Interleukin-12p35 knockout promotes macrophage differentiation, aggravates vascular dysfunction, and elevates blood pressure in angiotensin II-infused mice

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Aims
Numerous studies have demonstrated that inflammation is involved in the progression of hypertension. Inflammatory cytokines interleukin (IL)-12 and IL-35 belong to the IL-12 cytokine family and share the same IL-12p35 subunit. Accumulating evidence has demonstrated that IL-12p35 knockout (IL-12p35 KO) leads to cardiovascular disease by regulating the inflammatory response. This study aimed to investigate whether IL-12p35 KO elevates blood pressure in a hypertension mouse model.

Methods and results
Mice with angiotensin (Ang) II infusion showed marked aortic IL-12p35 expression; thus, aortic macrophages may be the main source of IL-12p35. Wild-type and IL-12p35 KO mice were infused with Ang II or saline. IL-12p35 KO promoted M1 macrophage differentiation, amplified the inflammatory response, aggravated vascular dysfunction, and elevated blood pressure in Ang II-treated mice. Then, some Ang II-infused mice were given phosphate buffer saline, mouse recombinant IL-12 (rIL-12), or rIL-35, and the results showed that rIL-12 but not rIL-35 treatment had an antihypertensive effect on Ang II-infused mice. In addition, detection of human plasma IL-12 levels in hypertensive patients and control subjects showed that IL-12 was significantly increased in hypertensive patients when compared with control subjects. In hypertensive patients, IL-12 levels were positively correlated with blood pressure.

Conclusion
IL-12p35 KO amplifies the inflammatory response and promotes blood pressure elevation in Ang II-treated mice. In addition, IL-12, but not IL-35, plays a protective role in the Ang II-induced hypertension model. Thus, IL-12 may be a novel therapeutic agent for the prevention and treatment of clinical hypertension.

Keywords
Hypertension • Angiotensin II • Interleukin-12p35 deficiency • Macrophages • Inflammatory response • Interleukin 12

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1. Introduction

Hypertension is one of the most notorious chronic diseases that lead to irreversible damage in important organs, including the cardiovascular system, brain, and kidney. In the cardiovascular system, hypertension is the most important risk factor for cardio-cerebrovascular disease and can lead to a variety of cardio-cerebrovascular complications, which account for approximately 30% of all deaths worldwide.1,2

Numerous evidence has demonstrated that interleukins (ILs) are critical in the progression of hypertension. Previous studies have reported that angiotensin (Ang) II-induced hypertension is attenuated in IL-6-deficient mice.3,4 The effect of IL-10 on blood pressure is controversial, as both increasing and decreasing effects on blood pressure have been observed.5,6 In addition, a study has also reported that IL-10 does not affect blood pressure.7 Other studies have reported that knockout or inhibition of IL-10 significantly reduces the elevated blood pressure in Ang II-infused mice.8–10 Interestingly, exogenous IL-17 treatment directly inhibits the IL-10 secretion and the Th1 immune response, which is critical for macrophage (Mø) differentiation.15–17 Meanwhile, IL-35 is an anti-inflammatory cytokine and negatively regulates the progression of atherosclerosis,18 autoimmune disease,19 autoimmune diabetes,20 arthritis,21,22 hepatitis,23 and other diseases by down-regulating the inflammatory response. However, whether IL-12 and IL-35 are involved in Ang II-induced inflammatory response and hypertension is still unknown. We hypothesize that IL-12 and/or IL-35 could regulate the differentiation of Mø which is closely related to inflammation and hypertension. In the present study, the interventions of IL-12p35 KO and exogenous cytokine treatment were used to investigate the effects of IL-12 and IL-35 on Ang II-induced Mø differentiation and hypertension.

2. Methods

2.1 Animals and animal models

Male WT mice (HFK Bioscience, Beijing, China) and IL-12p35 KO mice (Jackson Laboratory, Bar Harbor, ME, USA) with a C57BL/6 background, aged 10–11 weeks and weighting 24–26 g were used in this study. First, the WT mice were infused with Ang II for 4 weeks, and an equal volume of saline was infused in control animals (n = 6 in each group). In addition, both WT mice and IL-12p35 KO mice were infused with Ang II or saline for 4 weeks (n = 8 in each group). From the third day to the end of perfusion, some of the Ang II-treated WT and IL-12p35 KO mice were treated daily (ip) with phosphate buffer saline (PBS) (50 μL, n = 8), 37.5 ng of recombinant mouse IL-12 (rIL-12, purity >98%, Chimerigen, n = 8), or 0.75 ng of rIL-35 (purity >98%, PeproTech, n = 8).22 All mice used in this study were housed in the specific-pathogen-free mouse room in Beijing Anzhen Hospital of Capital Medical University. At the end of chronic infusion, mice were euthanized by CO2 inhalation, and the aortas were isolated for subsequent experiments. This study was reviewed and approved by the Institutional Animal Care and Use Committee at the Beijing Anzhen Hospital of Capital Medical University (Beijing, China) and conducted in accordance with the guidelines from directive 2010/63/EU of the European Parliament.

2.2 Chronic Ang II infusion

After mice were anaesthetized with 2% isoflurane. Osmostic mini-pumps (Alzet model 2004, USA) filled with Ang II (Enzo Life Sciences, USA) or saline were implanted subcutaneously in the nape of the neck as previously described.24. The rate of Ang II infusion was 750 ng/min/kg.

2.3 Western blot

After the aortas were lysed, total protein was collected and measured with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, USA). Approximately 12 μg of total protein was separated on SDS polyacrylamide gels and transferred to Immobilon-FL PVDF membranes (Millipore, USA). After blocking with 5% non-fat milk, the membranes were incubated with anti-IL-12p35 (Abcam, UK), anti-GAPDH, anti-signal transducer and activator of transcription (STAT) 1, anti-phospho-STAT1, anti-IL-17, anti-p65 (the preceding four antibodies were obtained from Cell Signaling Technology, USA) and anti-phospho-P65 (p-P65, Bioworld Technology, Minnesota, USA) antibodies at 4°C overnight. Afterward, the membranes were incubated with secondary antibodies at room temperature for 1 h. The blots were scanned using a two-colour infrared imaging system (Odyssy; LI-COR Biosciences, Lincoln, USA).

2.4 Histological analysis

The aortas were immediately fixed with 4% neutral paraformaldehyde. And were then embedded in paraffin, cut into approximately 4–5 μm slices and mounted onto slides. Histopathological analysis was performed by immunofluorescence staining. Anti-IL-12p35 antibody (R&D Systems, USA), anti-IL-12p40 antibody, anti-Epstein-Barr virus-induced gene3 (EB3) antibody, anti-smooth muscle 22a (SM22a) antibody, anti-osteopontin (OPN) antibody (Abcam), and anti-p-p65 antibody were used to label IL-12p35, IL-12p40, EB3, SM22a, OPN, and p-p65, respectively.

2.5 Quantitative polymerase chain reaction

Total mRNA was collected after the aortas and cells were lysed using TRIzol reagent. cDNA was synthesized using 2 μg of total mRNA using a reverse transcription kit. PCR amplification was performed using LightCycler 480 SYBR Green Master Mix (all from Roche, Roche Diagnostics GmbH, Mannheim, Germany). The IL-12p35, inducible nitric oxide synthase (iNOS), arginine 1 (Arg-1), IL-6, IL-17, tumour necrosis factor α (TNF-α), IFN-γ, SM22a, OPN, IL-12p15, and gp130 mRNA levels were investigated, and the results were normalized against the expression levels of GAPDH. The target mRNA and Quantitative polymerase chain reaction (RT-qPCR) primer sequences are shown in Table 1.

2.6 Blood pressure measurement

The systolic blood pressure (SBP) and heart rate (HR) of each mouse were measured using a tail-cuff system (CODA, Kent Scientific, USA) at...
different time points after Ang II or saline infusion as previously described. Briefly, the non-invasive tail-cuff system was assembled, and the heating plate of the system was pre-heated to approximately 37°C. After resting for 25–30 min, mice were placed in a fixator. The head of the mouse was placed in the nose fixator, and the tail was placed in a hole in the back of the device. The position of the total nasal fixator allowed the mice to remain comfortably positioned in the device but did not allow them to move backwards or forwards. The entire fixator was then placed on a heating plate to warm the mice. The pressurized tail sleeve was placed on the tail of the mouse, as close as possible to the tail root, and the sensor was positioned near the pressurized tail sleeve. Finally, the program was started, and measurements were obtained. In addition, the baseline SBP and HR values were recorded.

2.7 Vascular ring experiments

Aortas were isolated and immediately placed in cold physiological salt solution. To measure vascular reactivity, the aortic rings were cut into 3–4 mm sections and connected to an isometric force transducer in a custom-designed 15 mL organ chamber filled with 37°C cold physiological salt solution and bubbled with 95% O2 and 5% CO2. SM22α and OPN mRNA expression by RT-qPCR analysis.

2.8 Cell isolation and culture

Smooth muscle cells (SMCs) were isolated from WT mice as previously described. The bone marrow-derived Ma from WT mice (WT Ma) and IL-12p35 mice (KO Ma) were prepared as previously described. The spleen was isolated from WT mice, and a spleen cell suspension was prepared in Roswell Park Memorial Institute (RPMI) 1640 complete culture medium; B lymphocytes, T lymphocytes, and dendritic cells (DCs) were then positively selected using CD20, CD3, and CD11c magnetic beads (Miltenyi Biotech) and an autoMACS separator. In addition, CD4+ T lymphocytes from WT mice (WT CD4) and IL-12p35 KO mice (KO CD4) were positively selected using an autoMACS separator and CD4 magnetic beads. Isolated T lymphocytes were cultured in complete RPMI 1640 medium and activated by treatment with anti-CD3 antibody and anti-CD28 antibody.

First, the WT Ma, B lymphocytes, T lymphocytes, and DCs were treated with saline or Ang II (100 nmol/L) for 24 h; then, total mRNA was extracted for IL-12p35 mRNA analysis. In addition, for the co-culture of Ma and CD4+ T lymphocytes, the Ma and CD4+ T lymphocytes derived from WT and IL-12p35 KO mice were divided into eight groups as follows: (i) WT Ma; (ii) KO Ma; (iii) WT Ma+Ang II; (iv) KO Ma+Ang II; (v) WT Ma+WT CD4+Ang II; (vi) WT Ma+KO CD4+Ang II; (vii) KO Ma+WT CD4+Ang II; and (viii) KO Ma+KO CD4+Ang II. The total numbers of Ma and CD4+ T lymphocytes in each group were 5 × 10^6 and 2.5 × 10^5, respectively. After culturing for 24 h, the mRNA levels of iNOS in Ma of each group were measured. SMCs were cultured in complete DMEM and treated with Ang II; in addition, the medium described above was also added. The SMCs were used to detect SM22α and OPN mRNA expression by RT-qPCR analysis.

2.9 Collection of human blood samples and plasma IL-12 analysis

Fasting venous peripheral blood samples from hypertensive patients (n = 80) and control subjects (n = 40) were collected at the Beijing Anzhen Hospital of Capital Medical University from September 2015 to October 2016. After centrifugation, plasma was collected from each sample, and the plasma IL-12 levels in each sample were measured using human IL-12 ELISA kits (eBioscience) following the manufacturer’s instructions. This study followed the Declaration of Helsinki guidelines for biomedical research involving human subjects. This study was approved by the institutional ethics and clinical research committee at the Beijing Anzhen Hospital of Capital Medical University (Beijing, China), and all patients or their families provided informed consent. Clinical data of the hypertensive patients and control subjects are listed in Table 2.

2.10 Statistical analysis

All the data from the animal study are expressed as the mean ± standard deviation (SD). Differences between two groups were compared using Student’s t-tests, and differences between multiple groups were compared by one- or two-way analysis of variance (ANOVA), followed by Tukey’s multiple comparisons test. In the clinical study, data with a normal distribution are expressed as the mean ± SD and compared as in the animal study above. Data with abnormal distribution are expressed as

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>IL-12p35</td>
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<tr>
<td>IL-17</td>
<td>TCCAGAAGGCCTCAGACTA</td>
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<td>TNF-α</td>
<td>CCCAGGACCTCTTCTGATAC</td>
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<td>IFN-γ</td>
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<td>TGGCTCATGAAATGCTT</td>
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<tr>
<td>iNOS</td>
<td>TACGCTCCTGAAACTGTAGCA</td>
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<td>Arg-1</td>
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<td>OPN</td>
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<td>SM22α</td>
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<td>GAPDH</td>
<td>AACCTTGGCATTGTGGAAG</td>
<td>CACATTGGGGGTAGGAA</td>
</tr>
</tbody>
</table>

Table 1 RT-PCR primers used
3. Results

3.1 Ang II infusion increases IL-12p35 expression in aortic Mø in mice

Western blot and histological analyses were used to detect IL-12p35 expression in aortas, and the results showed that Ang II infusion significantly increased aortic IL-12p35 levels (Figure 1A and B). In addition, Ang II treatment did not affect IL-12p35 mRNA levels in B lymphocytes and T lymphocytes but did increase IL-12p35 mRNA levels in Mø and DCs, especially in the Mø (Figure 1C). Double staining with anti-IL-12p35 and anti-F4/80 antibodies showed that aortic Mø were the source of IL-12p35 (Figure 1D).

3.2 IL-12p35 deficiency increases Ang II-induced hypertension

At baseline, no differences in SBP and HR were observed among the four groups (Figure 2A and B). Ang II infusion significantly increased blood pressure in WT mice (Week 4 SBP: control group: 107 ± 5 mmHg vs. Ang II group: 157 ± 5 mmHg, Figure 2A), and knockout of IL-12p35 further promoted the blood pressure elevation (Week 4 SBP: Ang II group: 157 ± 5 mmHg vs. Ang II-12p35 KO group: 179 ± 5 mmHg, Figure 2A). Meanwhile, Ang II infusion and IL-12p35 KO did not affect the HR (Figure 2B).

3.3 IL-12p35 deficiency aggravates Ang II-induced vascular dysfunction

To investigate the effect of IL-12P35 on vascular reactivity, endothelium-dependent and endothelium-independent relaxation responses were investigated using endothelium-intact aortas isolated from mice that had been treated for 4 weeks. Ang II treatment significantly reduced ACh- and SNP-induced relaxation (Figure 3A and C) and increased PE-induced contraction (Figure 3B). The effect of Ang II on the relaxation and contraction of vascular rings was aggravated by IL-12p35 KO (Figure 3A–C). To investigate the effect of the inflammatory response on vascular dysfunction, some of the vascular rings were treated with indomethacin, revealing that indomethacin attenuates the effect of IL-12p35 KO on Ang II-induced vascular dysfunction (Figure 3D).

3.4 IL-12p35 deficiency promotes Mø1 differentiation in Ang II-infused mice

To investigate the effect of IL-12p35 KO on Mø differentiation, double immunofluorescence staining and RT-qPCR were performed. The results showed that infusion with Ang II increased the number of Mø in the aorta and that IL-12p35 KO further promoted Mø1 differentiation in Ang II-infused mice (Figure 4A). Ang II infusion promoted iNOS mRNA expression, and the effect of Ang II on iNOS mRNA expression was increased by IL-12p35 KO (Figure 4B). In addition, Ang II promoted the mRNA expression levels of Mø1-related cytokines, including IL-1β, IL-6, IL-17, TNF-α, and IFN-γ, and the mRNA expression levels of these cytokines were also increased by IL-12p35 KO (Figure 4C). Finally, IL-12p35 KO increased P65 pathway activation but did not affect STAT1 pathway activation (Figure 4D). The P65 activation in Mø was investigated, and the results showed that IL-12p35 KO increased p-P65 expression in Mø (Figure 4E).

3.5 IL-12p35 deficiency affects the smooth muscle phenotype in Ang II-treated mice

Expression levels of SM22α and OPN in aortas were measured by immunofluorescence staining, and the results showed that chronic Ang II infusion resulted in increased OPN expression in aortas. In addition, higher OPN levels were observed in the Ang II-12p35 KO group than in the Ang II group, whereas SM22α levels were reduced after chronic Ang II infusion and further deceased by IL-12p35 KO (Figure 5A). In vitro, Ang II treatment did not affect iNOS mRNA expression in CD4+ T cells. Co-culture with WT CD4+ T cells or IL-12p35 KO CD4+ T cells led to increased iNOS mRNA levels in Mø, which were more significantly increased in IL-12p35 KO Mø than in WT Mø (Figure 5A). For SMC

Table 2 Clinical characteristics of patients with or without hypertension

<table>
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<tr>
<th>Characteristics</th>
<th>Control</th>
<th>Hypertension</th>
<th>P-value</th>
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<tr>
<td>Old age (years), n (%)</td>
<td>15 (37.5)</td>
<td>28 (35)</td>
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<td>Male, n (%)</td>
<td>23 (57.5)</td>
<td>52 (65.0)</td>
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<td>Smoked, n (%)</td>
<td>12 (30)</td>
<td>34 (42.5)</td>
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<td>Drank alcohol, n (%)</td>
<td>9 (22.5)</td>
<td>24 (30)</td>
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<tr>
<td>BMI &gt;25, n (%)</td>
<td>26 (65.0)</td>
<td>62 (77.5)</td>
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<td>Family story, n (%)</td>
<td>13 (32.5)</td>
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<td>HDL-C, n (%)</td>
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<td>T2DM, n (%)</td>
<td>1 (2.5)</td>
<td>4 (5.0)</td>
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<tr>
<td>Age (years)</td>
<td>56 ± 13</td>
<td>55 ± 10</td>
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<td>BMI (kg/m²)</td>
<td>26.7 ± 2.3</td>
<td>27.7 ± 3.6</td>
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<td>SBP (mmHg)</td>
<td>120 (117–130)</td>
<td>151 (145–165)</td>
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<td>DBP (mmHg)</td>
<td>76 (70–80)</td>
<td>95 (85–101)</td>
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<td>HR (b.p.m.)</td>
<td>70 ± 7</td>
<td>71 ± 9</td>
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<td>CREA (µmol/L)</td>
<td>70 (61–77)</td>
<td>68 (59–84)</td>
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<td>Glu (mmol/L)</td>
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<td>TC (mmol/L)</td>
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<td>TG (mmol/L)</td>
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<td>HDL-C (mmol/L)</td>
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<td>LDL-C (mmol/L)</td>
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<td>56 (70.0)</td>
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<tr>
<td>Diuretic</td>
<td>0 (0)</td>
<td>17 (21.3)</td>
<td>&lt;0.05</td>
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</table>

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; CCB, calcium channel blockers; CREA, creatinine; CRP, C-reactive protein; DBP, diastolic blood pressure; Glu, fasting glucose; Hcy, homocysteine; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HLP, hyperlipidaemia; HR, heart rate; SBP, systolic blood pressure; T2DM, Type 2 diabetes mellitus; TC, total cholesterol; TG, total triglycerides.
3.6 IL-12 treatment reverses the blood pressure elevation induced by Ang II

IL-12p35 eliminated the effects of both IL-12 and IL-35. To clarify which IL mediated these effects, Ang II-infused mice were treated daily with rIL-12 and rIL-35. The results showed that rIL-12 treatment significantly reduced SBP (Week 4 SBP: Ang II + PBS group: 161 ± 7 mmHg vs. Ang II + rIL-12 group: 130 ± 5 mmHg, Figure 6A), while rIL-35 treatment had no effect on SBP (Week 4 SBP: Ang II + PBS group: 161 ± 7 mmHg vs. Ang II + rIL-35 group: 162 ± 5 mmHg, Figure 6A). In addition, the Ang II-induced reduction in ACh- and SNP-induced relaxation and increase in PE-induced contraction were restored by rIL-12 treatment, but not by rIL-35 treatment (Figure 6B–D).

3.7 IL-12 treatment increases IL-12p40 expression in aortas of Ang II-infused mice

After treatment with rIL-12 or rIL-35, expression of IL-12p40 and EBI3, subunits of IL-12, and IL-35, respectively, was investigated in the aorta by immunofluorescence staining. The results showed that rIL-12 treatment, but not rIL-35 treatment, increased aortic IL-12p40 expression, while neither rIL-12 nor rIL-35 affected aortic EBI3 levels (Figure 6E). In addition, the mRNA levels of IL-12Rβ1 and gp130, which are subunits of the IL-12 receptor and IL-35 receptor, respectively, were measured by RT-qPCR. Increased IL-12Rβ1 mRNA levels were observed in Ang II-infused mice.
mice treated with rIL-12 but not in those treated with rIL-35 (Figure 6F), and no significant difference in gp130 expression was observed after PBS, rIL-12, or rIL-35 treatment in Ang II-infused mice (Figure 6F).

3.8 Plasma IL-12 levels are increased in hypertensive patients

Compared with control subjects, plasma IL-12 levels were significantly increased in hypertensive patients (Figure 7A). Positive correlations of SBP and diastolic blood pressure (DBP) with plasma IL-12 levels were observed in hypertensive patients (Figure 7B and C). Furthermore, multivariate analysis taking into account the major risk factors for hypertension revealed higher levels of IL-12 in subjects who smoked, drank alcohol, and were male, while other risk factors, including old age, obesity and family history, did not affect plasma IL-12 expression. The plasma IL-12 levels, Type III sum of square, F-value and P-value in each group are listed in Table 3.

4. Discussion

Previous studies have reported that IL-12p35 levels are increased in different heart disease models, including Ang II-induced cardiac fibrosis and left anterior descending coronary artery ligation-mediated myocardial infarction, in response to the local inflammatory response in the heart. In addition, studies have shown that IL-12p35 KO aggravates cardiac fibrosis and protects against myocardial infarction. Thus, we hypothesized that IL-12p35 also participates in the progression of hypertension, which is also a chronic inflammatory disease.

To test this hypothesis, we first detected IL-12p35 protein expression in the aortas of Ang II-infused mice. The results showed that aortic IL-12p35 expression increased by approximately 0.9-fold following Ang II infusion for 4 weeks. IL-12 is mainly produced by antigen-presenting cells such as Mø, B lymphocytes, T lymphocytes, and DCs. Meanwhile, IL-35 is mainly secreted by CD4+ regulatory T cells, which is an important T lymphocyte. IL-35 is also secreted by activated DCs and Mø. Because IL-12p35 is the mutual subunit of IL-12 and IL-35, to determine its source, Mø, B lymphocytes, T lymphocytes, and DCs were treated with Ang II. The results showed that Ang II treatment increased IL-12p35 mRNA expression in Mø, B lymphocytes, T lymphocytes, and DCs by 5.5-fold, 1.2-fold, 1.1-fold, and 1.5-fold, respectively. These results suggest that aortic Mø may be the main source of IL-12p35 in the Ang II-induced inflammatory environment. To confirm this hypothesis, double immunofluorescence staining of F4/80 and IL-12p35 was performed, and the results confirmed that Mø were the main source of IL-12p35 following Ang II stimulation.

In the present study, we found that IL-12p35 KO significantly increased SBP in Ang II-infused mice. In addition, the aggravation of Ang II-induced vascular dysfunction by IL-12p35 KO could be completely reversed by treatment with indomethacin, an anti-inflammatory drug.
Previous studies have reported that IL-12p35 KO can regulate inflammation; therefore, we speculated that the regulatory effect of IL-12p35 KO on SBP is mediated by the amplification of inflammation. In Mø colony-stimulating factor-deficient mice, which exhibit defects in the maturation of Mø, the vascular dysfunction and arterial hypertension induced by Ang II treatment were attenuated.36 Data from experimental models and human studies are in concordance and support a central role for Mø differentiation in the pathogenesis of hypertension.37

Figure 4 Effect of IL-12p35 KO on M1 macrophage differentiation. (A) Double immunofluorescence staining of anti-F4/80 and anti-CD80 in the four groups (×200). (B–G) The mRNA expression levels of iNOS, IL-1β, IL-6, IL-17, TNF-α, and IFN-γ in each group were measured by RT-qPCR (two-way ANOVA). (D) The p-STAT1, T-STAT1, p-P65, T-P65, and GAPDH levels were measured by western blotting (two-way ANOVA). (E) Double immunofluorescence staining of anti-F4/80 and anti-p-P65 in the Ang II group and the Ang II+IL-12p35 group (200×). IL-12p35 KO = p35 KO. N = 5–6 in each group. *P < 0.05 vs. the control group. #P < 0.05 vs. the Ang II-treated group.
**Figure 5** Effect of IL-12p35 KO on SMC differentiation. (A) Aortic OPN and SM22a protein expression was detected by immunofluorescence staining (×200); n = 5 in each group. *P < 0.05 vs. the control group. **P < 0.05 vs. the Ang II-treated group. (B) iNOS mRNA levels in macrophages and OPN and SM22a mRNA levels in SMCs were measured by RT-qPCR (two-way ANOVA). N = 5 in each group. IL-12p35 KO = p35 KO. *P < 0.05 vs. the WT Mø+WT CD4+Ang II group. **P < 0.05 vs. the KO Mø+WT CD4+Ang II group.
Activated Mø can differentiate into Mø1 and Mø2 macrophages (Mø2), and both an increase and a decrease in the level of Mø2 have been observed in the vascular walls of Ang II-treated mice. Moore et al. even suggested that Ang II-induced hypertension was mediated by the increase in Mø2. Mø2 are associated with the induction of Th2 T lymphocyte responses and IL-4 secretion, while IL-4 has been demonstrated to reduce or have no effect on blood pressure in Ang II-treated mice. Due to these controversial findings, we suspected that changes in the level of Mø2 may be associated with vascular repair and reconstruction. The imbalance in Mø1 and Mø2 has been used to explain the causes of hypertension; specifically, enhanced inflammation and decreased anti-inflammatory effects are two of the most important mechanisms, in which the increase in Mø1 plays a dominant role. Many Mø1-related cytokines have been reported to be closely related to Ang II-induced hypertension, therefore, we concluded that the above explanation was reasonable. We then detected the level of aortic Mø1 and found that IL-12p35 KO increased the levels of aortic Mø1 in Ang II-treated mice. As a previous study reported that activation of the P65 and STAT1 pathways promoted Mø1 differentiation and inflammatory functions, we measured the levels of phosphorylated STAT1 and P65 and found that IL-12p35 KO increased the level of P65 phosphorylation rather than the level of P65 expression. Thus, we further detected p-P65 expression in aortic Mø, and higher p-P65 levels were found in Ang II + IL-12p35 KO mice than in other mice. These data indicate that knockout of IL-12p35 activates the aortic P65 pathway and promotes Mø1 differentiation, thereby aggravating the inflammatory response and elevating blood pressure.

In a previous study, Li et al. found that Ang II-induced cardiac fibrosis was aggravated by IL-12p35 deletion, and these effects were mediated by the differentiation of heart Mø2 and the activation of the transforming growth factor-β1 (TGF-β1) pathway. Although, the present study found IL-12p35 KO promoted Ang II-induced aortic Mø1 differentiation, our
The results seem to contradict these previous conclusions. In fact, Mø1-related cytokines, such as IFN-γ, IL-1β, and IL-17, were reported to aggravate Ang II-induced cardiac fibrosis. The number of Mø in the body is not fixed; conversion between Mø1 and Mø2 can occur in response to external stimulation. Because Mø1 play a dominant role in the inflammatory response, which is critical for cardiac fibrosis, one possible explanation is that the compensatory increase in Mø2 protects against the cardiac damage mediated by increased Mø1, and increased Mø2 levels result in the development of cardiac fibrosis in the Ang II-induced inflammatory microenvironment. In fact, this phenomenon has also been described for vascular diseases, such as aortic aneurysm and aortic dissection. Previous studies have found that when Mø1 activity was significantly enhanced, Mø2 activity showed compensatory enhancement but was significantly weaker than that of Mø1 and was not sufficient to counteract its pro-inflammatory activity. To confirm this speculation, additional hypertension model mice were generated by Ang II infusion, and the dynamic changes of aortic Mø1 and Mø2 were detected. No differences were found in the baseline iNOS and Arg-1 levels among these groups. Ang II infusion gradually increased the iNOS mRNA levels during Week 1; these levels continued to increase during Week 2, reached a peak, and then were continuously maintained at high levels through the end of Week 4. However, IL-12p35 KO mice showed increases in iNOS mRNA levels at each time point (Supplementary material online, Figure S1A). Interestingly, the Arg-1 levels were decreased by Week 1, increased rapidly and reached a peak at Week 2, and then were maintained until the end of Week 4 (Supplementary material online, Figure S1B). Surprisingly, IL-12p35 KO further decreased Arg-1 mRNA levels during Week 1 but further increased Arg-1 mRNA levels during Week 2 and Week 4 (Supplementary material online, Figure S1A and B). The changes in SBP were consistent with an increase in Mø1 but not Mø2, which further demonstrated that Mø1, rather than Mø2, play a dominant role in Ang II-induced hypertension. IL-12p35 KO promoted Mø1 differentiation at different time points, which further supports that IL-12p35 KO elevates blood pressure may be associate with the amplification of Mø1-mediated inflammation.

SMCs are the main components of arteries, and SMC phenotypic alterations are critical in the development of hypertension because vasoconstriction mainly depends on SMCs; specifically, the transformation of SMC phenotype leads to increased vasoconstriction and vascular resistance. SMCs of normal vessel walls are contractile; these SMCs...
differentiate in hypertension or after Ang II treatment. Therefore, we detected the levels of SM22a and OPN, which are a marker of contractility and a secreted SMC marker, respectively, in the aorta. We found that IL-12p35 KO decreased SM22a expression but increased OPN expression in Ang II-infused mice. The results were verified in the in vitro experiments, which showed that IL-12p35 KO increased iNOS levels when Mø and CD4<sup>+</sup> Th cells were co-cultured. Moreover, OPN mRNA expression could be increased, and the SM22a mRNA levels could be decreased by treating the medium. Therefore, the results suggest that the vascular SMC transition induced by Mø1 may underlie the effect of IL-12p35 KO on Ang II-induced hypertension.

Some of the Ang II-treated mice were treated with rIL-12 or rIL-35 to investigate whether its effect on blood pressure regulation would be abolished by IL-12p35 KO. We found that rIL-35 treatment did not affect SBP, while rIL-12 treatment decreased SBP in a dose-dependent manner (Supplementary material online, Figure S2A). Daily treatment with 300 ng of rIL-12 even reduced the SBP below the baseline level (Supplementary material online, Figure S2B). In addition, rIL-12 treatment prevented Ang II-induced vascular dysfunction and increased the expression of IL-12p40, the other subunit of IL-12, and IL-12Rβ1 in the aortas. Meanwhile, rIL-35 treatment did not affect Ang II-induced vascular dysfunction or the expression of EBI, the other subunit of IL-35, and gp130 in the aortas. These data suggest that IL-12 plays an antihypertensive role in Ang II-infused WT mice, while IL-35 has no effect on blood pressure. These results suggest that IL-12 plays an anti-inflammatory role in Ang II-treated mice, which has been reported in previous studies. To elucidate the mechanisms of the antihypertensive effect of IL-12, the effect of IL-12 on Mø differentiation at different time points in Ang II-infused WT mice was determined. IL-12 significantly reduced the iNOS mRNA levels at each time point. Interestingly, IL-12 decreased Areg-1 levels during Week 1 but rescued Areg-1 levels from Week 2 until Week 4 (Supplementary material online, Figure S1A and B). The evidence suggested that IL-12 inhibits Mø1 differentiation, plays an anti-inflammatory role and lowers blood pressure in Ang II-infused WT mice. To further explore the mechanisms of the antihypertensive effect of IL-12, Ang II-infused IL-12p35 KO mice were also treated with PBS, rIL-12, or rIL-35. We found that rIL-12 treatment, rather than rIL-35, lowered the SBP, reduced aortic iNOS expression at Week 4 and alleviated vascular dysfunction in Ang II-treated IL-12p35 KO mice (Supplementary material online, Figure S3A–E). Our results suggest that IL-12 plays an anti-inflammatory role in the Ang II-induced inflammatory microenvironment. However, the exact role of IL-12 appears to depend on the inflammatory microenvironment. Because of the antihypertensive effect of IL-12 on Ang II-induced hypertension, we also detected plasma IL-12 levels in hypertensive patients and are positively correlated with SBP and DBP. In addition, the major risk factors for hypertension, including smoking, drinking, and male sex, are also correlated with increased plasma IL-12 levels. These results suggest that elevated IL-12 positive feedback can protect against certain inflammatory environment-mediated damages.

5. Conclusion

In conclusion, we demonstrated that IL-12p35 KO promoted blood pressure elevation by up-regulating Mø1 differentiation, amplifying the inflammatory response and promoting SMC phenotypic transition in Ang II-treated mice. Additionally, IL-12, but not IL-35, plays a protective role in the Ang II-induced hypertension model. However, IL-12p35 KO abolishes the effect of IL-12 on blood pressure regulation. Nonetheless, IL-12 may be a novel therapeutic agent for the prevention and treatment of clinical hypertension.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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References


