Cytokine mRNA Degradation in Cardiomyocytes Restrains Sterile Inflammation in Pressure Overloaded Hearts

Running Title: Omiya et al.; mRNA Degradation and Heart Failure

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Abstract

Background: Proinflammatory cytokines play an important role in the pathogenesis of heart failure. However, the mechanisms responsible for maintaining sterile inflammation within failing hearts remain poorly defined. Although transcriptional control is important for proinflammatory cytokine gene expression, the stability of the mRNA also contributes to the kinetics of immune responses. Regnase-1 is an RNase involved in the degradation of a set of proinflammatory cytokine mRNAs in immune cells. The role of Regnase-1 in non-immune cells such as cardiomyocytes remains to be elucidated.

Methods: To examine the role of proinflammatory cytokine degradation by Regnase-1 in cardiomyocytes, cardiomyocyte-specific Regnase-1-deficient mice were generated. The mice were subjected to pressure overload by means of transverse aortic constriction (TAC) to induce heart failure. Cardiac remodeling was assessed by echocardiography as well as histological and molecular analyses 4 weeks after operation. Inflammatory cell infiltration was examined by immunostaining. Furthermore, interleukin-6 (IL-6) signaling was inhibited by the administration with its receptor antibody. Finally, overexpression of Regnase-1 in the heart was performed by adeno-associated viral vector-mediated gene transfer.

Results: Cardiomyocyte-specific Regnase-1-deficient mice showed no cardiac phenotypes under baseline conditions, but exhibited severe inflammation and dilated cardiomyopathy after 4 weeks of pressure overload compared to the control littersates. Four weeks after TAC, the Il6 mRNA level was upregulated, but not other cytokine mRNAs including tumor necrosis factor-α in Regnase-1-deficient hearts. Although the Il6 mRNA level increased 1 week after operation in both Regnase-1-deficient and control hearts, it showed no increase in control hearts 4 weeks after operation. Administration of anti-IL-6 receptor antibody attenuated the development of inflammation and cardiomyopathy in cardiomyocyte-specific Regnase-1-deficient mice. In severe pressure overloaded wild-type mouse hearts, sustained induction of Il6 mRNA was observed, even though the protein level of Regnase-1 increased. Adeno-associated virus 9-mediated cardiomyocyte-targeted gene delivery of Regnase-1 or administration of anti-IL-6 receptor antibody attenuated the development of cardiomyopathy induced by severe pressure overload in wild-type mice.

Conclusions: The degradation of cytokine mRNA by Regnase-1 in cardiomyocytes plays an important role in restraining sterile inflammation in failing hearts and the Regnase-1-mediated pathway might be a therapeutic target to treat patients with heart failure.

Key Words: heart failure; inflammation; Regnase-1; cytokine mRNA; interleukin-6

Nonstandard Abbreviations and Acronyms
TAC transverse aortic constriction
IL-6 interleukin-6
TNF-α tumor necrosis factor-α
MCP-1 monocyte chemotactic protein-1
MCPIP1 monocyte chemotactic protein-1-induced protein-1
Mlc2v myosin light chain 2v
sTAC severe transverse aortic constriction
ITR inverted terminal repeats
AAV adeno-associated virus
eGFP  enhanced green fluorescent protein
Reg-1  Regnase-1
Reg1-AAV9  AAV type9 encoding FLAG-tagged Regnase-1
eGFP-AAV9  AAV type9 encoding enhanced green fluorescent protein
GAPDH  glyceraldehyde 3-phosphate dehydrogenase
TUNEL  TdT-mediated dUTP-biotin nick end labeling
qRT-PCR  real-time quantitative reverse transcription PCR
IL-1b  interleukin 1 beta
Nppa  atrial natriuretic peptides
Nppb  brain natriuretic peptides
Col1a2  collagen type I α2 chain
Col3a1  collagen type III α1 chain
IL12b  interleukin 12b
ANOVA  one-way analysis of variance
LVIDd  end-diastolic left ventricular internal dimension
LVIDs  end-systolic left ventricular internal dimension
FS  fractional shortening
HW/TL  heart weight/tibia length
LungW/TL  lung weight/tibia length
Clinical Perspective

What is new?

- The degradation of cytokine mRNA by Regnase-1, a RNase, in cardiomyocytes plays an essential role in restraining inflammation in mouse pressure overload-induced failing hearts.
- The major target for Regnase-1-mediated mRNA degradation appears to be IL-6 in cardiomyocytes.
- Sustained increase in Il6 mRNA by deficiency or insufficient upregulation of Regnase-1 in pressure-overloaded hearts promotes cardiac remodeling and inflammation.

What are the clinical implications?

- Failure of appropriate induction of Regnase-1 may underlie the persistent and chronic inflammation seen in chronic heart failure.
- Upregulation of Regnase-1 function or IL-6 blockade may be a fruitful approach to therapeutic immunomodulation in heart failure patients with an increased level of IL-6.
Introduction

Heart failure is the major cause of death in the developed countries. Circulating levels of proinflammatory cytokines including tumor necrosis factor-α (TNF-α) are increased in patients with heart failure and related to the severity and prognosis of the disease, although infection of microorganisms is not involved in most cases.\(^1\) This suggests an important role of sterile inflammation in the pathogenesis of chronic heart failure. However, the targeted anti-TNF approaches were negative with respect to the primary end points of the trial or resulted in worsening heart failure or death.\(^2\) In addition to TNF-α, the pro-inflammatory cytokines that are elaborated in heart failure include other members of the tumor necrosis factor superfamily, members of the interleukin-1 family, and interleukin-6 (IL-6).\(^1\) Thus, the whole scheme how inflammation occurs in stressed hearts has to be elucidated to develop novel and effective therapeutics against heart failure.

We have previously reported that incomplete degradation of mitochondrial DNA by lysosomal DNase II in cardiomyocytes results in the initiation of inflammation and development of heart failure in pressure overload-induced mouse heart failure model.\(^3\) However, the mechanisms responsible for maintaining inflammatory responses within failing hearts remain poorly defined. Although transcriptional control is a determinant of the kinetics of proinflammatory cytokine gene expression, the stability of the mRNA also has a key function in coordinating immune responses.\(^4\)

Regnase-1 (also known as Zc3h12a and monocyte chemotactic protein-1 (MCP-1)-induced protein-1 (MCPIP1)) is an RNase that destabilizes a set of mRNAs, including IL-6 and IL-12b, through cleavage of their 3’ untranslated regions in macrophages.\(^5\) Regnase-1-deficient mice showed augmented serum immunoglobulin levels, autoantibody production and infiltration
of plasma cells to the lung. Macrophages isolated from Regnase-1-deficient mice showed increased production of IL-6 and IL-12p40 but not TNF. Although Regnase-1 is ubiquitously expressed, the role of Regnase-1 in non-immune cells such as cardiomyocytes has not been fully elucidated.

In this study, we generated cardiomyocyte-specific Regnase-1-deficient mice to elucidate the role of cytokine mRNA degradation in cardiomyocytes during cardiac remodeling. The results of this study indicate that cytokine mRNA degradation by Regnase-1 in cardiomyocytes is important in the maintenance of sterile inflammation and development of heart failure.

Methods

The data, analytic methods, and study materials are available from the corresponding author to other researchers on reasonable request for purposes of reproducing the results or replicating the procedure.

Study Approval

All in vivo and in vitro experimental protocols were approved by King’s College London Ethical Review Process Committee and UK Home Office (Project Licence No. PPL70/7260) and were performed in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 (UK Home Office).

Generation of Cardiomyocyte-specific Regnase-1-deficient Mice.

Mice bearing a Regnase-1^flox allele were crossed with knock-in mice expressing Cre recombinase under the control of myosin light chain 2v (Mlc2v) promoter to produce cardiomyocyte-specific Regnase-1-deficient (Regnase-1^flox/flox;Mlc2v-Cre^+) mice. All mice used were on the C57BL/6 and SV129 mixed background and 8- to 12-week-old male Regnase-1-
deficient mice. Their littermates were used as controls. Mice were given food and water *ad libitum.*

**Isolation of Mouse Adult Cardiomyocytes**

Adult cardiomyocytes were isolated from 10- to 12-week-old male mice using a Langendorff system and cultured as we previously reported.³

**Echocardiography and Transverse Aortic Constriction**

A Vevo 2100 system with a 22–55 MHz linear transducer (Visual Sonics) was used to perform echocardiography on conscious mice.⁸ Non-invasive measurement of tail blood pressure was also performed on conscious mice using a BP Monitor for Rats and Mice (Muromachi Kikai) as previously described.⁸ The mice were subjected to thoracic transverse aortic constriction (TAC) and severe TAC (sTAC) using 26- and 27-gauge needles for aortic constriction, respectively.³ In TAC, a small piece of a 6-0 silk suture was placed between the innominate and left carotid arteries. Two loose knots were tied around the transverse aorta and a 26-gauge needle was placed parallel to the transverse aorta. The knots were quickly tied against the needle and the needle was promptly removed to yield a 26-gauge stenosis. In sTAC, aortic constriction was performed by tying a 6-0 silk suture against 27-gauge needle to yield a more severe constriction. Sham surgeries were identical except for the aortic constriction.

**Administration of MR16-1**

After TAC operation, *Reg1⁺/⁺* and *Reg1⁻/⁻* mice received an intraperitoneal injection of 2 mg anti-mouse IL-6 receptor antibody MR16-1 (a kind gift from Chugai Pharmaceutical Co., Ltd.) or 2 mg control IgG (855951; MP Biomedicals). Afterwards they were injected intraperitoneally once a week with a total of three injections with either 0.5 mg MR16-1 or IgG.⁹ For the experiment to examine the effect of MR16-1 on cardiac remodeling in wild-type mice, sTAC-
operated C57BL/6 mice received weekly injection with 0.15 mg MR16-1 or IgG from 1 week after sTAC.

**Virus Production and Infection**

FLAG-tagged Reg1 was cloned into inverted terminal repeats (ITR)-containing adeno-associated virus (AAV) plasmid harboring the chicken cardiac troponin T promoter (PL-C-PV1967, Penn Vector Core, University of Pennsylvania) after removal of enhanced green fluorescent protein (eGFP) sequence. AAV type 9 encoding FLAG-tagged Reganase-1 (Reg1-AAV9) was generated by transient transfection of HEK293 cells using three plasmids (the cis ITR-containing plasmid, the transplasmid encoding AAV replicase and capsid genes and the adenoviral helper plasmid) in Penn Vector Core. As a control, AV-9-PV1967 (Penn Vector Core) (eGFP-AAV9) was used.

Eight- to 10-week-old C57BL/6 mice subjected to TAC operation were intraperitoneally injected with 1 x 10^{11} vector genomes of Reg1-AAV9 or eGFP-AAV9 1 week before surgery.

**Western Blot Analysis**

Total protein homogenates were subjected to western blot analysis using a monoclonal mouse antibody to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (G8795; SIGMA), a monoclonal rabbit antibody to Regnase-1 (generated by Prof. Akira), a monoclonal rabbit antibody to phospho-STAT3 (Tyr705) (#9145S; Cell Signaling) and a monoclonal mouse antibody to STAT3 (124H6) (#9139S; Cell Signaling). After incubation with secondary antibody, the blot was developed with an infrared imaging system, ODYSSEY CLx (LI-COR Biosciences). Image Studio software (LI-COR Biosciences) was used for quantitative analysis to evaluate protein expression levels.

**Histological Analysis**

Left ventricle samples were embedded in the OCT compound (Thermo Fisher Scientific Inc.),
then immediately frozen in liquid nitrogen. The samples were sectioned into 6 μm thickness and fixed with acetone. Hematoxylin-eosin staining and Masson's trichrome staining (Masson's Trichrome Stain Kit, Polysciences, Inc.) were performed on serial sections. For wheat germ agglutinin staining, heart samples were stained with FITC-conjugated lectin (SIGMA) to measure the cross-sectional area of cardiomyocytes. Quantitative analyses of fibrosis fraction and cardiomyocyte cross-sectional areas were examined in five different areas (magnification, x200) per section and measured using the NIH ImageJ software (National Institutes of Health). TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed using an In situ Apoptosis Detection Kit (Takara Bio Inc.). The number of TUNEL-positive nuclei and total nuclei was counted. For immunohistochemical staining, avidin-peroxidase (VECTASTAIN Elite ABC Kit, Vector Laboratories, Inc.) and DAB Peroxidase Substrate Kit (Vector Laboratories, Inc.) were used, followed by counterstaining with hematoxylin as described previously. Quantitative analyses of inflammatory cells were examined by counting the number of immunopositive cells in five different areas (magnification, x200) per section, and expressed as the number per millimeters squared. For these quantification in histology, two serial heart sections were prepared, then 3 different areas from mid-portion of free wall and 2 areas from mid-portion of septal wall in each sections were assessed. Images were analysed in a blinded fashion by two reviewers. The primary antibodies used were rat anti-CD45 (MAB114; R&D Systems, Inc.), rat anti-CD68 (MCA1957GA; AbD Serotec) or rabbit anti-CD68 antibody for immunofluorescence (ab125212; Abcam), rat anti-Ly6G/6C (550291; BD Biosciences), rabbit anti-CD3 (ab16669; Abcam), hamster anti-mouse CD11c antibody (MCA1369; Bio-Rad Laboratories, Inc.), and rat anti-mouse CD206 antibody (MCA2235; Bio-Rad Laboratories, Inc.). The secondary antibodies were goat anti-rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568...
(A-11036; Thermo Fisher Scientific Inc.), goat anti-hamster IgG (H+L) Secondary Antibody, Alexa Fluor 488 (A21110; Thermo Fisher Scientific Inc.), and goat anti-rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A11006; Thermo Fisher Scientific Inc.). DAPI (ProLong Gold Antifade Reagent with DAPI; Life Technologies) was used to detect nuclei.

**Real-time Quantitative Reverse Transcription PCR (qRT-PCR)**

Total RNA was isolated from the left ventricles or isolated cardiomyocytes using TRIzol reagent (Thermo Fisher Scientific Inc.). The mRNA expression levels were determined by qRT-PCR using SuperScript II Reverse Transcriptase (Thermo Fisher Scientific Inc.) for reverse transcription and a Power SYBR Green PCR Master Mix (Thermo Fisher Scientific Inc.) for qRT-PCR reaction with PCR primers designed as follows: forward 5’ -

GAGTGGAAACGCTTCATCGAG-3’ and reverse 5’-AGGAAGTTGTCCAGGCTAGG-3’ for Regnase-1 (Reg1), forward 5’-ACAACCACGGCcTCTCCCTACTT-3’ and reverse 5’-CACGATTTCCCAGAGACATGTC-3’ for interleukin 6 (Il6), forward 5’-TCCCAGGTCTCTTCAAGGGA-3’ and reverse 5’-GGTGAGGAGACGTAGTCCG-3’ for tumor necrosis factor alpha (Tnfa), forward 5’- AAGAGCTTCAGGCAGAGCATCA-3’ and reverse 5’-TAATGGGAACGTCACACACAGCA-3’ for interleukin 1 beta (Il1b), forward 5’-TCGTTCTGGCCTTGGCT-3’ and reverse 5’-TCCAGGTGCTTAGCAGGTCT-3’ for atrial natriuretic peptides (Nppa), forward 5’-AAGTCCTAGCCAGTCTCCAGA-3’ and reverse 5’-GAGCTGTCTCTGGGCCATTTC-3’ for brain natriuretic peptides (Nppb), forward 5’-ACGCGGACTCTGTTGCT-3’ and reverse 5’-CGGGACCCTCTTGTCACG-3’ for collagen type I alpha 2 (Col1a2), forward 5’-CCGGGTGCTCTGACAGA-3’ and reverse 5’-CACCCCTGAGGACCAGCCA-3’ for collagen type III alpha 1 (Col3a1), and forward 5’- ATGACAACTTTGTCAAGCTCATTT-3’ and reverse 5’-GGTCCACCACCTGTCTTGCT-3’ for
The PCR primers for interleukin 12b (Il12b) (Mm01288989_m1), interferon b1 (Ifnb1) (Mm_00439552_s1), interferon gamma (Ifng) (Mm01168134_m1), and interleukin 10 (Il10) (Mm01288386_m1) were purchased from Thermo Fisher Scientific Inc. The TaqMan Gene Expression Master Mix (Thermo Fisher Scientific Inc.) was used for amplification of Il12b, Ifnb1, Ifng, and Il10. PCR standard curves were constructed using the corresponding complementary DNA and all data were normalized to Gapdh mRNA content and are expressed as fold increase over the control group.

**In Situ Hybridization**

In situ hybridization was performed using the QuantiGene ViewRNA Chromogenic Signal Amplification Kit (Affymetrix eBioscience), the QuantiGene ViewRNA ISH Tissue 1-Plex Assay Kit (Affymetrix eBioscience), and the ViewRNA Probe MOUSE Il6 (Affymetrix eBioscience) according to the manufacturer’s instructions. After frozen tissue slides were fixed in 10% neutral buffered formalin at 4°C for 16 h, the slides were dehydrated and digested with protease. Hybridization with the probe for Il6 mRNA was performed, followed by signal amplification and signal detection steps. After washing in phosphate-buffered saline, the slides were incubated with an anti-α-sarcomeric actin (A2172; SIGMA) and chicken anti-mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A-21200; Thermo Fisher Scientific Inc.).

**Statistics**

Results are shown as mean ± SEM. Paired data were evaluated by Student's t-test which was used for two groups comparison and one-way analysis of variance (ANOVA) with the Bonferroni’s post hoc test for multiple comparisons. \( P < 0.05 \) was considered statistically significant.
Results

Regnase-1-deficient Mice Had No Cardiac Phenotypes at Baseline

To examine the in vivo role of Regnase-1 in cardiomyocytes, mice with a Regnase-1<sup>flox</sup> allele<sup>6</sup> were crossed with knock-in mice expressing Cre recombinase under the control of Mlc2v promoter<sup>7</sup>, to produce Regnase-1<sup>flox/flox</sup>,Mlc2v-Cre<sup>+</sup> (Reg1<sup>−/+</sup>) mice. Regnase-1<sup>flox/flox</sup>,Mlc2v-Cre<sup>−</sup> (Reg1<sup>+/−</sup>) littersmates were used as controls. The Reg1<sup>−/+</sup> mice were born at Mendelian frequency and grew to adulthood. In Reg1<sup>−/+</sup> hearts, there was a significant reduction in the protein level of Regnase-1 (Figure IA in the online-only Data Supplement). The deletion of Reg1 was confirmed in isolated cardiomyocytes by qRT-PCR (Figure IB in the online-only Data Supplement). There were no significant differences in physiological and echocardiographic parameters between Reg1<sup>+/−</sup> and Reg1<sup>−/+</sup> mice (Table I in the online-only Data Supplement), indicating that the Reg1<sup>−</sup> mice had normal global cardiac structure and function.

Regnase-1-deficient Mice Developed Heart Failure in Response to Pressure Overload.

To determine the role of Regnase-1 during cardiac remodeling, Reg1<sup>+/−</sup> and Reg1<sup>−/+</sup> mice were subjected to pressure overload by means of TAC<sup>11</sup>. There was no difference in the survival ratio between TAC-operated Reg1<sup>−/+</sup> mice (8.3%, 1 out of 12 mice) and TAC-operated Reg1<sup>+/−</sup> (0%, 0 out of 13 mice). The Reg1<sup>+/−</sup> and Reg1<sup>−/+</sup> mice exhibited left chamber dilatation and cardiac dysfunction 4 weeks after TAC (Figure 1A and B). However, the extent was more severe in Reg1<sup>−/+</sup> mice. Although TAC induced an increase in the heart weight-to-tibia length ratio in both groups, the ratio was larger in Reg1<sup>−/+</sup> mice (Figure 1C). The lung weight-to-tibia length ratio was elevated in TAC-operated Reg1<sup>−/+</sup> mice, but not in Reg1<sup>+/−</sup> mice (Figure 1C). TAC-operated Reg1<sup>−/+</sup> hearts exhibited intermuscular cell infiltration (Figure 1D). Interstitial fibrosis was present in TAC-operated Reg1<sup>−/+</sup> hearts (Figure 1E). The cardiomyocyte cross-sectional area in
TAC-operated $Reg^{1+}$ mice was larger than in TAC-operated $Reg^{+/+}$ mice (Figure 1F). The mRNA levels of $Nppa$ and $Nppb$ increased in both TAC-operated $Reg^{1+}$ and $Reg^{+/+}$ hearts, but were higher in TAC-operated $Reg^{1-/-}$ hearts (Figure 1G). The mRNA expression of $Col3a1$ in TAC-operated $Reg^{1+}$ hearts was higher than in TAC-operated $Reg^{+/+}$ hearts (Figure 1G). The number of apoptotic cardiomyocytes increased in TAC-operated $Reg^{1-/-}$ hearts (Figure II A and B in the online-only Data Supplement). These data suggest that ablation of Regnase-1 in cardiomyocytes resulted in severe cardiac chamber dilatation, dysfunction, hypertrophy and fibrosis and lung congestion in response to pressure overload.

**Cardiomyocyte-specific Deletion of Regnase-1 in Pressure-overloaded Hearts Resulted in the Development of Inflammation with a Specific Increase in $Il6$ mRNA.**

Four weeks after TAC, the $Il6$ mRNA level was upregulated, but not other cytokine mRNAs including $Tnfa$ and $Il12b$ (a known Regnase-1 target)\(^5\), in TAC-operated $Reg^{1-/-}$ hearts compared to the corresponding sham-operated and TAC-operated $Reg^{+/+}$ hearts (Figure 2A). The $Il6$ mRNA level was not significantly increased in TAC-operated $Reg^{+/+}$ hearts compared to sham-operated $Reg^{+/+}$ hearts (Figure 2A). A higher number of CD45\(^+\), CD68\(^+\), and CD3\(^+\), but not Ly6G\(^+\) cells infiltrated TAC-operated $Reg^{1-/-}$ hearts than TAC-operated $Reg^{+/+}$ hearts (Figure 2B–E). Most inflammatory cells were CD68\(^+\) macrophages, especially CD206\(^+\) M2-macrophages (Figure 3A–C). The *in situ* hybridization analysis indicates that a higher number of cardiomyocytes expressed $Il6$ mRNA in $Reg^{1-/-}$ hearts compared to $Reg^{+/+}$ hearts under pressure overload (Figure 3D). In contrast to the results 4 weeks after TAC, $Il6$ and $Tnfa$ mRNAs increased in both TAC-operated $Reg^{+/+}$ and $Reg^{1-/-}$ hearts and there was no significant difference in the level of the cytokine mRNAs between the two groups 1 week after TAC (Figure 3E). Phosphorylation of STAT3, a downstream of IL-6 signaling pathway, was increased in
TAC-operated Reg1−/− hearts compared to TAC-operated Reg1+/+ hearts (Figure IIC in the online-only Data Supplement). Thus, deficiency of Regnase-1 in cardiomyocytes caused sustained induction of Il6 mRNA with severe infiltration of inflammatory cells in the heart in response to pressure overload.

**IL-6 Blockade Attenuated Inflammation and Heart Failure in Regnase-1-deficient Mice**

To examine whether the persistent elevation of Il6 mRNA is a cause for pressure overload-induced heart failure in Reg1−/− mice, IL-6 signaling was blocked using a monoclonal antibody against the IL-6 receptor (MR16-1) after TAC surgery. Control IgG or MR16-1 had no effect on cardiac chamber size and function and heart and lung weight in sham-operated Reg1+/+ and Reg1−/− mice (Figure IIE and F in the online-only Data Supplement). MR16-1 attenuated the chamber dilatation, cardiac dysfunction and hypertrophy induced by TAC in Reg1−/− mice (Figure 4A–C). Furthermore, MR16-1 attenuated non-cardiomyocyte cell infiltration, fibrosis, the increase in cardiomyocyte cross-sectional area, the upregulation of Nppa mRNA, and the increase in number of apoptotic cardiomyocytes (Figure 4D–G and Figure IID in the online-only Data Supplement). Infiltration of CD45+ and CD68+ cells was also inhibited by MR16-1 (Figure 5A–D). In contrast, MR16-1 had no beneficial effect on cardiac abnormalities observed in Reg1+/+ mice, which exhibited no increase in Il6 mRNA 4 weeks after TAC (Figure 4A–C).

**Severe Pressure Overload Induced Sustained Il6 mRNA Upregulation in Hearts**

Since the plasma level of IL-6 in the patients with heart failure was related with its severity, severe pressure overload, sTAC, may increase the level of Il6 mRNA in mouse failing hearts. The wild-type C57BL/6 mice showed chamber dilatation, cardiac dysfunction and lung congestion 1 and 4 weeks after sTAC (Figure IIIA and B in the online-only Data Supplement). Non-cardiomyocyte infiltration, fibrosis, infiltration of CD45+ and CD68+ cells and upregulation
of Nppa, Nppb, Col1a2 and Col3a1 mRNAs were observed 4 weeks after sTAC (Figure IIIC and D in the online-only Data Supplement). The levels of Il6 and Tnfa mRNA in the hearts increased 1 week after sTAC compared to those in sham-operated hearts and the level of II1b mRNA was not different between sTAC- and sham-operated hearts 1 week after surgery (Figure IIIE–G in the online-only Data Supplement). The level of Il6 mRNA was higher in sTAC-operated hearts than that in sham-operated hearts 4 weeks after sTAC, whereas the levels of Tnfa and II1b mRNA showed no difference between sTAC- and sham-operated hearts 4 weeks after surgery (Figure IIIE–G in the online-only Data Supplement). Thus, we switched to a sTAC model to examine the effect of overexpression of Regnase-1 or administration of MR16-1 in wild-type mice, in which Il6 mRNA was upregulated.

Reg1+– mice exhibited more severe left chamber dilatation and cardiac dysfunction 4 weeks after sTAC, compared to Reg1+/+ mice (Figure IIH and I in the online-only Data Supplement) as observed in TAC-operated Reg1+/+ and Reg1–/– mice.

Regnase-1 Overexpression or IL-6 Blockade in Wild-type Hearts Attenuated Heart Failure

Upper and lower bands on Western blot represent phosphorylated and non-phosphorylated Regnase-1 in sTAC-operated wild-type C57BL/6 hearts 4 weeks after surgery (Figure IIIJ in the online-only Data Supplement), respectively, which of both exhibited reduced density in Reg1+/– hearts (Figure IA in the online-only Data Supplement). Protein levels of non-phosphorylated Regnase-1 and total Regnase-1 significantly increased in sTAC-operated mouse hearts, but there was no significant difference in phosphorylated Regnase-1 between sham- and sTAC-operated hearts (Figure IIIJ in the online-only Data Supplement). To test whether insufficient induction of Regnase-1 during cardiac remodeling may lead to the sustained upregulation of Il6 mRNA, Reganse-1 was overexpressed in wild-type mouse cardiomyocytes by
infection of recombinant adeno-associated virus 9 encoding FLAG-tagged Reganase-1 (Reg1-AAV9) under the control of cardiac troponin T promoter. One week after intraperitoneal injection with AAV9 expressing Regnase-1 or eGFP (eGFP-AAV9), wild-type mice were subjected to sTAC. There were no significant differences in cardiac function 1 week after the infection between the two groups (Table II in the online-only Data Supplement). The mice were observed for 4 weeks after surgery. Infection of Reg1-AAV9 resulted in 8.4-fold increase in Regnase-1 protein level in the hearts compared to control infected with eGFP-AAV9 (Figure IVA in the online-only Data Supplement). There was no significant difference in the mortality in Reg1-AAV9-infected mice (21.1%, 4 out of 19 mice) and that in control vector-infected mice (21.4%, 3 out of 14 mice). Echocardiography revealed improvement in cardiac chamber dilatation and function in Reg1-AAV9-infected mice compared to control vector-infected mice (Figure 6A and B). Regnase-1 overexpression reduced the ratio of lung weight-to-tibia length (Figure 6C) and attenuated non-cardiomyocyte cell infiltration, fibrosis, upregulation of Nppa and Nppb mRNAs (Figure 6D–F) and infiltration of CD45+ and CD68+ cells (Figure 7A and B), and decreased Il6 mRNA level (Figure 7C) in sTAC-operated hearts. Interestingly, overexpression of Regnase-1 did not show beneficial effects on left chamber dilatation and cardiac dysfunction induced by TAC in wild-type mice, in which the level of Il6 mRNA was not significantly increased (Figure IVB–D in the online-only Data Supplement).

Next, we examined the effect of MR-1 on cardiac remodeling after sTAC in wild-type mice. Since IgG (0.5 mg) administration seems to have a non-specific cardioprotective effect (Figure 1 and 4), we examined the dose-dependent effect of IgG on cardiac remodeling. C57BL/6 mice were subjected to sTAC operation and received intraperitoneal injection of various doses of IgG (0, 0.15, or 0.50 mg) once a week from one week after operation. The 0.15
mg of IgG had no effect on left chamber dilation and cardiac dysfunction, while 0.5 mg of IgG attenuated the development of cardiac remodeling (Figure VA in the online-only Data Supplement). Thus, 0.15 mg IgG or MR-1 were injected to the mice to examine the effect of IL-6 blockade on cardiac remodeling. MR16-1 attenuated LV dilatation, cardiac dysfunction, hypertrophy, lung congestion and fibrosis induced by sTAC in wild-type mice (Figure VB–D in the online-only Data Supplement).

**Discussion**

Our data indicate that during normal embryonic development, there is no cardiac myocyte-autonomous requirement for the Regnase-1 signaling pathway. Furthermore, the Regnase-1-mediated pathway does not appear to be required for normal heart growth in the postnatal period. In response to pressure overload, Regnase-1 plays a protective role against the development of heart failure.

In Reg1−/− mice, Il6 mRNA levels increased 1 and 4 weeks after TAC, while in Reg1+/+ mice, the cytokine mRNA was upregulated 1 week after TAC, but not 4 weeks after TAC. Il6 mRNA is known to be a target of Regnase-1. Thus, Il6 mRNA degradation by Regnase-1 in cardiomyocytes regulates the time course of its expression level in the heart. Protective effects of MR16-1 in Reg1−/− mice indicate that the observed cardiac phenotypes in Reg1−/− mice is, at least in a part, due to the continuous elevation of Il6 mRNA. However, the lack of beneficial effect of MR16-1 on cardiac contractility in Reg1+/+ mice indicates that cardiac dysfunction observed in Reg1+/+ mice is IL6-independent. Inhibition of IL-6 reduced fibrosis and apoptosis in TAC-operated Reg1−/− mice and overexpression of Regnase-1 and inhibition of IL-6 reduced fibrosis in sTAC-operated wild-type mice, suggesting the loss of cardiomyocytes is involved in IL-6-
mediated cardiac dysfunction. It has been reported that IL-6 decreases cardiac contractility via STAT3-nitric oxide-dependent pathway.\textsuperscript{13} We observed the increased activation of STAT3 in TAC-operated \textit{Reg1}\textsuperscript{+} mice, indicating the negative inotropic effect of IL-6 may also be involved in the pathogenesis.

No significant increase in \textit{Il12b} mRNA (a known Regnase-1 target) was observed in \textit{Reg1}\textsuperscript{+/+} and \textit{Reg1}\textsuperscript{--} hearts 4 weeks after TAC. The major target for Regnase-1-mediated mRNA degradation appears to be IL-6 in cardiomyocytes. In T cells, Regnase-1 regulates the production of interferon-\(\gamma\) mRNA, while in macrophages, it regulates the degradation of \textit{Il6} and \textit{Il12p40}\textit{mRNAs}.\textsuperscript{5} The target of Regnase-1 seems to be cell type-specific.

Our data showed that the time course of \textit{Il6} mRNA level during cardiac remodeling depends on the strength of the stress. This is in agreement with the clinical study that the plasma level of IL-6 in the patients with heart failure was related with its severity.\textsuperscript{12} To know the role of myocardial Regnase-1 upregulation during cardiac remodeling, we employed the severe TAC model, in which \textit{Il6} mRNA showed continuous upregulation until 4 weeks after operation. Overexpression of Regnase-1 in cardiomyocytes decreased \textit{Il6} mRNA level in the heart and attenuated the development of myocardial inflammation and heart failure in sTAC-operated wild-type mouse model. Thus, upregulation of Regnase-1 is a protective mechanism to prevent hearts against pressure overload and the level of its upregulation is insufficient to suppress the development of inflammation and dilated cardiomyopathy. MR16-1 attenuated the development of cardiac remodeling in sTAC-operated wild-type mice. Practical point would be that targeting IL-6 might be a fruitful treatment for patients with a high level of IL-6. In addition, cytokine mRNA degradation in cardiomyocytes may be a new potential target for the therapy of heart failure.
Regnase-1 was reported to be MCPIP1.\textsuperscript{14} MCP-1 is the main chemotactic factor for migration of monocyte/macrophages and the pathogenesis of chronic inflammation.\textsuperscript{15} Cardiomyocyte-targeted expression of MCP-1 in mice resulted in the induction of MCPIP1 and development of cardiac dysfunction with an increased number of apoptotic cardiomyocytes.\textsuperscript{14} However, the present study shows that Reganase-1 protects the heart against hemodynamic stress, inconsistent with these reports showing the detrimental role of MCP-1-MCPIP1 pathways. Excessive overexpression of Regnase-1 from the embryonic stage might be detrimental to the heart.

In conclusion, the data suggest that the degradation of cytokine mRNA, as well as mitochondrial DNA, in non-immune cardiomyocytes is critical for restraining inflammation in failing hearts. The Regnase-1-related signaling pathway in cardiomyocytes is a potential therapeutic target to treat patients with heart failure.

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**Disclosures**

None.
References


Figure Legends

**Figure 1. Pressure overload-induced cardiomyopathy in Reg1^{+/−} mice.** The Reg1^{+/+} and Reg1^{−/−} mice were subjected to pressure overload by means of transverse aortic constriction (TAC). The mice were analyzed 4 weeks after TAC. Data were evaluated by one-way analysis of variance (ANOVA) with the Bonferroni’s post hoc test. Data are mean ± SEM. *P<0.05, **P<0.01, ***P<0.001. A, M-mode echocardiographic tracings from sham- or TAC-operated Reg1^{+/+} or Reg1^{−/−} mice. Scale bars, 0.1 sec and 2 mm. B and C, Echocardiographic (B) and physiological (C) parameters. N=7 (sham-Reg1^{+/+}), 7 (TAC-Reg1^{+/+}), 7 (sham-Reg1^{−/−}) or 6 (TAC-Reg1^{−/−}) per group. LVIDd and LVIDs, end-diastolic and end-systolic left ventricular internal dimension, respectively. FS, fractional shortening; HW/TL, heart weight/tibia length; LungW/TL, lung weight/TL. D–F, Hematoxylin-eosin-stained (D), Masson’s trichrome-stained (E), and wheat germ agglutinin-stained (F) heart sections. Scale bar, 100 µm. Fibrosis fraction was measured (N=3). Cardiomyocyte cross-sectional area was measured by tracing the outline of 70 myocytes in non-fibrotic area in each section (N=3). G, mRNA expression of Nppa, Nppb, Col1a2, and Col3a1. N=5 (sham-Reg1^{+/+}), 4 (TAC-Reg1^{+/+}), 5 (sham-Reg1^{−/−}) or 5 (TAC-Reg1^{−/−}) per group. Gapdh mRNA was used as the loading control. The averaged value in sham-operated Reg1^{+/+} hearts was set equal to 1.

**Figure 2. Inflammatory responses in pressure-overloaded Reg1^{−/−} hearts.** The Reg1^{+/+} and Reg1^{−/−} mice subjected to TAC were analyzed 4 weeks after TAC. Data were evaluated by one-way ANOVA with the Bonferroni’s post hoc test. Data are mean ± SEM. *P<0.05, **P<0.01, ***P<0.001. A, Inflammatory cytokine mRNAs including Tnfa, Il6, Il1b, Il12b, Ifnb1, Ifng and
**Figure 3. Production of *Il6* mRNA in *Reg1*⁻/⁻ hearts under pressure overload.** *Reg1*⁺/⁺ and *Reg1*⁻/⁻ hearts 4 weeks (A–D) or 1 week (E) after TAC were analyzed. Data were evaluated by Student’s *t*-test (C and D) or one-way ANOVA with the Bonferroni’s post hoc test (E). Data are mean ± SEM. *P*<0.05, ***P*<0.001. A, Double staining of TAC-operated *Reg1*⁻/⁻ heart sections with anti-CD68 (red) and anti-CD11c (green) antibodies. B, Double staining of TAC-operated *Reg1*⁻/⁻ heart sections with anti-CD68 (red) and anti-CD206 (green) antibodies. Scale bar, 100 μm. C, The ratio of CD11c- or CD206-positive to CD68-positive cell number (N=3). D, *In situ* hybridization for *Il6* mRNA (red) in *Reg1*⁺/⁺ or *Reg1*⁻/⁻ hearts, followed by immunostaining with α-sarcomeric action antibody (green). Scale bar, 100 μm. Right graph shows the number of red dots in cardiomyocytes. E, *Tnfa* and *Il6* mRNA levels 1 week after TAC. N=5 (sham-*Reg1*⁺/⁺), 5 (TAC-*Reg1*⁺/⁺), 5 (sham-*Reg1*⁻/⁻) or 6 (TAC-*Reg1*⁻/⁻) per group. *Gapdh* mRNA was used as the loading control. The averaged value in sham-operated *Reg1*⁺/⁺ mice was set equal to 1.

**Figure 4. IL-6 blockade ameliorated TAC-induced cardiomyopathy in *Reg1*⁻/⁻ mice.** After TAC operation, *Reg1*⁺/⁺ and *Reg1*⁻/⁻ mice received an intraperitoneal injection of anti-mouse IL-6 receptor antibody MR16-1 or control IgG. Afterwards they were injected intraperitoneally once a week with a total of three injections with either MR16-1 or IgG. The mice were analyzed 4
weeks after TAC. Data were evaluated by one-way ANOVA with the Bonferroni's post hoc test. Data are mean ± SEM. *P<0.05, **P< 0.01, ***P<0.001. A, M-mode echocardiographic tracings from IgG-treated or MR16-1-treated Reg1+/+ or Reg1−/− mice. Scale bars, 0.1 sec and 2 mm. B and C, Echocardiographic (B) and physiological (C) parameters. N=7 (IgG-Reg1+/+), 8 (IgG-Reg1−/−), 6 (MR16-1-Reg1+/+) or 8 (MR16-1-Reg1−/−) per group. D–F, Hematoxylin-eosin-stained (D), Masson’s trichrome-stained (E), and wheat germ agglutinin stained (F) heart sections. Scale bar, 100 µm. Fibrosis fraction (N=5) and cross-sectional area of cardiomyocytes (N=3) were measured. G, mRNA expression of Nppa and Nppb. N=6 (IgG-Reg1+/+), 5 (IgG-Reg1−/−), 5 (MR16-1-Reg1+/+) or 5 (MR16-1-Reg1−/−) per group. Gapdh mRNA was used as the loading control. The averaged value in TAC-operated Reg1+/+ hearts treated with IgG was set equal to 1.

**Figure 5.** IL-6 blockade inhibited infiltration of inflammatory cells. TAC-operated Reg1+/+ and Reg1−/− hearts treated with anti-mouse IL-6 receptor antibody MR16-1 or control IgG were analyzed. Data were evaluated by one-way ANOVA with the Bonferroni’s post hoc test. Data are mean ± SEM. *P<0.05, **P< 0.01. A–D, Immunohistochemical analysis for CD45 (A), CD68 (B), CD3 (C), and Ly6G (D). Scale bar, 100 µm. Bottom graphs show quantitative analysis of each infiltrating inflammatory cell-type (N=3).

**Figure 6.** Overexpression of Regnase-1 protein attenuated severe TAC-induced heart failure. Wild-type C57BL/6 mice were intraperitoneally injected with AAV9 expressing eGFP (eGFP-AAV9) or Regnase-1 (Reg1-AAV9) and were subjected to severe TAC (sTAC) 1 week after infection. Sham- or sTAC-operated wild type mice infected with eGFP-AAV9 (eGFP-sham...
or eGFP-sTAC) or Reg1-AAV9 (Reg1-sham or Reg1-sTAC) were analyzed 4 weeks after operation. Data were evaluated by one-way ANOVA with the Bonferroni’s post hoc test. Data are mean ± SEM. *P<0.05, **P< 0.01, ***P<0.001. A, M-mode echocardiographic tracings from eGFP-sham, eGFP-sTAC, Reg1-sham or Reg1-sTAC wild-type mice. Scale bars, 0.1 sec and 2 mm. B and C, Echocardiographic (B) and physiological (C) parameters. N=16 (eGFP-sham), 11 (eGFP-sTAC), 16 (Reg1-sham) or 15 (Reg1-sTAC). D and E, Hematoxylin-eosin-stained (D) and Masson’s trichrome-stained (E) heart sections. Scale bar, 100 μm. Fibrosis fraction was evaluated. N=3 (eGFP- or Reg1-sham) or 4 (eGFP- or Reg1-sTAC). F, mRNA expressions of Nppa and Nppb. N=7 (eGFP-sham), 6 (eGFP-sTAC), 7 (Reg1-sham) or 7 (Reg1-sTAC). Gapdh mRNA was used as the loading control. The averaged value in eGFP-sham group was set equal to 1.

Figure 7. Induction of Regnase-1 protein suppressed the extent of inflammatory responses in severe TAC-induced heart failure. Severe TAC-operated wild-type C57BL/6 mice infected with AAV9 expressing eGFP (eGFP-sTAC) or Regnase-1 (Reg1-sTAC) were analyzed. Data were evaluated by one-way ANOVA with the Bonferroni’s post hoc test. Data are mean ± SEM. *P<0.05, ***P<0.001. A and B, Immunohistochemical analysis for CD45 (A) and CD68 (B). Scale bar, 100 μm. Bottom graphs show quantitative analysis of each infiltrating inflammatory cell-type in eGFP-sham, eGFP-sTAC, Reg1-sham or Reg1-sTAC hearts (N=3). C, Il6 mRNA expressions. N=7 (eGFP-sham), 6 (eGFP-sTAC), 7 (Reg1-sham) or 7 (Reg1-sTAC). Gapdh mRNA was used as the loading control. The averaged value in eGFP-sham group was set equal to 1.
Figure 2
The number of Il6 mRNA-positive dots in cardiomyocytes (mm²):

TAC
Reg1⁺⁺
Reg1⁻⁻

Figure 3

Overlay CD68/CD11c

The ratio of CD11c and 206 positive cells (%):

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Figure 4
The number of Ly6G positive cells (/mm²)

The number of CD3 positive cells (/mm²)

The number of CD68 positive cells (/mm²)

Figure 5
Figure 6
Figure 7