determine the role of macrophage-derived IL-12 in regulating TC-dependent macrophage differentiation, we performed coculture experiments with macrophages and CD4+ TCs with Ang II stimulation and detected the expression of CD206 (M2 surface marker) by flow cytometry. With WT or p35-KO CD4+ TCs coculture, CD206 expression in macrophages was significantly increased compared with no coculture (Figure 4D), which suggests that macrophage differentiation depends on CD4+ TCs. CD206 expression was significantly higher in p35-KO than WT macrophages, regardless of the coculture with WT or p35-KO CD4+ TCs. Thus, IL-12 deficiency from macrophages but not CD4+ TCs stimulated CD4+ TC–dependent M2 macrophage differentiation. Immunostaining revealed similar results (percentage of CD206-positive macrophages: WT MΦ+ Ang II: 0.81±0.21%; KO MΦ+ Ang II: 0.92±0.28%; WT MΦ+ WT CD4+ TCs + Ang II: 3.80±1.01%; KO MΦ+KO CD4+ TCs + Ang II: 9.52±2.51%) (Figure 4E). Therefore, IL-12 deficiency in macrophages causes the transition of CD4+ TCs to Th2 cells, thus leading to M2 macrophage polarization.

**IL-12p35 Deficiency–Induced M2 Macrophages Promote Cardiac Fibroblast Activation via TGF-β Signaling**

Macrophages are found in close proximity to collagen-producing myofibroblasts and play a key role in fibrosis.32 M2 macrophages secrete TGF-β and IL-10.33 To examine the effect of IL-12 deficiency on the expression of TGF-β and IL-10 in macrophages, we cocultured macrophages from p35-KO or WT mice with CD4+ TCs and found the level of TGF-β to be significantly higher, and that of IL-10 to be higher but not significantly, in p35-KO than WT macrophages (Figure 5A). Moreover, the increased mRNA and protein expression of TGF-β was confirmed in both p35-KO and WT hearts after Ang II infusion (Figure 5B–5D), with no difference in IL-10 expression in Ang II–treated p35-KO and WT hearts (Figure 5E and 5F). Thus, IL-12 deficiency increases the expression of TGF-β in macrophages.

TGF-β plays a critical role in modulating fibroblast activation.34 To investigate whether p35-KO macrophages activate CF differentiation into myofibroblasts, we pretreated macrophages from WT and p35-KO mice with CD4+ TCs and then
cocultured them with CFs in 3-dimensional nanofiber gels for 72 hours. qRT-PCR, Western blot analysis, and immunostaining demonstrated that the mRNA and protein levels of the myofibroblast marker α-SMA were significantly higher in CFs cocultured with p35-KO than WT macrophages (Figure 6A–6C). Furthermore, collagen I expression of myofibroblasts was increased in these cells (Figure 6D). To further determine whether IL-12 deficiency–induced activation of myofibroblasts is through TGF-β, we added TGF-β neutralizing antibody to block TGF-β in the coculture. TGF-β neutralizing antibody significantly decreased the expression of α-SMA in a dose-dependent manner (Figure 6E and 6F). Therefore, IL-12 deficiency–induced M2 macrophages directly activated CFs into myofibroblasts through TGF-β production.

**Discussion**

IL-12, composed of p35 and p40 subunits, is critical for the differentiation of naïve TCs into IFN-γ–producing TCs, which leads to differentiation of macrophages. p40 is a common subunit of IL-12 and IL-23; the paradoxical functional roles of IL-12 and IL-23 have been clarified in inflammation and tumors. We found that Ang II infusion markedly...
Figure 4. Interleukin-12 (IL-12) p35 deficiency promotes macrophages to differentiate into M2 phenotype in the presence of CD4+ T cells. 

A, Quantitative real-time polymerase chain reaction (qRT-PCR) of mRNA expression of interferon-γ (IFN-γ) and IL-13 in CD4+ T cells with and without macrophages. Data are from 3 independent experiments. *P<0.05 vs wild-type (WT) CD4+ T. 

B, qRT-PCR analysis of mRNA expression of IFN-γ and IL-13 in hearts from WT and p35-knockout (KO) mice with angiotensin II (Ang II) infusion (n=5 per group). *P<0.05 vs WT. 

C, Flow cytometry of IFN-γ cells and IL-13 in infiltrated CD4+ T cells in hearts with saline or Ang II infusion (n=3 per group). *P<0.05 vs WT+Ang II. 

D, Flow cytometry and E, immunofluorescence analysis of CD206 expression in macrophages cocultured with or without CD4+ T cells with Ang II stimulation. Proportion of CD206+F4/80+ cells to total cells (right). Data are from 3 independent experiments. *P<0.05 vs WT MΦ + WT CD4+ TCs + Ang II. Bars=100 μm.
upregulated IL-12p35/p40/p70 expression. IL-12p35 deficiency increased cardiac fibrosis in response to Ang II infusion. The deficiency in IL-12 stimulated a CD4 \(^+\) T-cell–dependent differentiation of M2 macrophages and TGF-\(\beta\) production, which led to cardiac myofibroblast activation and collagen I deposition. Thus, IL-12 is an important endogenous regulator for T-cell–dependent macrophage differentiation and a potential therapeutic target for hypertensive cardiac fibrosis.

IL-12 is the first cytokine predominantly produced by antigen-presenting cells such as monocytes, neutrophils, DCs, or macrophages in response to pathogens and their products.\(^35\) Bacterial products and proinflammatory cytokines are strong inducers of IL-12 expression. The expression of IL-12 is primarily upregulated by LPS, IFN-\(\gamma\), or p43 in monocytes, macrophages, or DCs by activating an NF-\(\kappa\)B or p38 mitogen-activated protein kinase pathway.\(^36-38\) Administration of *Lactobacillus salivarius* also resulted in sustained increase in the production of IL-12 and IFN-\(\gamma\) in human macrophage-like cell line THP-1 (human acute monocyte leukemia cell line).\(^39\) However, recent studies showed that noninfectious stimuli, such as treatment with Ang II–converting enzyme inhibitor or type 1 Ang II receptor blocker, affect the production of IL-12 from monocytes or macrophages.\(^40-42\) Here, we demonstrated that Ang II directly induced IL-12 expression in infiltrated macrophages and not TCs in hearts (Figure 1).

IL-12 can prime CD4 \(^+\) T-cell differentiation into Th1 cells and stimulate the expression of Th1 cytokines such as IFN-\(\gamma\).\(^43\) As shown in Figure 4A, when cocultured with macrophages, Th1 cytokine IFN-\(\gamma\) and Th2 cytokine IL-13 mRNA levels in WT or p35-KO CD4 \(^+\) TCs were increased compared with that of CD4 \(^+\) TCs alone, suggesting macrophages have effect on CD4 T-cell differentiation. Our data demonstrated that macrophages were the main source of IL-12 production in response to Ang II (Figure 1D–1H). Moreover, IFN-\(\gamma\) level was lower and IL-13 level was higher in CD4 \(^+\) TCs when IL-12p35 deletion took place either in vivo or in vitro as shown in Figure 4A to 4C. This conclusion was supported by studies that showed that heat-killed *Listeria monocytogenes* induced Th1 development both in vitro and in vivo through macrophage production of IL-12.\(^6\) Along with these studies, we showed that IL-12 KO promoted a CD4 \(^+\) T-cell–dependent M2 macrophage differentiation and fibrosis. Therefore, in Ang II–induced

![Figure 5](http://atvb.ahajournals.org/)

**Figure 5.** p35-knockout (KO) macrophages express transforming growth factor-\(\beta\) (TGF-\(\beta\)). A, Macrophages from p35-KO or wild-type (WT) mice were cocultured with CD4 \(^+\) T cells. Quantitative real-time polymerase chain reaction (qRT-PCR) of the mRNA expression of TGF-\(\beta\) and IL-10. *P<0.05 vs WT. Data are from 3 independent experiments. B, qRT-PCR of mRNA expression of TGF-\(\beta\) in cardiac tissue with saline or angiotensin II (Ang II) infusion (\(n=5\) per group). *P<0.05 vs WT+saline, #P<0.05 vs WT+Ang II. C, Immunohistochemistry of TGF-\(\beta\)-positive cells with saline or Ang II infusion. Bars=50 \(\mu\)m. D, Western blot analysis of protein expression of TGF-\(\beta\) with saline or Ang II infusion (top) and quantitative analysis (bottom, \(n=5\) per group). *P<0.05 vs WT+saline, #P<0.05 vs WT+Ang II. E, qRT-PCR analysis and F, immunohistochemistry of IL-10 level with saline or Ang II infusion (\(n=5\) per group). *P<0.05 vs WT+saline.
cardiac fibrosis, IL-12 produced from macrophages can prime TCs differentiation into Th1 cells and stimulate the expression of Th1 cytokines such as IFN-γ to induce M1 macrophage polarization.

IL-12 plays a proinflammatory role in the immune response by increasing natural killer cells and TCS IFN-γ production and favoring cytotoxic T-lymphocyte generation.44 This finding is consistent with our results of IL-12 KO suppressing IFN-γ production (Figure 4). A previous study found severe cardiac fibrosis associated with severe myocarditis in IFN-γ KO mice in experimental autoimmune myocarditis.45 IFN-γ played an antifibrotic role in animal experiments and a clinical trial.46

With lack of IL-12, interaction between infiltrated TCS and macrophages promotes M2-sustained expression of TGF-β and IL-13 (Figure 5). Elevated TGF-β level plays a central role in the pathogenesis of fibrosis by promoting the activation, proliferation, and differentiation of fibroblasts and collagen-producing myofibroblasts,34 which is consistent with our results (Figures 5 and 6). Moreover, studies with transgenic mice specifically overexpressing IL-13 in the lung confirmed IL-13 as a profibrotic mediator by influencing the activation of myofibroblasts,35 which is consistent with our results of increased IL-13 levels in p35-KO mice (Figure 4).

Activated NF-κB p65 binds to the IL-12 promoter to induce IL-12 expression in macrophages.38 Moreover, loss of Nur77 (nuclear receptor subfamily 4 group A member 1) in hematopoietic cells elevated M1 and reduced M2 macrophages, which was associated with increased phosphorylation of p65. Inhibition of NF-κB suppressed M1 macrophage activation,49 which is consistent with our results of increased number of M2 polarization macrophages in p35-KO mice accompanied by suppressed NF-κB level (Figure 3E).

The functional roles of IL-12 in fibrosis are unclear. Here, we showed that IL-12p35 deficiency markedly aggravated cardiac fibrosis in response to Ang II infusion (Figure 2). IL-12 plays an important role in the development of efficient innate immunity and Th1-type adaptive immunity.35 The major role of IL-12 is to induce proliferation of preactivated TCS and natural killer cells, thus leading to production of Th1 cytokines such as IFN-γ, tumor necrosis factor-α, IL-8, and IL-2.50 IL-12 has various in vivo activities, including antitumor and antiangiogenic activities and antifibrotic effects through IFN-γ.15,51 Indeed, our results showed IL-12 KO to be associated with reduced expression of IFN-γ (Figure 4A–4C), which establishes an important link to cardiac fibrosis. The causal effect of IFN-γ in cardiac fibrosis had been shown by a study of IFN-γ KO mice with increased fibrosis in experimental autoimmune myocarditis.45

Here, we demonstrated that IL-12p35 deficiency markedly aggravated Ang II–induced cardiac fibrosis (Figure 2). Even though Ang II infusion for 7 days induced expression of IL-12...
Activation of CFs is a critical event in the initiation of fibrosis, with macrophages having a central role in this process.32 Macrophages interact with other immune cells, such as CD4+ TCs, and release proinflammatory cytokines that activate quiescent CFs.59 M2 macrophages express and secrete the cytokines to modulate fibrosis.233 Our results demonstrated that the deletion of IL-12p35 stimulated a CD4+ TC–dependent differentiation of M2 macrophages and TGF-β production. This observation is consistent with the studies which showed that systemic anti-IL-12 treatment was associated with augmented TGF-β secretion and IL-12p35 suppressed TGF-β production.60,61 M2 macrophages have been divided into M2a, M2b, and M2c cells. M2a was strictly activated by Th2–derived cytokines (IL-13 or IL-4), identified by high level of Arg-1, Fizz1, and CD206 expression, and associated with fibrotic property. M2b was elicited by stimulation of LPS and IL-1β through IL-1R/Toll-like receptor superfamily. M2c was distinct from M2a because they produced much higher levels of IL-10 and exerted immunoregulatory functions. M2c cells generated by stimulation with IL-10, glucocorticoids, or TGF-β and that expressed CD206 and played an immunosuppressive role.56,63 In Ang II–infused IL-12p35-KO mice, IL-13 production was increased in CD4+ TCs, whereas Arg-1, Fizz1, TGF-β, and CD206 but not IL-10 expression was increased in macrophages; these results suggested that IL-12p35 deficiency promotes macrophage differentiation into M2a in Ang II–induced cardiac fibrosis.

In this study, TGF-β expression with CD4+ TCs coculture was higher in IL-12p35-deficient than WT macrophages (Figure 5A), and the increased TGF-β expression was further confirmed in p35-KO mouse hearts with Ang II infusion (Figure 5B–5D). Various cell types, such as fibroblasts, cardiomyocytes, endothelial cells, TCs, and macrophages, can produce TGF-β after heart injury. However, recent studies suggested that TGF-β can positively and negatively regulate fibrosis, which is governed by surrounding inflammatory cytokines.2 For example, regulatory TC-producing TGF-β ameliorated bleomycin-induced fibrosis in WT mice with intranasal TGF-β plasmid administration.54 We revealed the role of M2 macrophage–producing TGF-β in CFs by coculture experiments. Macrophages promoted CFs differentiation into myofibroblasts. This profibrotic role was more powerful in macrophages from p35-KO mice, as shown by increased α-SMA and collagen expression (Figure 6A–6D). Administration of neutralizing anti-TGF-β antibody attenuated the activation of CFs in p35-KO macrophages (Figure 6E and 6F). Thus, IL-12p35 deficiency may promote TGF-β production by M2 macrophages, which contributes to myofibroblast formation and cardiac fibrosis. However, TGF-β signaling is not related to the NF-κB pathway in Ang II–induced cardiac fibrosis. NF-κB activation is in the early stage of Ang II–induced inflammatory damages through regulation of M1 differentiation and expression of M1 proinflammatory cytokines, whereas TGF-β signaling is involved in M2 macrophage function and regulation of CFs activation and fibrosis at the late stage of Ang II–induced cardiac inflammation and fibrosis.

In conclusion, we provide novel insights into the roles of IL-12 in regulating Ang II–induced cardiac inflammation and
fibrosis, which it achieves by modulating CD4+ TC-dependent differentiation of M2 macrophages. M2 macrophages stimulate TGF-β expression, which activate CFs to produce collagen. Our characterization of IL-12 as a modulator of macrophage phenotypic transition and cardiac fibrosis presents a previously unknown inflammatory paradigm for understanding hypertensive heart disease.

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Disclosures
None.

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Interleukin-12p35 Deletion Promotes CD4 T-Cell–Dependent Macrophage Differentiation and Enhances Angiotensin II–Induced Cardiac Fibrosis

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