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Inflammation and NLRP3 Inflammasome Activation Initiated in Response to Pressure Overload by Ca²⁺/Calmodulin-Dependent Protein Kinase II δ Signaling in Cardiomyocytes Are Essential for Adverse Cardiac Remodeling

BACKGROUND: Inflammation is associated with cardiac remodeling and heart failure, but how it is initiated in response to nonischemic interventions in the absence of cell death is not known. We tested the hypothesis that activation of Ca²⁺/calmodulin-dependent protein kinase II δ (CaMKII δ) in cardiomyocytes (CMs) in response to pressure overload elicits inflammatory responses leading to adverse remodeling.

METHODS: Mice in which CaMKII δ was selectively deleted from CMs (cardiac-specific knockout [CKO]) and floxed control mice were subjected to transverse aortic constriction (TAC). The effects of CM-specific CaMKII δ deletion on inflammatory gene expression, inflammasome activation, macrophage accumulation, and fibrosis were assessed by quantitative polymerase chain reaction, histochemistry, and ventricular remodeling by echocardiography.

RESULTS: TAC induced increases in cardiac mRNA levels for proinflammatory chemokines and cytokines in ≤ 3 days, and these responses were significantly blunted when CM CaMKII δ was deleted. Apoptotic and necrotic cell death were absent at this time. CMs isolated from TAC hearts mirrored these robust increases in gene expression, which were markedly attenuated in CKO. Priming and activation of the NOD-like receptor pyrin domain-containing protein 3 inflammasome, assessed by measuring interleukin-1 β and NOD-like receptor pyrin domain-containing protein 3 mRNA levels, caspase-1 activity, and interleukin-18 cleavage, were increased at day 3 after TAC in control hearts and in CMs isolated from these hearts. These responses were dependent on CaMKII δ and associated with activation of Nuclear Factor-kappa B and reactive oxygen species. Accumulation of macrophages observed at days 7 to 14 after TAC was diminished in CKO and, by blocking Monocyte Chemoattractant Protein-1 signaling, deletion of CM Monocyte Chemoattractant Protein-1 or inhibition of inflammasome activation. Fibrosis was also attenuated by these interventions and in the CKO heart. Ventricular dilation and contractile dysfunction observed at day 42 after TAC were diminished in the CKO. Inhibition of CaMKII, Nuclear Factor-kappa B, inflammasome, or Monocyte Chemoattractant Protein-1 signaling in the first 1 or 2 weeks after TAC decreased remodeling, but inhibition of CaMKII after 2 weeks did not.

CONCLUSIONS: Activation of CaMKII δ in response to pressure overload triggers inflammatory gene expression and activation of the NOD-like receptor pyrin domain-containing protein 3 inflammasome in CMs. These responses provide signals for macrophage recruitment, fibrosis, and myocardial dysfunction in the heart. Our work suggests the importance of targeting early inflammatory responses induced by CM CaMKII δ signaling to prevent progression to heart failure.

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Clinical Perspective

What Is New?

- We establish a mechanism by which cardiac inflammation is initiated in response to hemodynamic stress in the absence of significant cardiomyocyte cell death.
- This is the first study to reveal a central and critical role for cardiomyocytes and signaling through CaMKII δ in initiating inflammatory responses in the heart.
- The NOD-like receptor pyrin domain-containing protein 3 inflammasome is activated in cardiomyocytes and contributes to macrophage recruitment, fibrosis, and ventricular dysfunction in response to pressure overload.

What Are the Clinical Implications?

- Results of recent clinical trials implicate interleukins, generated through NOD-like receptor pyrin domain-containing protein 3 inflammasome, in adverse cardiac effects associated with coronary artery disease and heart failure.
- Our studies reveal sites and mechanisms of proinflammatory gene and inflammasome activation within cardiomyocytes that could serve as targets for early intervention or disease prevention.

Hear failure is associated with adverse ventricular remodeling and remains a leading cause of death worldwide. Inflammation is an accepted driver of adverse remodeling after acute myocardial infarction.^{1,2} Cardiac inflammation is triggered, in response to ischemic stress, by the release of alarmins or damage associated molecular patterns (DAMPs) from dying cardiomyocytes (CMs). These induce expression of genes for proinflammatory chemokines and cytokines, resulting in immune cell recruitment and a cascade of inflammatory events.²⁻⁵ The NOD-like receptor pyrin domain containing protein 3 (NLRP3) inflammasome, a multiprotein signaling complex, is also assembled and activated in response to DAMPs to catalyze generation of the active forms of the potent proinflammatory cytokines interleukin [IL]-1 β and IL-18 and is thus another critical component of cardiac inflammation.^{6,7}

Growing evidence indicates that cardiac inflammation can also occur under nonischemic conditions. Early studies demonstrated that inflammatory genes and inflammatory cell infiltration occurred in hearts of mice after transverse aortic constriction (TAC),^{8,9} and other work shows that striking increases in the accumulation of macrophages and T-cells in the heart within days to weeks of TAC, are linked to fibrosis and adverse ventricular remodeling.¹⁰⁻¹⁴ The question of how pressure overload triggers inflammatory gene expression and

immune cell recruitment in the absence of ischemia-induced cell death and accompanying release of factors from dying cells has not, however, been addressed. Recent papers have also linked activation of the NLRP3 inflammasome to ischemic stress and subsequent adverse remodeling.¹⁵⁻¹⁷ Whether the inflammasome is assembled and activated in CMs is controversial, and whether or how this might be triggered in the absence of cell damage signals is unclear.

The multifunctional Ca²⁺/calmodulin regulated kinase δ (CaMKII δ) is rapidly activated in response to pressure overload.¹⁸ However our studies and those of others using CaMKII δ knockout (KO) mice revealed that CaMKII δ is not required for the development of pathological hypertrophy but instead for the progression from hypertrophy to heart failure.¹⁹⁻²¹ We also reported that CM CaMKII δ overexpression induces heart failure and that the resulting phenotypic changes are not rescued by normalizing sarcoplasmic reticulum calcium handling, inhibiting the mitochondrial permeability transition pore or blocking β -adrenergic receptors.²²⁻²⁴ These findings suggest that there is an as yet undefined pathway by which CaMKII δ activation mediates progression to heart failure.

Work from our group and others established that CaMKII δ contributes to inflammatory gene expression in response to ischemic injury and that this occurs through activation of Nuclear Factor-kappa B (NF κ B).²⁵⁻²⁷ This finding lead us to hypothesize that inflammation could be initiated in response to nonischemic stress in the absence of significant CM cell death and DAMP release by activation of CaMKII δ in CMs. In the present study, we used conditional CM-specific CaMKII δ KO (CKO) mice to demonstrate that CM generate inflammatory chemokines and cytokines and are the initial site of NLRP3 inflammasome activation. We further identify a causal role for CaMKII δ -mediated activation of the NLRP3 inflammasome and inflammatory responses in macrophage recruitment, cardiac fibrosis, and development of heart failure induced by pressure overload.

METHODS

The data, analytic methods, and study materials will be made available to other researchers as requested for purposes of reproducing the results, replicating the procedures, or for collaborative studies. Detailed Materials and Methods are presented in the [online-only Data Supplement](#).

Statistical Analysis

All results are presented as mean \pm SEM. Comparisons of 2 groups were accomplished using Mann-Whitney U test or unpaired Student's *t* test where appropriate. Experiments with >2 groups were compared by 1-way or 2-way ANOVA followed by Tukey's or Bonferroni's multiple comparisons test. All statistics were calculated using GraphPad Prism 7 (GraphPad Software Inc). *P* values <0.05 were considered statistically significant.

Study Approval

All animal protocols were approved by the Institutional Animal Care and Use Committee of University of California San Diego. Investigations using human heart were approved by Stritch School of Medicine, Loyola University, and the Veterans Administration San Diego.

RESULTS

TAC Induces Inflammatory Genes Through CM CaMKII δ

To determine whether inflammation is an early response to pressure overload-induced CaMKII δ activation, we examined the time course of inflammatory gene expression in response to TAC in CaMKII δ fl/fl (control [CTL]) and CKO mice. CKO mice generated by cre expression in CMs show nearly complete loss of CM CaMKII δ (Figure 1A and 1B in the online-only Data Supplement). There were no baseline differences in ventricular structure or function between CaMKII δ ^{fl/fl} CTL and CKO mice.²⁶ Heart rate and pressure gradients after TAC were also equivalent in the 2 lines (Table in the online-only Data Supplement). Hearts were isolated at various times after TAC, and mRNA prepared for quantitative polymerase chain reaction qPCR analysis of a range of proinflammatory genes (Figure 1). The mRNA levels for Monocyte Chemoattractant Protein-1 (MCP-1), Macrophage inflammatory Protein 1-alpha, C-X-C motif ligand 1 (CXCL1), and IL-6 were significantly elevated by day 1.5 and peaked at day 3, with 5- to 35-fold increases in CTL versus sham. All of these responses were decreased by \approx 40% in CKO hearts. Other genes evaluated, including C-X-C motif ligand 2 (CXCL2), IL-1 β , IL-18, and tumor necrosis factor α , increased somewhat more slowly and less transiently but also showed significantly attenuated responses in the hearts of CKO mice.

TAC Activates CaMKII δ and NF κ B Signaling Required for Early Regulation of Inflammatory Gene Expression

CaMKII activation was assessed using a ³²P enzymatic activity assay to extend our previous work using autophosphorylation as a readout.¹⁸ The fraction of autonomously active (CaM independent) CaMKII δ activity was demonstrated to be increased 1.6-fold (Figure 1I in the online-only Data Supplement) at 1.5 days after TAC. We next determined whether NF κ B, a master regulator of inflammation, was activated through CaMKII δ as an early response to TAC. NF κ B activation, assessed by immunoblotting for increases in the p65 NF κ B subunit in nuclear fractions, was increased in CTL hearts at days 1.5 and 3 after TAC, and this response was significantly attenuated in CKO hearts (Figure 2A). To assess the involvement of NF κ B activation in the induction of inflammatory genes, mice were injected with BMS-345541, an inhibitor of I κ B

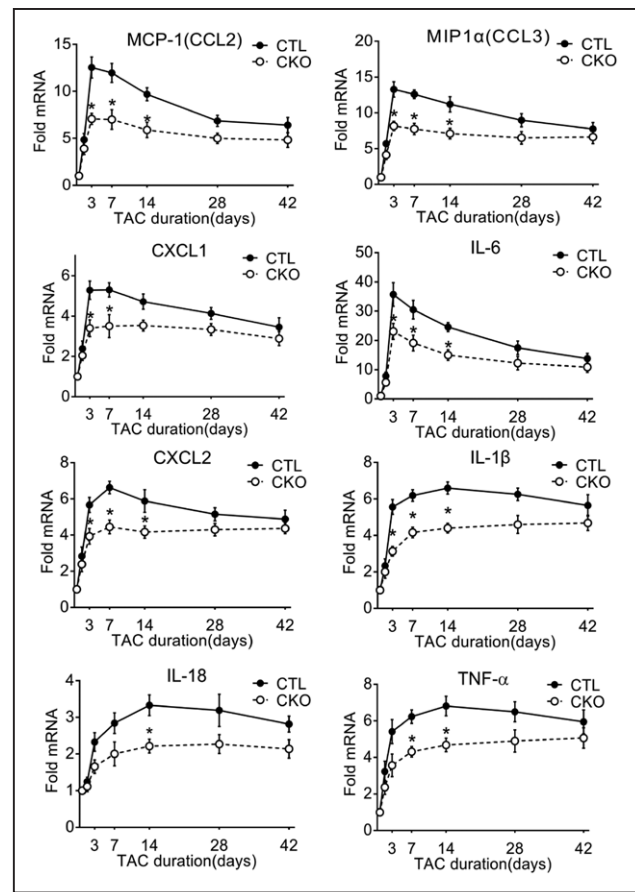


Figure 1. Pressure overload induces inflammatory genes through cardiomyocyte CaMKII δ .

Monocyte chemoattractant protein 1 (MCP-1/CCL2), macrophage inflammatory protein 1 α (MIP1 α /CCL3), C-X-C motif ligand 1 (CXCL1), C-X-C motif ligand 2 (CXCL2), interleukin 6 (IL-6), interleukin 1 β (IL-1 β), interleukin 18 (IL-18), and tumor necrosis factor α (TNF α) mRNA in whole ventricular lysates as measured by qPCR, normalized for the internal control GAPDH and expressed as fold increase over sham. N=6 to 8 per group (male=3–4, female=3–4). Data are presented as mean \pm SEM. * P <0.05 versus control (CTL) (fl/fl) TAC by 2-way ANOVA followed by Bonferroni's multiple comparisons test. CKO indicates cardiomyocyte-specific CaMKII δ KO mice; CTL, control floxed CaMKII δ mice; CXCL1, C-X-C motif ligand 1; CXCL2, C-X-C motif ligand 2; IL-6, interleukin 6; IL-1 β , interleukin 1 β ; IL-18, interleukin 18; MCP-1(CCL2), monocyte chemoattractant protein 1; MIP1 α (CCL3), macrophage inflammatory protein 1 α ; TAC, transverse aortic constriction; TNF- α , tumor necrosis factor α ; qPCR, quantitative polymerase chain reaction.

kinase, an upstream regulator of NF κ B. This led to nearly complete inhibition of TAC-induced increases in inflammatory gene mRNA (Figure 2B).

CaMKII δ -Dependent Inflammatory Gene Expression Is Initiated in CMs Versus Non-CMs

To demonstrate that CMs are the initial site of inflammatory gene expression in the heart, we isolated both adult mouse ventricular CMs and non-CMs from CTL and CKO mice at day 3 after TAC. The isolation procedures, detailed in Materials and Methods, yielded CM preparations with minimal contamination with immune cells, endothelial

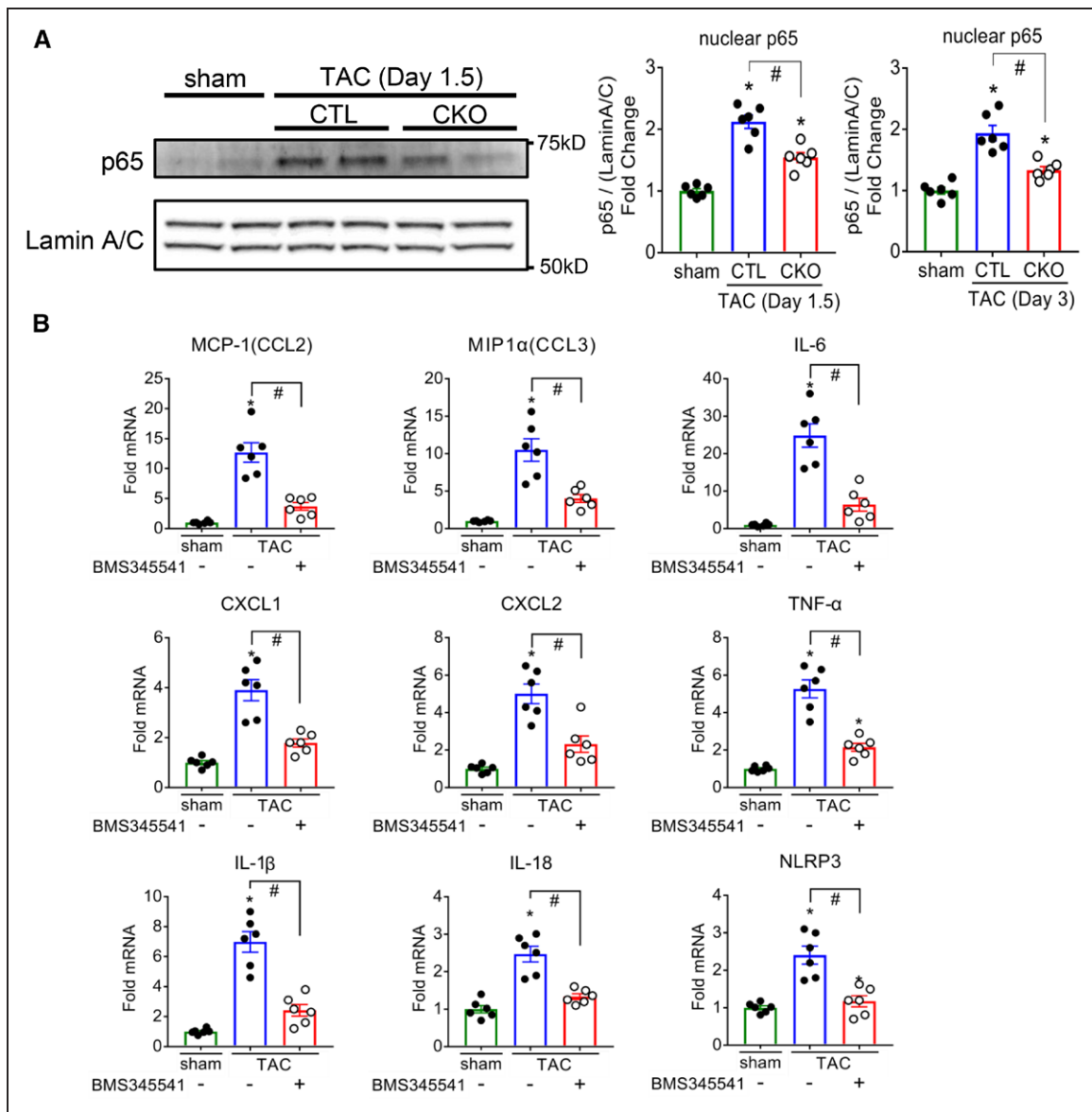


Figure 2. Cardiomyocyte CaMKII δ mediates pressure overload-induced NF κ B activation, which is required for inflammatory gene expression.

A, Nuclear fractions were prepared from ventricles and immunoblotted for p65 NF κ B subunit. Lamin A/C was used as a nuclear loading control. Representative immunoblot image and quantification of pooled data. N=6 per group (male=3, female=3). **B**, Mice were injected intraperitoneally every 12 hours with saline (–) or 45 mg/kg BMS345541(+) starting 1 hour before TAC surgery and for the 3 days before termination of the experiment. Inflammatory gene expression in whole ventricular lysates as measured by qPCR, normalized for the internal control GAPDH and expressed as fold increase over sham. N=6 per group (male=3, female=3). Data are presented as mean \pm SEM. * P <0.05 versus sham; # P <0.05 versus control (CTL) TAC by 1-way ANOVA followed by Tukey's multiple comparisons test. CKO indicates cardiomyocyte-specific CaMKII δ KO mice; CTL, control flox/flox CaMKII δ mice; CXCL1, C-X-C motif ligand 1; CXCL2, C-X-C motif ligand 2; IL-6, interleukin 6; IL-1 β , interleukin 1 β ; IL-18, interleukin 18; MCP-1(CCL2), monocyte chemoattractant protein 1; MIP1 α (CCL3), macrophage inflammatory protein 1 α ; NLRP3, NOD-like receptor pyrin domain containing protein 3; qPCR, quantitative polymerase chain reaction; TAC, transverse aortic constriction; and TNF- α , tumor necrosis factor α .

cells, or fibroblasts, as assessed by Western blotting for CD45, CD31(PECAM-1), and PDGFR α , respectively (Figure IC in the online-only Data Supplement). Inflammatory gene mRNA levels in the isolated CMs were increased to an extent similar in magnitude to that observed in the whole heart lysate and attenuated by 70% to 80% in CKO versus CTL cells (Figure 3). In contrast, significant increases in MCP-1, Macrophage inflammatory Protein 1-alpha, and IL-6 gene expression were not seen in the non-CM cells separated from the CMs at day 3 after TAC (Figure III in the online-only Data Supplement). These data

provide evidence that TAC regulates inflammatory gene expression within the CMs and, through activation of CaMKII δ , at the onset of pressure overload stress.

TAC Does Not Induce Apoptotic or Necrotic Cell Death \leq 7 Days

CM cell death leads to the release of DAMPs, which stimulate inflammation.^{2,4} Thus, we sought to rule out the possibility that TAC induces inflammation through such a mechanism. We examined hearts after 3 and 7

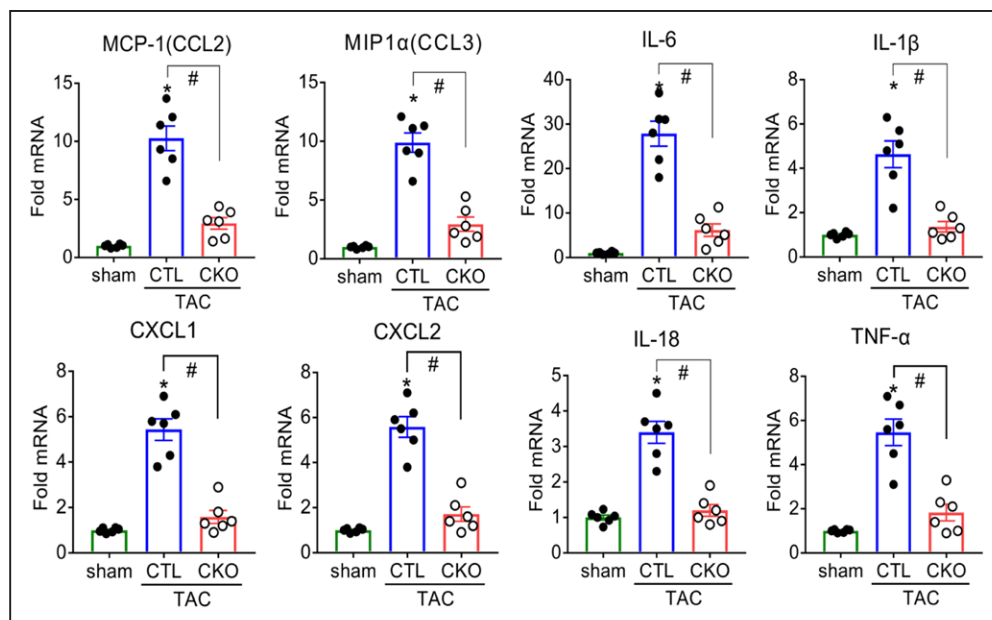


Figure 3. Robust CaMKII δ dependent inflammatory gene expression is evident in ventricular myocytes isolated from hearts at day 3 after TAC. Monocyte chemoattractant protein 1 (MCP-1/CCL2), macrophage inflammatory protein 1 α (MIP1 α /CCL3), C-X-C motif ligand 1 (CXCL1), C-X-C motif ligand 2 (CXCL2), interleukin 6 (IL-6), interleukin 1 β (IL-1 β), interleukin 18 (IL-18), and tumor necrosis factor α (TNF α) mRNA in adult mice ventricular cardiomyocytes (CMs) isolated at day 3 of TAC as measured by qPCR, normalized for the internal control GAPDH, and expressed as fold increase over sham. * P <0.05 versus sham; # P <0.05 versus CTL TAC. N =6 per group (male=3, female=3). Data are presented as mean \pm SEM. * P <0.05 versus sham; # P <0.05 versus CTL TAC by 1-way ANOVA followed by Tukey's multiple comparisons test. CKO indicates cardiomyocyte-specific CaMKII δ KO mice; CTL, control flox/flox CaMKII δ mice; CXCL1, C-X-C motif ligand 1; CXCL2, C-X-C motif ligand 2; IL-6, interleukin 6; IL-1 β , interleukin 1 β ; IL-18, interleukin 18; MCP-1(CCL2), monocyte chemoattractant protein 1; MIP1 α (CCL3), macrophage inflammatory protein 1 α ; qPCR, quantitative polymerase chain reaction; TAC, transverse aortic constriction; and TNF- α , tumor necrosis factor α .

days of TAC and observed no increase in Evans blue dye uptake, TUNEL staining, caspase-3 cleavage, or caspase-3 activation (Figure IV in the online-only Data Supplement). Only at later times of TAC were caspase-3 cleavage and caspase-3 activity increased (Figure IVB and IVC in the online-only Data Supplement). We also examined the Interferon regulatory factor 3-dependent gene program shown to be activated in response to cytosolic dsDNA released from CMs after myocardial infarction (MI).²⁸ Increases in these genes were seen after MI surgery but not at either day 3 or 7 after TAC (Figure V in the online-only Data Supplement).

Deletion of CaMKII δ in CMs Attenuates TAC-Induced Accumulation of CD68+ Macrophages

To determine whether macrophage recruitment was elicited by TAC through activation of CaMKII δ in CMs, we immunostained cardiac tissue sections with the macrophage marker CD68. CD68+ macrophage accumulation was markedly increased at days 7 and 14 after TAC and significantly attenuated in CKO mice (Figure 4A and 4B). A similar response was observed using F4/80 staining (Figure VIA and VIB in the online-only Data Supplement). Treatment with the CaMKII inhibitor KN-93 for the first week of TAC also reduced TAC-induced CD68-positive cell accumulation at day 14 (Figure 4C), confirming that the role of CaMKII δ in TAC-induced macrophage

accumulation was not because of secondary effects of CaMKII δ gene deletion. There was also upregulation of mRNA for IL-10 and transforming growth factor- β , cytokines considered to be products of macrophages^{29,30} that were attenuated in the CKO versus CTL (Figure 4D).

The NLRP3 Inflammasome Is Activated in Response to TAC Through CM CaMKII δ

The NLRP3 inflammasome is a critical mediator of inflammatory responses, but whether it is activated in CMs remains to be established. We observed (Figures 2B and 3) that mRNA levels for IL-1 β and IL-18, cytokines processed by the NLRP3 inflammasome, were increased through CaMKII δ and NF κ B signaling. NLRP3, a main component of the NLRP3 inflammasome, was also increased at the mRNA level in whole heart homogenates at days 1.5 and 3 after TAC and attenuated in CKO (Figure 5A) or when NF κ B was inhibited using BMS345541 (Figure 2B). NLRP3 protein was also increased at day 3 after TAC and significantly lower in CKO mice (Figure VII in the online-only Data Supplement).

Activation of inflammasomes leads to cleavage of pro-caspase-1 to its catalytically active form; thus, increases in caspase-1 activity are indicative of inflammasome activation. We observed significant increases in caspase-1 activity in whole heart homogenates at days 1.5 and 3 after TAC (Figure 5B), and this response was decreased in hearts of CKO mice. A proposed mechanism for activation

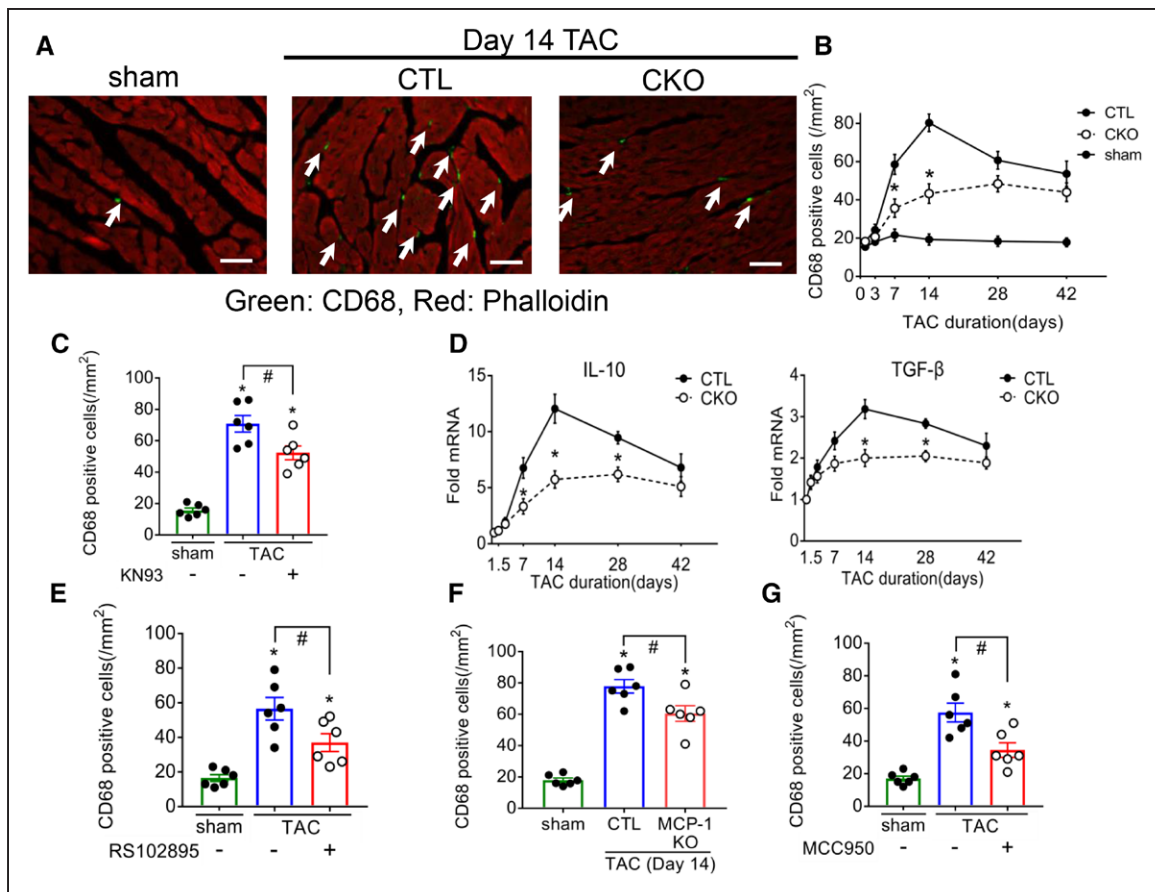


Figure 4. Deletion of CaMKII δ in cardiomyocytes attenuates pressure overload-induced accumulation of CD68+ macrophages and their products in the heart.

A, Representative image of cardiac section stained with CD68+ antibody (green: marked by arrows) for macrophage and phalloidin (red) for cardiomyocyte, obtained at day 14 of TAC. Scale bar=50 μ m. **B**, Quantification of CD68+ cells in cardiac sections from sham, control (CTL), and CKO mice subjected to 3, 7, 14, 28, and 42 days of TAC. Data were quantified from 4 to 6 images per sample. N=6 per group (male=3, female=3). Data are presented as mean \pm SEM. * P <0.05 versus CTL (fl/fl) TAC by 2-way ANOVA followed by Tukey's multiple comparisons test. **C**, Mice injected with saline (-) or 5 mg/kg of the CaMKII inhibitor KN93 (+) starting 1 hour before and daily up to 7 days TAC were harvested at day 14, and sections were stained for CD68, N=6 per group (male=3, female=3). Data are presented as mean \pm SEM. * P <0.05 versus sham; # P <0.05 versus CTL TAC by 1-way ANOVA followed by Tukey's multiple comparisons test. **D**, TGF- β and IL-10 mRNA in whole ventricular lysates as measured by qPCR, normalized for the internal control GAPDH and expressed as fold increase over sham. N=6 per group (male=3, female=3). Data are presented as mean \pm SEM. * P <0.05 versus CTL (fl/fl) TAC by 2-way ANOVA followed by Bonferroni's multiple comparisons test. **E–G**, CD68-positive cells quantitated at day 14 of TAC. **E**, Mice were injected intraperitoneally with saline (-) or 10 mg/kg RS102895 (+) 1 hour before TAC and every 6 hours for 3 days to block the MCP-1 receptor. **F**, Cardiac-specific MCP-1 KO mice were subjected to TAC. **G**, Mice were injected intraperitoneally with saline (-) or 10 mg/kg MCC950 (+) 1 hour prior to TAC and twice daily for 7 days to inhibit the inflammasome. N=6 per group (male=3, female=3). Data are presented as mean \pm SEM. * P <0.05 versus sham; # P <0.05 versus CTL TAC by 1-way ANOVA followed by Tukey's multiple comparisons test. CKO indicates cardiomyocyte-specific CaMKII δ KO mice; CD68, cluster of differentiation 68; CTL, control flox/flox CaMKII δ mice; IL-10, interleukin 10; qPCR, quantitative polymerase chain reaction; TAC, transverse aortic constriction; and TGF- β , transforming growth factor β .

of the inflammasome is through as yet undefined effects of reactive oxygen species (ROS).³¹ Accordingly, we tested the hypothesis that CaMKII δ regulates inflammasome activation after pressure overload through ROS generation. DCFDA fluorescence staining of CTL and CKO mouse ventricle sections indicated that 3-day TAC increases cardiac ROS and that this occurs through CaMKII δ (Figure VIII A in the online-only Data Supplement). We also assessed and quantitated mitochondrial ROS generation by isolating mitochondria from hearts after TAC and measuring mitochondrial protein carbonylation as an indicator of mitochondrial ROS production (Figure VIII B in the online-only Data Supplement). Mitochondrial protein carbonylation was increased by TAC in CTL but not in CKO. To test the role of mitochondrial ROS in cardiac inflamma-

some activation, mice were injected with the mitochondrial targeted ROS scavenger MitoTEMPO. TAC-induced ROS accumulation was inhibited (Figure VIII A and VIII B in the online-only Data Supplement), concomitant with significant inhibition of TAC-induced caspase-1 activation (Figure VIII C in the online-only Data Supplement).

To assess the extent to which the observed inflammasome activation occurs in CMs, we separated CMs and non-CMs from hearts harvested at day 3 after TAC. NLRP3 mRNA and caspase-1 activity were both increased in CMs but not in the non-CM fraction expected to contain fibroblasts, immune cells, and endothelial cells (Figure 5C and 5D). Changes in this fraction did occur later, as shown for day 7 after TAC (Figure IX in the online-only Data Supplement). IL-18 cleavage to its active product by caspase-1

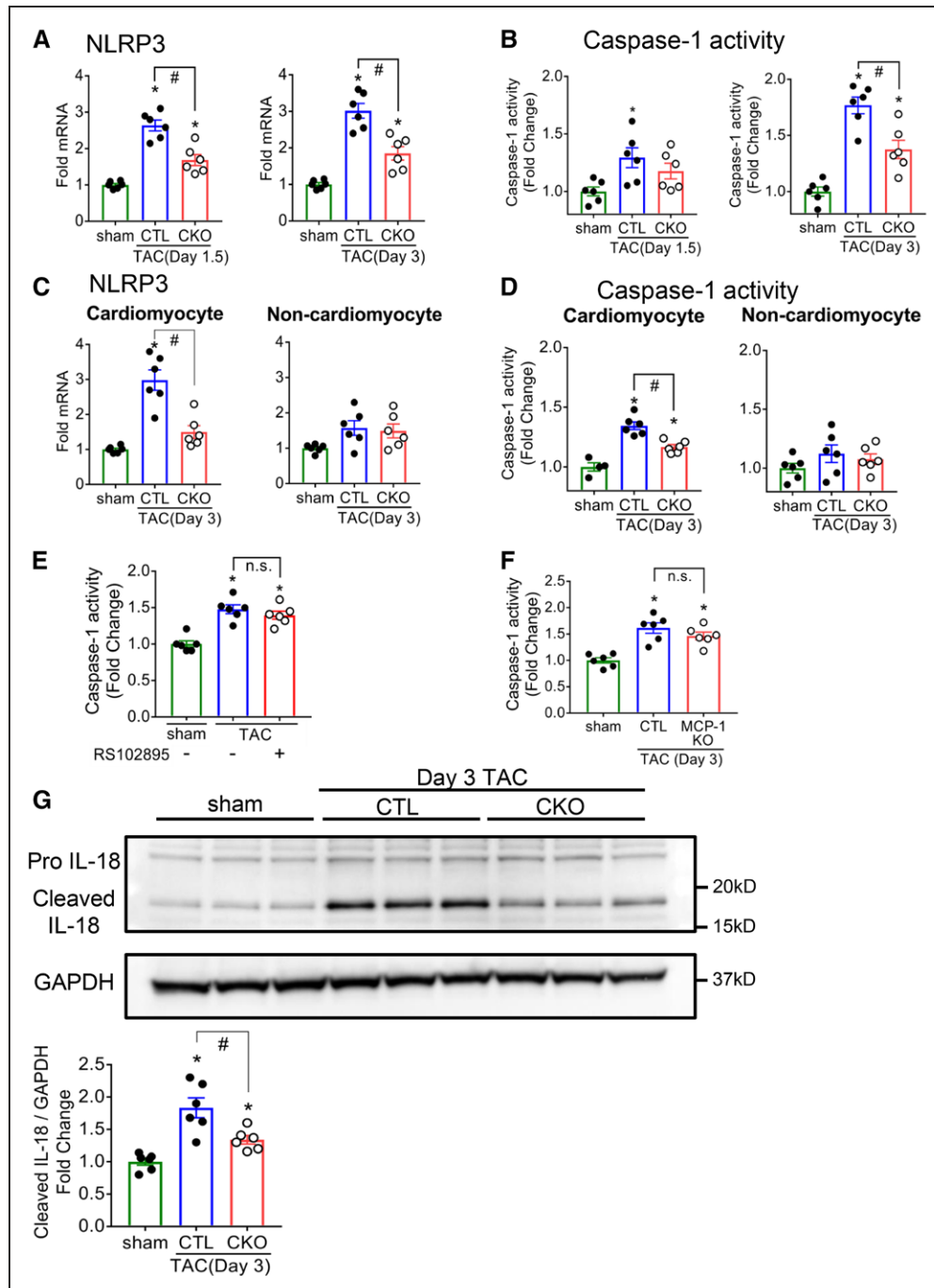


Figure 5. Inflammasome is activated in response to pressure overload through cardiomyocyte (CM) CaMKII δ .

NOD-like receptor family pyrin domain-containing protein 3 (NLRP3) mRNA in whole-heart homogenate (A) or isolated CMs (C) as measured by qPCR, normalized for the internal control GAPDH and expressed as fold increase over sham. Caspase-1 activity in whole-heart homogenate (B, E, and F) or isolated CMs (D) was assessed using a fluorometric caspase-1 activity assay. G, Pro and cleaved IL-18 was determined by immunoblotting in isolated CMs. E, Control (CTL) mice were injected intraperitoneally with saline (-) or 45 mg/kg BMS345541 (+) 1 hour before TAC surgery and twice daily for 3 days. F, Cardiac-specific MCP-1 KO mice were subjected to TAC. N=6 per group (male=3, female=3). Data are presented as mean \pm SEM. * P <0.05 versus sham; # P <0.05 versus CTL TAC by 1-way ANOVA followed by Tukey's multiple comparisons test. CKO indicates cardiomyocyte-specific CaMKII δ KO mice; CTL, control flox/flox CaMKII δ mice; GAPDH, glyceraldehyde-phosphate dehydrogenase; IL-18, interleukin 18; NLRP3, NOD-like receptor pyrin domain containing protein 3; MCP-1, monocyte chemoattractant protein 1; qPCR, quantitative polymerase chain reaction; and TAC, transverse aortic constriction.

is an additional indicator of inflammasome activation. Western blotting of CMs isolated from CTL mouse heart revealed that a significant fraction of IL-18 protein was present in its cleaved form at day 3 after TAC (Figure 5G), and this response was diminished in CMs from CKO mice.

To directly visualize activation of caspase-1 after TAC, we used a fluorescence indicator of caspase-1 activity (FAM-FLICA caspase-1) to image caspase activation in tissue sections (Figure 6A) and quantitated this in isolated CMs (Figure 6B and 6C). Caspase-1 activation was

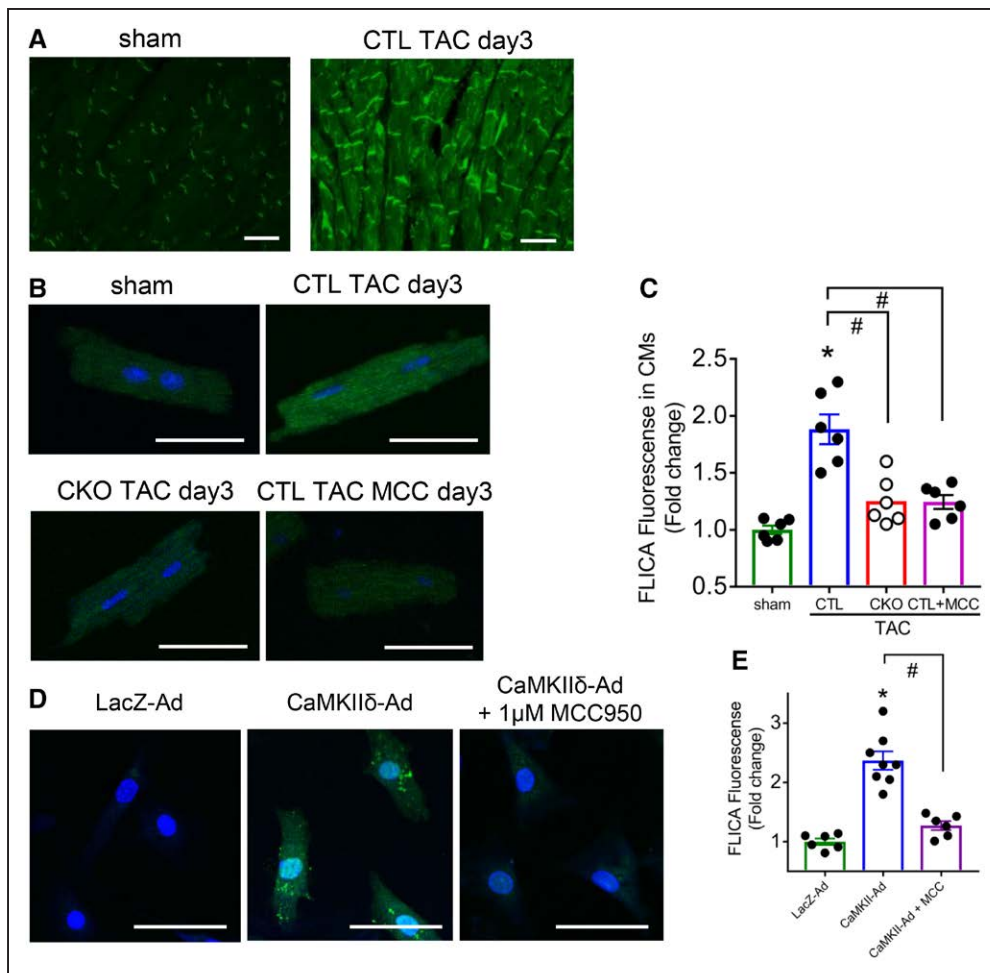


Figure 6. Visualization of inflammasome activation in cardiomyocytes (CMs) in response to pressure overload.

Representative image in cardiac section (A) and CMs (B) at day 3 of TAC. FAM-FLICA Caspase-1 assay kit was used to visualize caspase-1 activity. Scale bar=50 μ m. Mice were injected intraperitoneally with saline (-) or 10 mg/kg MCC950 (+) 1 hour before TAC and twice daily for 3 days. C, Quantification of FAM-FLICA Caspase-1 fluorescent signal in CMs. Data were quantified from 4 to 8 images per sample. N=6 per group (male=3, female=3). Data are presented as mean \pm SEM. * P <0.05 versus sham; # P <0.05 versus control (CTL) TAC by 1-way ANOVA followed by Tukey's multiple comparisons test. D, Representative image and (E) quantification of fluorescence in NRVMs infected with AdLacZ or the constitutively active δ C isoform of CaMKII (AdCaMKII δ) at a multiplicity of infection of 50. NRVMs were starved for 5 hours before a 3-hour infection and then washed with serum-free medium and cultured overnight. MCC950 (1 μ mol/L) was applied at the time of virus infection and medium exchange. FAM-FLICA Caspase-1 assay kit was used to visualize caspase-1 activity. Scale bar=50 μ m. N=6 to 8 per group. Data are presented as mean \pm SEM. * P <0.05 versus LacZ-Ad; # P <0.05 versus CaMKII δ -Ad by 1-way ANOVA followed by Tukey's multiple comparisons test. CaMKII δ -Ad indicates adenovirus expressing CaMKII δ ; CKO, cardiomyocyte-specific CaMKII δ KO mice; CTL, control flox/flox CaMKII δ mice; LacZ-Ad, adenovirus expressing β -galactosidase; MCC, MCC950; NRVM, neonatal rat ventricular myocyte; qPCR, quantitative polymerase chain reaction; and TAC, transverse aortic constriction.

significantly increased by TAC in CTL mouse CMs and virtually abolished in those from CKO (Figure 6B and 6C). The specificity of the FLICA fluorescence signal for caspase-1 was demonstrated by loss of this response in mice treated with MCC950, an inhibitor of NLRP3 inflammasome activation. Finally, expressing constitutively active CaMKII δ in neonatal rat ventricular myocytes at a multiplicity of infection that does not induce cell death increased FLICA fluorescence in an NLRP3-dependent manner (Figure 6D and 6E).

Chronic Models of Heart Failure

To extend the findings obtained using the relatively acute TAC model, we also examined cardiac inflammatory responses in a chronic mouse model of heart fail-

ure achieved by 4 weeks of daily isoproterenol injection. We have demonstrated that development of fibrosis and contractile dysfunction in this model depends on CaMKII δ .³² The possibility that inflammatory responses also play a role in CaMKII δ signaling to heart failure in the isoproterenol model is suggested by our finding that the inflammatory genes MCP-1 and IL-6, as well as the inflammasome component NLRP3 and cleaved IL-18, are significantly upregulated in a CaMKII δ -dependent manner (Figure X in the online-only Data Supplement). As further validation that similar inflammatory responses occur in a clinically relevant chronic heart failure model, we compared human nonischemic cardiomyopathy and normal hearts (Figure XI in the online-only Data Supplement). We detected increased MCP-1, IL-6, IL-10, and IL-1 β mRNA, as well as increased NLRP-3 mRNA and

cleaved IL-18, in association with upregulated CaMKII δ in the heart failure samples.

Signaling to Macrophage Recruitment

MCP-1, which was upregulated by TAC through CaMKII δ in CMs (Figures 1 and 3), is known to recruit monocytes to tissues where they differentiate to macrophages. Monocyte recruitment by MCP-1 occurs through its interaction with its receptor C-C chemokine receptor type 2. To determine whether MCP-1 plays a major role in regulation of macrophage accumulation after TAC, we treated CTL mice with RS102895, a highly selective small-molecule antagonist of C-C chemokine receptor type 2 (CCR2). This treatment decreased TAC-induced CD68-positive cell accumulation assessed at day 14 by 50% (Figure 4E). To directly determine whether the MCP-1 involved in this response was derived from CMs, we generated CM-specific MCP-1 KO, as shown in [Figure ID in the online-only Data Supplement](#), by crossing α MHC-Cre to MCP-1^{fl/fl} (The Jackson Laboratory) mice. CD68-positive cell accumulation in response to TAC was also significantly attenuated in these mice (Figure 4F). Inflammasome activation also contributes to macrophage recruitment as we found that treatment with MCC950 leads to \approx 60% inhibition of the TAC-induced accumulation of CD68-positive macrophages (Figure 4G). Thus, inflammasome-generated cytokines, as well as MCP-1, contribute to TAC- and CaMKII δ -induced macrophage recruitment. These data support our hypothesis that proinflammatory gene expression and inflammasome activation in CMs are the initiating CaMKII δ -mediated responses in the pathway leading to macrophage recruitment. Blocking macrophage recruitment with MCP-1 or inflammasome inhibitors did not decrease inflammatory gene expression assessed at day 3 after TAC ([Figure XIIA, XIIB, and XIIC in the online-only Data Supplement](#)), and inhibition of MCP-1 did not decrease caspase-1 activation (Figure 5E and 5F) indicating limited involvement of recruited macrophages in these early responses.

Deletion of CaMKII δ in CMs Attenuates TAC-Induced Fibrosis

Pressure overload leads to robust induction of myocardial fibrosis, as demonstrated by Masson's trichrome staining (Figure 7A). Fibrosis was diminished by 50% in hearts from CKO versus CTL mice (Figure 7A). The fibrotic gene markers collagen type 1 α 1, collagen type 3 α 1, and periostin were also upregulated by TAC in CTL hearts, and these increases, evident at days 7, 14s and 28, were significantly attenuated in CKO hearts (Figure 7B). Inhibition of CM MCP-1 signaling assessed in cardiac MCP-1 KO or reduction of inflam-

masome activation (by administering MCC950) also attenuated TAC-induced fibrosis (Figure 7C and 7D), paralleling the effects of these inhibitors on macrophage accumulation. These results support a critical role for CM CaMKII δ signaling in the development of fibrosis in the heart.

Early CaMKII δ Activation in CMs Initiates TAC-Induced Cardiac Remodeling

We implicated CaMKII δ in the transition of pressure overload-induced hypertrophy to ventricular dilation and dysfunction in the global CaMKII δ KO mouse.¹⁹ Here we demonstrate that ventricular dilation and decreases in ejection fraction at day 42 of TAC are also significantly diminished when CaMKII δ is selectively deleted from CMs (Figure 8A). To implicate early activation of CaMKII δ signaling pathways in the development of TAC-induced fibrosis and subsequent ventricular remodeling and dysfunction, we inhibited downstream mediators of the effects of CaMKII δ : NF κ B, NLRP3, and MCP-1. Pharmacological inhibition of NF κ B with BMS345541 or NLRP-3 with MCC950 1 hour before and for 7 days after TAC attenuated development of cardiac dilation and contractile dysfunction at 42 days after TAC ([Figure XIII A and XIII B in the online-only Data Supplement](#)), as did specific deletion of MCP-1 from CMs ([Figure XIII C in the online-only Data Supplement](#)).

To provide further evidence that early signaling actions of CaMKII δ in CMs trigger the ensuing maladaptive inflammation and remodeling, we compared the effects of blocking CaMKII δ signaling at various times. Mice treated with the CaMKII inhibitor KN-93 beginning 1 hour before and daily up to day 14 after TAC (Figure 8B, Protocol I) showed significantly inhibited fibrosis assessed at day 28 (ie, 2 weeks after KN93 treatment was discontinued) (Figure 8C). Ventricular dilation was also diminished, as was contractile dysfunction (Figure 8D). In contrast, when the 2-week treatment with KN-93 was delayed (Protocol II) until 14 days after initiation of TAC (a time at which macrophage accumulation is maximal), neither fibrosis nor cardiac dysfunction were attenuated (Figure 8C and 8E).

DISCUSSION

A small but compelling body of evidence has accumulated in recent years implicating inflammation in cardiac remodeling in response to pressure overload.^{10,13,14,33–36} The mechanism(s) by which maladaptive inflammatory responses to pressure overload are initiated, however, remain unknown. The studies presented here lead to several important new concepts relevant to this question. As summarized in [Figure XIV in the online-only Data Supplement](#), our data show that (1) signals generated in CMs, as opposed to the non-CM cell population

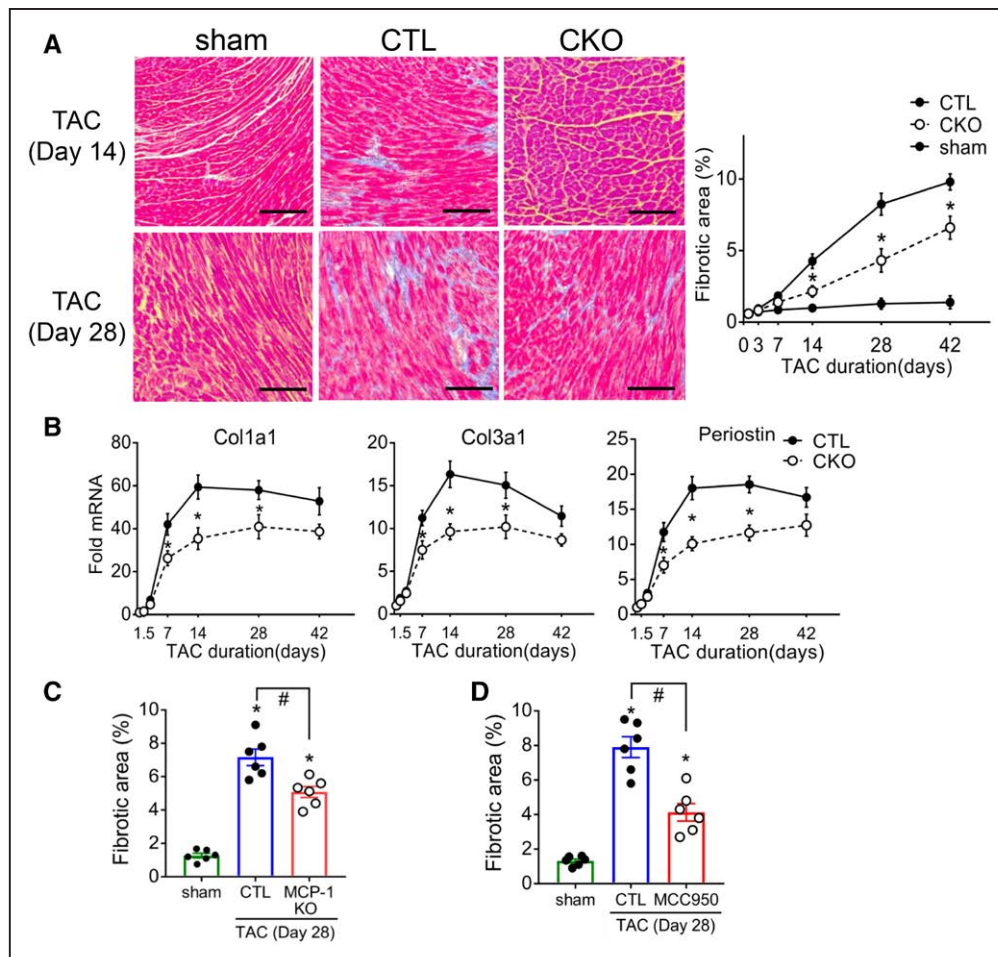


Figure 7. Deletion of CaMKII δ in cardiomyocytes attenuates pressure overload-induced fibrosis.

A, Representative image of cardiac sections stained with Masson's trichrome at 2 or 4 weeks after TAC. Connective tissue is stained blue. Scale bar=100 μ m. Quantification of % area showing fibrosis was calculated using ImageJ software. Data were quantified from 6 to 8 images per sample. N=6 per group (male=3, female=3). Data are presented as mean \pm SEM. * P <0.05 versus CTL (fl/fl) TAC by 2-way ANOVA followed by Tukey's multiple comparisons test. **B**, Fibrotic gene expression, Collagen Type I Alpha 1 Chain (Col1a1), Collagen Type III Alpha 1 Chain (Col3a1), and Periostin in whole ventricular lysates as measured by qPCR, normalized for the internal control GAPDH and expressed as fold increase over sham. N=6 per group (male=3, female=3). Data are presented as mean \pm SEM. * P <0.05 versus CTL (fl/fl) TAC by 2-way ANOVA followed by Bonferroni's multiple comparisons test. **C** and **D**, Cardiac sections quantitated for % area showing fibrosis at day 28 of TAC. **C**, Cardiac-specific MCP-1 KO mice were subjected to TAC. **D**, Mice were injected with saline or 10 mg/kg MCC950 1 hour before TAC and twice daily for 7 days. N=6 per group (male=3, female=3). Data are presented as mean \pm SEM. * P <0.05 versus sham; # P <0.05 versus CTL TAC by 1-way ANOVA followed by Tukey's multiple comparisons test. CKO indicates cardiomyocyte-specific CaMKII δ KO mice; Col1a1, collagen 1 α 1; Col3a1, collagen 3 α 1; CTL, control flox/flox CaMKII δ mice; GAPDH, glyceraldehyde-phosphate dehydrogenase; MCP-1, monocyte chemoattractant protein 1; qPCR, quantitative polymerase chain reaction; and TAC, transverse aortic constriction.

in the heart, are the initiators of inflammatory gene expression in response to pressure overload; (2) activation of CaMKII δ in CMs triggers NF κ B-mediated proinflammatory gene expression; (3) activation of CM CaMKII δ primes and activates the inflammasome through NF κ B and ROS signaling; (4) chemokines (eg, MCP-1) and products of inflammasome activation produced in CMs contribute to macrophage infiltration in the heart; (5) activation of CM CaMKII δ signaling pathways contributes to the development of fibrosis and adverse remodeling in response to pressure overload; and (6) fibrosis, ventricular dilation, and dysfunction can be attenuated by blocking CaMKII δ inflammatory signaling within the first 2 weeks of pressure overload but not later, after the onset of inflammatory cell accumulation.

Evidence That the TAC-Induced Inflammatory Pathway Is Unique From That Triggered by Cell Death

In response to ischemia, as occurs in MI, dying CMs release DAMPs or alarmins that trigger robust inflammatory cascades.^{2,4,5,37,38} We present several lines of evidence which argue that CM death does not occur during the first days to week after TAC, when activation of NF κ B, inflammatory gene expression, and the NLRP3 inflammasome are evident. CM necrosis and apoptosis, assessed by Evans blue dye uptake and TUNEL staining, as well as measurement of caspase-3 activity, were not changed during the first week, although cell death does occur at later

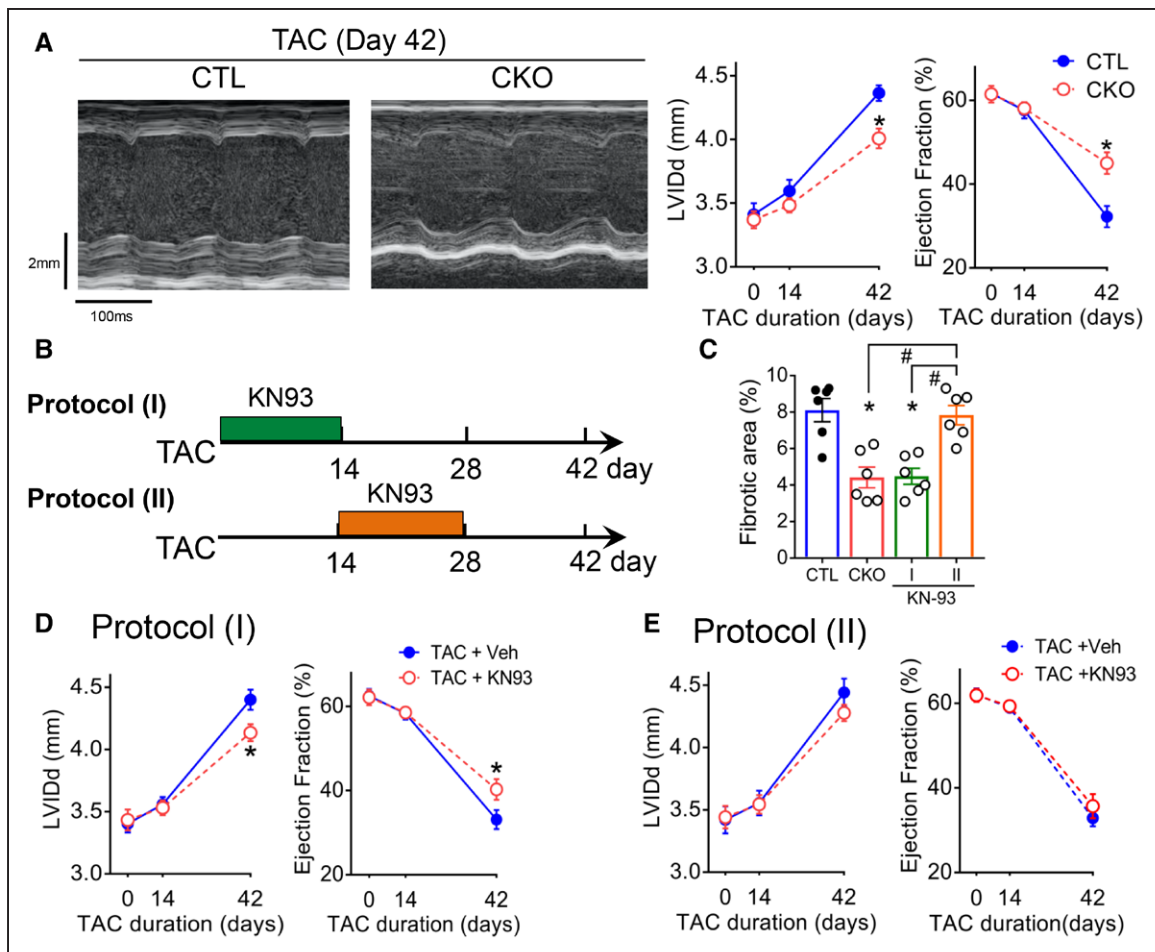


Figure 8. CaMKII δ activation at early times after TAC is required for subsequent cardiac remodeling.

A, left, Representative echocardiographs from CTL and CKO at 6 weeks of TAC. Scale bars=vertical 2.0 mm and horizontal 100 ms. **A, right,** Echocardiographic analysis of ventricular dilation and contractile function at various times after TAC. Cardiac dilation was evaluated by left ventricular end-diastolic internal dimension (LVIDd) and contractile function by ejection fraction. Heart rate values ranged from 495 to 540 bpm and were not significantly different between CTL TAC and CKO TAC. N=6 per group (male=3, female=3). Data are presented as mean \pm SEM. * P <0.05 versus CTL (fl/fl) TAC by 2-way ANOVA followed by Bonferroni's multiple comparisons test. **B,** Mice were injected intraperitoneally with 5 mg/kg of KN93 starting 1 hour before TAC and daily for 14 days (Protocol I) or injected with 5 mg/kg of KN93 starting at 14 days and daily up to 28 days (Protocol II). **C,** Quantification of % fibrotic area at day 28 of TAC. Data are presented as mean \pm SEM. * P <0.05 versus CTL; # P <0.05 versus protocol (II) by 1-way ANOVA followed by Tukey's multiple comparisons test. **D and E,** Echocardiographic analysis of ventricular dilation and contractile function at various times after TAC. Heart rate values ranged from 490 to 530 bpm and were not significantly different between TAC+Veh and TAC+KN93. Data are presented as mean \pm SEM. * P <0.05 versus TAC+Veh by 2-way ANOVA followed by Bonferroni's multiple comparisons test. CKO indicates cardiomyocyte-specific CaMKII δ KO mice; CTL, control flox/flox CaMKII δ mice; LVIDd, left ventricular internal diameter at diastole; TAC, transverse aortic constriction; and Veh, vehicle.

times as previously reported.¹⁹ Work from the Otsu laboratory, using mice in which DNase was deleted, provided evidence that TAC can induce the release of mitochondrial DNA, which, if not eliminated by autophagy-mediated degradation, can lead to inflammation and heart failure.³⁹ This outcome did not occur in wild-type mice; nonetheless, we considered the possibility of dsDNA serving as a DAMP after TAC. Interferon regulatory factor 3-dependent gene products, recently shown to result from signaling through the release of dsDNA in the mouse heart after MI,²⁸ were examined and found to not be induced at day 3 or 7 after TAC. Thus, although CaMKII activation has been associated with CM cell death,⁴⁰ the attenuated inflammation that we see in the CKO is unlikely to reflect diminished CaMKII δ signaling to apoptotic or necrotic responses.

Evidence That CMs Are the Initiating Site of Inflammatory Gene Expression

There is considerable evidence that cardiokines released from the heart can serve autocrine and paracrine functions.^{38,41} Although CMs could serve as sites of cardiokine generation, most studies have considered cardiac fibroblasts, endothelial cells, or resident immune cells as those that contribute to the production of inflammatory mediators. Our studies considered the question of whether the CM is the predominant site at which proinflammatory chemokines and cytokines are generated at early times after TAC. CMs isolated from hearts at day 3 after TAC showed TAC-induced increases in cytokine and chemokine mRNA expression levels equivalent to those seen in the whole heart homogenate and dependent on

CaMKII δ . In contrast, an increase in mRNA for these mediators was not observed in the non-CM fraction (eg, fibroblasts, endothelial cells, and immune cells) isolated from the heart subjected to 3-day TAC. Thus, although a portion of the increase in inflammatory gene expression seen in the whole heart homogenate at day 3 after TAC and at later times occurs independent of CM CaMKII δ signaling, the CM, through CaMKII δ , serves as a significant generator of proinflammatory mediators.

Studies using CM-specific MCP-1 KO mice provide evidence for the role of CMs in subsequent inflammation and cardiac remodeling. Specifically, we demonstrate that TAC-induced macrophage recruitment and fibrosis are diminished and heart failure development is attenuated when CM MCP-1 is deleted. Although loss of these TAC-mediated responses is not complete, these findings support the conclusion that an early increase in MCP-1 gene expression in CMs plays a role in initiating inflammatory responses and subsequent cardiac remodeling.

Site and Mechanism of Activation of the NLRP3 Inflammasome

There are established mechanisms by which NLRP3 inflammasome signaling in the heart would be triggered in response to ischemic cell death,^{2,4,42} when dying cells release substances that activate NF κ B (eg, dsDNA HMGB-1) and mediators of inflammasome activation (eg, ATP, ROS) are generated. How this might occur in the absence of cell death is not clear,²⁹ but recent publications demonstrate inflammasome activation in the heart after pressure overload and acute isoproterenol treatment.^{35,43} These reports suggest that the inflammasome is activated in CMs, specifically demonstrating increased CM-associated apoptosis-associated speck-like protein containing a caspase recruitment domain, NLRP3, and cleaved IL-18. Here we use visualization and enzymatic activity of caspase-1, as well as isolated CMs, to provide stronger evidence that CMs are the site at which the inflammasome is initially primed and activated in response to pressure overload and further demonstrate a mechanistic role for CaMKII δ in this response.

Using an enriched preparation of CMs isolated from hearts after TAC, we show that NLRP-3 induction and caspase-1 activation, assessed by a caspase-1 enzymatic activity and visualization of activated caspase-1 in CMs, are evident in the CM compartment at day 3. We also demonstrate increases in cleaved IL-18 in the CM compartment at this time. Neither caspase-1 activity nor NLRP-3 mRNA are significantly elevated in the non-CM fraction at day 3 after TAC. Thus, neither resident nor recruited macrophages appear to contribute to inflammasome activation at this early time nor do cardiac fibroblasts appear

to be involved. These data provide strong support for the concept that the CM is the site at which inflammasome activation is initiated to recruit immune cells and propagate inflammatory signaling in noncardiac cells.

Mechanistically, we demonstrate that the induction of IL-1 β , IL-18, and NLRP-3, as well as the activation of caspase-1 and cleavage of IL-18 apparent in the heart within 3 days of TAC, are attenuated when CaMKII δ is deleted from CMs. In addition, our studies using BMS345541 show that CaMKII δ activation in myocytes primes the inflammasome through the activation of NF κ B. We also elucidate the mechanism by which the second step, inflammasome activation, occurs. TAC increases mitochondrial ROS, an event that we show to be diminished in the CKO (ie, to be dependent on CaMKII δ signaling in CMs). The mechanism by which CaMKII δ increases ROS levels has not been clearly established, but earlier work from our group and studies from the Anderson laboratory demonstrate effects of CaMKII signaling on mitochondria that can affect Ca²⁺ uptake and thus mitochondrial ROS generation.^{21,44} Our experiments using Mito-TEMPO show that scavenging mitochondrial ROS attenuates TAC-induced caspase-1 activation, implicating ROS in this process. We also recently reported that the NLRP3 inflammasome is activated in response to angiotensin II-induced CaMKII δ activation and resultant ROS generation in CM.⁴⁵ Our conclusion that CaMKII δ mediates inflammasome activation, in addition to the extensive evidence that this occurs in CMs, substantially advance understanding of the sites and pathways by which cardiac inflammation is initiated in response to nonischemic stress.

The products of the NLRP3 inflammasome, IL-1 β and IL-18, are potent proinflammatory cytokines that have been implicated in macrophage recruitment, amplifying and sustaining cardiac inflammation.⁴³ These cytokines, as well as IL-10, have been shown to contribute to systolic and diastolic dysfunction through indirect effects on the development of myocardial fibrosis and CM apoptosis, as well as via direct effects on cardiac contractile function.^{30,42} Thus, NLRP3 inflammasome activation could function not only as an initiator of cardiac inflammation but also as a generator of cytokines that play a local role in causing CM dysfunction. In this regard, it is interesting to note that we also observe increases in IL-1 β , active cleaved IL-18, and IL-10 in hearts from patients with nonischemic cardiomyopathy, concomitant with elevated levels of CaMKII expression.

Temporal Aspects of Inflammatory Responses and Their Therapeutic Implications

To support our conclusion that the activation of CaMKII δ in CMs triggers events that initiate ensuing inflammatory responses and cardiac remodeling, we

asked whether blocking CaMKII δ signaling at early times in the course of pressure overload affected the subsequent development of heart failure. When mice received daily injections of inhibitors of either NF κ B or the NLRP3 inflammasome during the first week after TAC, contractile dysfunction and dilation monitored 6 weeks later were significantly attenuated. Additionally, treatment with the CaMKII inhibitor, KN93, during the first 2 weeks of TAC blocked the fibrotic response seen at 6 weeks after TAC to an extent equivalent to that seen in the CKO (ie, when CaMKII δ activation was abolished throughout the entire time period). In contrast, delayed treatment with KN93 beginning 2 weeks after the onset of pressure overload had no significant effect on the development of fibrosis, dilation, or contractile dysfunction. Consistent with these findings, a study published by the Prabhu laboratory demonstrated that when macrophage function was inhibited by inducible macrophage deletion (in macrophage Fas-induced apoptosis transgenic mice) at 2 weeks after TAC, subsequent cardiac fibrosis and remodeling were unaffected,⁴⁶ whereas earlier blockade of CCR2+ macrophage recruitment and expansion prevented remodeling.⁴⁷ These data are consistent with and support the notion that early inhibition of inflammatory signals is most effective at preventing heart failure development after TAC. This could also explain why attempts to treat established heart failure by blocking inflammation have been largely unsuccessful.⁴⁸

We conclude that CaMKII δ activation in CMs is responsible for initiating cardiac inflammation through cytokine generation and NLRP3 inflammasome activation. Although we have focused on MCP-1 actions and generation of IL-18, other products generated through NF κ B and inflammasome signaling could also contribute to cardiac dysfunction. These include IL-6, which has been reported to mediate pressure-overload induced cardiac remodeling in the mouse heart,^{33,36} as well as IL-1 β , the target of blockade by canakinumab, which was shown to diminish adverse cardiac events in patients with coronary artery disease,⁴⁹ and anakinra, an inhibitor of the IL-1 receptor, which improved cardiac performance in patients with decompensated systolic HF.⁵⁰

STUDY LIMITATION

The genetic deletion of CaMKII δ did not completely normalize the TAC induced phenotype; thus, other signaling pathways not delineated here must also contribute to the response to pressure overload. How CMs signal to other non-CM and which specific immune cells are involved has not been determined. We also acknowledged the possibility that other isoforms of CaMKII may be upregulated in the human failing heart and contribute to disease.

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Disclosures

None.

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