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RESEARCH ARTICLE

CaMKIIδ-mediated inflammatory gene expression and inflammasome activation in cardiomyocytes initiate inflammation and induce fibrosis

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Inflammation accompanies heart failure and is a mediator of cardiac fibrosis. CaMKIIδ plays an essential role in adverse remodeling and decompensation to heart failure. We postulated that inflammation is the mechanism by which CaMKIIδ contributes to adverse remodeling in response to nonischemic interventions. We demonstrate that deletion of CaMKIIδ in the cardiomyocyte (CKO) significantly attenuates activation of NF-κB, expression of inflammatory chemokines and cytokines, and macrophage accumulation induced by angiotensin II (Ang II) infusion. The inflammasome was activated by Ang II, and this response was also diminished in CKO mice. These events occurred prior to any evidence of Ang II–induced cell death. In addition, CaMKII-dependent inflammatory gene expression and inflammasome priming were observed as early as the third hour of infusion, a time point at which macrophage recruitment was not evident. Inhibition of either the inflammasome or monocyte chemoattractant protein 1 (MCP1) signaling attenuated macrophage accumulation, and these interventions, like cardiomyocyte CaMKIIδ deletion, diminished the fibrotic response to Ang II. Thus, activation of CaMKIIδ in the cardiomyocyte represents what we believe to be a novel mechanism for initiating inflammasome activation and an inflammatory gene program that leads to macrophage recruitment and ultimately to development of fibrosis.

Introduction

Inflammation has been widely associated with human heart failure, but attempts to therapeutically target inflammatory mediators to treat heart failure have met with limited success (1, 2). This may reflect the inability of a single inhibitor to block the self-regenerative and feed-forward inflammatory cascade already in progress in the failing heart. In addition, while inflammation has been demonstrated to trigger development of fibrosis (3–6), a key component of adverse remodeling in heart failure (7), established cardiac fibrosis may not be resolved by inhibiting inflammation. Accordingly, elucidation of the most proximal molecular events responsible for initiating the inflammatory signals that progress to more sustained and global inflammation, fibrosis, and heart failure is needed. The initiating causes of cardiac inflammation in the context of ischemic insults that result in myocardial infarction have been the focus of considerable research. Necrotic cell death leads to release of intracellular molecules referred to as damage-associated molecular patterns (DAMPs) (8, 9) which act on cardiac cells to stimulate proinflammatory transcriptional pathways (10–14). Inflammation can also occur, however, in nonischemic heart disease where cell death is not induced. Angiotensin II (Ang II) signals through an evolutionarily conserved neurohormonal pathway that plays a role in nearly all cardiovascular diseases. Ang II infusion is the most common model used to study inflammatory and fibrotic changes induced by a hypertensive nonischemic stress (5, 15–18). Recent studies have demonstrated that inflammatory responses and recruitment of immune cells that contribute to adverse cardiac remodeling also occur with pressure overload (19–23). The molecular mechanism by which cardiac inflammation is initiated in response to hypertensive stresses such as infusion of Ang II or pressure overload have not been established.

Previous work from our laboratory and those of others demonstrated that calcium/calmodulin-dependent protein kinase II δ (CaMKIIδ) and the transcription factor NF-κB play a regulatory role in development of inflammation induced by ischemic injury (24–26). Cardiomyocyte CaMKIIδ is also activated in response to

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Ang II and in pressure overload (27–31), but it is not known whether CaMKIIδ signaling in cardiomyocytes is responsible for initiation of NF-κB activation and inflammatory responses in the heart subjected to nonischemic stress. The NOD-like pyrin domain–containing protein 3 (NLRP3) inflammasome catalyzes caspase-1–dependent maturation of the proinflammatory cytokines IL-1β and IL-18 and is a regulator of inflammatory cascades (32, 33). There is recent evidence for inflammasome activation in the heart following ischemic interventions, suggested to be mediated by cell death and DAMPs (34–40). It is not clear, however, whether and how the inflammasome is activated by nonischemic interventions in the heart, i.e., independent of cell death–generated mediators.

In the studies reported here, we used cardiomyocyte-specific CaMKIIδ KO (CKO) mice to test the hypothesis that CaMKIIδ, activated in cardiomyocytes in response to Ang II, initiates NF-κB–dependent inflammatory gene expression and inflammasome activation. We demonstrate a critical role for CaMKIIδ signaling in the cardiomyocyte in inducing expression of inflammatory cytokines and chemokines and in priming and activating the inflammasome in the heart. Specifically the present study provides evidence that macrophage recruitment is regulated through cardiomyocyte CaMKIIδ activation, the chemokine monocyte chemoattractant protein 1 (MCP1), and inflammasome activation. We also demonstrate that these inflammatory responses contribute to development of fibrosis induced by Ang II. Our findings provide evidence that acute CaMKIIδ activation in cardiomyocytes by a nonischemic intervention can initiate cardiomyocyte autonomous inflammatory responses that contribute significantly to inflammatory cell recruitment and development of fibrosis in the heart.

Results

Ang II infusion activates CaMKIIδ and NF-κB. To determine whether activation of CaMKIIδ and subsequent NF-κB activation are early initiating steps in the development of Ang II–induced inflammatory and fibrotic responses, we used osmotic minipumps to deliver Ang II at 1.5 μg/kg/min, the standard protocol used by others (5, 18, 41). Hearts were isolated 1 and 3 hours after the start of infusion, and activation of CaMKII was assessed using a ^{32}P enzymatic CaMKII activity assay. Activated CaMKIIδ was increased by 1.3- and 1.6-fold at 1 and 3 hours of infusion, respectively (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.97054DS1). The observation that CaMKIIδ activation in the heart is an early response to in vivo Ang II infusion extends previous findings that this occurs after more prolonged Ang II treatment and in isolated cardiomyocytes (27, 29–31). NF-κB activation was assessed by immunoblotting for increases in the p65 NF-κB subunit in nuclear fractions. Nuclear p65 was increased in control (*Camk2d*$^{fl/fl}$) mouse hearts at 3 hours of Ang II infusion. This response was significantly attenuated in hearts of cardiomyocyte-specific CaMKIIδ KO mice (Supplemental Figure 1B), indicating that it is initiated in the cardiomyocyte through CaMKIIδ.

CaMKIIδ activation in cardiomyocytes mediates Ang II-induced cardiac inflammatory chemokine and cytokine expression. To examine the involvement of cardiomyocyte CaMKIIδ in Ang II–induced inflammatory gene expression, we infused control and cardiomyocyte-specific CaMKIIδ KO mice with 1.5 μg/kg/min Ang II. Hearts were isolated at 1 day of Ang II infusion and lysates used for preparation of mRNA. Robust increases in expression of *Ccl2*/MCP1, macrophage inflammatory protein 1 (*Ccl3*/MIP1α), *Cxcl1*, *Cxcl2*, *Il1b*, and *Il6* mRNA were observed in control hearts. Remarkably, these responses were attenuated by 70%–90% in hearts from CKO mice (Figure 1A). We assessed the involvement of NF-κB activation in the induction of inflammatory genes by Ang II infusion at this early time by injecting mice with BMS-345541 (BMS), an inhibitor of IκB kinase, the upstream regulator of NF-κB (42). This treatment led to nearly complete suppression of the Ang II–stimulated increases in inflammatory gene mRNA (Supplemental Figure 2).

The proinflammatory chemokine MCP1 has been shown to play a role in Ang II–induced inflammation and fibrosis, with the major source of MCP1 upregulation and generation suggested to be endothelial cells (18, 43, 44). To demonstrate that the cardiomyocyte can also act as a source of MCP1 expression, we isolated adult mouse ventricular myocytes (AMVMs) from hearts of mice following 1-day Ang II infusion. There was a 12-fold increase in *Ccl2*/MCP1 mRNA in the AMVMs isolated from control Ang II–infused mice (Figure 1B). Cardiomyocyte CaMKIIδ signaling was implicated by the observation that the increase in *Ccl2*/MCP1 mRNA was significantly attenuated in myocytes isolated from CKO mice (Figure 1B). AMVMs from untreated mice also responded to in vitro addition of Ang II (1 μmol/l), with CaMKIIδ-dependent increases in *Ccl2*/MCP1 expression (data not shown). In addition, we showed by Western blotting that cardiac MCP1 protein was increased by Ang II infusion and that this response was inhibited in hearts lacking CaMKIIδ in the cardiomyocyte (Figure 1C).

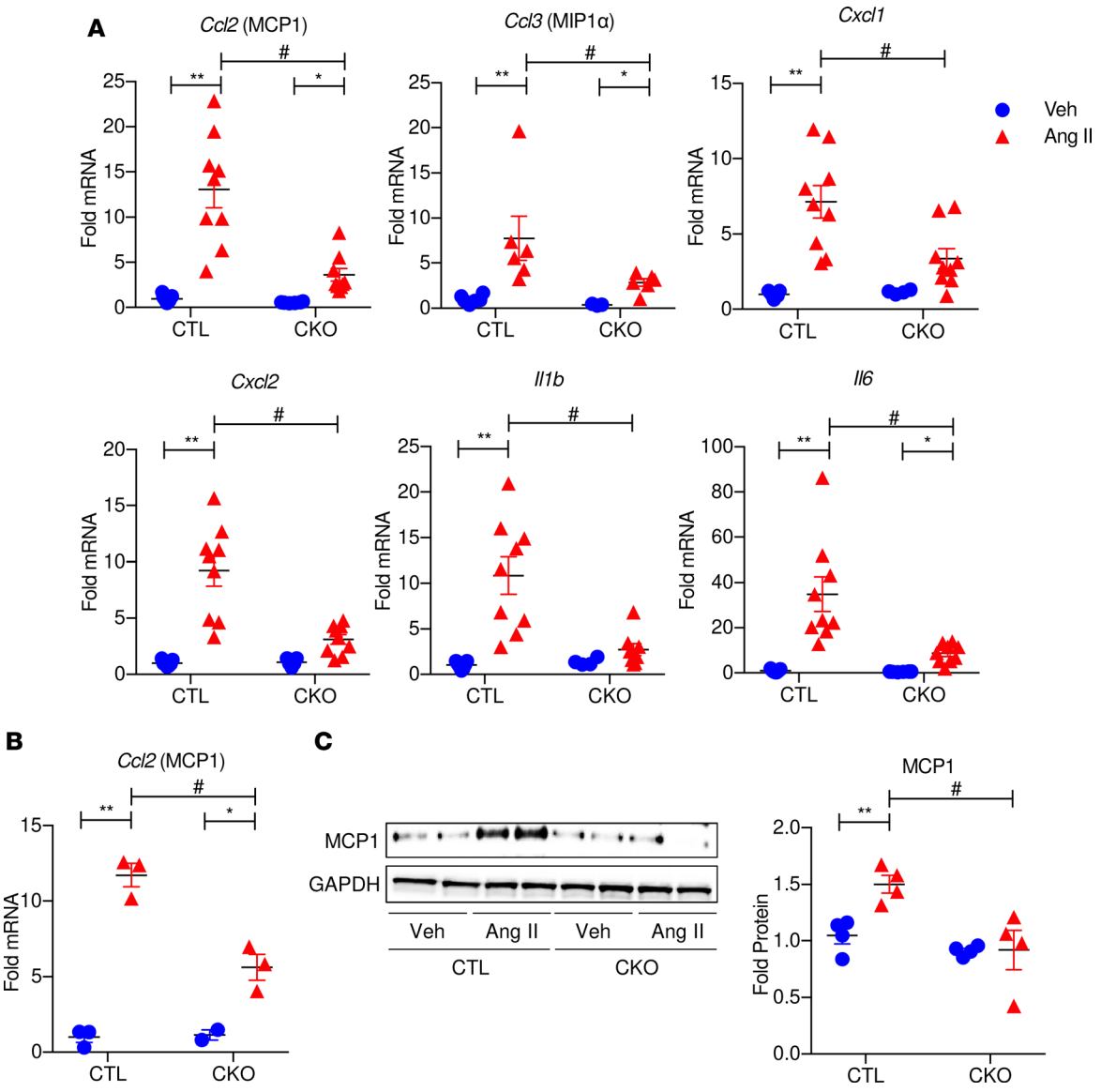

Figure 1. Deletion of CaMKIIδ in the cardiomyocyte attenuates Ang II–induced inflammatory gene expression in the heart and monocyte chemotactic protein 1 expression in the myocyte. (**A**) mRNA expression of monocyte chemoattractant protein 1 (*Ccl2*/MCP1), macrophage inflammatory protein 1α (*Ccl3*/MIP1α), CXC motif ligand 1 (*Cxcl1*), CXC motif ligand 2 (*Cxcl2*),IL-1β (*Il1b*), and IL-6 (*Il6*) in ventricular lysates of *Camk2d*$^{fl/fl}$ control (CTL) and cardiomyocyte-specific CaMKIIδ KO (CKO) mice infused with saline (vehicle [Veh]) or Ang II (1.5 μg/kg/min) for 1 day as measured by qPCR (n = 6–9/group). (**B**) *Ccl2*/MCP1 mRNA expression in adult mouse ventricular myocytes isolated from control and CKO mice after 1-day saline or Ang II infusion as measured by qPCR. Hearts were enzymatically digested, and resultant cell suspension was allowed to sediment by gravity. Supernatant was aspirated and pellet washed 2 times before being processed for RNA extraction, cDNA synthesis, and qPCR (n = 3/group). (**C**) Western blot and quantitation of MCP1 protein in ventricular lysates of control and CKO mice at 1 day of Ang II infusion. GAPDH, loading control (n = 4/group). Two-way ANOVA was used for all comparisons. *P < 0.05 vs. Veh; **P < 0.01 vs. Veh; #P < 0.05 CTL Ang II vs. CKO Ang II.

Ang II–induced early inflammatory responses are not secondary to cell death or hypertension. Cell death can lead to release of factors that stimulate inflammation (13), raising the possibility that inflammation is diminished in the CKO mouse heart due to decreased CaMKIIδ-mediated cell death. Notably, however, we observed no increase in Evans blue dye penetration (Figure 2A), TUNEL staining (Figure 2B), caspase-3 cleavage (Figure 2C), or caspase-3 activity as measured by a fluorometric activity assay (Figure 2D) in 1-day Ang II–infused versus vehicle-infused mouse hearts. Thus, neither necrotic nor apoptotic cell death were significantly elicited in response to the short term (1 day) Ang II infusion that robustly increases inflammatory gene expression. We also tested possible differences in hypertensive effects of Ang II infusion in control and CKO mice. Ang II infusion increased blood pressure to a

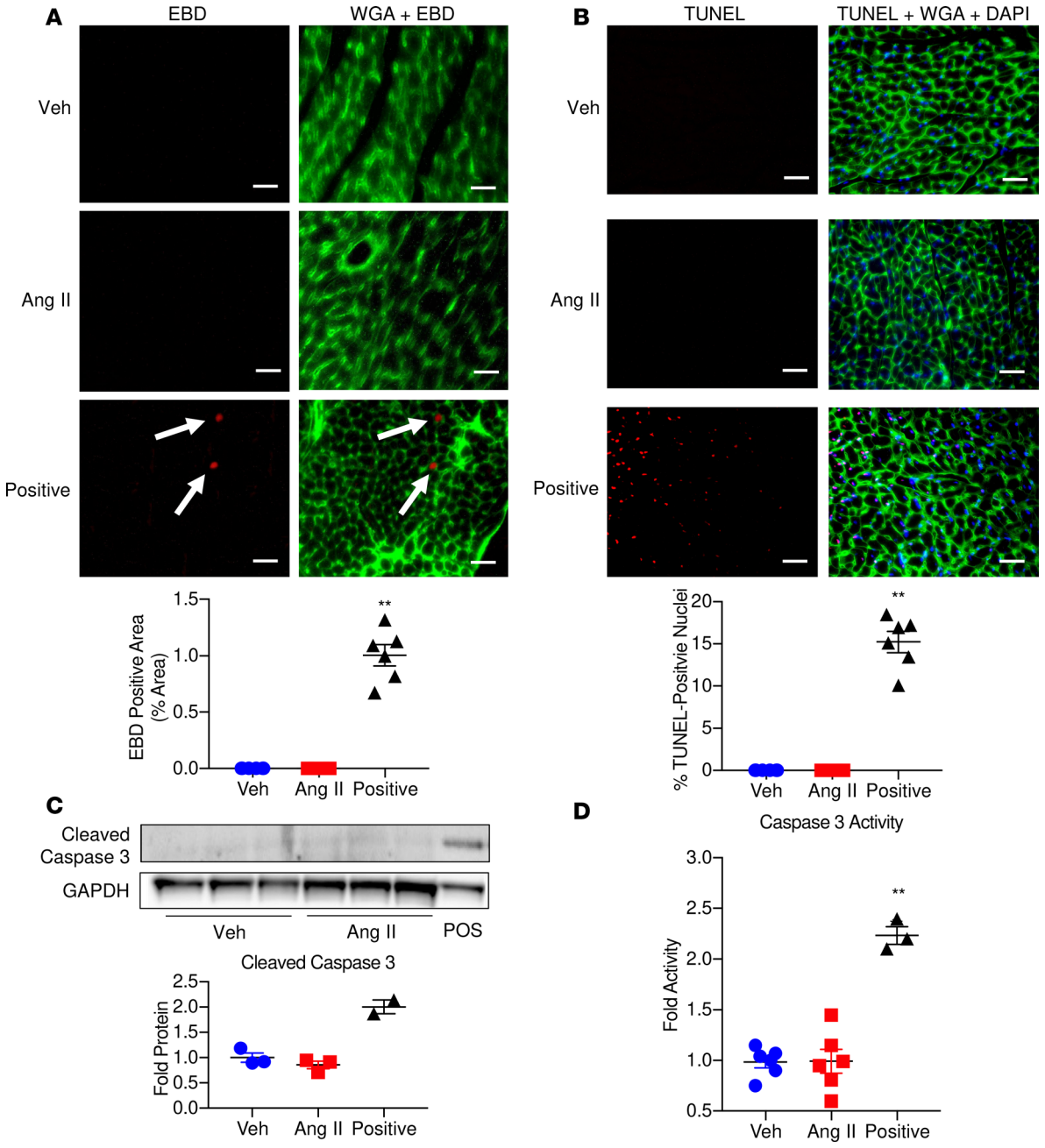

Figure 2. Angiotensin II infusion for 1 day does not cause cardiomyocyte cell death. (**A**) Representative pictures and quantitation of cryosections stained with wheat germ agglutinin (WGA; green) taken from control mice infused with saline (vehicle [Veh]) Ang II (1.5 μg/kg/min) for 1 day and injected with 100 mg/kg Evans blue dye (EBD) 18 hours before the end of the 1-day Ang II infusion. Positive control is 20 mg/kg doxorubicin injection 3 days before sacrifice. After sacrifice, hearts were immediately cryosectioned and necrotic cells visualized under fluorescence microscopy. Necrotic cells fluoresce red (arrows) (n = 6/group). (**B**) Representative pictures and quantitation of paraffin-embedded sections taken from control mice infused with saline or Ang II for 1 day and stained using TUNEL to visualize apoptotic nuclei. Positive control is Ang II infusion for 7 days. Apoptotic nuclei fluoresce red (n = 6/group). (**C**) Western blot and quantitation of cleaved caspase-3 protein in ventricular lysates of control mice infused with saline or Ang II for 1 day. Positive control is neonatal rat ventricular myocytes treated with 5 μmol/l staurosporine for 3 hours. GAPDH, loading control (n = 2–3/group). (**D**) Caspase-3 activity in fresh ventricular lysates of mice infused with saline or Ang II for 1 day as measured by a prevalidated fluorometric caspase-3 activity kit. Positive control is ventricular lysates of mice subjected to transaortic constriction for 7 days (n = 3–6/group). One-way ANOVA was used for all comparisons. $^{**}P < 0.01$ vs. Veh. Scale bars: 50 μm.

similar extent in both lines over 7 days, with only a modest increase at 1 day of infusion (Supplemental Figure 3). These data support a direct role of cardiomyocyte CaMKIIδ in transcriptional control of inflammatory gene expression rather than effects of CaMKIIδ on cardiomyocyte viability or systemic neurohumoral and vascular responses.

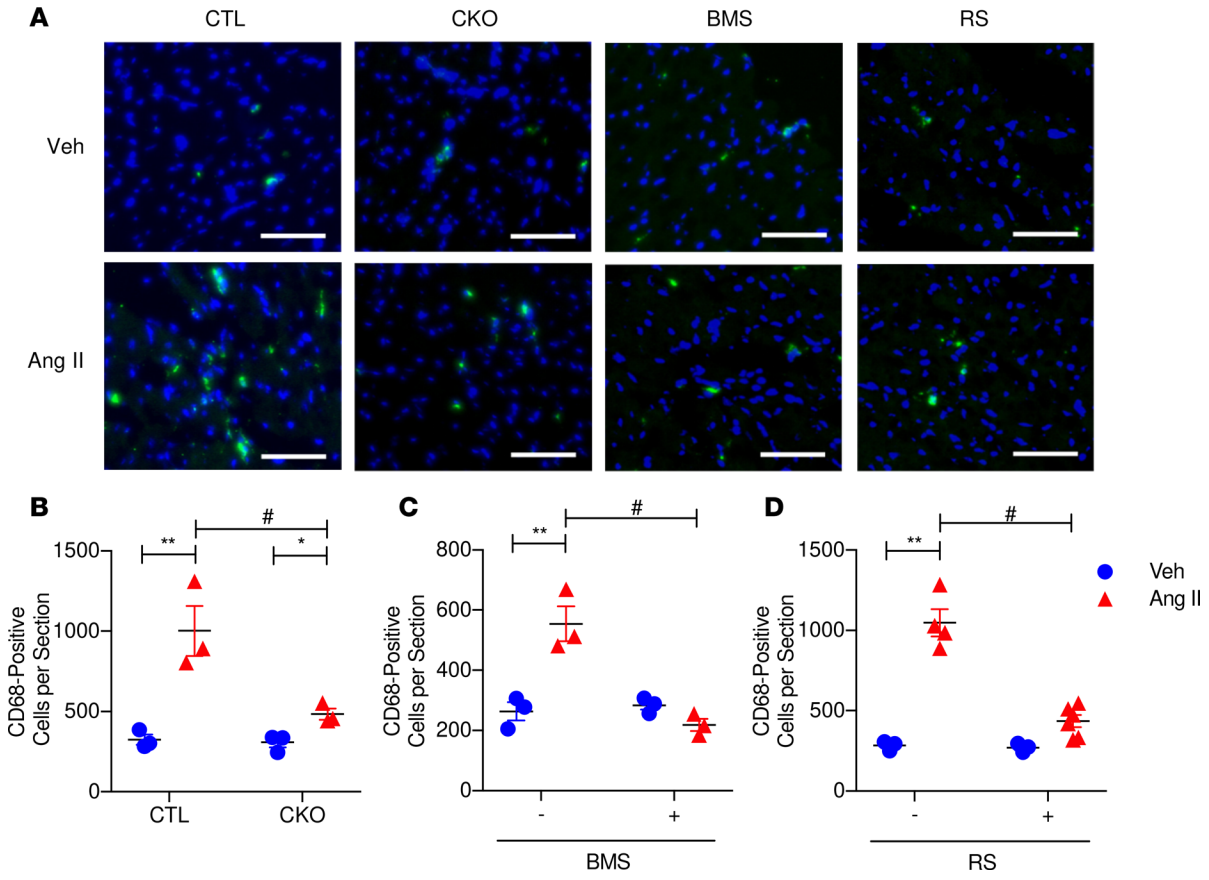

Figure 3. Ang II–induced macrophage accumulation in the heart is attenuated by CaMKIIδ gene deletion in the cardiomyocyte, inhibition of NF-κB activation, and MCP1 receptor blockade. (**A**) Representative pictures of cardiac cryosections stained with antibody to the pan-macrophage marker CD68 taken from mice infused with saline (vehicle [Veh]) or Ang II (1.5 μg/kg/min) for 1 day (n = 3–6/group). Mouse groups include *Camk2d*$^{fl/fl}$ control (CTL) mice, cardiomyocyte-specific CaMKIIδ KO (CKO) mice, control mice injected intraperitoneally with the inhibitor of NF-κB activation BMS-345541 (BMS), and control mice injected with the MCP1 receptor antagonist RS102895 (RS). CD68, green; DAPI, blue. Mice in the BMS group were injected with water (–) or 45 mg/kg of BMS-345541 (+) at 3 time points throughout the 1-day infusion. Doses and injection times were 30 mg/kg at time 0 hours of infusion, 15 mg/kg at time 5 hours, and 60 mg/kg at time 8 hours infused with. Mice in the RS group were injected intraperitoneally with 1:1 DMSO/water (–) or 10 mg/kg RS102895 every 6 hours for 24 hours (+). (**B**) Quantitation of CD68 staining in cryosections taken from control and CKO mice infused with saline or Ang II. (**C**) Quantitation of CD68 staining in cryosections taken from control mice infused with saline or Ang II and injected with water or BMS. (**D**) Quantitation of CD68 staining in cryosections taken from control mice infused with saline or Ang II and injected with 1:1 DMSO/water or RS102895. Two-way ANOVA was used for all comparisons. *P < 0.05 vs. Veh; **P < 0.01 vs. Veh; #P < 0.05, CTL Ang II vs. CKO Ang II, Ang II vs. Ang II + BMS, or Ang II vs. Ang II + RS. Scale bars: 50 μm.

Ang II recruits macrophages to the heart through CaMKIIδ, NF-κB, and MCP1. To determine whether Ang II infusion also elicits accumulation of macrophages in the heart through its effects on cardiomyocyte CaMKIIδ, we immunostained cardiac sections from Ang II–infused control and CKO mice with the macrophage markers CD68 and F4/80. Some CD68- and F4/80-positive cells were evident in both control and CKO sections under basal conditions (Figure 3, A and B, and Supplemental Figure 4). The number of both CD68- and F4/80-positive cells increased approximately 3-fold in control hearts at 1 day of Ang II infusion. Notably these increases were nearly fully abolished in hearts from cardiomyocyte-specific CaMKIIδ KO mice (Figure 3, A and B, and Supplemental Figure 4). To determine if macrophage accumulation was secondary to the observed increases in inflammatory gene expression, we blocked inflammatory gene expression by inhibiting NF-κB signaling. BMS treatment significantly decreased CD68 macrophage accumulation in response to Ang II infusion (Figure 3, A and C). Since MCP1 has been specifically implicated in Ang II–induced monocyte/macrophage recruitment (18, 43), we considered MCP1 generated in cardiomyocytes through CaMKIIδ and NF-κB as a mediator of macrophage recruitment. Mice were treated with RS102895, an antagonist of CCR2, the monocyte/macrophage receptor for MCP1 (45). RS102895 treatment decreased Ang II–induced CD68 staining by more than 70% (Figure 3, A and D).

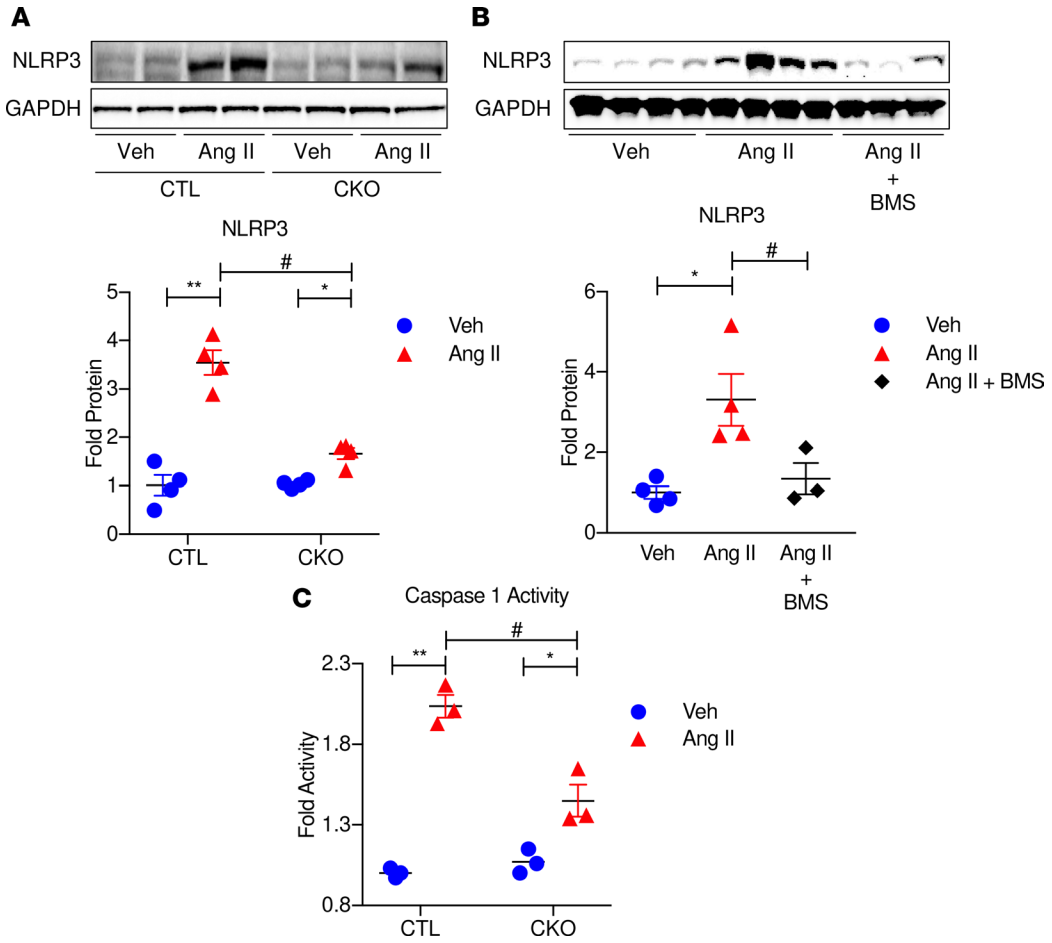

Figure 4. Ang II–induced priming and activation of the inflammasome is mediated through cardiomyocyte CaMKIIδ and NF-κB. (**A**) Western blot and quantitation of NLRP3 protein in ventricular lysates of *Camk2d*$^{fl/fl}$ control (CTL) and cardiomyocyte-specific CaMKIIδ KO (CKO) mice after 1-day saline (vehicle [Veh]) or Ang II (1.5 μg/kg/min) infusion. GAPDH, loading control (n = 4/group). (**B**) Western blot and quantitation of NLRP3 protein in ventricular lysates of control mice infused with saline or Ang II for 1 day and injected intraperitoneally with BMS-345541 (BMS) at 3 time points throughout the 1-day infusion. Doses and injection times were 30 mg/kg at time 0 hours of infusion, 15 mg/kg at time 5 hours, and 60 mg/kg at time 8 hours (n = 3–4/group). (**C**) Caspase-1 activity in fresh ventricular lysates of mice infused with saline or Ang II for 1 day as measured by a prevalidated fluorometric caspase-1 activity kit. n = 3/group. Two-way ANOVA was used in **A** and **C**. One-way ANOVA was used in **B**. $^{*}P < 0.05$ vs Veh, $^{**}P < 0.01$ vs Veh; $^{\#}P < 0.05$, CTL Ang II vs. CKO Ang II or Ang II vs. Ang II + BMS.

Ang II regulates NLRP3 inflammasome signaling in the heart through CaMKIIδ and NF-κB. Il1b mRNA was induced at 1-day Ang II infusion, and this was the gene most highly dependent on cardiomyocyte CaMKIIδ (Figure 1A). Upregulation of *Il1b* mRNA is considered a priming step for the NLRP3 inflammasome, since it encodes a precursor protein that is activated through processing by caspase-1 in the inflammasome complex (33). Expression of NLRP3, a key component of the inflammasome, is also upregulated through NF-κB as part of inflammasome priming (33); thus, we determined whether this occurred in response to Ang II through activation of cardiomyocyte CaMKIIδ. Mice infused with Ang II for 1 day showed a 3-fold increase in cardiac NLRP3 protein, and this response was largely attenuated in mice lacking CaMKIIδ in the cardiomyocyte (Figure 4A) or in mice in which NF-κB activation was inhibited by treatment with BMS (Figure 4B). Activation of the inflammasome follows its priming, and a standard measure of this critical step is an increase in caspase-1 activity (46). Ang II infusion for 1 day significantly increased caspase-1 activity as determined using a fluorometric activity assay (Figure 4C). This response was significantly attenuated in hearts of CKO mice. The observed increases in NLRP3 and caspase-1 are evidence that Ang II regulates inflammasome assembly and activation in the heart and that cardiomyocyte CaMKIIδ plays a critical role in orchestrating these responses.

Active CaMKIIδ regulates NLRP3 inflammasome priming and ROS-dependent inflammasome activation in cardiomyocytes. To provide evidence that inflammasome priming occurs in cardiomyocytes, we isolated

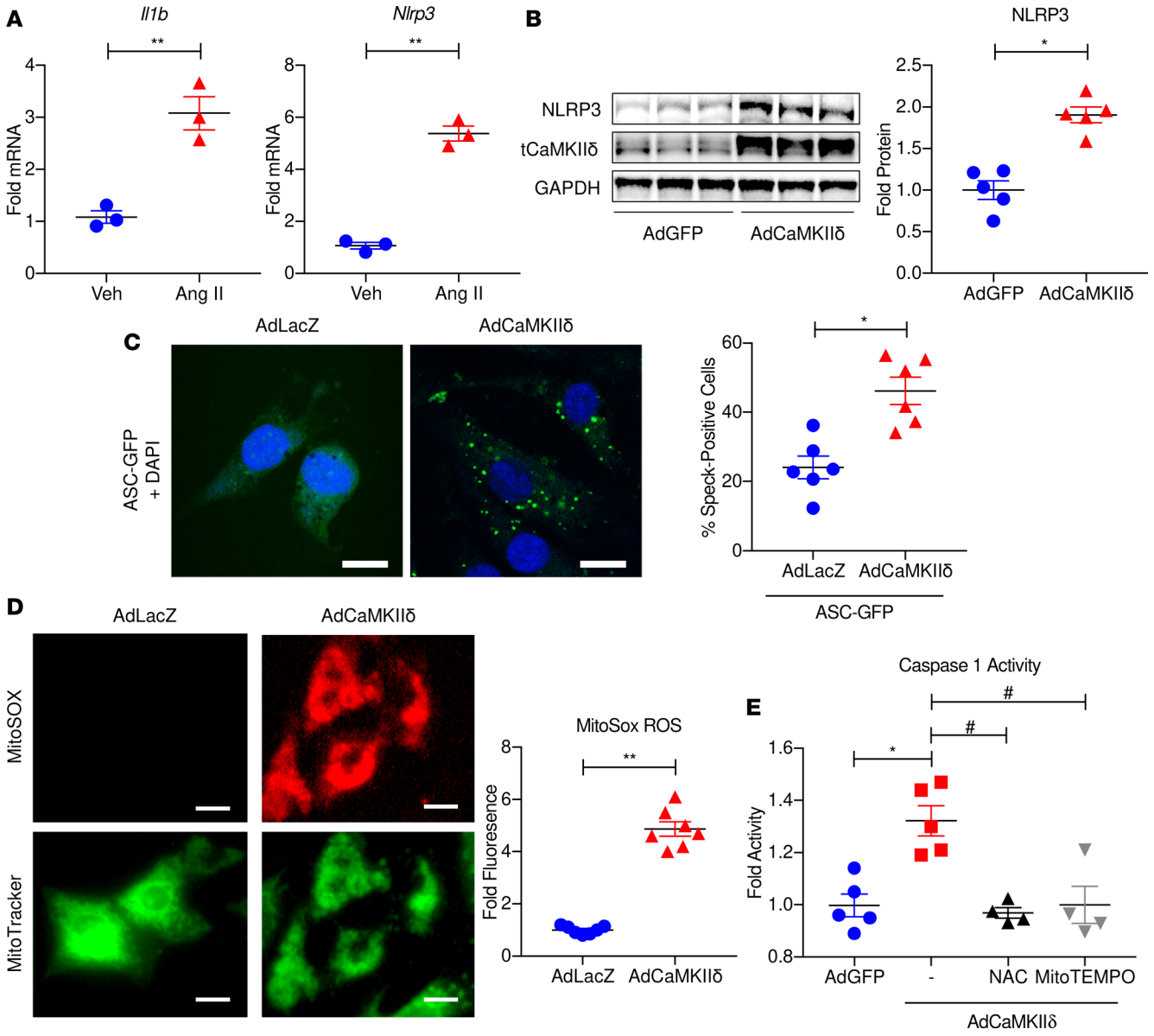

Figure 5. Ang II and CaMKIIδ prime and induce ROS-dependent inflammasome activation in cardiomyocytes. (**A**) mRNA expression of *Il1b* and *Nlrp3* as measured by qPCR in adult mouse ventricular cardiomyocytes isolated from mice after 1 day of Ang II infusion (n = 3/group). (**B**) Western blot and quantitation of NLRP3 protein in NRVMs infected with adenovirus expressing GFP (AdGFP) or the active δ_C isoform of CaMKII (AdCaMKIIδ) at an MOI of 50. NRVMs were starved for 5 hours prior to a 3-hour infection, then washed with serum-free medium and cultured overnight. tCaMKIIδ, total CaMKIIδ; GAPDH, loading control (n = 5/group). (**C**) Fluorescence microscopy of NRVMs transfected with cDNA encoding GFP-tagged ASC and infected with adenovirus expressing β-galactosidase (AdLacZ) or AdCaMKIIδ at an MOI of 50. Quantitation represents percentage of all transfected cells that were speck-positive from 2 independent experiments, with 220–588 total transfected cells counted per group (n = 6/group from 2 independent experiments). (**D**) Live cell images and quantitation of fluorescence in NRVMs infected with AdLacZ or AdCaMKIIδ and cultured as described above. MitoSOX (5 μmol/l) and MitoTracker green (1 mmol/l) were used to visualize mitochondrial ROS and mitochondria. Data were quantified from 10 images per sample (n = 7/group from 2 independent experiments). (**E**) Caspase-1 activity measured in NRVMs infected with AdGFP or AdCaMKIIδ with or without a 1-hour pretreatment with 10 mmol/l *N*-acetylcysteine (NAC) or 10 μmol/l MitoTEMPO (n = 4–5/group from 2 independent experiments). Student's t test was used in **A**–**D**, and 1-way ANOVA was used in **E**. $^*P < 0.05$ vs. Veh, AdGFP, or AdLacZ; $^{**}P < 0.01$ vs. Veh or AdLacZ; $^{\#}P < 0.05$, AdCaMKIIδ alone vs. NAC or MitoTEMPO pretreatment. Scale bars: 15 μm.

AMVMs from 1-day Ang II–infused mice. Expression of both *Nlrp3* and *Il1b* mRNA was increased 3- to 5-fold in AMVMs isolated from Ang II–infused mice (Figure 5A). To directly demonstrate that CaMKIIδ can regulate the NLRP3 inflammasome in cardiomyocytes, we infected neonatal rat ventricular myocytes (NRVMs) with control adenovirus expressing AdGFP or adenovirus expressing the constitutively active δ_C isoform of CaMKIIδ (AdCaMKIIδ) at a dose that we determined did not induce cell death. Expression

of CaMKIIδ led to a robust increase in NLRP3 protein (Figure 5B). ASC, an adapter protein required for the NLRP3 inflammasome assembly, forms speck-like puncta following inflammasome activation (47). We determined whether active CaMKIIδ induces ASC speck formation in cardiomyocytes by transfecting NRVMs with cDNA encoding GFP-tagged ASC. ASC-GFP was diffusely localized in control cells infected with adenovirus expressing β-galactosidase (AdLacZ) (Figure 5C); however, when active CaMKIIδ was expressed, ASC speck formation was clearly evident (Figure 5C), further demonstrating that CaMKIIδ can mediate inflammasome activation in cardiomyocytes. CaMKIIδ has been shown to regulate processes that would promote generation of mitochondrial ROS in cardiomyocytes (48, 49), and NRVMs infected with AdCaMKIIδ showed a robust increase in mitochondrial ROS as assessed by MitoSOX fluorescence (Figure 5D). Since ROS are known activators of the inflammasome (50, 51), we extended our studies to determine whether CaMKIIδ regulates inflammasome activation through ROS. AdCaMKIIδ expression led to activation of the NLRP3 inflammasome, as determined by increased cardiomyocyte caspase-1 activity (Figure 5E). Blocking ROS accumulation with the general or mitochondria-targeted ROS scavengers *N*-acetylcysteine or MitoTEMPO led to complete inhibition of this response (Figure 5E). These results indicate that CaMKIIδ activation can regulate the NLRP3 inflammasome in cardiomyocytes through effects on both its priming and activation.

Relationship among inflammatory gene expression, inflammasome activation, and macrophage signaling. The results above demonstrated that 1-day Ang II treatment induces inflammatory gene expression, priming and activation of the NLRP3 inflammasome, and macrophage recruitment, all of which are dependent on CaMKIIδ activation in cardiomyocytes. Macrophages are considered to be a source of cytokines as well as a major site of inflammasome signaling, raising the possibility that macrophage recruitment to the heart contributes to these responses. To assess the involvement of recruited immune cells, we tested a shorter Ang II infusion time (3 hours), at which we observed no increase in CD68 staining (Figure 6A). Robust increases in mRNA levels of *Ccl2*/MCP1, *Il1b*, and *Il6* were nonetheless detectable at this time and were highly dependent on the presence of CaMKIIδ (Figure 6B). Expression of the inflammasome component *Nlrp3* was also increased in the heart by 3 hours of Ang II treatment (Figure 6C), when macrophages were not yet recruited. The induction of *Nlrp3* and *Il1b* was also unaffected by inhibition of MCP1 signaling with RS102895 (Figure 6C). These data suggest that early increases in cytokines and inflammasome priming are independent of macrophage recruitment and are initiated through CaMKIIδ and NF-κB signaling in cardiomyocytes. Notably, by 1 day (versus 3 hours) of Ang II infusion, NLRP3 protein upregulation (Figure 7A) and caspase-1 activity (Figure 7B) were partially but significantly inhibited when MCP1 signaling was blocked, implicating MCP1-dependent macrophage recruitment in propagating the increases in inflammasome activity seen in the heart at this time.

Inhibition of the inflammasome attenuates Ang II–induced inflammatory responses. Inflammasome activation promotes and sustains proinflammatory cytokine expression and can also contribute to recruitment of macrophages (34). To test the role of inflammasome function in the recruitment of macrophages and induction of inflammatory genes, we injected mice with MCC950, an inhibitor of NLRP3 activation (52), beginning 1 hour prior to infusion with Ang II. Treatment with MCC950 led to nearly complete inhibition of the Ang II–induced accumulation of CD68-positive macrophages in the heart (Figure 8A), a result that was recapitulated using NLRP3-KO mice (Figure 8B). These findings demonstrate a critical role for NLRP3 inflammasome products in Ang II–induced macrophage accumulation in the heart. MCC950 treatment also significantly attenuated *Ccl2*/MCP1, *Ccl3*/MIP1α, *Cxcl1*, *Cxcl2*, and *Il6* mRNA expression induced by 1-day Ang II infusion (Figure 8C). Thus, inflammasome-induced macrophage recruitment and/or local signaling through inflammasome generated mediators (e.g., IL-1β) contribute to sustaining inflammatory gene expression in the heart.

Cardiomyocyte CaMKIIδ signaling contributes to Ang II–induced fibrosis. Ang II infusion leads to robust induction of myocardial fibrosis (5, 16, 18, 53–55). The increase in fibrosis observed by Masson's trichrome staining at 7 days of Ang II infusion was diminished by 60% in cardiomyocyte-specific CaMKIIδ KO mice (Figure 9A). The fibrotic gene markers collagen type 1α1 (*Col1a1*), *Col3a1*, and periostin (*Postn*) were also upregulated in control Ang II–infused mouse hearts, and these increases, evident at both 3 and 7 days, were significantly attenuated in hearts of mice lacking cardiomyocyte CaMKIIδ (Figure 9B). Expression of mRNA for profibrotic mediators including CCN family member 1/cysteine-rich angiogenic factor 61 (*Cyr61*), CCN family member 2/connective tissue growth factor (*Ctgf*), and TGF-β1 (*Tgfb1*) was also increased at 7 days of Ang II infusion and attenuated in the cardiomyocyte CKO (Supplemental Figure 5).

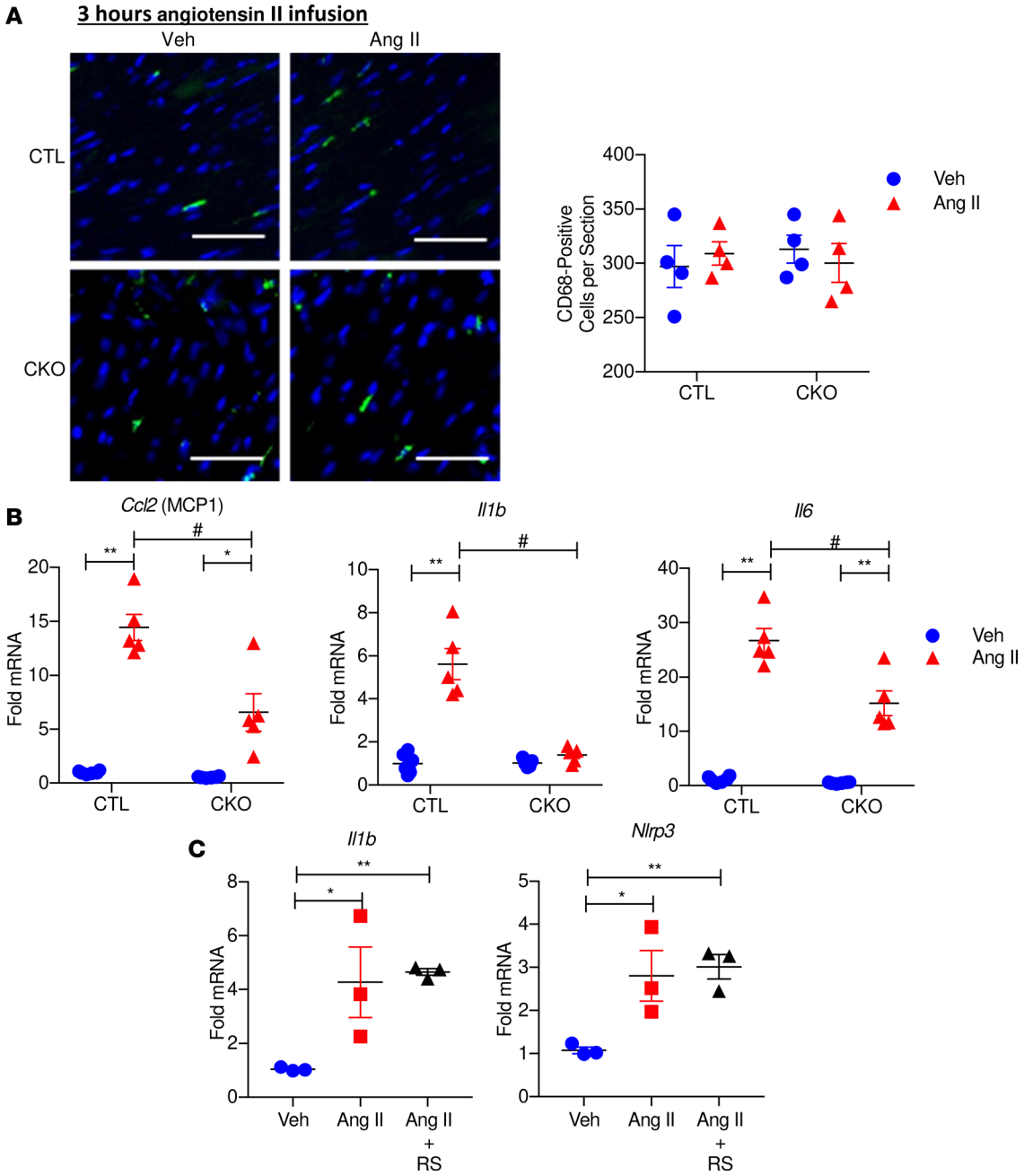

Figure 6. Ang II increases cardiac inflammatory gene expression through cardiomyocyte CaMKIIδ but not macrophage recruitment at 3 hours of infusion. (**A**) Representative images and quantitation of cardiac cryosections stained with antibody to CD68 from 3-hour saline- (vehicle [Veh]) or Ang II–infused (1.5 μg/kg/min) *Camk2d*$^{fl/fl}$ control (CTL) and cardiomyocyte-specific CaMKIIδ KO (CKO) mice (n = 4/group). (**B**) mRNA expression of monocyte chemoattractant protein 1 (*Ccl2*/MCP1), IL-1β (*Il1b*), and IL-6 (*Il6*) in ventricles of CTL and CKO mice infused with saline or Ang II for 3 hours as measured by qPCR (n = 5–6/group). (**C**) mRNA expression of *Il1b* and *Nlrp3* in ventricles of control mice infused with saline or Ang II and injected once intraperitoneally with 10 mg/kg RS102895 (RS) starting 1 hour prior to a 3-hour infusion as measured by qPCR (n = 3/group). Two-way ANOVA was used in **A** and **B**. One-way ANOVA was used in **C**. *P <0.05 vs. Veh; **P < 0.01 vs. Veh; #P < 0.05 CTL Ang II vs. CKO Ang II. Scale bars: 50 μm.

MCP1 and the inflammasome are involved in angiotensin II–induced fibrosis. There is considerable evidence that inflammation plays a role in development of cardiac fibrosis (3–6). Accordingly, we asked whether interventions that attenuated Ang II–induced inflammation and macrophage recruitment also diminished fibrosis. We tested both the MCP1 and NLRP3 inflammasome inhibitors for their effects on the fibrotic responses seen at 3 days of Ang II infusion. Treatment with RS102895 decreased the area of Ang II–induced fibrosis by 40% (Figure 10A) and significantly attenuated *Col1a1*, *Col3a1*, and *Postn* expression (Figure 10B). Treatment with MCC950 likewise attenuated Ang II–induced fibrosis (Figure 10C) and

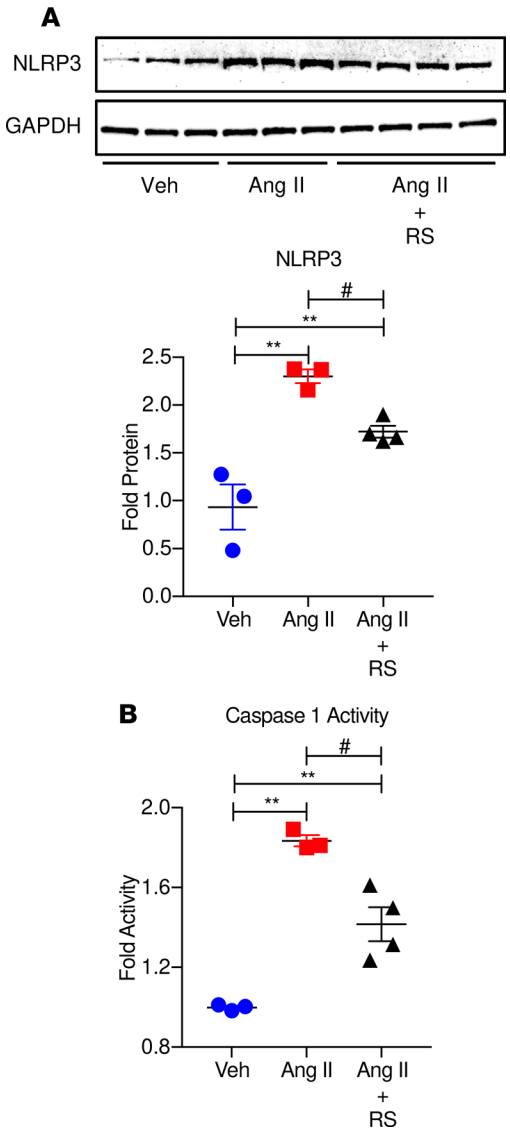

Figure 7. Inhibition of macrophage recruitment attenuates Ang II–induced inflammasome activation at 1 day of infusion. (**A**) Western blot and quantitation of NLRP3 protein in ventricular lysates of *Camk2d*$^{fl/fl}$ control mice infused for 1 day with saline (vehicle [Veh]) or Ang II (1.5 μg/kg/min) and injected intraperitoneally with 1:1 DMSO/water (Veh) or 10 mg/kg RS102895 (RS) every 6 hours during the 24-hour infusion. GAPDH, loading control (n = 3–4/group). (**B**) Caspase-1 activity in fresh ventricular lysates of mice infused with saline or Ang II and injected with RS102895 as described (n = 3–4/group). One-way ANOVA was used for all comparisons. $^{**}P < 0.01$ vs. Veh; $^{\#}P < 0.05$ Ang II vs. Ang II + RS.

significantly attenuated expression of the fibrotic gene markers *Col1a1*, *Col3a1*, and *Postn* (Figure 10D). Thus, the critical role of cardiomyocyte CaMKIIδ in initiating MCP1 signaling and inflammasome activation appears to underlie its contribution to Ang II–induced fibrosis.

Discussion

Cardiac inflammation occurs as a result of the necrotic cell death that accompanies myocardial infarction or ischemia/reperfusion injury (10, 44, 56). The studies presented here provide evidence that cardiac inflammatory responses can also be initiated, in the absence of acute loss of cardiomyocytes, through signaling pathways involving CaMKIIδ. Moreover, our work demonstrates an unexpectedly major role for cardiomyocytes in Ang II–induced inflammatory gene expression and inflammasome activation. It is increasingly accepted that "cardiokines" are generated in cells of the heart (57–59); however, the cardiomyocyte (versus, e.g., endothelial cells and fibroblasts) is not generally considered to be an essential generator of inflammatory mediators (other than secondary to its necrotic cell death). The observation that hearts lacking expression of CaMKIIδ in cardiomyocytes show markedly blunted induction of cardiac proinflammatory genes at 3 hours to 1 day of Ang II infusion identifies the cardiomyocyte as the site at which Ang II initiates proinflammatory signaling.

CaMKIIδ-mediated inflammatory gene expression accompanied by macrophage accumulation both occur within 1 day of Ang II treatment, a time point shown here and in the literature to not be associated with death of cardiomyocytes (60). Thus, while CaMKIIδ has been implicated in processes that elicit various forms of cardiomyocyte cell death (61–65), the modest level of CaMKIIδ activation observed at early times of Ang II infusion serves to elicit inflammatory responses through a rapidly activated transcriptional pathway in cardiomyocytes, rather than through factors released by dying cells. There may also be direct effects of Ang II on gene expression in resident non-cardiomyocytes (e.g., fibroblasts, macrophages, and endothelial cells). We observe increases in *Ccl2*/MCP1, *Il6*, and *Ccl3*/MIP1α expression in the non-myocyte fraction separated from the washed and sedimented cardiomyocytes (data not shown). Fibroblasts, macrophages, and endothelial cells present in this fraction may represent the small component of the Ang II–induced inflammatory gene expression that is CaMKIIδ independent. Nonetheless, increased expression of *Ccl2*/MCP1, *Nlrp3*, and *Il1b* is clearly evident in AMVMs isolated from hearts at 1 day of Ang II infusion, substantiating our conclusion that CaMKIIδ-mediated activation of inflammatory gene expression initiated in cardiomyocytes is a major mechanism by which Ang II induces inflammation in the absence of cell death.

The NLRP3 inflammasome has been implicated in the inflammatory response to myocardial infarction and ischemia/reperfusion (34, 35). NLRP3 inflammasome activation can be driven by release of DAMPs from necrotic cells, which could underlie inflammasome involvement in cardiac ischemic injury (39, 40). We demonstrate, however, that inflammasome activation also occurs in response to Ang II, in the absence of cell death, and that it is initiated through cardiomyocyte CaMKIIδ and activation of NF-κB. Specifically, our studies establish that Ang II treatment increases *Il1b* mRNA and NLRP3 expression in the mouse heart, that these increases are significantly attenuated when CaMKIIδ is deleted from cardiomyocytes, and that similar changes are seen in AMVMs isolated from Ang II–treated hearts. In addition, caspase-1 is activated in response to Ang II infusion, and this response is markedly diminished in the CaMKIIδ KO. Other studies presented here provide direct evidence that CaMKIIδ signaling in cardiomyocytes can

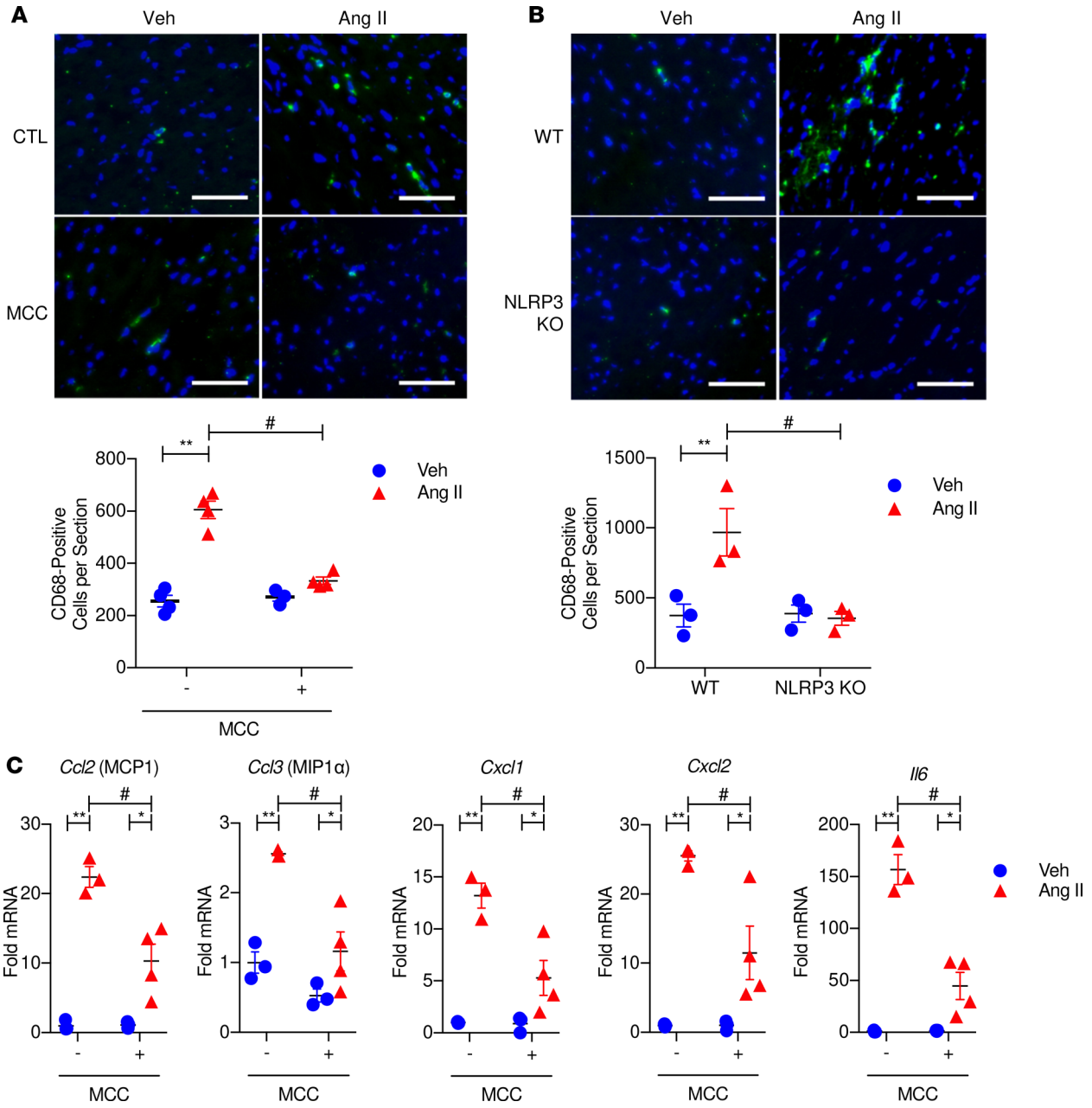

Figure 8. Inflammasome inhibition and NLRP3 gene deletion attenuate Ang II–induced inflammatory responses in the heart. (**A**) Representative images and quantitation of cardiac cryosections stained with antibody to CD68 taken from mice infused with saline (vehicle [Veh]) or Ang II (1.5 μg/kg/min) for 1 day and injected intraperitoneally with saline (–) or 10 mg/kg MCC950 (MCC) every 12 hours starting 1 hour prior to infusion (n = 3–4/group). (**B**) Representative images and quantitation of cardiac cryosections stained with antibody to CD68 from WT or *Nlrp3*$^{-/-}$ (NLRP3 KO) mice infused with saline (Veh) or Ang II (1.5 μg/kg/min) for 1 day (n = 3/group). (**C**) mRNA expression of monocyte chemoattractant protein 1 (*Ccl2*/MCP1), macrophage inflammatory protein 1α (*Ccl3*/MIP1α), CXC motif ligand 1 (*Cxcl1*), CXC motif ligand 2 (*Cxcl2*), and IL-6 (*Il6*) in ventricles of control mice infused with saline or Ang II and injected with MCC950 as described above (n = 3–4/group). Two-way ANOVA was used for all comparisons. *P < 0.05 vs. Veh or MCC; **P < 0.01 vs. Veh; $^{\#}P$ < 0.05, Ang II vs. Ang II + MCC or WT Ang II vs. NLRP3 KO Ang II. Scale bars: 50 μm.

lead to inflammasome activation; i.e., expression of constitutively active CaMKIIδ in myocytes increases NLRP3 protein, ASC speck formation, and caspase-1 activity. We further delineate a mechanism by which CaMKIIδ activation in cardiomyocytes can activate the NLRP3 inflammasome. Specifically, we show that CaMKIIδ expression increases mitochondrial ROS, consistent with published evidence for a role of CaMKIIδ in mitochondrial ROS generation (48, 49). CaMKIIδ has been demonstrated to interact with the mitochondrial calcium uniporter (49), a critical player in ROS generation at the mitochondria, and thus is suggested to be the mechanism by which CaMKIIδ activation leads to ROS production. We further provide

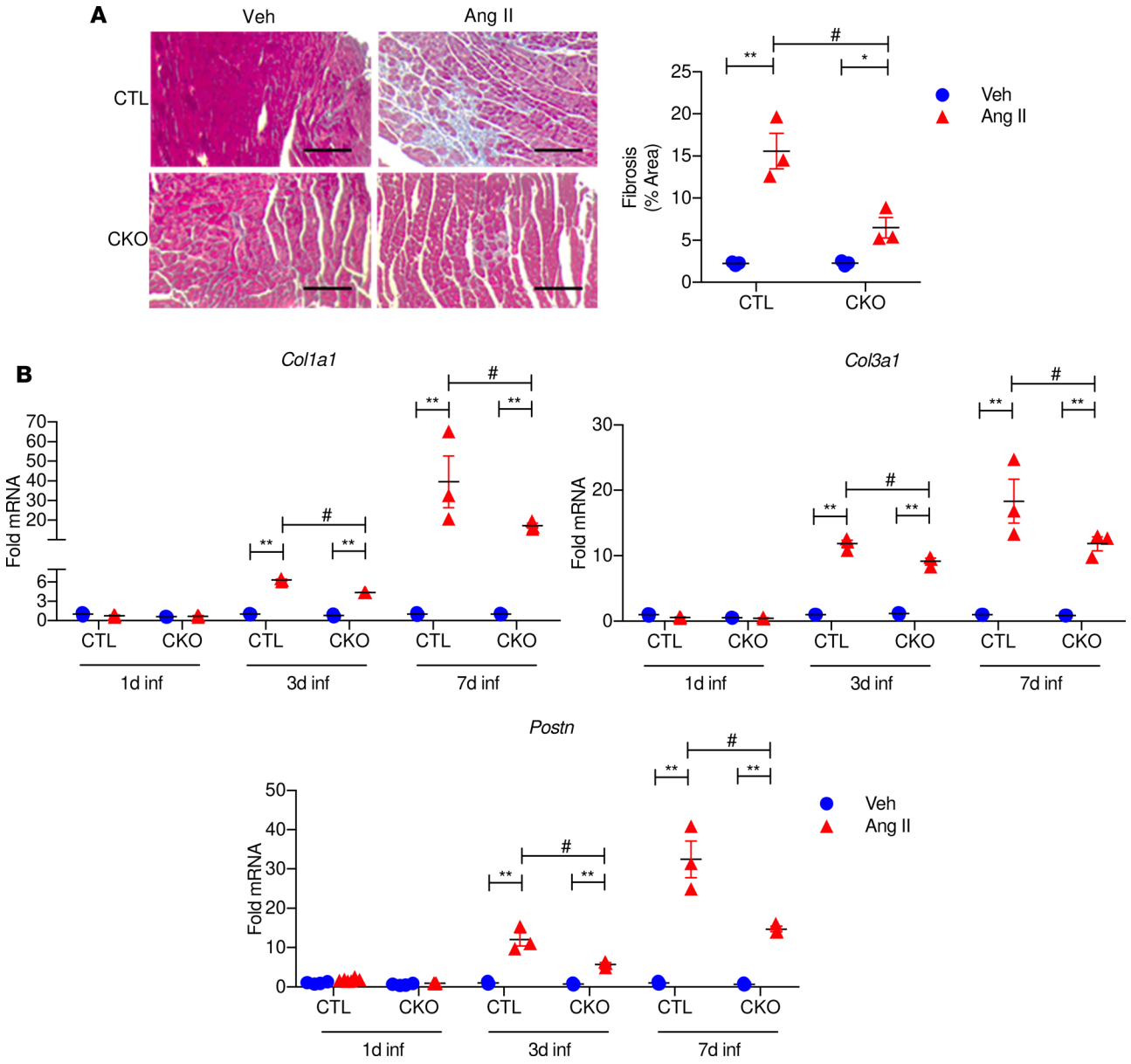

Figure 9. Cardiac fibrosis induced by 7-day Ang II infusion is attenuated in mice lacking CaMKIIδ in the cardiomyocyte. (**A**) Representative pictures and quantitation of paraffin-embedded sections stained with Masson's trichrome taken from *Camk2d*$^{fl/fl}$ control (CTL) and cardiomyocyte-specific CaMKIIδ KO (CKO) mice infused with saline (vehicle [Veh]) or Ang II (1.5 μg/kg/min) for 7 days (n = 3/group). (**B**) mRNA expression of fibrotic markers collagen 1α1 (*Col1a1*), collagen 3α1 (*Col3a1*), and periostin (*Postn*) in ventricles of CTL and CKO mice infused with saline or Ang II for 1, 3, and 7 days as measured by qPCR (n = 3/group). Two-way ANOVA was used for all comparisons. *P < 0.05 vs. Veh; **P < 0.01 vs. Veh; #P < 0.05, CTL Ang II vs. CKO Ang II. Scale bars: 100 μm.

evidence that mitochondrial ROS is required for caspase-1 activation, as previously reported in non-cardiac (immune) cells (50, 51). Thus, our studies extend those of others, suggesting a role for the cardiomyocyte in inflammasome signaling (35, 66, 67) and identify CaMKIIδ activation as a necessary and sufficient mediator of these responses.

Macrophages recruited to the heart in response to 1-day Ang II infusion also clearly promote and sustain inflammasome activation, as evidenced by our observation that blocking macrophage accumulation by inhibiting MCP1 receptor signaling decreases NLRP3 expression and caspase-1 activation at this time. However, *Il1b* and *Nlrp3* mRNA were significantly increased through cardiomyocyte CaMKIIδ at 3 hours of Ang II infusion, a time point at which no increased macrophage staining was evident. In addition, a significant component of inflammasome activation even at 1 day of Ang II treatment was still evident when MCP1 signaling/macrophage recruitment was blocked. Fibroblasts may also contribute to inflammasome

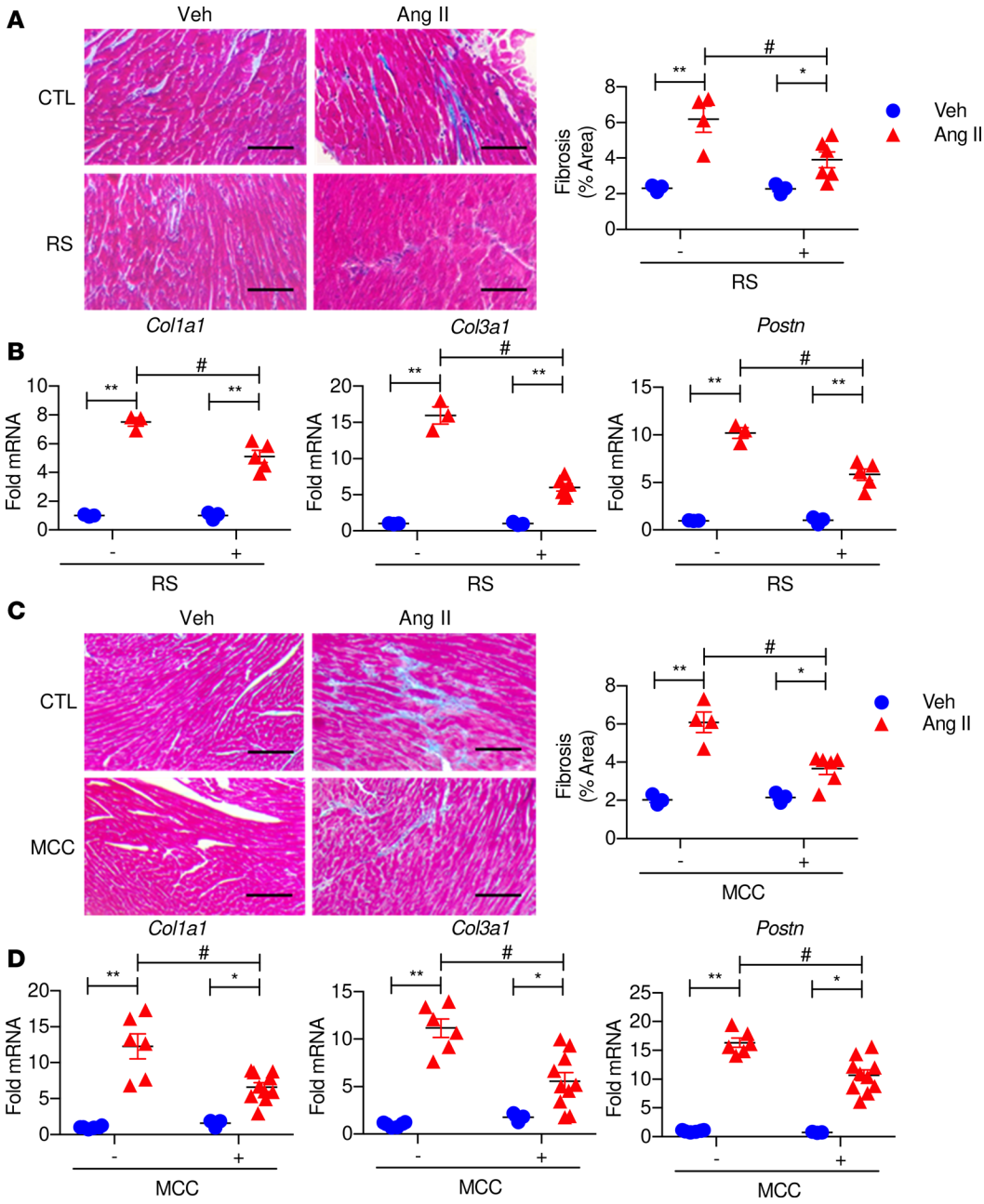

Figure 10. Inhibition of MCP1 or inflammasome signaling attenuates cardiac fibrosis induced by Ang II infusion. *Camk2d*$^{fl/fl}$ control mice were injected intraperitoneally with either 10 mg/kg RS102895 (RS) every 6 hours (**A** and **B**) or 10 mg/kg MCC950 (MCC) every 12 hours (**C** and **D**) starting 1 hour prior to a 3-day saline (vehicle [Veh]) or Ang II (1.5 μg/kg/min) infusion (inf). (**A**) Representative pictures and quantitation of paraffin-embedded sections stained with Masson's trichrome (n = 3–6/group). (**B**) mRNA expression of fibrotic markers collagen 1α1 (*Col1a1*), collagen 3α1 (*Col3a1*), and periostin (*Postn*) in ventricles as measured by qPCR (n = 3–6/group). (**C**) Representative pictures of paraffin-embedded sections stained with Masson's trichrome (n = 3–4/group). (**D**) mRNA expression of fibrotic markers *Col1a1*, *Col3a1*, and *Postn* in ventricles as measured by qPCR (n = 3–6/group). Two-way ANOVA was used for all comparisons. *P < 0.05 vs. Veh; **P < 0.01 vs. Veh; $^{\#}P$ < 0.05, Ang II vs. Ang II + RS/MCC. Scale bars: 100 μm.

signaling in the heart, as suggested by others (34, 68, 69), and these resident cells could be activated either directly by Ang II signaling or via cytokines or chemokines released from cardiomyocytes. It is also notable that blocking inflammasome signaling using pharmacological inhibition or genetic deletion of NLRP3 led to significant inhibition of both macrophage accumulation and inflammatory gene expression in the heart, suggesting a primary role for the inflammasome in sustaining cardiac inflammation.

CaMKIIδ has been implicated in development of cardiac fibrosis induced by pressure overload, Gαq overexpression, sustained β-adrenergic stimulation, and myocardial infarction (28, 48, 70–72), but the

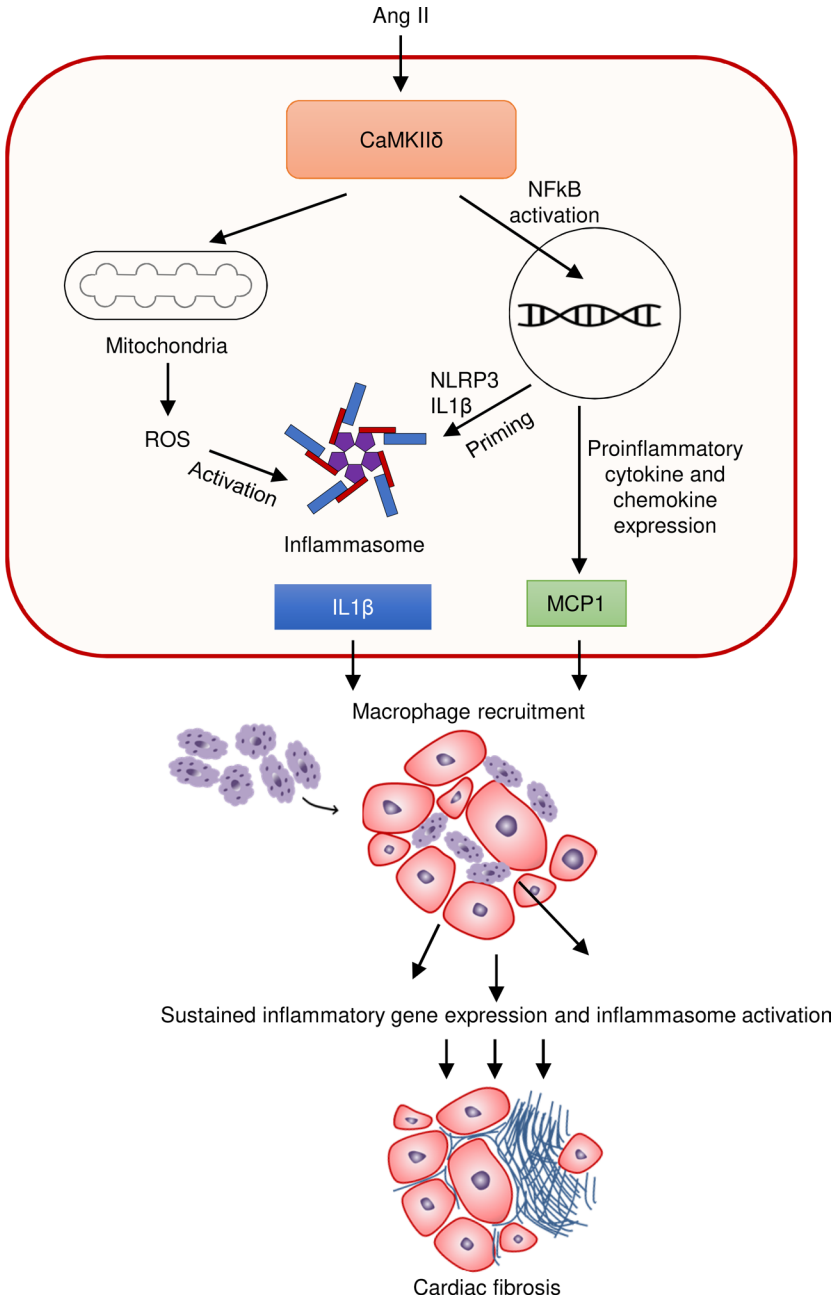

Figure 11. Interplay between Ang II–induced inflammatory events in the heart triggered by cardiomyocyte CaMKIIδ. Cardiomyocyte CaMKIIδ is activated by Ang II infusion resulting in initiation of an early transcriptional proinflammatory gene program. This event contributes to recruitment of macrophages through MCP1 and inflammasome activation, both of which produce a feed-forward proinflammatory signal that culminates with an outcome of cardiac fibrosis.

mechanism by which CaMKIIδ regulates cardiac fibrosis has not been determined. The data presented here link cardiomyocyte CaMKIIδ activation to MCP1 signaling and to inflammasome activation, and demonstrate through the use of pharmacologic inhibitors that these processes are critical for development of fibrosis. Notably, Ang II also increased expression of the profibrotic mediators *Cyr61*, *Ctgf*, and *Tgfb1*, which could act directly on fibroblasts to induce fibrosis. While it is not clear whether the mRNA for these profibrotic proteins is upregulated in cardiomyocytes, fibroblasts, or other cardiac cells, what is significant is that these changes are also secondary to the actions of Ang II on cardiomyocyte CaMKIIδ signaling.

In conclusion, our studies reveal an unexpected central role for CaMKIIδ signaling in the cardiomyocyte as a trigger for inflammatory responses, immune cell recruitment, and cardiac fibrosis. Findings from our study identify a pathway by which cardiomyocyte CaMKIIδ activated by Ang II generates a feed-forward proinflammatory signal that involves the inflammasome and recruitment of macrophages, both of which contribute to fibrosis as depicted in the schematic in Figure 11. The involvement of the inflammasome is particularly intriguing, since products of inflammasome activation could orchestrate the global

inflammatory cascades observed in the failing heart. We are currently extending this work to examine the role of CaMKIIδ-mediated inflammatory responses in development of adverse remodeling and dysfunction in pressure overload–induced heart failure. Treatment of cardiac inflammation through pharmacological inhibition of CaMKIIδ, or the products of the early inflammatory genes that it regulates, may provide significant benefits in preventing heart failure progression.

Methods

Animals. Mice in which CaMKIIδ was genetically deleted in the cardiomyocyte (cardiomyocyte-specific CaMKIIδ KO mice) were generated by crossing Black Swiss homozygous floxed CaMKIIδ (*Camk2d*$^{fl/fl}$) mice (generated in our laboratory) with C57BL/6 MLC2v-Cre mice (provided by Ju Chen, UCSD). Mice were backcrossed 10 times to achieve a Black Swiss background. There were no baseline changes in ventricular structure or function as compared with WT and *Camk2d*$^{fl/fl}$ (control) mice (25). *Nlrp3*$^{-/-}$ mice (generated by our research team) were created as previously described (73) and backcrossed 10 times to a C57BL/6 background. Male *Camk2d*$^{fl/fl}$ control, cardiomyocyte-specific CaMKIIδ KO, and *Nlrp3*$^{-/-}$ mice 8–12 weeks of age were infused with 1.5 μg/kg/min Ang II or vehicle (saline) using unprimed ALZET model 1007D micro-osmotic pumps for up to 7 days. There were no differences in inflammatory responses to Ang II among WT, *Camk2d*$^{fl/fl}$, and the unfloxed MLC2v-Cre mice (data not shown). Accordingly, we used *Camk2d*$^{fl/fl}$ mice as controls for this study. In some experiments, mice were injected intraperitoneally every 6 hours with vehicle (1:1 water/DMSO) or 10 mg/kg RS102895 (Tocris) starting 1 hour prior to a 1- or 3-day Ang II infusion. This dose and time course are shown in the literature to achieve a plasma concentration sufficient to inhibit macrophage recruitment (45). Other mice were also injected intraperitoneally every 12 hours with vehicle (saline) or 10 mg/kg MCC950 (Millipore) starting 1 hour prior to a 1- or 3-day Ang II infusion. Mice were also injected intraperitoneally with vehicle (water) or BMS at 3 time points throughout a 1-day Ang II infusion, a regimen calculated using published pharmacokinetic parameters (42). Doses and injection times were 30 mg/kg at time 0 hours of Ang II infusion, 15 mg/kg at time 5 hours, and 60 mg/kg at time 8 hours.

AMVM isolation. AMVMs were isolated from male control and cardiomyocyte-specific CaMKIIδ KO mice using a modified published protocol (74). In brief, mice were anesthetized with ketamine/xylazine, and hearts removed, cannulated, and perfused at 37 °C at a rate of 3 ml/min. Initially, hearts were perfused for 3 minutes with a calcium-free buffer. Hearts were then digested with buffer containing 12.5 mol/l calcium and 0.34 mg/ml Liberase TH (Roche) for 7–10 minutes. Hearts were removed from the cannula, atria and vessels removed, and ventricles manually dissociated using micro-forceps and by multiple passages through a Pasteur pipette. Cells were strained through a 100-μm strainer, and the Liberase TH was deactivated by resuspending the cells in perfusion buffer containing 10% fetal bovine serum (STOP buffer). Cardiomyocytes were pelleted by gravity sedimentation, supernatant was aspirated, and cardiomyocytes were washed with STOP buffer 2 times. After the final wash, supernatant was aspirated, and pelleted cells were immediately lysed for RNA extraction. To ensure purity of cardiomyocyte fractions, samples of the cell suspensions before the final sedimentation were collected and cultured on laminin-coated plates for 4 hours at 37°C in M199 medium (supplemented with 5% bovine calf serum, 1 mg/ml 2,3-butanedione monoxime, 100 U/ml penicillin, and 100 μg/ml streptomycin). Some cultures were washed with ice-cold PBS, fixed in 4% PFA for 10 minutes, blocked in 5% goat serum for 1 hour, stained with 1:100 CD68 (Abcam, ab53444), and visualized with 1:750 Alexa Fluor 488 secondary antibody (Thermo Fisher Scientific, A11008) on a Zeiss Axio Observer Z1 inverted microscope. Other cultures were incubated for an additional 2 days in DMEM supplemented with 20% fetal bovine serum and examined under light microscopy for fibroblasts. The adult myocyte-enriched cultures we isolated using this protocol did not contain fibroblasts or cells positive for CD68.

NRVM isolation and culture conditions. Hearts were isolated from 1- to 2-day-old Sprague-Dawley rat pups (Envigo) and digested in collagenase II (Worthington). Resultant myocytes were purified by passage through a Percoll gradient and plated at a density of 3.5×10^4 cells/cm^2 overnight at 37°C on gelatin-coated plates in DMEM supplemented with 10% horse serum, 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. After overnight plating, cells were washed with serum-free medium and starved for 5 hours. Adenovirus expressing the constitutively active δ_C isoform of CaMKIIδ (AdCaMKIIδ) or GFP (AdGFP) was applied to cultures for 3 hours at an MOI of 50. Cultures were washed with serum-free medium and incubated overnight before processing for Western blotting or caspase-1 activity. To inhibit ROS

formation, 10 mmol/l *N*-acetylcysteine (Sigma-Aldrich) or 10 μmol/l MitoTEMPO (Enzo Life Sciences) was added to cultures 1 hour prior to infection with adenovirus and once again after washing with serum-free medium. To visualize ROS generation, live cells infected with AdCaMKIIδ or adenovirus expressing β-galactosidase (AdLacZ) at an MOI of 50 were loaded with MitoSOX (5 μmol/l) or MitoTracker green (1 mmol/l), and fluorescence was measured using a Zeiss Axio Observer Z1 inverted microscope.

ASC-GFP transfection. NRVMs were isolated and plated as described above on gelatin-coated chamber slides (Thermo Fisher Scientific). After overnight plating, cells were transfected using Lipofectamine 2000 (Life Technologies) and 0.44 μg/chamber slide of cDNA encoding ASC-GFP (generated by our research group) in a 3:1 ratio, respectively. Lipofectamine and cDNA were incubated alone in Opti-MEM media (Gibco) at room temperature for 5 minutes, followed by mixing and incubating further for 10 minutes. The mixture was added to plates containing fresh media. Following overnight incubation at 37°C, cells were washed with serum-free medium and starved for 5 hours. AdCaMKIIδ or AdLacZ was applied to cells at an MOI of 50 for 3 hours, followed by washing with serum-free medium and overnight incubation. Fluorescence was visualized using a Leica SP5 confocal microscope. Quantitation of speck formation was calculated as percent of total transfected cells (diffuse and speck fluorescence) that were speck-positive from 2 independent experiments, with 220–588 total transfected cells counted per group.

Histological analyses. Hearts were fixed in 4% PFA for 1 day, cryoprotected in 30% sucrose, frozen in O.C.T. compound (Thermo Fisher Scientific), and cryosectioned (5 μm). Sections were stained with 1:100 F4/80 (Serotec, MCA497) or 1:100 CD68 (Abcam, ab53444) and visualized with 1:750 Alexa Fluor 488 secondary antibody (Thermo Fisher Scientific, A11008) on a Zeiss Axio Observer Z1 inverted microscope. The degree of CD68 and F4/80 staining was calculated as the total number of CD68- and F4/80-positive cells per cardiac section. Paraffin-embedded sections (5 μm) were used to measure fibrosis and cell death. Sections were stained with Masson's trichrome to visualize fibrosis. Percentage area fibrosis was calculated from greater than 10 randomly acquired images per cardiac section (20×) using ImageJ (NIH) software.

Cell death. Necrotic cells were examined by intraperitoneally injecting mice with 100 mg/kg Evan's blue dye (Sigma Aldrich), which accumulates in necrotic cells and autofluoresces red, 18 hours prior to sacrifice (64, 75). Apoptotic cells were examined using a TUNEL kit (Roche). Western blotting for cleaved caspase-3 (Cell Signaling Technology, 9661) and fluorometric assay of caspase-3 activity (Abcam, ab39383) in ventricular lysates were used as additional measures of apoptotic cell death.

RNA extraction, cDNA synthesis, and quantitative PCR. Total RNA was isolated from ventricles and adult mouse ventricular myocytes using TRIzol reagent as described by the manufacturer (Thermo Fisher Scientific). Single-stranded cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression of *Ccl2*, *Ccl3*, *Cxcl1*, *Cxcl2*, *Il1b, Il6*, *Nlrp3*, *Col1a1*, *Col3a1*, *Postn*, *Tgfb1*, *Cyr61*, and *Ctgf* was measured using a prevalidated Taqman Gene expression assay (Integrated DNA Technologies) on a Fast 7500 quantitative PCR (qPCR) machine (Applied Biosystems).

Caspase-1 activity assay. Caspase-1 activity was measured in ventricular homogenates and NRVMs using a prevalidated Fluorometric Caspase-1 Activity Kit as described by the manufacturer (Abcam, ab39412).

Immunoblotting. Western blot analysis was performed on snap-frozen ventricular tissue. Tissue samples were lysed in radioimmunoprecipitation assay buffer and protein content measured via a bicinchoninic acid assay (Thermo Fisher Scientific). Equal amounts of cardiac protein (30 μg) were heated in lithium dodecyl sulfate with 100 mM dithiothreitol at 70°C for 10 minutes. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. Blots were blocked in 5% milk 1× Tris-buffered saline/Tween 20 (TBST) before incubation overnight at 4°C with 1:250 cleaved caspase-3 (Cell Signaling Technology, 9661), 1:1,000 GAPDH (Cell Signaling Technology, 2118), 1:1,000 lamin A/C (Cell Signaling Technology, 2032), 1:500 MCP1 (Cell Signaling Technology, 2029), 1:500 NLRP3 (Cell Signaling Technology, 15101), 1:1,000 P65 (Cell Signaling Technology, 8242), 1:1,000 RhoGDI (Cell Signaling Technology, 2564), and 1:5,000 CaMKIIδ (provided by Donald Bers Laboratory, UC Davis, Davis, California, USA). Blots were washed with 1× TBST, incubated with HRP-conjugated secondary antibody (Sigma-Aldrich) at room temperature for 1 hour, washed with 1× TBST, and visualized by chemiluminescence (SuperSignal West Femto, Thermo Fisher Scientific). See complete unedited blots in the supplemental material.

Statistics. All results are presented as mean ± SEM, and groups were compared using *t* test, or 1-way or 2-way ANOVA. All statistics were calculated using GraphPad Prism 7. *P* values less than 0.05 were considered significant. For additional details regarding the methods used, see Supplemental Methods.

Study approval. Animal studies were performed in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee of UCSD.

Author contributions

AW, TS, SM, and JHB designed experiments. Data were analyzed and generated by AW, TS, and AN. HMH provided experimental resources. AW, SM, and JHB wrote the manuscript.

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1. Dick SA, Epelman S. Chronic heart failure and inflammation: what do we really know? *Circ Res*. 2016;119(1):159–176.
2. Heymans S, et al. Inflammation as a therapeutic target in heart failure? A scientific statement from the Translational Research Committee of the Heart Failure Association of the European Society of Cardiology. *Eur J Heart Fail*. 2009;11(2):119–129.
3. Hartupee J, Mann DL. Role of inflammatory cells in fibroblast activation. *J Mol Cell Cardiol*. 2016;93:143–148.
4. Prabhu SD, Frangogiannis NG. The biological basis for cardiac repair after myocardial infarction: from inflammation to fibrosis. *Circ Res*. 2016;119(1):91–112.
5. Duerrschmid C, Trial J, Wang Y, Entman ML, Haudek SB. Tumor necrosis factor: a mechanistic link between angiotensin-II-induced cardiac inflammation and fibrosis. *Circ Heart Fail*. 2015;8(2):352–361.
6. Kania G, Blyszczuk P, Eriksson U. Mechanisms of cardiac fibrosis in inflammatory heart disease. *Trends Cardiovasc Med*. 2009;19(8):247–252.
7. Segura AM, Frazier OH, Buja LM. Fibrosis and heart failure. *Heart Fail Rev*. 2014;19(2):173–185.
8. Krysko DV, et al. Emerging role of damage-associated molecular patterns derived from mitochondria in inflammation. *Trends Immunol*. 2011;32(4):157–164.
9. Schaefer L. Complexity of danger: the diverse nature of damage-associated molecular patterns. *J Biol Chem*. 2014;289(51):35237–35245.
10. Arslan F, de Kleijn DP, Pasterkamp G. Innate immune signaling in cardiac ischemia. *Nat Rev Cardiol*. 2011;8(5):292–300.
11. Ma Y, et al. Temporal neutrophil polarization following myocardial infarction. *Cardiovasc Res*. 2016;110(1):51–61.
12. Zhang X, Mosser DM. Macrophage activation by endogenous danger signals. *J Pathol*. 2008;214(2):161–178.
13. Zhang W, et al. Necrotic myocardial cells release damage-associated molecular patterns that provoke fibroblast activation in vitro and trigger myocardial inflammation and fibrosis in vivo. *J Am Heart Assoc*. 2015;4(6):e001993.
14. Liu L, et al. Up-regulated TLR4 in cardiomyocytes exacerbates heart failure after long-term myocardial infarction. *J Cell Mol Med*. 2015;19(12):2728–2740.
15. Wu Y, et al. S100a8/a9 released by CD11b+Gr1+ neutrophils activates cardiac fibroblasts to initiate angiotensin II-Induced cardiac inflammation and injury. *Hypertension*. 2014;63(6):1241–1250.
16. Benigni A, Cassis P, Remuzzi G. Angiotensin II revisited: new roles in inflammation, immunology and aging. *EMBO Mol Med*. 2010;2(7):247–257.
17. Epelman S, et al. Embryonic and adult-derived resident cardiac macrophages are maintained through distinct mechanisms at steady state and during inflammation. *Immunity*. 2014;40(1):91–104.
18. Haudek SB, et al. Monocytic fibroblast precursors mediate fibrosis in angiotensin-II-induced cardiac hypertrophy. *J Mol Cell Cardiol*. 2010;49(3):499–507.
19. Damilano F, et al. Distinct effects of leukocyte and cardiac phosphoinositide 3-kinase γ activity in pressure overload-induced cardiac failure. *Circulation*. 2011;123(4):391–399.
20. Nevers T, et al. Left Ventricular T-Cell Recruitment Contributes to the Pathogenesis of Heart Failure. *Circ Heart Fail*. 2015;8(4):776–787.
21. Xia Y, Lee K, Li N, Corbett D, Mendoza L, Frangogiannis NG. Characterization of the inflammatory and fibrotic response in a mouse model of cardiac pressure overload. *Histochem Cell Biol*. 2009;131(4):471–481.

22. Laroumanie F, et al. CD4+ T cells promote the transition from hypertrophy to heart failure during chronic pressure overload. *Circulation*. 2014;129(21):2111–2124.
23. Patel B, Ismahil MA, Hamid T, Bansal SS, Prabhu SD. Mononuclear phagocytes are dispensable for cardiac remodeling in established pressure-overload heart failure. *PLoS One*. 2017;12(1):e0170781.
24. Gray CB, et al. CaMKIIδ subtypes differentially regulate infarct formation following ex vivo myocardial ischemia/reperfusion through NF-κB and TNF-α. *J Mol Cell Cardiol*. 2017;103:48–55.
25. Ling H, et al. Ca2+/Calmodulin-dependent protein kinase II δ mediates myocardial ischemia/reperfusion injury through nuclear factor-κB. *Circ Res*. 2013;112(6):935–944.
26. Singh MV, et al. Ca2+/calmodulin-dependent kinase II triggers cell membrane injury by inducing complement factor B gene expression in the mouse heart. *J Clin Invest*. 2009;119(4):986–996.
27. Erickson JR, et al. A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. *Cell*. 2008;133(3):462–474.
28. Ling H, et al. Requirement for Ca2+/calmodulin-dependent kinase II in the transition from pressure overload-induced cardiac hypertrophy to heart failure in mice. *J Clin Invest*. 2009;119(5):1230–1240.
29. Purohit A, et al. Oxidized Ca(2+)/calmodulin-dependent protein kinase II triggers atrial fibrillation. *Circulation*. 2013;128(16):1748–1757.
30. Wagner S, et al. NADPH oxidase 2 mediates angiotensin II-dependent cellular arrhythmias via PKA and CaMKII. *J Mol Cell Cardiol*. 2014;75:206–215.
31. Zhao Z, Fefelova N, Shanmugam M, Bishara P, Babu GJ, Xie LH. Angiotensin II induces afterdepolarizations via reactive oxygen species and calmodulin kinase II signaling. *J Mol Cell Cardiol*. 2011;50(1):128–136.
32. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. *Nat Rev Immunol*. 2013;13(6):397–411.
33. Guo H, Callaway JB, Ting JP. Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nat Med*. 2015;21(7):677–687.
34. Kawaguchi M, et al. Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury. *Circulation*. 2011;123(6):594–604.
35. Mezzaroma E, et al. The inflammasome promotes adverse cardiac remodeling following acute myocardial infarction in the mouse. *Proc Natl Acad Sci USA*. 2011;108(49):19725–19730.
36. Marchetti C, et al. Pharmacologic inhibition of the NLRP3 inflammasome preserves cardiac function after ischemic and nonischemic injury in the mouse. *J Cardiovasc Pharmacol*. 2015;66(1):1–8.
37. Toldo S, et al. Inhibition of the NLRP3 inflammasome limits the inflammatory injury following myocardial ischemia-reperfusion in the mouse. *Int J Cardiol*. 2016;209:215–220.
38. Toldo S, Mezzaroma E, Mauro AG, Salloum F, Van Tassell BW, Abbate A. The inflammasome in myocardial injury and cardiac remodeling. *Antioxid Redox Signal*. 2015;22(13):1146–1161.
39. Iyer SS, et al. Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. *Proc Natl Acad Sci USA*. 2009;106(48):20388–20393.
40. de Haan JJ, Smeets MB, Pasterkamp G, Arslan F. Danger signals in the initiation of the inflammatory response after myocardial infarction. *Mediators Inflamm*. 2013;2013:206039.
41. Ma F, et al. Macrophage-stimulated cardiac fibroblast production of IL-6 is essential for TGF β/Smad activation and cardiac fibrosis induced by angiotensin II. *PLoS One*. 2012;7(5):e35144.
42. Burke JR, et al. BMS-345541 is a highly selective inhibitor of I kappa B kinase that binds at an allosteric site of the enzyme and blocks NF-kappa B-dependent transcription in mice. *J Biol Chem*. 2003;278(3):1450–1456.
43. Xu J, et al. CCR2 mediates the uptake of bone marrow-derived fibroblast precursors in angiotensin II-induced cardiac fibrosis. *Am J Physiol Heart Circ Physiol*. 2011;301(2):H538–H547.
44. Ghigo A, Franco I, Morello F, Hirsch E. Myocyte signalling in leucocyte recruitment to the heart. *Cardiovasc Res*. 2014;102(2):270–280.
45. Mitchell LA, Hansen RJ, Beaupre AJ, Gustafson DL, Dow SW. Optimized dosing of a CCR2 antagonist for amplification of vaccine immunity. *Int Immunopharmacol*. 2013;15(2):357–363.
46. He Y, Hara H, Núñez G. Mechanism and regulation of NLRP3 inflammasome activation. *Trends Biochem Sci*. 2016;41(12):1012–1021.
47. Stutz A, Horvath GL, Monks BG, Latz E. ASC speck formation as a readout for inflammasome activation. *Methods Mol Biol*. 2013;1040:91–101.
48. Westenbrink BD, et al. Mitochondrial reprogramming induced by CaMKIIδ mediates hypertrophy decompensation. *Circ Res*. 2015;116(5):e28–e39.
49. Joiner ML, et al. CaMKII determines mitochondrial stress responses in heart. *Nature*. 2012;491(7423):269–273.
50. Heid ME, Keyel PA, Kamga C, Shiva S, Watkins SC, Salter RD. Mitochondrial reactive oxygen species induces NLRP3-dependent lysosomal damage and inflammasome activation. *J Immunol*. 2013;191(10):5230–5238.
51. Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. *Nature*. 2011;469(7329):221–225.
52. Coll RC, et al. A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nat Med*. 2015;21(3):248–255.
53. Campbell SE, Katwa LC. Angiotensin II stimulated expression of transforming growth factor-beta1 in cardiac fibroblasts and myofibroblasts. *J Mol Cell Cardiol*. 1997;29(7):1947–1958.
54. Duerrschmid C, et al. TNF receptor 1 signaling is critically involved in mediating angiotensin-II-induced cardiac fibrosis. *J Mol Cell Cardiol*. 2013;57:59–67.
55. Gao X, He X, Luo B, Peng L, Lin J, Zuo Z. Angiotensin II increases collagen I expression via transforming growth factor-beta1 and extracellular signal-regulated kinase in cardiac fibroblasts. *Eur J Pharmacol*. 2009;606(1-3):115–120.
56. Frangogiannis NG. Inflammation in cardiac injury, repair and regeneration. *Curr Opin Cardiol*. 2015;30(3):240–245.
57. Shimano M, Ouchi N, Walsh K. Cardiokines: recent progress in elucidating the cardiac secretome. *Circulation*.

2012;126(21):e327–e332.
58. Aoyagi T, Matsui T. The cardiomyocyte as a source of cytokines in cardiac injury. *J Cell Sci Ther*. 2011;2012(S5):003.
59. Doroudgar S, Glembotski CC. The cardiokine story unfolds: ischemic stress-induced protein secretion in the heart. *Trends Mol Med*. 2011;17(4):207–214.
60. Li TT, et al. Endoplasmic reticulum stress in bone marrow-derived cells prevents acute cardiac inflammation and injury in response to angiotensin II. *Cell Death Dis*. 2016;7(6):e2258.
61. Feng N, Anderson ME. CaMKII is a nodal signal for multiple programmed cell death pathways in heart. *J Mol Cell Cardiol*. 2017;103:102–109.
62. Timmins JM, et al. Calcium/calmodulin-dependent protein kinase II links ER stress with Fas and mitochondrial apoptosis pathways. *J Clin Invest*. 2009;119(10):2925–2941.
63. Yang Y, et al. Calmodulin kinase II inhibition protects against myocardial cell apoptosis in vivo. *Am J Physiol Heart Circ Physiol*. 2006;291(6):H3065–H3075.
64. Zhang T, et al. CaMKII is a RIP3 substrate mediating ischemia- and oxidative stress-induced myocardial necroptosis. *Nat Med*. 2016;22(2):175–182.
65. Zhu WZ, et al. Linkage of beta1-adrenergic stimulation to apoptotic heart cell death through protein kinase A-independent activation of Ca2+/calmodulin kinase II. *J Clin Invest*. 2003;111(5):617–625.
66. Zhou Z, et al. PEDF Inhibits the activation of NLRP3 inflammasome in hypoxia cardiomyocytes through PEDF receptor/phospholipase A2. *Int J Mol Sci*. 2016;17(12):E2064.
67. Kalbitz M, et al. Complement-induced activation of the cardiac NLRP3 inflammasome in sepsis. *FASEB J*. 2016;30(12):3997–4006.
68. Sandanger Ø, et al. The NLRP3 inflammasome is up-regulated in cardiac fibroblasts and mediates myocardial ischaemia-reperfusion injury. *Cardiovasc Res*. 2013;99(1):164–174.
69. Zhang W, et al. Cardiac fibroblasts contribute to myocardial dysfunction in mice with sepsis: the role of NLRP3 inflammasome activation. *PLoS One*. 2014;9(9):e107639.
70. Grimm M, et al. CaMKIIδ mediates β-adrenergic effects on RyR2 phosphorylation and SR Ca(2+) leak and the pathophysiological response to chronic β-adrenergic stimulation. *J Mol Cell Cardiol*. 2015;85:282–291.
71. He BJ, et al. Oxidation of CaMKII determines the cardiotoxic effects of aldosterone. *Nat Med*. 2011;17(12):1610–1618.
72. Weinreuter M, et al. CaM Kinase II mediates maladaptive post-infarct remodeling and pro-inflammatory chemoattractant signaling but not acute myocardial ischemia/reperfusion injury. *EMBO Mol Med*. 2014;6(10):1231–1245.
73. Kanneganti TD, et al. Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature*. 2006;440(7081):233–236.
74. O'Connell TD, Rodrigo MC, Simpson PC. Isolation and culture of adult mouse cardiac myocytes. *Methods Mol Biol*. 2007;357:271–296.
75. Li S, et al. Nrf2 deficiency exaggerates doxorubicin-induced cardiotoxicity and cardiac dysfunction. *Oxid Med Cell Longev*. 2014;2014:748524.