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Characterization of NLRP3 in the Maintenance of Cardiac Tissue Homeostasis

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Research Paper

The Nlrp3 inflammasome promotes myocardial dysfunction in structural cardiomyopathy through interleukin-1β

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New findings

- **What is the central question of this study?**
  Heart failure is associated with persistent sterile inflammation that worsens disease severity; however, the molecular mechanisms behind cytokine recruitment and their relevance in the diseased myocardium remain unknown.
- **What is the main finding and its importance?**
  We show that interleukin-1β is activated downstream of the Nlrp3 inflammasome in calcineurin-transgene-induced structural heart disease. Genetic deletion of *Nlrp3* abrogated inflammasome signalling and interleukin-1β release, improving function. The role of Nlrp3 in non-ischaemic cardiomyopathy and the utility of inflammasome antagonism have not yet been explored, revealing potential for translational application.

Heart failure is associated with a low-grade and chronic cardiac inflammation that impairs function; however, the mechanisms by which this sterile inflammation occurs in structural heart disease remain poorly defined. Cardiac-specific heterozygous overexpression of the calcineurin transgene (CNTg) in mice results in cardiac hypertrophy, inflammation, apoptosis and ventricular dilatation. We hypothesized that activation of the Nlrp3 inflammasome, an intracellular danger-sensing pathway required for processing the pro-inflammatory cytokine interleukin-1β (IL-1β), may contribute to myocardial dysfunction and disease progression. Here we report that Nlrp3 mRNA was increased in CNTg mice compared with wild-type. Consistent with inflammasome activation, CNTg animals had increased conversion of pro-caspase-1 to cleaved and activated forms, as well as markedly increased serum IL-1β. Blockade of IL-1β signalling via chronic IL-1 receptor antagonist therapy reduced cardiac inflammation and myocyte pathology in CNTg mice, resulting in improved systolic performance. Furthermore, genetic ablation of Nlrp3 in CNTg mice reduced pro-inflammatory cytokine maturation and cardiac inflammation, as well as improving systolic performance. These findings indicate that activation of the Nlrp3 inflammasome in CNTg mice promotes myocardial inflammation and systolic dysfunction through the production of pro-inflammatory IL-1β. Blockade of IL-1β signalling with the IL-1 receptor antagonist reverses these phenotypes and offers a possible therapeutic approach in the management of heart failure.

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Chronic heart disease is associated with a persistent low-grade inflammatory response that promotes adverse cardiac remodelling and is correlated with disease progression (Borja *et al.* 2006; Kalogeropoulos *et al.* 2012). Heart failure is the most common cause for admission to hospital, and the medical management/survival have not changed significantly over the last 5–10 years (Curtis *et al.* 2008). There is an urgent need to increase our understanding of sterile inflammatory processes that underlie chronic heart disease in order to provide new avenues for therapeutic intervention.

The failing myocardium is characterized by extensive myocyte and interstitial cell death with an inflammatory infiltrate (Whelan *et al.* 2010; González *et al.* 2011). Fibroblasts and infiltrating cells release a host of pro-inflammatory cytokines into the tissue microenvironment and circulation (Hedayat *et al.* 2010; Lecour & James, 2011). A growing body of evidence has revealed that the nucleotide-binding domain and leucine-rich repeat-containing (NLR) family of cytosolic pattern recognition receptors plays a critical role in promoting sterile inflammation through the recognition of danger signals released from dying cells. While many NLRs participate in the host innate immune response to infectious stimuli, Nlrp3 regulates chronic inflammation in response to intrinsic host signals. Nlrp3 oligomerizes with the adaptor molecule 'apoptosis-associated speck-like protein containing a CARD domain' (ASC) in response to danger signals such as ATP, urate and membrane lipids (Mariathasan *et al.* 2006; Martinon *et al.* 2006; Luheshi *et al.* 2012). Nlrp3 and ASC subsequently recruit the cysteine protease pro-caspase-1 to generate a caspase-1-activating platform called the inflammasome (Martinon *et al.* 2002; Pétrilli *et al.* 2007). Activation of the inflammasome results in the caspase-1-dependent proteolytic cleavage of the 31 kDa precursor cytokine pro-interleukin-1β (pro-IL-1β) into the 17 kDa mature form, which participates in the pathogenesis of a range of chronic diseases such as diabetes, kidney injury and gouty arthritis (Martinon *et al.* 2006; Hirota *et al.* 2010; Schroder *et al.* 2010; Vilaysane *et al.* 2010).

Interleukin-1β (IL-1β) is a pyrogenic cytokine found typically within the serum during inflammatory stimuli. It is secreted mainly by blood monocytes and has tremendous effects on immunological function at very low concentrations (Pétrilli *et al.* 2007). Within the cardiovascular system, cardiac-derived IL-1β impairs contractility by inducing calcium leak from the sarcoplasmic reticulum and ultimately promotes cell death and tissue remodelling in a nitric-oxide-dependent pathway (Schulz *et al.* 1995; Ing *et al.* 1999; Duncan *et al.* 2010). Interleukin-1β is elevated in the serum from human patients following myocardial infarction and is prospectively and independently associated with poor outcomes in cardiovascular disease (Guillén *et al.* 1995). The mechanisms behind IL-1β production and release in the heart and the utility of its blockade at the level of the inflammasome during structural heart disease, however, remain unknown.

The inflammasome has recently been described in the pathogenesis of myocardial ischaemia–reperfusion injury and infarction; however, the significance of Nlrp3 specifically and its signalling within the context of chronic heart disease have not been addressed (Kawaguchi *et al.* 2011; Mezzaroma *et al.* 2011). Cardiac myocyte-specific expression of the constitutively active calcineurin transgene (CNTg) results in an inflammatory and hypertrophic cardiomyopathy that is characterized by a progressive and time-dependent cardiac dysfunction (Somers *et al.* 2008). Importantly, this model represents a chronically established cardiac defect, allowing for dissection of the mechanisms promoting chronic inflammation in longstanding hypertrophy similar to that seen clinically in patients with heart failure (Kalogeropoulos *et al.* 2012). We therefore sought to assess the relevance of IL-1β and its upstream signalling mechanisms in CNTg-induced heart failure. Here we report that the Nlrp3 inflammasome is activated in the failing myocardium of CNTg mice. Treatment with the soluble IL-1 receptor antagonist (IL-1-ra) demonstrates improved systolic function and reduced infiltrate and interstitial apoptosis. Additionally, we show for the first time that Nlrp3 genetic ablation reduces serum IL-1β and myocardial caspase-1 activation, producing a similar phenotype to IL-1β pharmacological antagonism. These results provide a mechanistic platform for therapeutic exploitation of the inflammasome in the treatment of established structural heart disease.

Methods

Ethical approval

All experiments were performed under the approval of the Animal Care Committee at the University of Calgary.

Animal studies

Calcineurin-transgenic (CNTg) mice were obtained from Molkentin's original 10 independent founder lines and backcrossed on the C57Bl/6 background (Somers *et al.* 2008). Every line demonstrated the same phenotype. The constitutively active heterozygous calcineurin gene product is expressed behind the α-myosin heavy chain promoter, resulting in cardiac myocyte-specific expression. The CNTg and wild-type (WT) mice were separated using PCR analysis. Primer sequences used were as follows: forwards 5′-GTCTGACTAGGTGTCCTTCT-3′ and reverse 5′-CGTCCTCCTGCTGGTATTAC-3′. *Nlrp3*$^{-/-}$ and CNTg mice, both on the C57Bl/6

background, were bred (Vilaysane *et al.* 2010). Their heterozygous progeny were crossed to obtain mixed *Nlrp3*$^{+/-}$ CNTg/*Nlrp3*$^{+/+}$ CNTg groups, which were used to assess cardiac function compared with wild-type ($n = 4$–5 per group). Colonies were maintained through mating *Nlrp3*$^{-/-}$ CNTg homozygotes. All mice were housed in a specific pathogen-free facility.

Osmotic pumps (Alzet, Cupertino, CA, USA) were filled with either drug-containing solution or sterile saline ($n = 5$ per group) and surgically implanted into the dorsum under general anaesthesia with isoflurane delivered by inhalation. Briefly, a small 1–2 cm incision was made in the skin and separated using blunt dissection of subcutaneous fascia. After implantation of the pump the skin was closed with a 7–0 silk suture and mice were allowed to recover with standard food and water.

Echocardiograms were obtained in conscious, restrained mice to assess cardiac structure and function using a SONOS 5500 from Agilent Technologies (Andover, MA, USA) with a 15 MHz paediatric probe. Echocardiograms were obtained between 14 and 20 weeks of age to allow for significant disease progression. Cardiac physiological data were independently reviewed by a cardiologist in a blinded fashion.

Tissue preparation

Hearts were excised following cervical dislocation, rinsed in PBS, fixed in 10% buffered formalin and embedded in paraffin for staining with Haematoxylin and Eosin (H&E) following standard protocols. Inflammation was assessed by a pathologist blinded to the identity of the specimens. Myocyte pathology was qualified in a blinded manner by assessing the following features: vacuolated cytoplasm; myofibril dropout; and markedly hypertrophic and pleomorphic nuclei with mononuclear cellular infiltrates. CD3 staining was performed on frozen sections using rat anti-CD3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Terminal deoxynucleotidyl transferasedUTP nick end labeling (TUNEL) stain was performed on paraffin-embedded sections using the *in situ* cell death detection kit according to the manufacturer's protocol (Roche, San Francisco, CA, USA). Positive cells were quantified by counting and averaged over five random fields of view (×40 objective).

Hearts were additionally cut and flash frozen in liquid nitrogen for storage until further processing. For protein isolation, cardiac tissue was minced in lysis buffer [150 mM NaCl, 20 mM Tris (pH 7.5), 1 mM EDTA and 1% Triton X-100] containing protease inhibitors (Roche Complete Minitab; Roche), then homogenized by hand on ice. Samples were cleared by centrifugation at 13.2*g* at 4°C and diluted with 3× Laemmli sample buffer. Lysates were separated on 15% gels by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% skim milk powder, immunoblotting was performed using the following antibodies: polyclonal rabbit anti ASC (AL177; Adipogen, San Diego, CA, USA), polyclonal mouse anti Nlrp3 (clone Cryo2; Adipogen), polyclonal rabbit anti-mouse caspase-1 (Santa Cruz Biotechnology), polyclonal rabbit anti-phospho-nuclear factor-κB (anti-phospho-NFκB; Cell Signaling, Beverly, MA, USA), polyclonal rabbit anti-total NFκB (Cell Signaling) and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH; Santa Cruz). Serum IL-1β and tumour necrosis factor-α (TNF-α) were measured using a luminex XMap assay according to the manufacturer's protocol (Luminex Corp., Toronto, Ontario, Canada).

RNA was prepared by homogenization of mouse tissue and extraction with RNeasy Mini kit (Qiagen, Germantown, MD, USA), according to the manufacturer's instructions. Quantitative real-time PCR was performed as previously described (Hirota *et al.* 2010; Vilaysane *et al.* 2010). Briefly, primers used were as follows: Nlrp3 forward 5′-AGAGCCTACAGTTGGGTGAAATG-3′; probe 6-FAM-5′-CGTGCCTTAGAAGCG-3′-MGB; and reverse 5′-CCACGCCTACCAGGAAATCTC-3′; and 20X Mouse GAPDH-FAM/MGB Probe (Applied Biosystems, Foster City, CA, USA) was used as the endogenous control.

Cell culture

C57Bl/6 mice were killed between postnatal day 0 and 3, and hearts were excised, rinsed in saline and minced. Tissue was digested in 0.16 mg ml^{-1} collagenase (Yakult, Tokyo, Japan), 10 mg ml^{-1} bovine serum albumin and 20 mM taurine. Tissue was stirred continuously in a dry bath heater, and solution was replaced every 5 min. Supernatant was diluted in M199 culture medium (Sigma) plus 26 mM $NaHCO_3$, 10 mM Hepes, 15% fetal bovine serum and 100 U ml^{-1} penicillin/streptomycin with pH adjusted to 7.40. Cells were plated in 24-well plates. Fibroblasts were allowed to adhere for 20–40 min and then the myocytes were removed and placed in separate wells. Fibroblasts were cultured for an additional 24 h to 70–80% confluency in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 100 U ml^{-1} penicillin/streptomycin. Myocyte layers showed spontaneous beating following collagenase dispersion and cell culture.

NIH-3T3 fibroblasts, HL-1 atrial myocytes (ATCC) and primary myocytes and fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin. Lysates were prepared at 70–80% confluency. Cell culture was maintained at 37°C in air supplemented with 5% CO_2.

Statistics and analysis

Statistical analyses were performed using Microsoft Excel software. All data are expressed as means ± SEM unless otherwise indicated. The results were analysed for statistical variance using Student's paired or unpaired *t* test or one-way ANOVA where appropriate. Statistical significance of data was considered at $P < 0.05$.

Results

The Nlrp3 inflammasome is activated in calcineurin-induced heart failure

The CNTg phenotype is characterized by progressive deterioration in cardiac function. After 3 months of age the fractional shortening, a metric of systolic performance measured as [left ventricular internal dimension at end diastole (LVIDd) minus left ventricular internal dimension at peak systole (LVIDs)]/LVIDd × 100, was significantly reduced compared with WT mice (Fig. 1*A*; $P < 0.001$). The CNTg hearts demonstrated extensive myocyte pathology, with increased cellularity and a diffuse infiltrate on H&E staining (Fig. 1*B*). In addition, serum from CNTg mice contained elevated levels of IL-1β ($P < 0.05$). Given that these phenotypes all involve chronic, sterile inflammation, we sought to assess whether the development of myocardial dysfunction involves activation of the Nlrp3 inflammasome (Obasanjo-Blackshire *et al.* 2006; Somers *et al.* 2008).

Neonatal mouse cardiac fibroblasts and myocytes were compared with NIH-3T3 fibroblasts and HL-1 atrial myocytes for expression of inflammasome components at the protein level (Fig. 2*A*). Nlrp3 was expressed in both fibroblasts and myocytes, as was pro-caspase-1. We also detected ASC in all cell types tested, although cardiac fibroblasts expressed less in comparison with both neonatal myocytes and HL-1 cells. These results identify resident cells of the heart as capable of assembling functional inflammasomes.

We examined heart tissue from WT and CNTg mice for activation of the inflammasome. While hearts from WT and CNTg mice both demonstrated Nlrp3 and ASC protein, only CNTg hearts showed robust conversion of pro-caspase-1 into the mature and active enzyme, consistent with inflammasome activation (Fig. 2*B*). We detected a fourfold induction of Nlrp3/GAPDH mRNA transcripts in the hearts of CNTg mice compared with

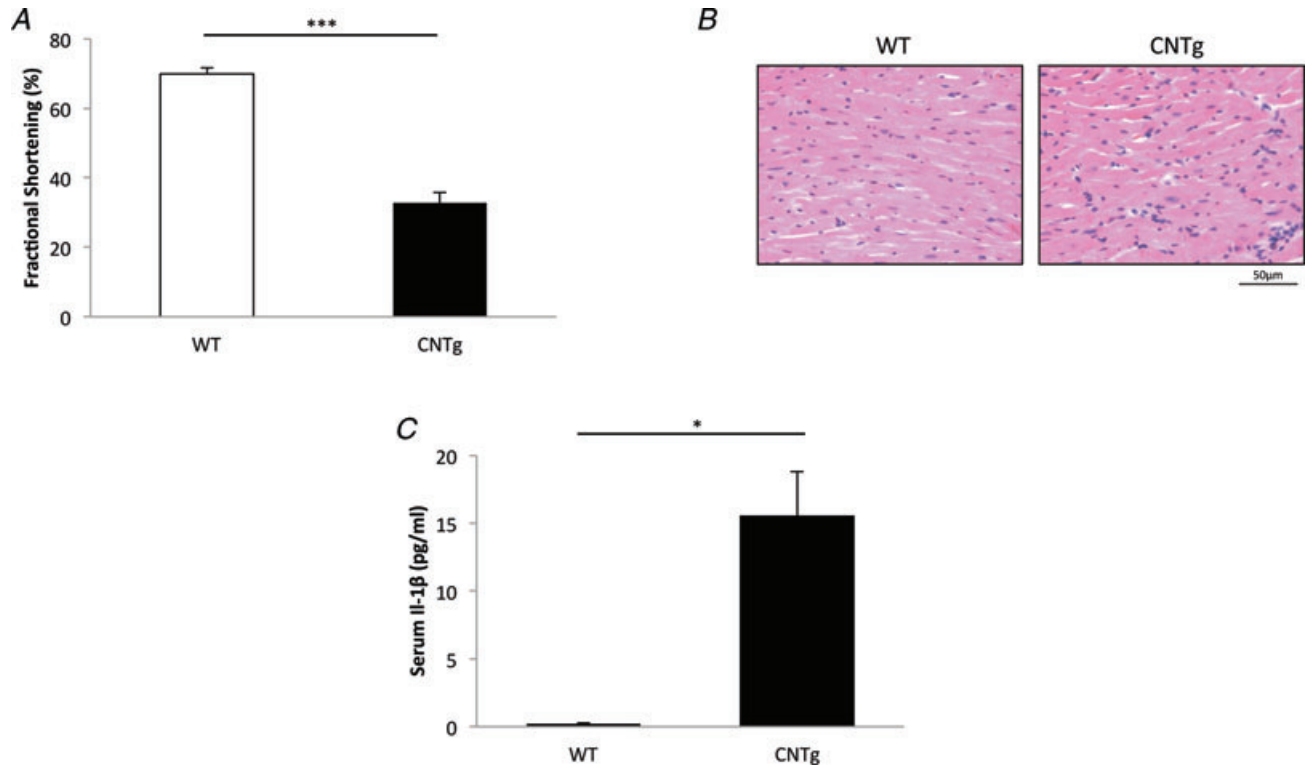

Figure 1. Calcineurin transgenic (CNTg) mice demonstrate myocardial inflammation with impaired cardiac function
A, CNTg fractional shortening was significantly reduced compared with wild-type (WT; $P < 0.001$, $n = 4$). *B*, Haematoxylin and Eosin (H&E)-stained sections of CNTg myocardium revealed a diffuse interstitial inflammation with increased cellularity and myocyte nuclear pleomorphism. *C*, serum interleukin-1β (IL-1β) measured by Luminex was significantly higher in CNTg mice compared with WT ($P < 0.05$, $n = 5$).

WT using quantitative real-time PCR on whole cardiac tissue-derived RNA (Fig. 2*C*; $P < 0.05$). Elevated cardiac Nlrp3 mRNA in the absence of significant changes in Nlrp3 protein could further implicate activation of the inflammasome. Professional immune cells typically undergo regulated inflammatory cell death by pyroptosis following Nlrp3 inflammasome activation, and Nlrp3 has been suggested to undergo ubiquitination during autophagy in some cell types following cytokine release (Shi *et al.* 2012). In this way, minor changes in Nlrp3 levels could reflect persistent inflammatory signalling. To further explore Nlrp3 regulatory mechanisms in CNTg hearts we probed whole-heart protein lysates for phosphorylation of the NF-κB negative regulatory protein IκBα. Hearts from CNTg mice showed increased phosphorylation of IκBα at Ser32/36, consistent with NF-κB-regulated *Nlrp3* gene expression (Fig. 2*D*; Bauernfeind *et al.* 2010). Taken together, these results show activation of the inflammasome, and strongly implicate Nlrp3 as an important chronic inflammatory responsive pattern recognition receptor in the hearts of CNTg mice.

Interleukin-1β regulates progression of cardiac dysfunction in CNTg mice

We next looked to evaluate the consequences of IL-1β pharmacological antagonism *in vivo* in the CNTg heart failure model. The IL-1 receptor antagonist (IL-1-ra) competitively binds to the IL-1 receptor with high affinity and is used clinically in the treatment of rheumatoid arthritis and various auto-inflammatory disorders (Carter *et al.* 1990; Martinon *et al.* 2007). The CNTg mice were surgically implanted with mini-osmotic pumps (Alzet), and Il-1-ra (10 mg kg^{-1} day^{-1}) was delivered for 14 days. Table 1 shows the mean echocardiographic data of conscious, unsedated mice at baseline and following treatment. After 2 weeks of saline, there was a progressive reduction in fractional shortening (FS) from 30 to 18% (Fig. 3*A*; $P < 0.01$). This is consistent with our previous work demonstrating a stepwise impairment of cardiac function (Semeniuk *et al.* 2003).

Interleukin-1-ra treatment significantly reduced left ventricular dilatation (Table 1). The LVIDd was 0.49 ± 0.05 mm with IL-1-ra, compared with 0.53 ± 0.01 mm in saline-treated mice ($P < 0.05$). The LVIDs was also improved, measuring 0.32 ± 0.08 mm after IL-1-ra *versus* 0.45 ± 0.05 mm after saline ($P < 0.05$). As a result, the FS was $34 \pm 3\%$ with IL-1-ra treatment; significantly greater than the $20 \pm 3\%$ in the saline-treated group (Fig. 3*A*; $P < 0.05$). We did not detect changes in septal wall thickness and heart weight/body weight ratios (not shown), indicating that IL-1β does not significantly participate in hypertrophic signalling downstream of the calcineurin transgene.

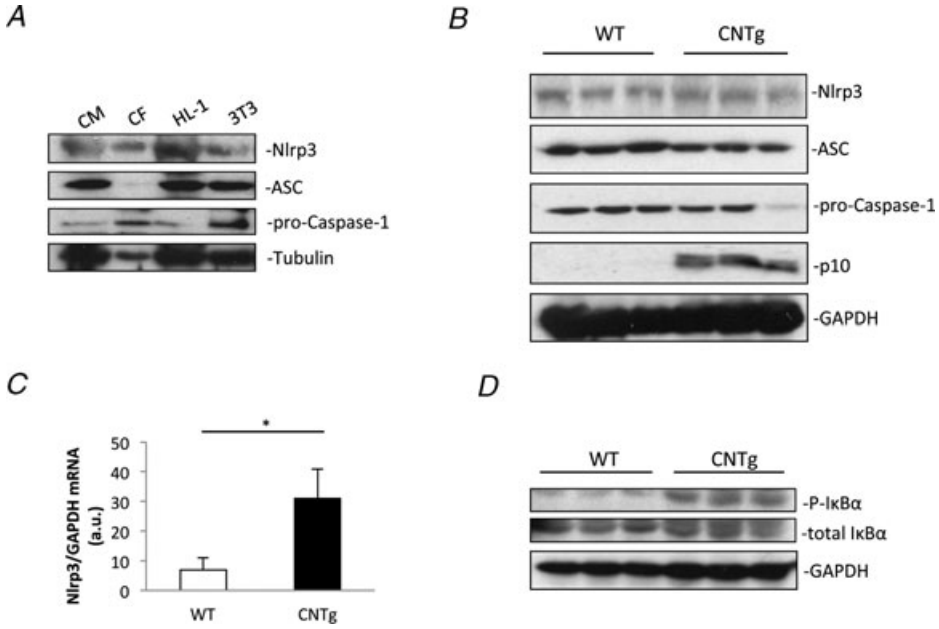

Figure 2. The inflammasome is activated in CNTg myocardium
A, primary neonatal cardiac fibroblast (CF), cardiac myocyte (CM), NIH 3T3 fibroblast and HL-1 atrial myocyte whole-cell lysates were immunoblotted for inflammasome components. *B*, WT and CNTg whole-heart lysates were immunoblotted for inflammasome components, demonstrating increased maturation of pro-caspase-1. *C*, quantitative real-time PCR analysis of whole-heart RNA from WT and CNTg mice revealed increased *Nlrp3* transcripts in CNTg compared with WT ($P < 0.05$, $n = 3$). *D*, WT and CNTg whole-heart lysates were immunoblotted for phosphorylated IκBα at Ser32/36, consistent with increased nuclear factor-κB signalling.

Table 1. Conscious, non-sedated echocardiographic results of IL-1-ra treatment in CNTg mice

Parameter	Baseline	After 2 weeks of saline	Baseline	After 2 weeks of IL-1-ra
HR (beats min^{-1})	471 ± 110	536 ± 60	447 ± 81	463 ± 181
SWT (cm)	0.092 ± 0.005	0.082 ± 0.007	0.09 ± 0.01	0.089 ± 0.01
LVIDd (cm)	0.47 ± 0.05	0.53 ± 0.01*†	0.45 ± 0.05	0.49 ± 0.05*
PWT (cm)	0.092 ± 0.005	0.084 ± 0.004	0.092 ± 0.01	0.09 ± 0.01
LVIDs (cm)	0.33 ± 0.05	0.45 ± 0.05†	0.31 ± 0.07	0.32 ± 0.08*

One-way ANOVA was used to evaluate the differences between groups statistically. $^*P < 0.05$, $\dagger P < 0.05$ comparing vehicle *versus* IL-1-ra at matched time points ($n = 5$). Data are presented as means ± SEM. Abbreviations are as follows: CNTg, calcineurin transgene; HR, heart rate; IL-1-ra, interleukin-1 receptor antagonist; LVIDd, left ventricular internal dimension at end diastole; LVIDs, left ventricular internal dimension at peak systole; PWT, posterior wall thickness; and SWT, septal wall thickness.

Interleukin-1β antagonism improves myocardial structure and pathology in CNTg mice

We observed an increased mononuclear cellular infiltrate in the interstitium of CNTg hearts, with associated myocyte pathology consistent with previous studies (Fig. 3*B*; Somers *et al.* 2008). Specifically, CNTg hearts treated with saline demonstrated significant areas of myofibril dropout, many with prominent eosinophilic

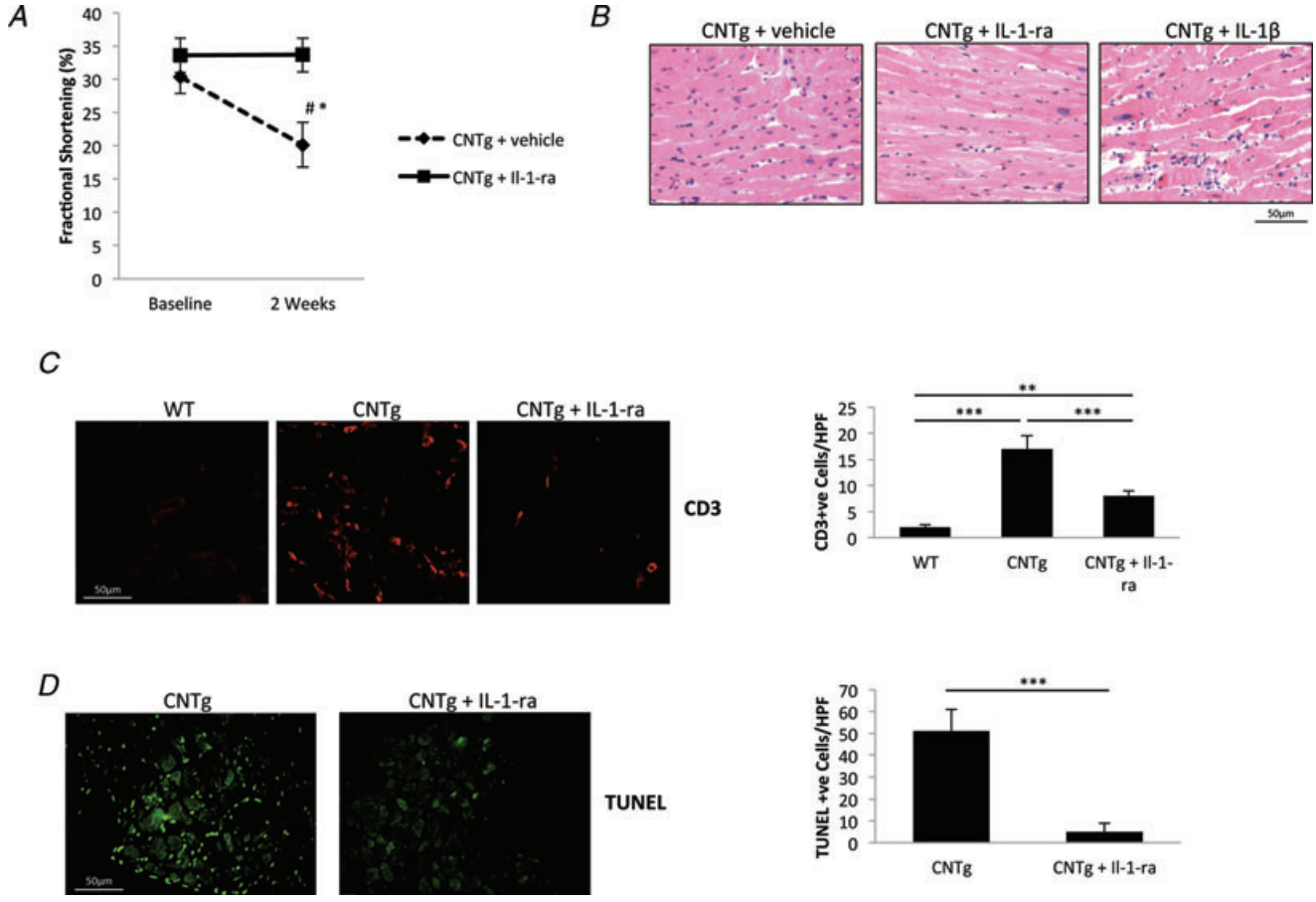

Figure 3. Interleukin-1β blockade in CNTg mice improves cardiac structure and function
A, fractional shortening was measured at baseline and following 2 weeks of treatment with either vehicle or IL-1 receptor antagonist (IL-1-ra; $\#P < 0.05$ compared with baseline in same group, $^*P < 0.05$ between groups at the same time point, one-way ANOVA, $n = 5$). *B*, H&E-stained myocardial sections of CNTg mice treated with vehicle, IL-1-ra or recombinant mature IL-1β for 14 days. *C*, CD3-positive cells present in CNTg hearts were significantly reduced with IL-1-ra treatment ($n = 4$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's unpaired *t* test). *D*, TUNEL staining on CNTg cardiac tissue demonstrated reduced intracardiac apoptotic cells with IL-1-ra treatment ($^{***}P < 0.001$, $n = 4$, Student's unpaired *t* test).

Table 2. Conscious, non-sedated echocardiographic results of *Nlrp3* genetic deletion in CNTg mice

Parameter	Wild-type	*Nlrp3*$^{-/-}$	CNTg	*Nlrp3*$^{-/-}$ CNTg
HR (beats min^{-1})	494 ± 40	594 ± 17	469 ± 24	355 ± 16
SWT (cm)	0.083 ± 0.003	0.083 ± 0.007	0.091 ± 0.03	0.073 ± 0.002
LVIDd (cm)	0.35 ± 0.01	0.31 ± 0.01	0.51 ± 0.02	0.41 ± 0.05
PWT (cm)	0.065 ± 0.005	0.078 ± 0.005	0.090 ± 0.02	0.083 ± 0.004
LVIDs (cm)	0.11 ± 0.01	0.11 ± 0.01	0.34 ± 0.02	0.21 ± 0.04†

Student's unpaired *t* test was used to evaluate differences between groups statistically ($n = 4$–5 mice per group). $P < 0.05$, †$P < 0.001$ compared with CNTg mice. Data are presented as means ± SEM. Abbreviations used are as follows: CNTg, calcineurin transgene; HR, heart rate; LVIDd, left ventricular internal dimension at end diastole; LVIDs, left ventricular internal dimension at peak systole; PWT, posterior wall thickness; and SWT, septal wall thickness.

nuclear inclusions and increased mononuclear cellular infiltrate on H&E staining. These changes were greatly reduced on treatment with IL-1-ra. Additionally, the delivery of exogenous mature IL-1β to CNTg mice by osmotic pump for 7 days (3 μg kg^{-1} day^{-1}) dramatically exaggerated the CNTg histological phenotype (Fig. 3*B*).

Immunostaining revealed that the mononuclear infiltrate in CNTg hearts consisted largely of CD3-positive cells, which were significantly reduced following IL-1-ra treatment (Fig. 3*C*). TUNEL staining further showed that the interstitial bare areas contained abundant apoptotic nuclei (Fig. 3*D*). These cells were greatly reduced on IL-1-ra treatment ($P < 0.05$).

As we observed increased inflammatory signalling in CNTg hearts, we sought to assess the consequences of IL-1β blockade on inflammasome components within the heart. Interleukin-1-ra did not affect levels of ASC; however, Nlrp3 was reduced in CNTg mice following 14 days of treatment (Fig. 4*A*). Additionally, IL-1-ra reduced serum IL-1β compared with untreated CNTg mice (Fig. 4*B*; $P < 0.05$). The reduced Nlrp3 and IL-1β were paralleled by a reduction in IκBα phosphorylation (Fig. 4*C*). These results are consistent with feedforward NFκB-mediated Nlrp3 expression downstream of the IL-1 receptor, with resultant changes in inflammatory transcriptional programmes.

Interleukin-1β-induced cardiac dysfunction in CNTg mice occurs downstream of the Nlrp3 inflammasome

We next sought to determine the upstream signalling mechanisms behind IL-1β processing and release in calcineurin-transgene-induced heart failure. To this end, CNTg mice were bred into the *Nlrp3*$^{-/-}$ background.

Mean echocardiographic measurements are presented in Table 2. Similar to IL-1-ra treatment, we observed an

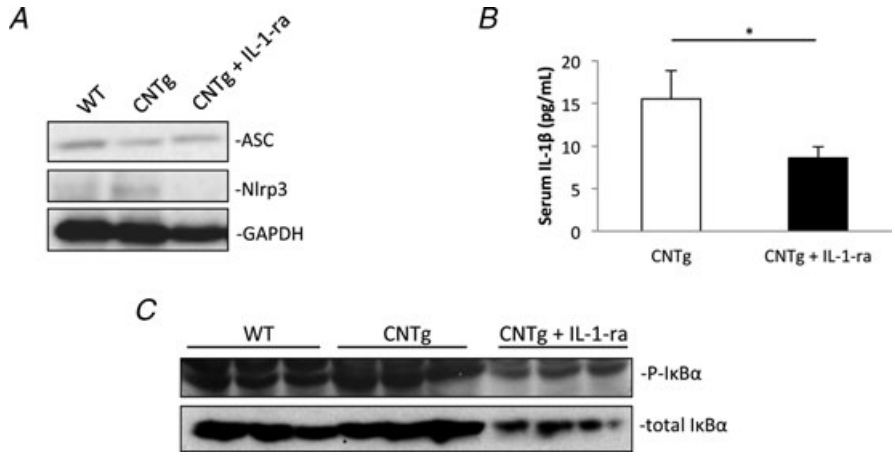

Figure 4. Interleukin-1β blockade reduces inflammatory signalling in CNTg hearts
A, WT, CNTg and IL-1-ra-treated CNTg whole-heart lysates were immunoblotted for inflammasome components. *B*, serum IL-1β measured by Luminex was significantly reduced following 2 weeks of IL-1-ra treatment in CNTg mice (*$P < 0.05$, $n = 5$, Student's unpaired *t* test). *C*, WT, CNTg and IL-1-ra-treated CNTg whole-heart lysates were immunoblotted for phosphorylated IκBα at Ser32/36, suggesting reduced nuclear factor-κB signalling following IL-1β blockade.

improvement in left ventricular systolic performance in *Nlrp3*$^{-/-}$ CNTg mice. Fractional shortening was 50 ± 6% in *Nlrp3*$^{-/-}$ CNTg mice, compared with 33 ± 3% in CNTg mice (Fig. 5*B*; $P < 0.05$). Also in accordance with IL-1-ra treatment, there was a reduction in the degree of left ventricular dilatation on abrogation of Nlrp3. The LVIDs was significantly improved, measuring 0.21 ± 0.04 mm in *Nlrp3*$^{-/-}$ CNTg mice, compared with 0.34 ± 0.02 mm in CNTg mice ($P < 0.01$). Again, there was no significant change in the degree of left ventricular wall thickness in *Nlrp3*$^{-/-}$ CNTg mice.

We observed a general reduction in myocardial cellularity and inflammation in *Nlrp3*$^{-/-}$ CNTg hearts compared with CNTg (Fig. 5*C*). Specifically, there were fewer regions of myofibre dropout and interstitial bare areas in the *Nlrp3*$^{-/-}$ CNTg hearts, reflective of maintained myocyte organization and structure, and a reduced mononuclear cellular infiltrate.

We assessed activation of the inflammasome in *Nlrp3*$^{-/-}$ CNTg hearts by Western blotting and serum analysis. Genetic deletion of *Nlrp3* produced no changes in ASC or pro-caspase-1 expression (Fig. 6*A*); however, there was a significant reduction in serum-IL-1β in *Nlrp3*$^{-/-}$ CNTg mice compared with CNTg mice (Fig. 6*B*; $P < 0.05$). In accordance with serum IL-1β measurements, myocardial activation of pro-caspase-1 was reduced in *Nlrp3*$^{-/-}$ CNTg hearts, suggesting that IL-1β intracellular processing occurs downstream of Nlrp3-dependent caspase-1 activation (Fig. 6*A*). In addition to IL-1β reduction, *Nlrp3*$^{-/-}$ CNTg mice had significantly reduced serum TNF-α compared with CNTg mice (Fig. 6*C*), consistent with a reduction in systemic inflammation on Nlrp3 and IL-1β antagonism.

Discussion

We have previously assessed the role of inducible nitric oxide synthase signalling in the cardiac inflammation in CNTg mice, noting an improvement in cardiac structure and function with a resolution of inflammation on genetic ablation of inducible nitric oxide synthase (Somers *et al.* 2008). There is growing evidence to suggest that a significant component of IL-1β-induced cardiac dysfunction results downstream of inducible nitric oxide synthase (Tatsumi *et al.* 2000; Csont *et al.* 2005); therefore,

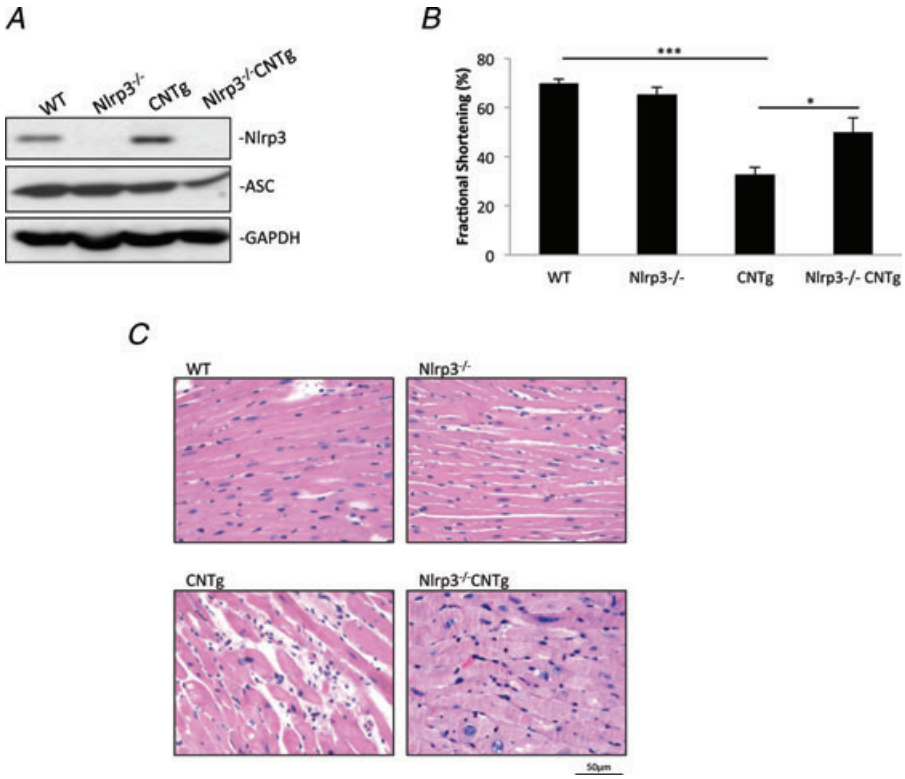

Figure 5. Genetic deletion of *Nlrp3* in CNTg mice improves cardiac structure and function
A, WT, *Nlrp3*$^{-/-}$, CNTg and *Nlrp3*$^{-/-}$ CNTg whole-heart lysates were immunoblotted for Nlrp3 and ASC. *B*, fractional shortening was significantly improved in *Nlrp3*$^{-/-}$ CNTg mice compared with CNTg mice ($^{*}P < 0.05$, $^{***}P < 0.001$, $n = 3$–6 per group, Student's unpaired *t* test). *C*, H&E-stained myocardial sections from WT, *Nlrp3*$^{-/-}$, CNTg and *Nlrp3*$^{-/-}$ CNTg mice revealed improved cardiac architecture on *Nlrp3* deletion.

we sought to expand on our previous work and examine the impact of IL-1β antagonism in the CNTg model.

The importance of pro-inflammatory IL-1β in the pathogenesis of heart disease has been explored in animal experiments, where exogenous administration both *in vivo* and *in vitro* has resulted in impaired calcium transients, fibrosis and structural remodelling with reduced cardiac function (Bujak & Frangogiannis, 2009; Duncan *et al.* 2010). Subsequent studies in both mice and humans have validated these results (Abbate *et al.* 2008). Recently, IL-1β has additionally been suggested to induce systolic dysfunction and exercise intolerance in heart failure patients, further supporting a cardiodepressant role for this cytokine in chronic heart disease (Van Tassell *et al.* 2012).

The administration of IL-1-ra (anakinra) in clinical trials post-myocardial infarction resulted in a reduction in adverse remodelling, improved cardiac function and normalization of serum inflammatory markers (Abbate *et al.* 2010). However, current work examining the efficacy of IL-1β and inflammasome blockade has focused on ischaemic cell death and the resulting cardiac remodelling processes. As such, previous studies have been unable to distinguish inflammatory remodelling from expansion/development of infarction (Kawaguchi *et al.* 2011; Mezzaroma *et al.* 2011). Here we sought to assess the impact of Nlrp3 inflammasome signalling on established structural heart disease. We elaborate on the mechanisms upstream of IL-1β processing, identifying a critical role for Nlrp3 in pro-inflammatory cytokine production and the progression of systolic dysfunction. Moreover, using a model of structural and inflammatory cardiomyopathy in the absence of infarction allows for a greater understanding of how the Nlrp3 inflammasome participates in the pathogenesis of heart failure without the confounding effects of infarct size.

The associated chronic inflammation in heart failure is known to have deleterious consequences for cardiac contractility. Dying cells release danger signals within the injured tissue microenvironment, which are subsequently recognized by danger-sensing systems. Nlrp3 oligomerization with pro-caspase-1 and ASC generates active caspase-1, which processes pro-IL-1β for secretion to drive sterile inflammation (Hirota *et al.* 2010; McDonald *et al.* 2010; Schroder *et al.* 2010; Vilaysane *et al.* 2010) The improvement in systolic performance on Nlrp3 blockade demonstrates that inflammasome/IL-1β antagonism possesses utility not only in the context of an

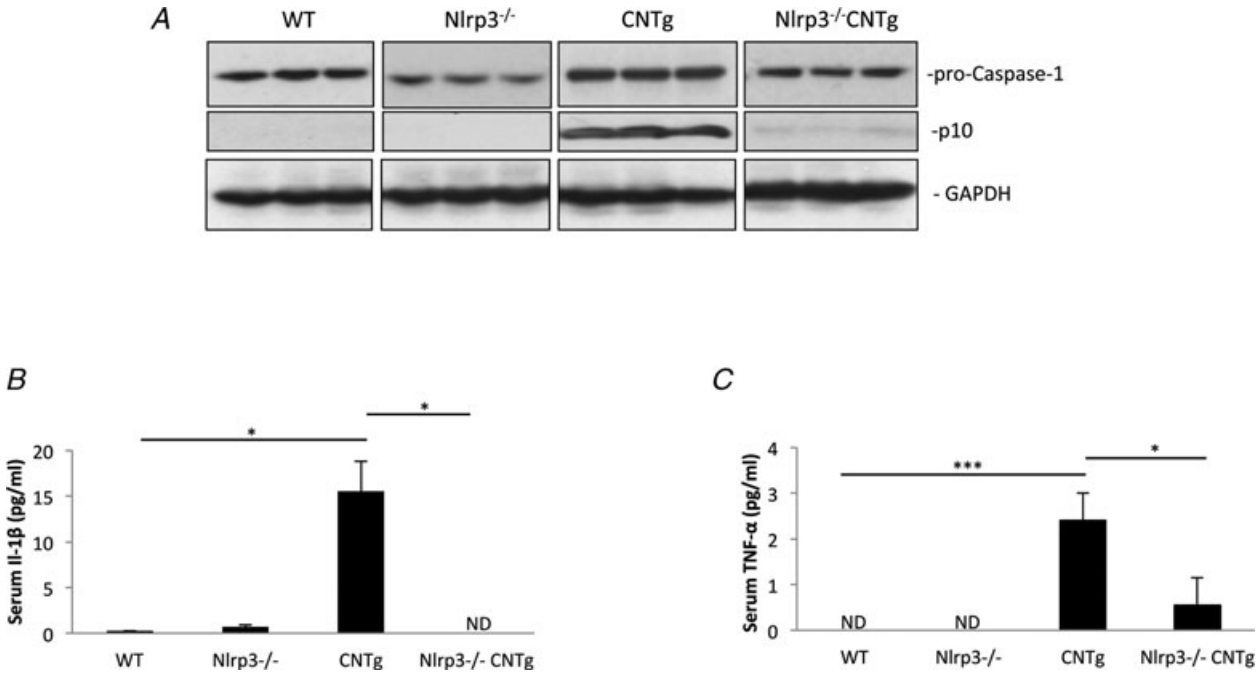

Figure 6. Genetic deletion of *Nlrp3* in CNTg mice reduces inflammasome activation and pro-inflammatory cytokine production
A, WT, *Nlrp3*$^{-/-}$, CNTg and *Nlrp3*$^{-/-}$CNTg whole-heart lysates were immunoblotted for caspase-1 maturation. There was virtually no caspase-1 activation, evidenced by reduced p10 production present in hearts from *Nlrp3*$^{-/-}$ mice. *B*, serum cytokine analysis by Luminex revealed significant reduction in IL-1β. *C*, analysis of serum further revealed significantly reduced tumour necrosis factor-α (TNF-α) in *Nlrp3*$^{-/-}$ CNTg mice compared with CNTg mice ($^{*}P < 0.05$, $^{***}P < 0.001$, $n = 4$–5 per group, Student's unpaired *t* test).

acutely evolving injury such as ischaemia/reperfusion or infarction, but also in the progression of heart disease, where ongoing myocardial stress yields chronic tissue damage.

The progression of changes that occur in cardiac architecture in the CNTg model is well described, beginning with septal and posterior wall thickening, with a subsequent chamber dilatation and eventual thinning of the ventricular walls resulting in a dilated phenotype with markedly reduced systolic function (Semeniuk *et al.* 2003). Nlrp3 blockade and IL-1β antagonism prevented the decline in systolic performance and reduced the degree of chamber dilatation. These changes were seen in the absence of significant reductions in wall thickness or fibrosis, which is consistent with the recent work of Mezzaroma *et al.* (2011), who found improved contractility using Nlrp3 small interfering RNA in mice after myocardial infarction. The signalling pathways involved in myocyte hypertrophy are heterogeneous and complex, and it is becoming evident that synergistic actions among multiple cytokine mediators are likely to be involved. Instead, our findings support a role for the Nlrp3 inflammasome and IL-1β in cell death, because we observed a significant preservation of cardiac architecture and reduced interstitial CD3-positive cells on abrogation of inflammasome signalling. Much evidence has suggested that myocardial and interstitial apoptosis alone are sufficient to impair cardiac contractility and inhibit cardiac function *in vivo* (Merkle *et al.* 2007; Whelan *et al.* 2010). Nevertheless, we cannot discount a direct effect of inflammasome-derived pro-inflammatory cytokines on cardiac contractility, calcium homeostasis and excitation–contraction coupling. Likewise, we cannot determine specifically the causal relationship between myocardial apoptosis and infiltrating immune cells.

The cardiac-specific calcineurin-overexpressing mouse represents an experimental tool that is representative of the long-term sequelae of inflammatory and structural heart failure, including hypertrophy, regional ischaemia and progressive deterioration in both systolic and diastolic function (Lim *et al.* 2000; Somers *et al.* 2008). Our findings of improved cardiac structure and function with Nlrp3 blockade are limited, in that previous reports of IL-1β downstream of Nlrp3 have centred on myeloid cells. Our findings are consistent with intracardiac inflammasome activation; however, these observations remain correlational, because we were unable truly to distinguish between inflammatory and non-inflammatory cells for IL-1β production/secretion within the myocardium *in vivo*. Nevertheless, these results demonstrate that the Nlrp3 inflammasome is activated in the myocardium of CNTg mice with structural heart disease and that IL-1β maturation downstream of Nlrp3-dependent caspase-1 activation plays a critical role in the progression of systolic dysfunction. We show that the Nlrp3 inflammasome participates in chronic disease progression and identify its molecular constituents as potential targets in the clinical management of congestive heart failure.

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