Deletion of Interleukin-6 Attenuates Pressure Overload-Induced Left Ventricular Hypertrophy and Dysfunction


Rationale: The role of interleukin (IL)-6 in the pathogenesis of cardiac myocyte hypertrophy remains controversial. Interleukin (IL)-6 is produced by cardiac myocytes themselves, and persistent activation of gp130, a key component of IL-6 signaling, induced myocardial hypertrophy in mice.3 However, gp130 also transduces signaling by other members of the IL-6 family of cytokines, and hence not specific for IL-6. A subsequent study has shown that IL-6 infusion results in concentric hypertrophy in rats, although without increasing blood pressure.4 However, there is no direct evidence to date that IL-6 signaling is necessary for pressure overload–induced LV hypertrophy.

Objective: To conclusively determine whether IL-6 signaling is essential for the development of pressure overload–induced left ventricular (LV) hypertrophy and to elucidate the underlying molecular pathways.

Methods and Results: Wild-type and IL-6 knockout (IL-6−/−) mice underwent sham surgery or transverse aortic constriction (TAC) to induce pressure overload. Serial echocardiograms and terminal hemodynamic studies revealed attenuated LV hypertrophy and superior preservation of LV function in IL-6−/− mice after TAC. The extents of LV remodeling, fibrosis, and apoptosis were reduced in IL-6−/− hearts after TAC. Transcriptional and protein assays of myocardial tissue identified Ca2+/calmodulin-dependent protein kinase II (CaMKII) and signal transducer and activator of transcription 3 (STAT3) activation as important underlying mechanisms during cardiac hypertrophy induced by TAC. The involvement of these pathways in myocyte hypertrophy was verified in isolated cardiac myocytes from wild-type and IL-6−/− mice exposed to prohypertrophy agents. Furthermore, overexpression of CaMKII in H9c2 cells increased STAT3 phosphorylation, and exposure of H9c2 cells to IL-6 resulted in STAT3 activation that was attenuated by CaMKII inhibition. Together, these results identify the importance of CaMKII-dependent activation of STAT3 during cardiac myocyte hypertrophy via IL-6 signaling.

Conclusions: Genetic deletion of IL-6 attenuates TAC-induced LV hypertrophy and dysfunction, indicating a critical role played by IL-6 in the pathogenesis of LV hypertension in response to pressure overload. CaMKII plays an important role in IL-6–induced STAT3 activation and consequent cardiac myocyte hypertrophy. These findings may have significant therapeutic implications for LV hypertrophy and failure in patients with hypertension.

Key Words: 
Ca2+/calmodulin-dependent protein kinase type II ■ cardiac myocyte ■ hypertension ■ interleukin-6 ■ left ventricular hypertrophy ■ signal transducer and activator of transcription 3
control and IL-6−/− mice, indicating that IL-6 was not essential for pressure overload–induced hypertrophy in vivo.

Considering the above controversies, and the well-known association of IL-6 with cardiovascular pathologies, we sought to conclusively determine whether IL-6 is necessary for pressure overload–induced hypertrophy using an in vivo model of TAC. We also elucidated the molecular pathways involved in this process. Our results indicate that pressure overload–induced deleterious effects on LV structure and function are attenuated in the absence of IL-6, indicating an essential role played by this cytokine. Our molecular data further unravel several heretofore unknown signaling roles, including the contribution of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) toward signal transducer and activator of transcription 3 (STAT3) activation in this process.

Methods

An expanded methods section is available in the Online Data Supplement.

Animals

All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Kansas Medical Center. Male IL-6−/− mice (IL-6−/−/Kot, genetic background C57BL/6J, stock No: 002650) were purchased from the Jackson Laboratory (Bar Harbor, ME). Age and body weight–matched wild-type (WT) C57BL/6J mice were used as controls. Deletion of IL-6 was confirmed by polymerase chain reaction using mouse tail genomic DNA as template (Online Figure I).

Minimally Invasive TAC

Minimally invasive TAC was performed to induce LV pressure overload in age- and body weight–matched mice.

Histology

Standard methods were used for the assessment of fibrosis and apoptosis.

Measurement of Cardiac Myocyte Size

Cardiac myocytes were isolated from WT and IL-6−/− mice at 6 weeks after sham and TAC procedures.

Echocardiography

LV function and structure were assessed by serial transthoracic echocardiography before surgery and at 1, 2, 4, and 6 weeks after surgery using published methods.2,4

Invasive Hemodynamic Studies

Open chest in vivo hemodynamic studies were performed.1

Assessment of Apoptosis

Myocyte apoptosis was quantitated using Terminal dUTP Nick End-labeling (TUNEL) assay performed on myocardial sections at 6 weeks after TAC.

H9c2 Myoblast Culture

H9c2 cells were maintained according to suggested culture methods. Transfection of H9c2 cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and plasmids targeting CaMKII (Addgene, Cambridge, MA).

Cell Treatments

Isolated adult cardiac myocytes were stimulated with angiotensin II (Ang II), phenylephrine, or recombinant IL-6 (rIL-6); H9c2 myoblasts were exposed to rIL-6 and CaMKII or STAT3 inhibitors.

Assessment of Cell Surface Area

H9c2 cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet for assessment of cell surface area.

Quantitative Polymerase Chain Reaction

Total RNA was isolated from frozen tissues and cultured cells and processed for real-time quantitative reverse transcription polymerase chain reaction using standard protocols.

Protein Extraction and Western Blot Analysis

Standardized protocols were used as described in the Online Data Supplement.

Statistical Analysis

Data were expressed as mean±SEM. For comparison of 2 groups, Student t tests (2-tailed) were performed; multiple groups (≥3 groups) comparison was performed by 1-way ANOVA with Bonferroni post hoc test. Serial echocardiographic parameters were analyzed using 2-way (time and group) ANOVA followed by Student t tests with the Bonferroni correction. P values <0.05 were considered statistically significant. All statistical analyses were performed using the SPSS software version 22.0 (IBM, Armonk, NY).

Results

Experimental Protocol

The in vivo experimental protocol is summarized in Online Figure II. A total of 153 mice were enrolled. Fifteen mice were excluded for the reasons specified in Online Table II. After a baseline echocardiography, WT and IL-6−/− mice underwent either TAC (TAC group) or sham (sham group) surgery. Echocardiography was repeated at 1, 2, 4, and 6 weeks after the surgery for quantitative assessment of LV structural and functional parameters. Mice were euthanized at 6 weeks after surgery after a terminal hemodynamic study. Histological and molecular assays were performed using myocardial tissue harvested at 1, 2, and 6 weeks after surgery. In vitro experiments were conducted using cardiac myocytes isolated from WT and IL-6−/− hearts after TAC.

Genetic Deletion of IL-6 Attenuated LV Hypertrophy Induced by Pressure Overload In Vivo

Gross morphology after 6 weeks of surgery in the WT and IL-6−/− mice (Figure 1A) suggested attenuation of hypertrophy in IL-6−/− hearts. We used heart weight (HW)/tibia length ratio to assess changes in LV mass. As expected, HW/tibia length in sham-operated WT and IL-6−/− mice were similar. However, in the TAC groups, HW/tibia length in WT mice was significantly higher compared with IL-6−/− mice (10.1±0.44 versus 8.8±0.29 mg/mm, P<0.05) (Figure 1B; Online Table III). Next, we measured the

Nonstandard Abbreviations and Acronyms

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<thead>
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<th>Abbreviation</th>
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<tr>
<td>Ang II</td>
<td>angiotensin II</td>
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<tr>
<td>BW</td>
<td>body weight</td>
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<tr>
<td>CaMKII</td>
<td>Ca²⁺/calmodulin-dependent protein kinase II</td>
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<td>EF</td>
<td>ejection fraction</td>
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<td>FS</td>
<td>fraction shortening</td>
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<td>HW</td>
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<td>IL-6−/−</td>
<td>interleukin-6 knock out</td>
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<td>LV</td>
<td>left ventricle</td>
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<td>STAT3</td>
<td>signal transducer and activator of transcription 3</td>
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<tr>
<td>TAC</td>
<td>transverse aortic constriction</td>
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IL-6−/− knockout mice after sham and TAC procedures. As expected, HW/tibia length in sham-operated WT and IL-6−/− mice were similar. However, in the TAC groups, HW/tibia length in WT mice was significantly higher compared with IL-6−/− mice (10.1±0.44 versus 8.8±0.29 mg/mm, P<0.05) (Figure 1B; Online Table III). Next, we measured the
myocytes in sham-operated *IL-6−/−* hearts at 6 weeks after TAC were smaller compared with WT counterparts (135.1±13.2 μm² versus 167.4±6.7 μm², P<0.05; Figure 1C; Online Table IV). These findings suggest that IL-6 plays an important role in the normal development of cardiac myocytes. After TAC, the transverse area of myocytes increased in both WT and *IL-6−/−* mice; however, this increase was significantly attenuated in *IL-6−/−* mice compared with WT mice (173.4±12.3 μm² versus 256.4±17.7 μm², P<0.05; Figure 1C; Online Table IV). Because of differences in the transverse area of myocytes in *IL-6−/−* and WT mice in the sham group, we calculated the percent change in the area in respective TAC group. The increase in myocyte area in WT mice was significantly greater compared with *IL-6−/−* mice (53% versus 28%, P<0.05). These results indicate that the absence of IL-6 attenuates LV hypertrophy induced by pressure overload.
In addition, we measured surface area of cardiac myocytes isolated from WT and IL-6−/− mice at 6 weeks after surgery. Consistent with histological observations above, cardiac myocytes isolated from IL-6−/− mice were smaller than WT in the sham group (3126.78±73.33 μm² versus 3415.11±60.56 μm², P<0.05; Figure 1D and 1E). Cell surface area increased in both WT and IL-6−/− mice after TAC; however, the increase was significantly attenuated in IL-6−/− mice compared with WT (3904.30±21.24 μm² versus 5208.14±243 μm², P<0.05; Figure 1D and 1E). Similarly, the width of isolated cardiac myocytes was less in IL-6−/− mice compared with WT in the sham groups (20.9±0.32 μm versus 22.9±0.25 μm, P<0.05; Figure 1D and 1F); and the increase in myocyte width was significantly attenuated in IL-6−/− mice compared with WT mice (23.96±0.09 μm versus 26.14±0.61 μm, P<0.05; Figure 1D and 1F) in the TAC groups. Interestingly, there was no difference in length between WT and IL-6−/− myocytes in the sham groups (145.5±1.56 μm versus 143.7±2.8 μm, P=NS; Figure 1G). There was an increase in cardiac myocyte length in the TAC groups; however, the increase was significantly attenuated in IL-6−/− hearts compared with WT hearts (169.78±0.83 μm versus 191.93±5.38 μm, P<0.05; Figure 1G).

To confirm findings at the molecular level, we performed mRNA and protein characterization for hypertrophy-related markers using tissue samples isolated at 2 weeks after surgery. There was significantly higher mRNA expression of atrial natriuretic peptide (anp), brain natriuretic peptide (bnp), α-skeletal muscle actin (α-SK actin), and GATA-binding protein 4 (gata4) in WT hearts compared with IL-6−/− hearts after TAC. There was no significant difference between sham-operated WT and IL-6−/− hearts (Figure 1H). Consistently, protein expression for ANP was significantly higher in WT hearts compared with IL-6−/− hearts at both 2 and 6 weeks after TAC (Figure 1I and 1J). The continued suppression of ANP in IL-6−/− mice at 6 weeks after TAC suggests that the protective effect of IL-6 deletion is sustained.

Pressure Overload–Induced Ventricular Remodeling Was Ameliorated in the Absence of IL-6

Echocardiographic LV mass increased in both WT and IL-6−/− mice at 1 week after TAC; however, the increase was significantly greater in WT mice (101.77±4.87 mg versus 88.79±3.51 mg, P<0.05; Figure 2A). The other parameters of LV remodeling including posterior wall thickness at end-diastole, LV end-diastolic diameter, and LV end-diastolic volume were also significantly persevered in IL-6−/− mice compared with WT mice after TAC (Figure 2B–2D). The LV chamber diameter measured in hearts harvested at 6 weeks after surgery was significantly larger in WT compared with IL-6−/− mice in the TAC groups (4.2±0.05 mm versus 3.8±0.05 mm, P<0.05; Figure 2E). These results corroborated with the echocardiographic data.

Genetic Deletion of IL-6 Prevented Pressure Overload–Induced LV Dysfunction

There was no significant difference in LV ejection fraction (69.11±1.06% versus 70.56±0.92%, in WT and IL-6−/− mice, respectively, P=NS; Figure 3A) and LV fractional shortening (39.81±0.98% versus 39.21±2.06%, in WT and IL-6−/− mice, respectively, P=NS; Figure 3B) at baseline. LV ejection fraction decreased in both WT and IL-6−/− mice at 1 week after TAC; however, the reduction was significantly greater in
WT mice compared with IL-6−/− mice (64.21±0.83% versus 66.77±0.64%, P<0.05; Figure 3A). Similar results were noted for LV fractional shortening in the TAC group (35.42±0.72% versus 37.69±0.70%, P<0.05; Figure 3B). There was continued reduction in LV ejection fraction and LV fractional shortening in WT mice during follow-up at 2, 4, and 6 weeks after TAC. At each follow-up time point, cardiac function was better preserved in IL-6−/− mice compared with WT mice (Figure 3A and 3B). Consistently, the progressive increase in LV end-systolic volume was also less pronounced in IL-6−/− mice (Figure 3C).

These observations corroborated with data from invasive hemodynamic studies at 6 weeks after surgery. LV dP/dt max was significantly better in IL-6−/− mice compared with WT mice in the TAC groups, while there was no significant difference in the sham groups (Figure 3D; Online Table V). The end-systolic pressure–volume relationship also showed preserved systolic function in IL-6−/− mice compared with WT mice (Figure 3E and 3F). Consistently, the progressive increase in LV end-systolic volume was also less pronounced in IL-6−/− mice (Figure 3C).

Pressure Overload–Induced Myocardial Fibrosis Was Attenuated in IL-6−/− Hearts

Myocardial interstitial and perivascular fibrosis was assessed in picrosirius red–stained sections at 6 weeks after surgery. There was no significant difference in the extents of interstitial and perivascular fibrosis in WT and IL-6−/− mice in the sham groups. However, both interstitial and perivascular fibrosis increased in WT hearts after TAC, with more pronounced changes in perivascular areas (Figure 4A–4D). This increase in interstitial and perivascular fibrosis was significantly blunted in IL-6−/− hearts after TAC (Figure 4A–4D). To understand the molecular underpinnings of these results, we assessed gene and protein expression of fibrosis-related molecules in myocardial tissue. The mRNA levels for collagen type I α1 (Col1A1), collagen type III α1 (Col3A1), and periostin were measured in LV tissue samples harvested at 2 weeks after surgery. Consistent with data from quantitative analysis of fibrosis in picrosirius red–stained myocardial sections, the mRNA expression for Col1A1 and Col3A1 was significantly higher in WT compared with IL-6−/− mice after TAC (Figure 4E). The mRNA expression of periostin was also significantly higher in WT compared with IL-6−/− mice after TAC (Figure 4E). The TAC-induced increase in myocardial periostin expression was also confirmed at the protein level (Figure 4F). Importantly, increased expression of MMP9 (matrix metalloproteinase-9)
noted in WT hearts at 6 weeks after TAC was attenuated in IL-6−/− hearts (Figure 4F). These results from mRNA and protein characterization were consistent with our histological findings of improved remodeling in the absence of IL-6.

Genetic Deletion of IL-6 Attenuated Cardiac Myocyte Apoptosis Induced by Pressure Overload
To elucidate the role of IL-6 in the pressure overload–induced apoptosis, we performed TUNEL staining on myocardial samples harvested at 6 weeks after surgery. The percentage of apoptotic cardiac myocyte nuclei in WT hearts was significantly greater than IL-6−/− hearts in the TAC groups (0.074±0.012% versus 0.040±0.008%, P<0.05; Figure 5A and 5B), indicating that absence of IL-6 protects cardiac myocytes from pressure overload–induced apoptosis. Consistent with these observations, the expression of proapoptotic molecule Bcl-2–associated X protein in WT hearts was greater compared with IL-6−/− hearts at 6 weeks after TAC. Moreover, the levels of antiapoptotic molecule Bcl-2 were reduced in WT hearts compared with IL-6−/− hearts at 6 weeks after TAC (Figure 5C). The ratio of Bcl-2–associated X protein/Bcl-2 was significantly lower in IL-6−/− hearts compared with WT hearts in TAC groups (Figure 5D), suggesting that the absence of IL-6 promotes an antiapoptotic milieu.

Hypertrophic Response Was Attenuated in IL-6−/− Adult Cardiac Myocytes
To specifically examine the role of IL-6 in cardiac myocyte hypertrophy, we performed in vitro experiments using cardiac myocytes isolated from WT and IL-6−/− mice. These cardiac myocytes were exposed to rIL-6 for 24 hours followed by isolation of mRNA and quantitative polymerase chain reaction for hypertrophy-related genes (anp, bnp, and myh-7). The expression of these genes after rIL-6 stimulation was significantly higher in WT cardiac myocytes compared with IL-6−/− cardiac myocytes (Figure 6A). This prompted us to explore the expression of IL-6 receptors in WT and IL-6−/− cardiac myocytes. Indeed, the expression of IL-6 receptors was lower in IL-6−/− cardiac myocytes compared with WT cardiac myocytes (Figure 6B), offering a possible explanation for the lack of response to IL-6. Next, we tested the effects of Ang II on cardiac myocytes isolated from WT and IL-6−/− mice. Although Ang II increased the expression of anp, bnp, and myh-7 in both groups, the response was blunted in IL-6−/− cardiac myocytes (Figure 6C). When WT and IL-6−/− cardiac myocytes were stimulated with phenylephrine, another hypertrophy-promoting agent, the results showed similar trends (Figure 6D). Together, these results corroborate our in vivo observations about the requirement of IL-6 for pressure overload–induced cardiac hypertrophy.

Pressure Overload–Induced Activation of Mitogen-Activated Protein Kinase and Akt Signaling Pathways Was Suppressed by Genetic Deletion of IL-6
Because mitogen-activated protein kinase pathways are involved in gp130-mediated cardiac myocyte hypertrophy,9 we assessed the levels of phosphorylated ERK1/2 and JNK at 6 weeks after surgery. Although phosphorylated ERK1/2 increased in both WT and IL-6−/− mice after TAC, the increase was attenuated in IL-6−/− mice (Online Figure IIIA and IIIB),...
indicating that intact IL-6 signaling is necessary for pressure overload–induced activation of ERK1/2. Similarly, the increase in phosphorylated JNK levels noted in WT hearts after TAC was attenuated in IL-6−/− hearts (Online Figure IIIA and IIIC), albeit the difference was less pronounced. In addition, increased phosphorylation of Akt (Online Figure IIID and IIIE) and GSK-3β (Online Figure IIID and IIIF) was noted in WT hearts after TAC; both of which were significantly attenuated in IL-6−/− hearts. There was no significant difference in the levels of these proteins in sham-operated WT and IL-6−/− mice (Online Figure IIIA–IIIF).

CaMKII and STAT3 Contributed to IL-6–Induced Cardiac Myocyte Hypertrophy

Previous studies have suggested the involvement of STAT3 and CaMKII in pressure overload–induced cardiac hypertrophy. At 6 weeks after TAC, the levels of phosphorylated STAT3 (pTyr-STAT) and phosphorylated CaMKII (p-CaMKII) increased in both WT and IL-6−/− mice; however, the increase was significantly attenuated in IL-6−/− mice (Figure 7A–7C). These observations highlight a significant role of IL-6 signaling in phosphorylation of STAT3 and CaMKII in specifically the setting of TAC-induced hypertrophy. To further confirm the interaction of IL-6 signaling with STAT3 and CaMKII, we used rIL-6 for stimulation of H9c2 myoblasts in the presence and absence of inhibitors of STAT3 and CaMKII. Exposure to rIL-6 increased the surface area of H9c2 cells indicating hypertrophy; however, this increase was blocked in the presence of STAT3 inhibitor and CaMKII inhibitor (Figure 7D and 7E). Consistently, exposure to rIL-6 also increased gene expression of hypertrophy markers (anp and bnp) in H9c2 cells; and this was suppressed in the presence of STAT3 and CaMKII inhibitors (Figure 7F). To understand

Figure 5. Attenuation of pressure overload–induced cardiac myocyte apoptosis in the absence of interleukin (IL)-6. A and B, Terminal dUTP Nick End-labeling (TUNEL) staining was performed on myocardial sections from wild-type (WT) and IL-6−/− hearts harvested at 6 weeks after surgery. Apoptotic nuclei (white arrows) are visualized by green fluorescence. Nuclei are identified in blue (DAPI [4′,6-diamidino-2-phenylindole]). Sections were coimmunostained with anti-α-sarcomeric actin (red) to identify cardiac myocytes. TUNEL-positive myocyte nuclei were quantitated as a percentage of total cardiac myocyte nuclei, scale bar, 5 μm (n=10–12 per group). C, Representative Western immunoblots for Bcl-2–associated X protein (Bax) and Bcl-2. D, Bar graph showing the ratio of Bax to Bcl-2 (n=6 per group). Data represent mean±SEM. *P<0.05 vs Sham WT mice; #P<0.05 vs transverse aortic constriction (TAC) WT mice.

Figure 6. Requirement of interleukin (IL)-6 for adult cardiac myocyte hypertrophy. A, Expression of hypertrophy-related genes (anp, bnp, and myh-7) in adult mouse cardiac myocytes from wild-type (WT) and IL-6−/− mice after stimulation with recombinant IL-6 (50 ng/mL) for 24 h. B, Expression of IL-6 receptor in WT and IL-6−/− cardiac myocytes. C, Expression of anp, bnp, and myh-7 in adult cardiac myocytes from WT and IL-6−/− mice after stimulation with angiotensin II (Ang II; 1×10−7 mol/L) for 48 h. D, Expression of anp, bnp, and myh-7 in adult cardiac myocytes from WT and IL-6−/− mice after stimulation with phenylephrine (PE, 10 μmol/L) for 24 h. Data represent mean±SEM from 3 independent experiments. *P<0.05 vs WT cardiac myocytes; #P<0.05 vs WT cardiac myocytes treated with prohypertrophic stimulators.
the time-dependent nature of phosphorylation of STAT3 and CaMKII, we treated H9c2 cells with rIL-6 for variable durations. IL-6–induced phosphorylation of STAT3 and CaMKII peaked in 30 minutes and plateaued subsequently (Figure 7G–7I). Our observations indicate that phosphorylation of CaMKII and STAT3 follow a similar time course after exposure to IL-6. Together, these data from harvested tissues and H9c2 cells identify important roles of CaMKII and STAT3 in IL-6–induced cardiac hypertrophy.

**IL-6 Activated STAT3 via a CaMKII-Dependent Manner**

The relationship between CaMKII and STAT3 activation was further investigated by transiently transfecting H9c2 cells with a vector encoding CaMKII. H9c2 cells transfected with an empty vector served as controls. The levels of CaMKII, p-CaMKII, and pTyr-STAT3 increased significantly in CaMKII-transfected cells 48 hours later, suggesting that CaMKII may be an upstream activator of STAT3 (Figure 8A–8C). This relationship was further confirmed by using an inhibitor of CaMKII (KN-62) before stimulation of H9c2 cells with rIL-6. The level of pTyr-STAT3 was significantly attenuated in the presence of CaMKII inhibitor (Figure 8D–8F). Because STAT3 is translocated to the nucleus after phosphorylation, we examined phosphorylated protein levels in cytoplasmic and nuclear fractions. pTyr-STAT3 levels increased significantly in both fractions in H9c2 cells treated with rIL-6. However, the addition of CaMKII inhibitor resulted in significant reduction in pTyr-STAT3 levels in both nuclear and cytoplasmic fractions of rIL-6–exposed cells (Figure 8G–8I). Taken together, these observations confirm that CaMKII is upstream of STAT3 and plays important role in the activation of STAT3 by IL-6. Similar experiments using isolated adult cardiac myocytes yielded similar results (Online Figure IV) further confirming the role of CaMKII in IL-6–induced STAT3 activation.

**Discussion**

**Salient Findings**

Newer therapeutic approaches are urgently needed to improve the clinical outcomes in millions of patients with hypertensive heart disease. However, and despite intense research, the precise molecular pathways that can be modulated to prevent LV hypertrophy remain poorly understood. In particular, the involvement of IL-6, an inflammatory cytokine, in this process remains controversial. Our studies using a mouse model of pressure overload in vivo and isolated cardiac myocytes in vitro provide the following important observations: (1) genetic deletion of IL-6 attenuates pressure overload–induced cardiac hypertrophy.

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**Figure 7.** Signal transducer and activator of transcription 3 (STAT3) and Ca2+/calmodulin-dependent protein kinase II (CaMKII) contributed to interleukin (IL)-6–Induced cardiac myocyte hypertrophy. A–C, Representative Western immunoblots (A) and quantitative data for pTyr-STAT3 (B), and p-CaMKII (C) in wild-type (WT) and IL-6−/− mouse hearts at 6 weeks after surgery (n=6 per group). *P<0.05 vs Sham WT mice; #P<0.05 vs TAC WT mice. D, Representative images of H9c2 cells stained with 1% crystal violet after treatment with KN-62 (10 μmol/L) or WP1066 (6 μmol/L) in the presence and absence of recombinant IL (rIL)-6 (50 ng/mL) for 24 h, scale bar, 100 μm. E, Cell surface area measured after 24 h of exposure to rIL-6 in the presence or absence of inhibitors of CaMKII or STAT3. F, Levels of mRNA for anp and bnp as determined by quantitative polymerase chain reaction after 24 h of exposure to rIL-6 in the presence or absence of inhibitors of CaMKII and STAT3. G–I, Stimulation with rIL-6 (50 ng/mL) for variable duration with subsequent quantitation of pTyr-STAT3 (H) and p-CaMKII (I). Data represent mean±SEM from 3 independent experiments. *P<0.05 vs control; #P<0.05 vs rIL-6 treatment only.
hypertrophy; (2) pressure overload–induced LV remodeling and dysfunction are ameliorated in the absence of IL-6, indicating a critical role of this cytokine in the pathogenesis of these unfavorable outcomes; (3) pressure overload–induced myocardial fibrosis and apoptosis are ameliorated in the absence of IL-6; (4) IL-6 is required for hypertrophy of adult cardiac myocytes in response to Ang II and phenylephrine; and (5) CaMKII plays a heretofore unknown and important role in IL-6–induced STAT3 activation and cardiac myocyte hypertrophy. These signaling insights may be useful toward formulation of novel cardioprotective strategies in hypertensive patients.

IL-6 Deletion Prevents LV Hypertrophy in Response to Pressure Overload

Although gp130, a key component of IL-6 signaling, and the IL-6 family of cytokines collectively have been implicated in LV hypertrophy, the specific impact of IL-6 signaling with regard to LV hypertrophy remains unsubstantiated and controversial. Importantly, gp130 transduces signaling not only for IL-6 but also for the other members of this family of cytokines. As for IL-6 specifically, previous studies have shown that hypertrophy induced by Ang II and norepinephrine is significantly attenuated in IL-6−/− mice, suggesting a role of IL-6 in pathological hypertrophy.12,13 A previous study in rats showed that IL-6 infusion for 7 days induced LV hypertrophy, albeit without raising blood pressure.4 The direct impact of IL-6 deletion on pressure overload–induced LV hypertrophy has been investigated to date only in 1 study, which showed no effect of IL-6 deletion on pressure overload–induced LV dysfunction and remodeling at 2 and 4 weeks after TAC.6

Using a similar model of TAC, our study is the first to report that genetic deletion of IL-6 prevents LV hypertrophy, thereby identifying a critical role of this cytokine in the pathogenesis of hypertensive heart disease. The potential reasons for differences between observations made by Lai et al6 and our current findings include possible differences in the severity of TAC and the differences in follow-up durations. In the study by Lai et al,6 mice were followed for 4 weeks after TAC, whereas our observations were made at 6 weeks after TAC. Although it is possible that the protective effects of IL-6 deletion become more pronounced with longer follow-up after TAC, the duration of follow-up may not be the primary reason for the observed differences, because the benefits of IL-6 deletion were apparent even at 1 week after TAC. Another important consideration is the fact that IL-6−/− mice have been reported to exhibit smaller HW and HW/BW ratio with body weights of IL-6−/− starting to differ after 6 months of age.14 Other investigators did not find differences in HW and HW/BW ratio at younger ages.13,15,16 In light of these reports, and to avoid any potential confounding effects of IL-6−/− mice at baseline, we selected age- and body weight–matched male WT mice as controls. Importantly, our in vivo observations are further supported by observations in isolated cardiac myocytes (Figure 1D–1G) and molecular evidence that IL-6 deletion significantly curbs the induction of genes responsible

Figure 8. Interleukin (IL)-6–induced signal transducer and activator of transcription 3 (STAT3) phosphorylation and translocation in part via a Ca2+/calmodulin-dependent protein kinase II (CaMKII)–dependent manner. A–C, H9c2 cells were transfected with 2, 4, and 6 μg of plasmids encoding CaMKII. Representative Western immunoblots (A) and quantitative data for CaMKII (B) and pTyr-STAT3 (C). D–F, Representative Western immunoblots (D) and quantitative data for p-CaMKII (E) and pTyr-STAT3 (F) after stimulation with recombinant IL (rIL)-6 (50 ng/mL) in the presence or absence of KN-62 (5 or 10 μmol/L). G–I, Representative Western immunoblots (G) and quantitative data for cytoplasmic and nuclear contents of p-CaMKII (H) and pTyr-STAT3 (I) after stimulation with rIL-6 (50 ng/mL) in the presence or absence of KN-62 (5 or 10 μmol/L). Data represent mean±SEM from 3 independent experiments. *P<0.05 vs control; #P<0.05 vs IL-6 treatment only.
for cardiac hypertrophy (Figure 1H–IJ). In addition, the in vivo data indicating a direct role of IL-6 in pressure overload–induced hypertrophy was confirmed in our in vitro model of IL-6–induced cardiac myocyte hypertrophy (Figure 7D–7F). Together, these data provide conclusive evidence that IL-6 is essential for pressure overload–induced cardiac myocyte hypertrophy.

**Pressure Overload–Induced LV Remodeling, Fibrosis, and Failure Are Attenuated in the Absence of IL-6**

Persistent pressure overload induced by systemic hypertension or TAC results in LV hypertrophy that is preceded by diverse molecular changes and culminates in LV remodeling and eventual failure. The functional manifestation of LV remodeling is diastolic dysfunction during earlier stages and systolic impairment at later stages. Although increased IL-6 levels have been noted in patients with diastolic dysfunction, no direct evidence to date has linked IL-6 to the pathogenesis of hypertensive LV remodeling and failure. Although IL-6 infusion in a previous study resulted in LV hypertrophy, blood pressure was not elevated.4 Our results show that LV remodeling was evident in WT mice at 1 week after TAC. Subsequently, the LV underwent progressive hypertrophy with increasing duration of follow-up. IL-6 deletion was beneficial in preventing LV remodeling at every stage of follow-up after TAC (Figure 2). Concentric remodeling and hypertrophy are risk factors for development of heart failure with preserved ejection fraction. With increasing prevalence of heart failure with preserved ejection fraction, the functional manifestation of LV remodeling and eventual failure in vivo in a model of pressure overload.

LV remodeling is often associated with increased myocardial fibrosis. Increasing collagen deposition after persistent pressure overload or myocardial infarction contributes to the deterioration of LV compliance and development of diastolic dysfunction. Our data are the first to show that IL-6 deletion inhibits myocardial collagen deposition in both interstitial and perivascular areas after pressure overload, with greater impact on perivascular fibrosis (Figure 4A–4D). These histological quantitative data were further confirmed by concordant changes in expression of fibrosis-related genes. These observations suggest that IL-6 may also play an important role in the pathogenesis of hypertensive heart failure in humans.

With regard to LV failure, elevated IL-6 levels in patients with LV dysfunction even in the absence of the clinical syndrome of congestive heart failure have been reported. The current results suggest that IL-6 signaling plays an important role in the pathogenesis of LV dysfunction after pressure overload. The concordance of data from echocardiography and invasive hemodynamic studies is noteworthy in this regard. Furthermore, hemodynamic results also indicate that both LV systolic and diastolic function are preserved after TAC in mice with genetic deletion of IL-6 (Figure 3).

**IL-6 Deletion Attenuates Cardiac Myocyte Apoptosis Induced by Pressure Overload**

A low level of cardiac myocyte apoptosis has been proposed as a causal mechanism for heart failure.22 During persistent pressure overload, the molecular survival mechanisms are overwhelmed and cardiac myocyte apoptosis ensues.23 Because of the low rate of cardiac myocyte turnover, cumulative loss of cardiac myocytes through apoptosis eventually leads to detrimental cardiac remodeling and heart failure. Although cardiac myocyte apoptosis is commonly observed in cardiac hypertrophy, the effects of IL-6 signaling on myocyte apoptosis during cardiac hypertrophy remain unknown. A few reports have suggested that IL-6 exerts pleiotropic effects and controls the balance between anti- and proapoptotic pathways.24,25 Our results from histological quantitation of apoptosis as well as molecular characterization of apoptosis regulators show that cardiac myocyte apoptosis in response to pressure overload was significantly attenuated in IL-6−/− hearts at 6 weeks after TAC (Figure 5). However, the potentially complex interactions of IL-6 signaling with cardiac myocyte apoptosis pathways during cardiac hypertrophy and failure needs to be further investigated in future studies.

**Hypertrophic Response Is Attenuated in IL-6−/− Adult Cardiac Myocytes**

In addition to in vivo experiments, we also performed mechanistic studies using adult cardiac myocytes from WT and IL-6−/− mice to examine the impact of IL-6 on cardiac myocyte hypertrophy in response to various prohypertrophic agents. Previous studies have suggested an involvement of IL-6 in inflammation and dysfunction induced by Ang II26; however, its role in cardiac myocyte hypertrophy triggered by common inducers of myocyte hypertrophy remains unclear. Moreover, such observations have rarely been tested in adult cardiac myocytes isolated from IL-6−/− mice. Our data show that both Ang II- and phenylephrine-induced increase in hypertrophy genes is nearly abrogated in IL-6−/− cardiac myocytes (Figure 6C and 6D). The necessity of IL-6 in mediation of hypertrophy in response to otherwise unrelated hypertrophy-promoting agents highlights a central role played by IL-6 in cardiac myocyte hypertrophy. Interestingly, exposure to rIL-6 produced dissimilar responses with regard to hypertrophy gene expression in IL-6−/− and WT cardiac myocytes (Figure 6A). This unexpected observation may be potentially explained by the equally unexpected finding that IL-6 receptor expression is reduced in IL-6−/− cardiac myocytes (Figure 6B).

**Impact of IL-6 Deletion on Molecular Mediators of Pressure Overload–Induced Hypertrophy**

It is well known that the IL-6 family of cytokines activate JAK/STAT pathway via gp130 to mediate various cellular responses. Besides the JAK/STAT pathway, gp130 activation also leads to Akt and ERK1/2 phosphorylation. However, despite reported involvement of Akt, ERK1/2, and JNK in cardiac hypertrophy,27 little direct evidence exists about potential roles of these pathways in the context of IL-6 signaling and pressure overload. Our data from myocardial tissue samples at 6 weeks after TAC indicate that the activation of Akt, ERK, and JNK is blunted in IL-6−/− hearts, indicating
broad suppression of prohypertrophy signaling in the absence of IL-6. Furthermore, we noted increased phosphorylation of GSK-3β in WT hearts at 6 weeks after TAC, which was abrogated in IL-6−/− hearts (Online Figure IID and IIF). To our knowledge, this is the first evidence that phosphorylation and inhibition of GSK-3β in the setting of pressure overload requires intact IL-6 signaling.

With regard to STAT3, published evidence indicates that members of ‘IL-6 family’ are able to induce cardiac myocyte hypertrophy via gp130-mediated STAT3 activation. However, published evidence directly implicating IL-6 in this process is sparse and inconclusive. Moreover, in the study by Lai et al., myocardial STAT3 activation was not affected in IL-6−/− mice after TAC. Our results show that STAT3 activation was markedly attenuated in the absence of IL-6 at 6 weeks after TAC (Figure 7A and 7B). This is consistent with the paradigm that IL-6 itself plays an important and nonredundant role in pressure overload–induced activation of STAT3. These data also suggest that the absence of IL-6 is not functionally compensated by upregulation of other members of IL-6 family. In conjunction with the in vitro data with Ang II and phenylephrine stimulation, these results identify a central role of STAT3 activation during pressure overload.

Role of CaMKII in IL-6–Induced STAT3 Activation and Cardiac Myocyte Hypertrophy

CaMKII is known to play an important role during cardiac hypertrophy. Although CaMKII is upregulated after pressure overload, whether CaMKII plays any role in IL-6–induced cardiac hypertrophy remains unknown. Moreover, the participation of CaMKII signaling in heart failure is complex and may vary depending on etiology and other associated pathologies, such as diabetes mellitus. Our results show that myocardial p-CaMKII levels are markedly elevated in WT mice at 6 weeks after TAC, and this increase is abrogated in the absence of IL-6 (Figure 8A and 8C). Furthermore, the time-dependent increase in p-CaMKII levels in H9c2 cells after rIL-6 exposure confirmed the ability of IL-6 to directly activate CaMKII. Consistently, IL-6–induced increase in cell surface area and hypertrophy gene induction was blocked by inhibition of CaMKII, indicating a critical role of this kinase.

Interestingly, the time course of CaMKII activation was similar to STAT3 activation after IL-6 stimulation in vitro. When KN-62, a CaMKII inhibitor, was added in addition to IL-6, the inhibition of STAT3 activation was also mirrored changes in p-CaMKII levels, indicating that inhibition of CaMKII activation prevents STAT3 activation in response to IL-6. These results were further confirmed in assays using cytoplasmic and nuclear protein fractions. To our knowledge, these results from in vivo as well as in vitro experiments provide the first evidence that CaMKII is necessary for the activation of STAT3 by IL-6. These data also identify CaMKII as an essential component of IL-6 signaling toward inducing cardiac hypertrophy in response to pressure overload.

Conclusions

The current findings provide definitive evidence that IL-6 signaling is critically important toward the development of LV hypertrophy, remodeling, and dysfunction during pressure overload. Our results also identify a heretofore unknown role of CaMKII in IL-6–induced STAT3 activation and cardiac myocyte hypertrophy. These insights may be utilized to formulate novel pharmacological approaches to ameliorate hypertensive heart disease.

Sources of Funding

This work was supported, in part, by National Institutes of Health grant R01 HL-117730.

Disclosures

None.

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IL-6 in Pressure Overload–Induced Hypertrophy

What Is Known?
- Interleukin (IL)-6 has been implicated in several cardiovascular pathologies.
- IL-6 has been shown to induce cardiac myocyte hypertrophy, without increasing blood pressure; however, published reports also indicate that IL-6 is not essential for left ventricular (LV) hypertrophy.
- The role of IL-6 in pressure overload–induced cardiac hypertrophy and failure remains controversial and the underlying molecular signaling poorly understood.

What New Information Does This Article Contribute?
- Genetic deletion of IL-6 attenuates LV hypertrophy and remodeling induced by pressure overload in vivo and prevents cardiac myocyte hypertrophy in vitro after exposure to hypertrophy-promoting agents, indicating an essential role of IL-6 in cardiac myocyte hypertrophy.
- Cardiac myocyte apoptosis and LV dysfunction in response to pressure overload are attenuated in the absence of IL-6.
- Ca2+-calmodulin-dependent protein kinase II plays an important role in IL-6–induced STAT3 activation and consequent cardiac myocyte hypertrophy during pressure overload, and GSK-3β inhibition in the setting of pressure overload requires intact IL-6 signaling.

IL-6, a pleiotropic cytokine with multifaceted actions, has been implicated in cardiac hypertrophy; however, the precise role of IL-6 in this process remains unclear. Our echocardiographic and hemodynamic data show that LV hypertrophy, remodeling, and dysfunction in response to pressure overload induced by transverse aortic constriction in vivo are ameliorated in mice with genetic deletion of IL-6. These physiological changes are accompanied by reduced myocardial fibrosis and apoptosis at the tissue level. The necessity of IL-6 for cardiomyocyte hypertrophy is further evidenced by blunted enlargement of IL-6–deficient cardiomyocytes after stimulation with hypertrophy-promoting agents. The current data also provide evidence that Ca2+-calmodulin-dependent protein kinase II is necessary for the activation of STAT3 by IL-6 in the setting of pressure overload; and suggest that IL-6 signaling is important in phosphorylation and inhibition of GSK-3β. These findings suggest that IL-6 signaling is critical for ventricular hypertrophy and dysfunction during pressure overload. Thus, therapeutic modulation of IL-6 signaling could potentially benefit patients with hypertensive heart disease.
Deletion of Interleukin-6 Attenuates Pressure Overload-Induced Left Ventricular Hypertrophy and Dysfunction

Lin Zhao, Guangming Cheng, Runming Jin, Muhammad R. Afzal, Anweshan Samanta, Yu-Ting Xuan, Magdy Girgis, Harold K. Elias, Yanqing Zhu, Arash Davani, Yanjuan Yang, Xing Chen, Sheng Ye, Ou-Li Wang, Lei Chen, Jeryl Hauptman, Robert J. Vincent and Buddhadeb Dawn

Circ Res. 2016;118:1918-1929; originally published online April 28, 2016; doi: 10.1161/CIRCRESAHA.116.308688

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL MATERIALS AND METHODS

DETAILED METHODS

Minimally Invasive Transverse Aortic Constriction. Minimally invasive transverse aortic constriction (TAC) was performed on wild-type (WT) mice and IL-6 knockout (IL-6⁻/⁻) mice. Mice were anesthetized using 2% isoflurane mixed with 100% oxygen. Mice received sustained release buprenorphine (0.3-1.0 mg/kg SQ) prior to surgery. All surgeries were performed under aseptic protocol and pre- and postsurgical gentamicin (5mg/kg i.m.) was administered. Limited median sternotomy was performed from the suprasternal notch to the second rib using angled scissors under a surgical microscope. The aorta was freed by blunt dissection and a blunt 27-gauge needle was placed along the aortic arch. A 7-0 nylon suture was placed around the aorta and the needle between the innominate and the left common carotid artery. Next, the suture was tied and the blunt needle was promptly removed thereafter. The sham surgery was identical to the above description except that the knot was not tied around the aorta. The chest wall was closed using a purse-string suture and the surgical wound was closed with interrupted sutures. Mice were allowed to recover under intensive monitoring.

In order to measure pressure gradient post-TAC in both groups of mice, right and left carotid arteries were cannulated with flame stretched PE 50 tubing. Catheters were connected to pressure transducers accepting a 23-gauge needle bore at the spout, which was connected to the catheter. The transducer was connected to PowerLab 16/30 data acquisition system (AD Instruments, Colorado Springs, CO) to record arterial blood pressures. After the pressure monitoring system was ready, TAC procedure was conducted. Before aortic constriction, systolic pressures in both WT and IL-6⁻/⁻ mice were similar, there was no difference in the pressure gradients between the left carotid (distal to the suture) and right carotid (proximal to the suture) which remained constant throughout the subsequent 60-min period in both groups after TAC.

Tissue Harvest for Morphometric and Histological Studies. Each mouse was anesthetized and injected with 0.5 ml of 5% KCl (i.v.) to ensure the heart arrests in diastole. The heart was rapidly excised and the aorta was cannulated with a 20-gauge needle. This set-up was then mounted in a Langendorff apparatus to wash out blood using calcium and magnesium-free PBS and maintain diastolic fixation by formalin perfusion. Each heart was transversely cut into two halves near the mid-papillary region and the apical half was processed and embedded in paraffin. Tissue was harvested for analysis at several different pre-specified time-points after the TAC procedure.

Histology. The paraffin embedded-apex was sectioned at 4 µm intervals, and stained with Masson’s trichrome stain (Sigma Aldrich, St. Louis, MO). The mid-section was used to measure LV diameter.¹ ² These anatomical parameters were corrected to a uniform sarcomere length
(2.1µm).\(^1\) For determination of myocyte cross-sectional area, cardiac myocytes with a circular profile and a central nucleus were analyzed.\(^2\) For assessment of fibrosis, tissue sections were stained with picrosirius red (Sigma Aldrich, St. Louis, MO). To measure interstitial collagen percentage, 9-18 fields (without any blood vessels) of the left ventricle (excluding papillary muscles) were randomly imaged. Perivascular collagen ratio was calculated from 9-12 randomly chosen fields containing vasculature and it was expressed as the ratio of fibrotic area immediately surrounding the vessel to cross-sectional area of the vessel.\(^3\) All measurements were made using ImagePro Plus software version 7.0 (Media Cybernetics, Rockville, MD). All morphometric analyses were performed in a blinded fashion.

**Tissue Harvest for Gene Profiling and Protein Analysis.** Each heart was excised and weighed after the removal of great vessels and atria. The right ventricular free wall was removed to weigh the left ventricle, which was then cut into two parts longitudinally and snap frozen to be stored at -80°C for mRNA and protein assays.

**Echocardiography.** Echocardiograms were obtained using a Vevo 2100 Ultrasound System (VisualSonics, Toronto, Canada) equipped with a high-frequency (30 MHz) linear array transducer. Echocardiography was performed prior to TAC, and 1, 2, 4, and 6 weeks following surgery. The mice were anesthetized with isoflurane (3% for induction and 1-1.5% for maintenance) mixed in 1 L/min O₂ via a facemask. Hair was removed from the anterior chest using a chemical hair remover. Using a rectal temperature probe, body temperature was carefully maintained close to 37.0°C with a heating pad throughout the study. Modified parasternal long-axis and parasternal short-axis views were used to obtain two-dimensional (2-D), M-mode, and spectral Doppler images.\(^1\)\(^,\)\(^2\) Systolic and diastolic anatomic parameters were obtained from M-mode tracings at the mid-papillary level. LV mass was estimated by the area-length method. All views were digitally stored in cine loops consisting of >300 frames. Subsequent analysis was performed off-line on a workstation installed with Vevo LAB software (version 1.7.1) (VisualSonics, Toronto, Canada) by an experienced operator who was blind to the treatment allocation and was unaware of data from other modalities.

**Hemodynamic Studies.** Invasive hemodynamic measurements were performed at the 35-day follow-up before sacrifice. The mice were anesthetized using isoflurane (3% for induction and 1–1.5% for maintenance) and ventilated using a rodent ventilator. Heparinized saline (1ml) was injected intraperitoneally before the procedure to fluid load animals. Core body temperature was maintained at 37.5°C. The LV apex was exposed through an incision between the seventh and eighth ribs, and a 1.0 Fr PV conductance catheter (PVR-1045; Millar Instruments, Houston, TX) was advanced through the apex to lie along the longitudinal axis. Absolute volume was calibrated, and pressure–volume (PV) loop data were obtained from the 10–15 successive cardiac cycles at steady state and during transient reduction of venous return by occluding the inferior vena cava with a 6-0 silk snare suture using PowerLab 16/30 data acquisition system (AD instruments, Colorado Springs, CO) and analyzed with LabChart® 7 Pro (AD instruments, Colorado Springs, CO). Parallel conductance corrections were performed in duplicate for each animal by injecting 2µL of 30% NaCl solution into the jugular vein.
Terminal dUTP Nick End-labeling (TUNEL) Assay. Myocardial sections were deparaffinized, rehydrated and incubated with recombinant proteinase K (PCR grade, Roche, Basel, Switzerland) for 10 min at room temperature. After rinsing with PBS, the positive controls were incubated with recombinant DNase1 (Roche, Basel, Switzerland). Sections were then blocked with 10% donkey serum (Jackson ImmunoResearch, West Grove, PA). Apoptotic cells were detected using in situ cell death detection kit, fluorescein (Roche, Basel, Switzerland). All myocytes were stained with monoclonal anti-α-sarcomeric actin (Sigma Aldrich, St. Louis, MO) and mounted with Vectashield H-1500 (Vector Laboratory, Burlingame, CA) containing DAPI to counterstain the nuclei. While counting the apoptotic nuclei, only nucleated myocytes in the left ventricle were chosen excluding the papillary muscles and outer edges of tissue. The number of TUNEL-positive nuclei was expressed as a percentage of total cardiomyocytes in left ventricle.

Adult Cardiomyocyte Isolation and Culture. Each heart was digested using a buffer containing 168 U/ml collagenase type 2 (Worthington, Lakewood, NJ), 160 U/ml collagenase type 4 (Worthington, Lakewood, NJ), 0.02 mg/ml proteinase XIV (Sigma Aldrich, St. Louis, MO) and 40μM CaCl$_2$ (Sigma Aldrich, St. Louis, MO). The great vessels, atria, anulus, and right ventricular free wall were removed from the heart, and LV tissue was minced with fine scissors in the buffer and gently pipetted to cause individual cell separation. Myocytes were filtered through 150-μm nylon mesh, counted, and exposed to increasing doses of CaCl$_2$. Each heart yielded approximately 1x10$^6$ myocytes, more than 70% of which were rod-shaped. Myocytes were plated on laminin-coated dishes. Myocytes were either measured for size or cultured overnight for molecular studies.

Cardiomyocyte Size. Cardiomyocytes were isolated from WT and IL-6$^{-/-}$ mice six weeks after sham and TAC procedures. They were imaged using an Olympus IX 71 microscope (Olympus, Tokyo, Japan) fitted with a DP 72 digital camera (Olympus, Tokyo, Japan) under a 20X objective after 4 hours of plating. Only binuclear cardiomyocytes were assessed after exclusion of misshapen myocytes. Total surface areas, lengths and widths were calculated for a minimum of 100 representative cardiomyocytes per mouse and 6 mice for each group, using cellSens Standard software (Olympus, Tokyo, Japan) for analysis.

H9c2 Myoblast Culture. H9c2 myoblasts were purchased from the American Tissue Culture Collection (Manasas, VA, USA). Cells were cultured in 1x Dulbecco’s modified Eagle’s medium (DMEM; Cambrex Bio Science, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Rockville, MD, USA) and antibiotics (25U/ml penicillin and 25U/ml streptomycin) at 37°C in a humidified atmosphere containing 5% CO$_2$. Cells were plated and cultured for 24h in the medium. Prior to treatment, the cells were serum starved for 12 hours.

Cell Treatment. For isolated adult cardiomyocytes, which were treated with recombinant IL-6 (rIL-6) (R&D Systems, Minneapolis, MN) 50ng/ml for 24 hours, angiotensin II (Ang II) (Calbiochem, Darmstadt, Germany) 1x10$^{-7}$ mol/L for 48 hours and phenylephrine (PE) (Sigma Aldrich, St. Louis, MO) 10μmol/L for 24 hours, respectively. For studies of myocyte hypertrophy, H9c2 cells were treated with rIL-6 (50ng/ml) in presence or absence of CaMKII inhibitor KN-62
(10µmol/L) (Millipore, Darmstadt, Germany) and STAT3 inhibitor WP1066 (6µmol/L) (Millipore, Darmstadt, Germany) for 24 h. To evaluate the phosphorylation of STAT3 and CaMKII induced by rIL-6, H9c2 cells were treated with rIL-6 (50ng/ml) for several time periods up to 12 h. To determine the relationship between CaMKII and STAT3, H9c2 cells were pretreated with KN-62 (5µmol/L or 10µmol/L) for 2 h, followed by treatment with rIL-6 (50ng/ml) for 30 min.

Crystal Violet Staining. After washing with PBS, H9c2 cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 15 min and stained with 1% crystal violet (Thermo Fisher Scientific, Lenexa, KS) for 2 h at room temperature to assess cell surface area. Images were obtained using a DP 72 digital camera (Olympus, Tokyo, Japan) on an Olympus IX 71 microscope (Olympus, Tokyo, Japan). Ten random photographs were taken from each group, and at least 200 individual cells were examined in each group. Cell surface area was analyzed using cellSens standard software (Olympus, Tokyo, Japan).

Transfection. H9c2 cells were plated in culture medium on the day before transfection and were 80% confluent at the time of transfection. Using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), cells were transiently transfected with plasmid encoding with CaMKII (Addgene, Cambridge, MA) in a dose-dependent manner while empty vector was used as control. Cells were incubated for 18-24 hours in 5% CO2 at 37°C. The cells were then placed in fresh culture medium for another 24-30 hours prior to harvest.

Quantitative PCR. Total RNA was extracted from frozen tissues and cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. cDNA synthesis from 1μg RNA was carried out using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR (qPCR) was done using a SYBR® green PCR master mix kit (Applied Biosystems, Foster City, CA) on a ViiA™ 7 system (Applied Biosystems, Foster City, CA). GAPDH was used as control and the 2ΔΔCT method was used for data analysis. Primers used for qPCR are listed in Online Table I. The results from three independent experiments, each sample were performed duplicated in each experiment.

Protein Extraction and Western Blot Analysis. Cardiac tissue, cultured H9c2 myoblasts and adult cardiomyocytes were lysed in RIPA lysis buffer (Millipore, Darmstadt, Germany) with protease and phosphatase cocktail inhibitors (Roche, Basel, Switzerland) to yield total protein. Cytoplasmic and nuclear protein fractions were isolated using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Lenexa, KS) using the manufacturer’s protocol. Protein concentration was determined using Pierce® BCA protein assay kit (Thermo Fisher Scientific, Lenexa, KS). Proteins were separated using 8%-14% SDS-polyacrylamide gel electrophoresis and transferred to an Immobilon-FL membrane (Millipore, Darmstadt, Germany). After blocking with Odyssey® blocking buffer (LI-COR, Lincoln, NE), membranes were hybridized with anti-p-CaMKII, anti-CaMKII, anti-p-STAT3 (Try705), anti-p-ERK1/2, anti-ERK1/2, anti-p-JNK, anti-JNK, anti-p-Akt, anti-Akt, anti-p-GSK3β, anti-GSK3β, anti-Bcl-2 (Cell Signaling Technology, Danvers, MA), anti-ANP, anti-STAT3, anti-CaMKIIβ, anti-Bax, anti-MMP9, anti-periostin, anti-GAPDH, anti-laminA (Santa Cruz, Dallas, TX) overnight at 4°C. Secondary antibodies labelled with fluorescent dye (IRDye) (LI-COR, Lincoln, NE) were used for 1 hour at
room temperature and immunoblots were scanned using ODYSSEY® CLx infrared imaging system (LI-COR, Lincoln, NE). GAPDH is total protein loading control; LaminA is nuclear protein loading control. Arbitrary optical densities were measured by Image Studio™ Lite software version 4.0 (LI-COR, Lincoln, NE).

REFERENCES


ONLINE FIGURE LEGENDS

Online Figure I. Confirmation of IL-6 deletion in IL-6/− mice. Ladder is Promega 100bp (G2101), bright band is 500bp. One band (174bp) is wild type (WT); two bands (174 and 380bp) are IL-6 heterozygous allele (IL-6+/−); one band (380bp) is IL-6 homozygous mutated allele (IL-6−/−).

Online Figure II. Experimental protocol.

Online Figure III. Attenuation of pressure overload-induced activation of MAPK and Akt signaling pathways in the absence of IL-6. A-C, representative Western immunoblots (A) and quantitative data for p-ERK1/2 (B) and p-JNK (C) in WT and IL-6/− mouse hearts at 6 weeks after surgery (n=6 per group). D-E, representative Western immunoblots (D) and quantitative data for p-Akt (E) and p-GSK3β (F) in WT and IL-6/− mouse hearts at 6 weeks after surgery (n=6 per group). Data represent means ± SEM. *P<0.05 vs. Sham WT mice; # P<0.05 vs. TAC WT mice; § P<0.05 vs. Sham IL-6−/− mice.

Online Figure IV. CaMKII contributed to STAT3 activation in IL-6-induced cardiomyocyte hypertrophy. A-C, isolated cardiomyocytes were treated with IL-6 with or without CaMKII inhibitor KN-62. Representative Western blots (A) and quantitative data for p-CaMKII (B) and pTyr-STAT3 (C). Data represent means ± SEM from three independent experiments. *P<0.05 vs control; # P<0.05 vs IL-6 treatment only.
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## Online Table II. Reasons for excluding mice from studies in vivo

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<td>9.8</td>
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<tr>
<td>Mortality (%)</td>
<td>11.4</td>
<td>9.8</td>
<td>7.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Heart weight was less than 120 mg at 6 weeks of post TAC; Death, naturally dead after TAC; Technical problem, aortic rupture or pneumothorax.
Online Table III. Age, BW, HW, and TL analysis

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Age weeks</th>
<th>Pre-OPBW g</th>
<th>Post-OPBW g</th>
<th>HW mg</th>
<th>TL mm</th>
<th>HW/BW mg/g</th>
<th>HW/TL mg/mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham WT</td>
<td>10</td>
<td>11.1±0.05</td>
<td>24.7±0.75</td>
<td>26.9±0.81</td>
<td>101.2±4.23</td>
<td>16.9±0.17</td>
<td>3.8±0.06</td>
<td>6.0±0.21</td>
</tr>
<tr>
<td>Sham IL-6⁻⁻</td>
<td>10</td>
<td>11.0±0.04</td>
<td>24.6±0.41</td>
<td>26.6±0.28</td>
<td>100.7±1.97</td>
<td>16.9±0.09</td>
<td>3.8±0.06</td>
<td>6.0±0.14</td>
</tr>
<tr>
<td>TAC WT</td>
<td>12</td>
<td>11.3±0.23</td>
<td>24.5±0.33</td>
<td>28.5±0.42</td>
<td>174.4±7.40*</td>
<td>17.2±0.09</td>
<td>6.1±0.24*</td>
<td>10.1±0.44*</td>
</tr>
<tr>
<td>TAC IL-6⁻⁻</td>
<td>12</td>
<td>11.1±0.24</td>
<td>24.5±0.38</td>
<td>27.5±0.36</td>
<td>150.6±4.85§#</td>
<td>17.1±0.05</td>
<td>5.5±0.13§#</td>
<td>8.8±0.29§#</td>
</tr>
</tbody>
</table>

Pre-OPBW, pre-operative body weight; Post-OPBW, post-operative body weight at 6 weeks; HW, heart weight; TL, tibia length. *P<0.05 vs. Sham WT mice; § P <0.05 vs. Sham IL-6⁻⁻ mice; #P <0.05 vs. TAC WT mice.
Online Table IV. Morphometric measurement for transverse myocyte cell surface

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Cardiomyocyte size (μm²)</th>
<th>Nuclear area (μm²)</th>
<th>Nuc-cyto ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham WT</td>
<td>8</td>
<td>167.4±6.7</td>
<td>10.62±0.6</td>
<td>6.5±0.4</td>
</tr>
<tr>
<td>Sham IL-6⁻</td>
<td>8</td>
<td>135.1±13.2*</td>
<td>8.6±0.9*</td>
<td>6.8±0.5</td>
</tr>
<tr>
<td>TAC WT</td>
<td>12</td>
<td>256.4±17.7*</td>
<td>13.56±0.9*</td>
<td>5.5±0.2*</td>
</tr>
<tr>
<td>TAC IL-6⁻</td>
<td>12</td>
<td>173.4±12.3#</td>
<td>11.13±0.5#</td>
<td>6.5±0.4</td>
</tr>
</tbody>
</table>

Data are means ± SEM. *P<0.05 vs. Sham WT mice; #P <0.05 vs. TAC WT mice.
# Online Table V. Hemodynamics Analysis after 6 weeks of surgery

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>HR (bpm)</th>
<th>LVP (mmHg)</th>
<th>LVMP (mmHg)</th>
<th>LVESP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>dP/dt max (mmHg/s)</th>
<th>dP/dt min (mmHg/s)</th>
<th>Tau (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham WT</td>
<td>12</td>
<td>511±6</td>
<td>100±1</td>
<td>41±1</td>
<td>99±1</td>
<td>5.7±0.3</td>
<td>8515±142</td>
<td>-9115±148</td>
<td>6.5±0.1</td>
</tr>
<tr>
<td>Sham IL-6⁻/⁻</td>
<td>12</td>
<td>500±27</td>
<td>105±3</td>
<td>41±2</td>
<td>100±4</td>
<td>5.3±0.9</td>
<td>9310±532</td>
<td>-9452±338</td>
<td>6.0±0.2</td>
</tr>
<tr>
<td>TAC WT</td>
<td>12</td>
<td>498±7</td>
<td>141±2*</td>
<td>55±1*</td>
<td>138±2*</td>
<td>8.7±0.3*</td>
<td>6747±95*</td>
<td>-7715±119*</td>
<td>8.7±0.1*</td>
</tr>
<tr>
<td>TAC IL-6⁻/⁻</td>
<td>11</td>
<td>574±6§#</td>
<td>144±2§</td>
<td>54±1§</td>
<td>145±3§</td>
<td>5.9±0.1#</td>
<td>9034±197#</td>
<td>-9332±203#</td>
<td>6.7±0.2#</td>
</tr>
</tbody>
</table>

HR, heart rate; LVP, left ventricular pressure; LVMP, LV mean pressure; LVESP, LV end-systolic pressure; LVEDP, LV end-diastolic pressure; dP/dt max, LV maximum value of dP/dt; dP/dt min, minimum value of dP/dt; Tau, LV relaxation constant. *P<0.05 vs. Sham WT mice; § P <0.05 vs. Sham IL-6⁻/⁻ mice; #P <0.05 vs. TAC WT mice.
Online FIGURE I. Confirmation of \textit{IL-6} deletion in \textit{IL-6}^{-/-} mice. Ladder is Promega 100bp (G2101), bright band is 500bp. One band (174bp) is wild type (WT); two bands (174 & 380bp) are IL-6 heterozygous allele (\textit{IL-6}^{+/-}); one band (380bp) is IL-6 homozygous mutated allele (\textit{IL-6}^{-/-}).
Online Figure II. Experimental protocol
Online FIGURE III
Online FIGURE III. Attenuation of pressure overload-induced activation of MAPK and Akt signaling pathways in the absence of IL-6. A-C, representative Western immunoblots (A) and quantitative data for p-ERK1/2 (B) and p-JNK (C) in WT and IL-6−/− mouse hearts at 6 weeks after surgery (n=6 per group). D-E, representative Western immunoblots (D) and quantitative data for p-Akt (E) and p-GSK3β (F) in WT and IL-6−/− mouse hearts at 6 weeks after surgery (n=6 per group). Data represent means ± SEM. *P<0.05 vs. Sham WT mice; # P<0.05 vs. TAC WT mice; § P<0.05 vs. Sham IL-6−/− mice.
Online FIGURE IV. CaMKII contributed to STAT3 activation in IL-6-induced cardiomyocyte hypertrophy. A-C, isolated cardiomyocytes were treated with IL-6 with or without CaMKII inhibitor KN-62. Representative Western blots (A) and quantitative data for p-CaMKII (B) and pTyr-STAT3 (C). Data represent means ± SEM from three independent experiments. *P<0.05 vs control; # P<0.05 vs IL-6 treatment only.