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

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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 Ca^{2+}/calmodulin-dependent protein kinase II δ (CaMKIIδ) in cardiomyocytes (CMs) in response to pressure overload elicits inflammatory responses leading to adverse remodeling.

METHODS: Mice in which CaMKIIδ 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 CaMKIIδ 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 CaMKIIδ 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-1β 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 CaMKIIδ 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 Chemotactic Protein-1 signaling, deletion of CM Monocyte Chemotactic 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 Chemotactic 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 CaMKIIδ 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 CaMKIIδ signaling to prevent progression to heart failure.

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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), and other inflammatory cell infiltration can also occur under nonischemic conditions. Early 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. 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. 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 Ca\(^{2+}\)/calmodulin regulated kinase \(\delta\) (CaMKII\(\delta\)) is rapidly activated in response to pressure overload. However our studies and those of others using CaMKII\(\delta\) knockout (KO) mice revealed that CaMKII\(\delta\) is not required for the development of pathological hypertrophy but instead for the progression from hypertrophy to heart failure. We also reported that CM CaMKII\(\delta\) 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 \(\beta\)-adrenergic receptors. These findings suggest that there is an as yet undefined pathway by which CaMKII\(\delta\) activation mediates progression to heart failure.

Work from our group and others established that CaMKII\(\delta\) contributes to inflammatory gene expression in response to ischemic injury and that this occurs through activation of Nuclear Factor-kappa B (NFkB). 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\(\delta\) in CMs. In the present study, we used conditional CM-specific CaMKII\(\delta\) 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\(\delta\)-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±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 CaMKIIδ
To determine whether inflammation is an early response to pressure overload-induced CaMKIIδ activation, we examined the time course of inflammatory gene expression in response to TAC in CaMKIIδ fl/fl (control [CTL]) and CKO mice. CKO mice generated by cre expression in CMs show nearly complete loss of CM CaMKIIδ (Figure IA and IB in the online-only Data Supplement). There were no baseline differences in ventricular structure or function between CaMKIIδ 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 Chemotactic 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 ≈40% in CKO hearts. Other genes evaluated, including C-X-C motif ligand 2 (CXCL2), IL-1β, 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 CaMKIIδ and NFκB 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 activity was demonstrated to be increased 1.6-fold (Figure II in the online-only Data Supplement) at 1.5 days after TAC. We next determined whether NFκB, a master regulator of inflammation, was activated through CaMKIIδ as an early response to TAC. NFκB activation, assessed by immunoblotting for increases in the p65 NFκB 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 NFκB activation in the induction of inflammatory genes, mice were injected with BMS-345541, an inhibitor of IkB kinase, an upstream regulator of NFκB. This led to nearly complete inhibition of TAC-induced increases in inflammatory gene mRNA (Figure 2B).

CaMKIIδ-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
cells, or fibroblasts, as assessed by Western blotting for CD45, CD31 (PECAM-1), and PDGFRα, 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 CaMKIIδ, at the onset of pressure overload stress.

**TAC Does Not Induce Apoptotic or Necrotic Cell Death ≤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
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).

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

To determine whether macrophage recruitment was elicited by TAC through activation of CaMKIIδ 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 CaMKIIδ 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 CaMKIIδ in TAC-induced macrophage accumulation was not because of secondary effects of CaMKIIδ gene deletion. There was also upregulation of mRNA for IL-10 and transforming growth factor-β, cytokines considered to be products of macrophages that were attenuated in the CKO versus CTL (Figure 4D).

The NLRP3 Inflammasome Is Activated in Response to TAC Through CM CaMKIIδ

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-1β and IL-18, cytokines processed by the NLRP3 inflammasome, were increased through CaMKIIδ and NFκB 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 NFκB 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...
of the inflammasome is through as yet undefined effects of reactive oxygen species (ROS).31 Accordingly, we tested the hypothesis that CaMKIIδ 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 CaMKIIδ (Figure VIIIA 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 VIIIB 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 inflammasome activation, mice were injected with the mitochondrial targeted ROS scavenger MitoTEMPO. TAC-induced ROS accumulation was inhibited (Figure VIIIA and VIIIB in the online-only Data Supplement), concomitant with significant inhibition of TAC-induced caspase-1 activation (Figure VIIIC 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 of the inflammasome is through as yet undefined effects of reactive oxygen species (ROS).31 Accordingly, we tested the hypothesis that CaMKIIδ 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 CaMKIIδ (Figure VIIIA 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 VIIIB 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 inflammasome activation, mice were injected with the mitochondrial targeted ROS scavenger MitoTEMPO. TAC-induced ROS accumulation was inhibited (Figure VIIIA and VIIIB in the online-only Data Supplement), concomitant with significant inhibition of TAC-induced caspase-1 activation (Figure VIIIC in the online-only Data Supplement).
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 5. Inflammasome is activated in response to pressure overload through cardiomyocyte (CM) CaMKIIδ.
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±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 CaMKIIδ KO mice; CTL, control flox/flox CaMKIIδ 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.
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 failure achieved by 4 weeks of daily isoproterenol injection. FAM-FLICA Caspase-1 assay kit was used to visualize caspase-1 activity. Scale bar=50 µm. 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±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 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. FAM-FLICA Caspase-1 assay kit was used to visualize caspase-1 activity. Scale bar=50 µm. N=6 to 8 per group. Data are presented as mean±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.

We have demonstrated that development of fibrosis and contractile dysfunction in this model depends on CaMKII δ. 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-1β, 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-1β mRNA, as well as increased NLRP-3 mRNA and
cleaved IL-18, in association with upregulated CaMKIIδ in the heart failure samples.

**Signaling to Macrophage Recruitment**

MCP-1, which was upregulated by TAC through CaMKIIδ 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 αMHC-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 ≥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 CaMKIIδ-induced macrophage recruitment. These data support our hypothesis that proinflammatory gene expression and inflammasome activation in CMs are the initiating CaMKIIδ-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, XIII B, and XIIIC 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 CaMKIIδ 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 1a1, collagen type 3a1, 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 inflammasome 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 CaMKIIδ signaling in the development of fibrosis in the heart.

**Early CaMKIIδ Activation in CMs Initiates TAC-Induced Cardiac Remodeling**

We implicated CaMKIIδ in the transition of pressure overload-induced hypertrophy to ventricular dilation and dysfunction in the global CaMKIIδ KO mouse. Here we demonstrate that ventricular dilation and decreases in ejection fraction at day 42 of TAC are also significantly diminished when CaMKIIδ is selectively deleted from CMs (Figure 8A). To implicate early activation of CaMKIIδ signaling pathways in the development of TAC-induced fibrosis and subsequent ventricular remodeling and dysfunction, we inhibited downstream mediators of the effects of CaMKIIδ: NFκB, NLRP3, and MCP-1. Pharmacological inhibition of NFκB 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 CaMKIIδ in CMs trigger the ensuing maladaptive inflammation and remodeling, we compared the effects of blocking CaMKIIδ 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. 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...
in the heart, are the initiators of inflammatory gene expression in response to pressure overload; (2) activation of CaMKIIδ in CMs triggers NFκB-mediated proinflammatory gene expression; (3) activation of CM CaMKIIδ primes and activates the inflammasome through NFκB 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 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 CaMKIIδ 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. 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 NFκB, 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
times as previously reported. 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. 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, 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, the attenuated inflammation that we see in the CKO is unlikely to reflect diminished CaMKIIδ 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. 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
CaMKIIδ. 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 CaMKIIδ signaling, the CM, through CaMKIIδ, 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 NFkB (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 CaMKIIδ 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-1β, 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 CaMKIIδ is deleted from CMs. In addition, our studies using BMS345541 show that CaMKIIδ activation in myocytes primes the inflammasome through the activation of NFkB. 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 CaMKIIδ signaling in CMs). The mechanism by which CaMKIIδ increases ROS levels has not been clearly established, but earlier work from our group and studies from the Anderson laboratory demonstrate effects of CaMKIIδ 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 CaMKIIδ activation and resultant ROS generation in CM.45 Our conclusion that CaMKIIδ 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-1β 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-1β, 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 CaMKIIδ in CMs triggers events that initiate ensuing inflammatory responses and cardiac remodeling, we...
asked whether blocking CaMKIIδ 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 NFκB 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 CaMKIIα 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 depletion (in macrophage Fas-induced apoptosis transgenic mice) at 2 weeks after TAC, subsequent cardiac fibrosis and remodeling were unaffected, whereas earlier blockade of CCR2+ macrophage recruitment and expansion prevented remodeling. 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.

We conclude that CaMKIIδ 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-1β, other products generated through NFκB 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 as well as IL-1β, the target of blockade by canakinumab, which was shown to diminish adverse cardiac events in patients with coronary artery disease, and anakinra, an inhibitor of the IL-1 receptor, which improved cardiac performance in patients with decompenated systolic HF.

**STUDY LIMITATION**

The genetic deletion of CaMKIIδ 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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