# Proinflammatory Protein CARD9 Is Essential for Infiltration of Monocytic Fibroblast Precursors and Cardiac Fibrosis Caused by Angiotensin II Infusion

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### BACKGROUND

Angiotensin II (Ang II)–induced cardiac remodeling with the underlying mechanisms involving inflammation and fibrosis has been well documented. Cytosolic adaptor caspase recruitment domain 9 (CARD9) has been implicated in the innate immune response. We aimed to examine the role of CARD9 in inflammation and cardiac fibrosis induced by Ang II.

### METHODS

Two-month-old CARD9-deficient (CARD9<sup>-/-</sup>) and wild-type (WT) male mice were infused with Ang II (1,500 ng/kg/min) or saline for 7 days. Heart sections were stained with hematoxylin and eosin and Masson trichrome and examined by immunohistochemistry; and activity and protein levels were measured in macrophages obtained from mice.

#### RESULTS

WT mice with Ang II infusion showed a marked increase in CARD9<sup>+</sup> macrophages in the heart, but CARD9<sup>-/-</sup> mice showed

Hypertension is a major risk factor of cardiovascular morbidity and the most common cause of congestive heart failure. Hypertensive cardiac remodeling is characterized by left ventricular hypertrophy and cardiac fibrosis.<sup>1–3</sup> Activation of the rennin-angiotensin system plays an important role in blood pressure control, cardiovascular regulation and remodeling. Angiotensin II (Ang II), a critical hormone of this system, has been implicated in the development of cardiac fibrosis and inflammation. Progressive cardiac fibrosis contributes to increased cardiac muscle stiffness, leading to cardiac dysfunction and heart failure.<sup>4</sup> However, the mechanisms of the regulation of cardiac fibrosis and inflammation by Ang II remain to be explored.

Received 17 September 2010; first decision 2 November 2010; accepted 16 February 2011.

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significantly suppressed macrophage infiltration and expression of proinflammatory cytokines, including interleukin-1 $\beta$  (IL-1 $\beta$ ) and connective tissue growth factor (CTGF). Importantly, Ang II–induced cardiac fibrosis (extracellular matrix and collagen I deposition) was diminished in CARD9<sup>-/-</sup> hearts, as was the expression of transforming growth factor- $\beta$  (TGF- $\beta$ ) and level of myofibroblasts positive for  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA). Furthermore, Ang II activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), JNK and p38 mitogenactivated protein kinases (MAPKs) in WT macrophages was reduced in CARD9<sup>-/-</sup> macrophages.

### CONCLUSION

CARD9 plays an important role in regulating cardiac inflammation and fibrosis in response to elevated Ang II.

*Keywords:* blood pressure; CARD9; cardiac fibrosis; hypertension; inflammation; myofibroblast; NF-кВ

American Journal of Hypertension, advance online publication 24 March 2011; doi:10.1038/ajh.2011.42

The role of inflammation in fibrosis has been shown by recent studies describing an important role for blood-borne, monocytic fibroblast precursors that express CD34 and CD45 in Ang II-induced or other pathological interstitial cardiac fibrosis.<sup>5</sup> Ang II infusion resulted in the appearance of bone marrow-derived CD34<sup>+</sup>/CD45<sup>+</sup> fibroblast precursor cells that expressed collagen type I and the cardiac fibroblast marker DDR2, and structural fibroblasts were negative for CD34/CD45.<sup>6</sup> Moreover, knockout (KO) of monocyte chemoattract-ant protein 1 prevented Ang II-induced infiltration of CD34<sup>+</sup>/CD45<sup>+</sup> fibroblast precursor cells and cardiac fibrosis.

Growing evidence indicates that Ang II influences all stages of the inflammatory response involved in inflammatory gene expression. Via its AT1 receptor, Ang II activates NADPH oxidase and produces reactive oxygen species, thus leading to activation of the transcriptional factors Ets-1, early growth response 1, and nuclear factor-κB (NF-κB). We previously showed that in vascular smooth muscle cells. Ang II activates NF-κB via an IKK- and a mitogen-activated protein kinase (MAPK)-dependent mechanism.<sup>7–9</sup> However, how Ang II activates NF-κB in inflammatory cells remains unknown.

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The caspase-recruitment domain (CARD) is a protein-binding module that mediates the assembly of CARD-containing proteins. Cytosolic adaptor caspase recruitment domain 9 (CARD9) has received much attention.<sup>4,10</sup> It consists of an amino-terminal CARD and a carboxyterminal coiled-coil domain and is closely related to the well-characterized CARDcontaining adaptor CARMA1 (also called CARD11 and Bimp3).<sup>4,10</sup> CARD9 is expressed in various tissues, including spleen, liver, placenta, lung, leukocytes, 4,10 with highest expression in macrophages and dendritic cells.<sup>11</sup> Several studies of CARD9-deficient mice demonstrated that CARD9 is involved in inducing cytokine activity and in in vivo protection against fungal and bacterial infection.<sup>11-16</sup> CARD9 has disparate functions in these two pathways, linking tyrosine kinases to activation of the transcription factor NF-KB and toll-like receptors to MAPKs, including p38 and JNK.<sup>11,17,18</sup> Although such data suggest that CARD9 plays a central role in the innate immune response, no studies have examined the effects of CARD9 on inflammation and cardiac fibrosis.

Given the role of CARD9 in inflammation, we sought to determine the role of CARD9 signaling in Ang II–induced inflammation and cardiac fibrosis. We used a mouse model with KO of the *CARD9* gene and Ang II infusion. Cardiac fibrosis and inflammatory response induced by Ang II infusion was markedly inhibited in CARD9<sup>-/-</sup> mice.

### **METHODS**

Animal model. CARD9-KO (CARD9-/-) mice were kindly supplied by Dr Lin (Department of Molecular and Cellular Oncology, University of Texas, Houston, TX). Two-monthold CARD9<sup>-/-</sup> mice and littermate wild-type (WT) male mice of the same C57BL/6 background were used. Mice were implanted with osmotic pumps(Alzet MODEL 1007D; DURECT, Cupertino, CA) filled with Ang II and infused at 1,500 ng/kg/min in Ringer's solution (0.01 mol/l acetic acid in saline). Control mice received infusion of saline of comparable volume. WT and CARD9-/- mice were randomly assigned to the control group or Ang II-treated group. Systolic blood pressure was measured by the tail-cuff system (Softron BP-98A; Softron, Tokyo, Japan), and values were derived from an average of 6-8 measurements per animal at each time point. The animals were handled according to the animal welfare regulations of Capital Medical University, Beijing, China, and the study protocol was approved by the Animal Subjects Committee of Capital Medical University.

Histology and immunohistochemistry. Animals were killed 7 days after Ang II or saline infusion. Hearts were isolated and fixed in 4% paraformaldehyde solution. The samples were cut into 5- $\mu$ m sections and stained with hematoxylin and eosin by standard procedures. To measure fibrotic areas, sectioned hearts were stained with Masson trichrome. The interstitial fibrotic areas were calculated as the ratio of total area of interstitial fibrosis to total area of the section. Immunohistochemical analysis involved antibodies against CARD9 (1:200 dilution), Mac-2 (1:400 dilution), interleukin-1 $\beta$  (IL-1 $\beta$ ) (1:200 dilution),

connective tissue growth factor (CTGF) (1:200 dilution) and transforming growth factor- $\beta$  (TGF- $\beta$ ) (1:300 dilution; all Santa Cruz Biotechnology, Santa Cruz, CA); and collagen I (1:1,000 dilution). Anti-F4/80 (1:100 dilution; Abcam, Cambridge, MA), which recognizes the mouse F4/80 antigen, a 160kDa glycoprotein expressed a wide range of mature tissue macrophages including Kupffer cells, Langerhans cells, microglia, macrophages located in the gut lamina propria, peritoneal cavity, lung, thymus, bone marrow stroma, and macrophages in the red pulp of the spleen. The macrophages we used in *in vitro* study were from peritoneal cavity. a-Smooth muscle actin ( $\alpha$ -SMA) (1:200 dilution) described previously.<sup>19</sup> Images were viewed and captured by use of a Nikon Labophot 2 microscope equipped with a Sony CCD-Iris/RGB color video camera attached to a computerized imaging system and analyzed by ImagePro Plus 3.0 (ECLIPSE80i/90i; Nikon, Tokyo, Japan).

*Cell culture*. Isolation of mouse peritoneal macrophages was as described.<sup>20</sup> Briefly, 8–10-week-old mice were injected with 2 ml of 4% thioglycollate medium. Then, 4 days later, mice were killed and the peritoneal cavity was flushed with 5-ml cold phosphate-buffered solution. The phosphate-buffered solution with macrophages was removed with a syringe, and the process was repeated twice. Macrophages from each mouse were plated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1:100 penicillin/ streptomycin. Nonadherent cells were removed after 4h, and the medium was replaced with fresh medium.<sup>21</sup>

*NF-κB luciferase reporter assay.* NF-κB transcriptional activity was measured by use of adenovirus infection with NF-κB luciferase reporter (Ad.NF-κB-Luc) as described.<sup>22</sup> Briefly, peritoneal macrophages from WT and CARD9<sup>-/-</sup> mice were infected with Ad.NF-κB-Luc at a multiplicity of infection of 5 for 24 h before 100 nmol/l Ang II treatment; from each sample, 5 µg total protein underwent luciferase activity assay according to the manufacturer's protocol (Promega, Madison, WI).

Western blot analysis. Cells were lyzed with lysis buffer (20 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/lEGTA, 1% Triton X-100, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerophosphate, 1 mmol/l Na<sub>3</sub>VO<sub>4</sub>, 4 µg/ml aprotinin, 4µg/ml leupeptin, 4µg/ml pepstatin, and 1mmol/l PMSF). Western blot analysis was performed as described.<sup>23</sup> In total, 50 µg cell protein was loaded on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, transferred to nitrocellulose membrane, and probed with the primary antibodies against CARD9, β-actin (Santa Cruz Biotechnology), phospho-p65 (Ser536), p65, phospho-JNK1/2 (Thr183/ Tyr185), JNK1/2, phospho-p38 (Thr180/Tyr182), and p38 (all Cell Signaling Technology, Danvers, MA). After being washed extensively, the membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1h at room temperature. The signals were visualized by use of an enhanced chemiluminescence kit (Amersham, Piscataway, NJ). The levels of signaling proteins were normalized to that of  $\beta$ -actin.

*Statistical analysis.* Data are expressed as mean  $\pm$  s.e.m. Statistical analysis involved one-way ANOVA followed by LSD *t*-test for multiple comparisons within treatment groups. Analysis involved use of SPSS v13.0 (SPSS, Chicago, IL). *P* < 0.05 was considered statistically significant.

#### RESULTS

### Ang II infusion stimulates infiltration of CARD9<sup>+</sup> inflammatory cells into mouse hearts

To investigate the role of CARD9 in the development of Ang II–induced cardiac remodeling, WT mice were infused with Ang II, 1,500 ng/kg/min, for 7 days. The systolic blood pressure, heart weight/body weight (HW/BW) and heart weight/ tibia length (HW/TL) ratios were measured (**Table 1**). Baseline BP, HW/BW, and HW/TL ratios were similar in both WT and KO mice. After Ang II treatment, these parameters tended to be increased in KO mice compared with WT mice, but the difference was not statistically significant. Immunohistochemistry revealed that Ang II infusion significantly increased the number of CARD9<sup>+</sup> cells in mice hearts as compared with the control group (P < 0.05; **Figure 1a**).

CARD9 is known to be highly expressed in macrophages.<sup>11</sup> We then examined whether CARD9 is expressed in macrophages from myocardial tissues or from the peritoneal cavity using double immunostaining for F4/80 (for macrophages) and CARD9. CARD9 was expressed in macrophages, and its expression was markedly induced after Ang II infusion (Figure 1b,c).

### CARD9 deficiency inhibits myocardial inflammation induced by Ang II in mouse hearts

Inflammatory cell infiltrates were markedly increased in WT mice after Ang II infusion as compared with untreated WT mice (**Figure 2a**). The infiltration of inflammatory cells after Ang II was lower in CARD9<sup>-/-</sup> mice than in WT mice (P < 0.05). Furthermore, immunohistochemical staining of myocardial sections demonstrated the expression of Mac-2 (macrophage marker). IL-1 $\beta$  and CTGF significantly increased in Ang II-treated WT mice as compared with untreated WT mice, with the increased expression significantly lower in CARD9<sup>-/-</sup> mice than in WT mice after Ang II infusion (**Figure 2b-d**). The protein expression of Mac-2, IL-1 $\beta$ , and CTGF did not differ between WT and CARD<sup>-/-</sup> mice without Ang II treatment (**Figure 2b-d**). Moreover, the numbers of TUNEL-positive cardiomyocytes were comparable between WT and KO mice following 7 days of Ang II infusion (data not shown).

Table 1	Body weight, s	ystolic BP, HW/BW and HW/TL ratios
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	WT	KO	WT + Ang II	KO + Ang II		
Parameters	( <i>n</i> = 6)					
BP (mm Hg)	97.33±6.03	95.33 ± 4.62	$135 \pm 10$	$144 \pm 12$		
HW/BW (mg/g)	$5.03\pm0.35$	$5.22 \pm 0.26$	$5.74\pm0.81$	$6.00\pm0.77$		
HW/TL (mg/mm)	$7.01 \pm 0.4$	$6.86\pm0.37$	$8.12\pm0.99$	9.31 ± 1.82		
Ang II, angiotensin II; BP, blood pressure; BW, body weight; HW, heart weight; KO,						

Ang II, angiotensin II; BP, blood pressure; BW, body weight; HW, heart weight; KC knockout; TL, tibia length; WT, wild type.

### CARD9 deficiency attenuates cardiac fibrosis induced by Ang II in mouse hearts

We then determined the role of CARD9 in the development of cardiac fibrosis. Systolic blood pressure was markedly increased in both WT and CARD9<sup>-/-</sup> mice after Ang II infusion, with no significant difference between WT and CARD9-/- mice (data not shown). Moreover, heart sections stained with Masson trichrome for measurement of fibrotic areas showed that Ang II infusion significantly increased cardiac fibrotic areas in Ang II-treated WT mice as compared with untreated WT mice (Figure 3a). However, in CARD9<sup>-/-</sup> mice, the fibrotic areas were significantly smaller than in WT mice with Ang II treatment. WT and CARD9<sup>-/-</sup> mice did not differ in cardiac fibrotic areas without Ang II treatment. Moreover, immunohistochemistry confirmed that with Ang II treatment, the increased protein levels of collagen I and TGF-B in WT mice were markedly suppressed in CARD<sup>-/-</sup> mice (Figure 3b,c). With Ang II treatment, the protein expression of α-SMA, a marker of myofibroblasts, was markedly elevated in WT mice but significantly reduced in CARD<sup>-/-</sup> mice (P < 0.01; Figure 3d).

## Effect of CARD9 on the activation of NF-κB and MAPK signaling pathways in mouse peritoneal macrophages

To determine the mechanisms of the inhibition of cardiac inflammation and fibrosis in CARD9<sup>-/-</sup> mice after Ang II infusion, we first examined the CARD9 downstream target NF- $\kappa$ B activity in peritoneal macrophages using NF- $\kappa$ B luciferase reporter assay. Ang II treatment dose-dependently increased NF- $\kappa$ B luciferase activity in WT macrophages (**Figure 4a**), which was significantly suppressed in CARD<sup>-/-</sup> macrophages.



**Figure 1** Angiotensin II (Ang II) infusion induces cytosolic adaptor caspase recruitment domain 9 (CARD9) expression in macrophages of cardiac tissues. Wild-type (WT) mice were infused with Ang II at 1,500 ng/kg/min for 7 days, and heart tissues were harvested. (**a**) Heart sections from saline- and Ang II-treated mice stained with anti-CARD9 antibody (Ab) (left). Bar = 50 µm. F4/80 (macrophages) and CARD9 immunofluorescence staining of (**b**) cardiac tissues or (**c**) peritoneal macrophages from both WT and CARD9<sup>-/-</sup> mice infused with or without Ang II.

### **ORIGINAL CONTRIBUTIONS**



**Figure 2** | Cytosolic adaptor caspase recruitment domain 9 (CARD9) deficiency inhibits angiotensin II (Ang II) infusion–induced proinflammatory cell infiltration and expression of inflammatory cytokines. (**a**) Heart sections from wild-type (WT) and CARD9<sup>-/-</sup> mice treated with saline or Ang II at 1,500 ng/kg/min for 7 days were stained with hematoxylin and eosin (HE). (**b**–**d**) Heart sections from WT and CARD9<sup>-/-</sup> mice were detected by immunohistochemistry with antibodies against Mac-2, interleukin-1 $\beta$  (IL-1 $\beta$ ), and connective tissue growth factor (CTGF) (top). Bar graph shows quantified areas (bottom). Bar = 50 µm. Data represent mean ± s.e.m. (*n* = 5). \**P* < 0.01 vs. WT+Ang II.

We next examined the activation of NF- $\kappa$ B and MAPKs in peritoneal macrophages by Western blot analysis. Without Ang II treatment, levels of NF- $\kappa$ B/p65, p38, and JNK1/2 phosphorylation were lower in CARD9<sup>-/-</sup> than WT macrophages (Figure 4b). However, Ang II markedly activated NF- $\kappa$ B/p65, p38, and JNK1/2 in WT macrophages as compared with untreated WT cells. Activation of NF- $\kappa$ B/p65, p38, or JNK1/2 was similar in CARD9<sup>-/-</sup> macrophages before and after Ang II treatment (Figure 4b).



**Figure 3** | Cytosolic adaptor caspase recruitment domain 9 (CARD9) knockout prevents angiotensin II (Ang II) infusion–induced cardiac fibrosis. (**a**) Representative Masson trichrome staining of heart sections from wild-type (WT) and CARD9<sup>-/-</sup> mice (top). Bar graph shows quantified fibrotic areas on histological sections (bottom). (**b**–**d**) Heart sections from WT and CARD9<sup>-/-</sup> mice were detected by immunohistochemistry with antibodies against collagen I, transforming growth factor- $\beta$  (TGF- $\beta$ ) and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) (top). Bar graph shows quantified areas (bottom). Bar = 50 µm. Data were represented as the mean ± s.e.m. (n = 5). \*P < 0.01 vs. WT+Ang II.



**Figure 4** | Effect of cytosolic adaptor caspase recruitment domain 9 (CARD9) knockout on the activation of nuclear factor-κB (NF-κB) and mitogenactivated protein kinases. (**a**) Peritoneal macrophages were stimulated with different concentrations of angiotensin II (Ang II) for 24 h NF-κB activity was determined by luciferase assay (n = 3). \*P < 0.01 vs. control. (**b**) Peritoneal macrophages were stimulated with 100 nmol/l Ang II for 24 h. The protein levels of NF-κB/p65, JNK1/2, p38, and GAPDH were determined by Western blot analysis (top). Bar graph shows densitometric analysis of intensities of phosphorylated NF-κB/p65, JNK1/2, and p38 relative to that of β-actin (bottom, n = 3). \*P < 0.01 vs. WT, \*P < 0.05 vs. WT+Ang II. WT, wild type.

### DISCUSSION

Although Ang II has powerful proinflammatory effects on cardiac fibrosis and inflammation, the precise molecular mechanisms for these effects are not fully understood.<sup>24–28</sup> In the present study, we found that Ang II significantly stimulated the infiltration of CARD9<sup>+</sup> macrophages into hearts. Importantly, CARD9<sup>-/-</sup> prevented Ang II–induced inflammation and cardiac fibrosis. These effects were associated with reduced activation of the NF- $\kappa$ B/MAPK signaling pathway, expression of proinflammatory cytokines and accumulation of  $\alpha$ -SMA<sup>+</sup> myofibroblast cells with Ang II infusion. Thus, CARD9 is important in hypertensive cardiac remodeling induced by Ang II infusion.

Cardiac fibrosis and inflammation are the major events of Ang II–induced myocardial remodeling. CARD9 is a caspase recruitment-domain–containing signaling protein expressed in various tissues.<sup>10,11</sup> It is closely associated with several inflammatory diseases, including tuberculosis, inflammatory bowel disease, tuberculosis, and ankylosing spondylitis.<sup>13,29,30</sup> In CARD9<sup>-/-</sup> mice, stimulation of dendritic cells caused a marked decrease in the production of IL-2, -6, and -10, and TNF- $\alpha$  and decreased numbers of Th17 cells.<sup>31–33</sup> Humans with CARD9 mutation show defects in inflammation responses.<sup>34</sup> Given the important role of CARD9 in inflammation, we investigated the role of CARD9 in cardiac inflammation and fibrosis after Ang II infusion. We found infiltrated macrophages expressing CARD9 in response to Ang II infusion. The critical role of CARD9 was demonstrated by Ang II–induced macrophage infiltration, and the expression of TGF- $\beta$ , collagen I,  $\alpha$ -SMA, IL-1 $\beta$ , and CTGF was significantly inhibited in CARD9<sup>-/-</sup> mice with Ang II infusion (Figures 2 and 3).

Myofibroblast formation is controlled by a number of growth factors, cytokines, and mechanical stimuli.<sup>35</sup> In the present study, the number of  $\alpha$ -SMA<sup>+</sup> myofibroblasts in myocardial tissues of CARD9<sup>-/-</sup> mice was significantly lower than that of WT mice after Ang II infusion (**Figure 3**). Moreover, the expression of collagen I and TGF- $\beta$  was markedly decreased in CARD9<sup>-/-</sup> mice (**Figure 3**). Therefore, CARD9 deficiency inhibits cardiac fibrosis by inhibiting myofibroblast formation.

Increasing evidence has demonstrated that Ang II, via its type 1 receptor, activates a number of signaling pathways, including reactive oxygen species, MAPKs, receptor- or nonreceptor-associated tyrosine kinases, and transcriptional factors such as NF-KB, Ets-1, and early growth response 1 to regulate cardiac hypertrophy, inflammation, and fibrosis.<sup>36</sup> Among these, NF-KB and MAPK signaling pathways play pivotal roles in regulating the inflammatory, immune, and apoptotic responses in mammals.<sup>37</sup> Ang II, via AT1R activation, also increases production of aldosterone which is a steroid hormone with mineralocorticoid activity. Beyond its effects on renal sodium reabsorption, aldosterone exerts effects on the kidney, blood vessels, and the heart, and plays an important role in renal and cardiovascular injury, including inflammation, tissue remodeling and fibrosis, has been demonstrated in various animal models of hypertension.<sup>38,39</sup> The aldosterone increases tissue angiotensin-converting enzyme activity and upregulates AT1, indicating a vicious forward feedback loop resulting in increased production of both Ang II and aldosterone.<sup>40–42</sup> Furthermore, Rickard *et al.* demonstrated macrophage-specific KO of mineralocorticoid receptors protected against DoCa-induced cardiac fibrosis and hypertension.<sup>43</sup> However, question remains of how is proinflammatory transcriptional factor NF-KB activated in macrophage in response to Ang II.

Interestingly, CARD9 is selectively involved in the activation of NF-κB and MAPKs, which is required for the production of proinflammatory cytokines in innate immune responses to intracellular pathogens.<sup>11,44–46</sup> CARD9 signals allow the Tolllike receptor and Nod2 pathways to induce MAPK activation (including p38 and JNK1/2) and tyrosine kinase pathways to induce NF-κB activation. In the present study, Ang II treatment resulted in a marked activation of NF-κB and MAPK signaling pathways, as demonstrated by increased NF-κB luciferase activity (**Figure 4a**) and increased levels of phosphorylated NF-κB/p65, p38, and JNK1/2 in macrophages from WT mice. In contrast, macrophages from CARD9<sup>-/-</sup> mice showed no significant activation of NF-κB, p38, JNK1/2 with Ang II treatment (**Figure 4b**). Thus, CARD9 is involved in Ang II–mediated regulation of NF-κB and MAPKs in the heart. In conclusion, we revealed an important role of CARD9 in Ang II infusion–induced cardiac remodeling. CARD9 deficiency inhibited inflammatory cells infiltration, expression of proinflammatory cytokines, accumulation of myofibroblasts, and cardiac fibrosis with Ang II treatment. These effects might be a result of inhibiting the activation of NF- $\kappa$ B and MAPKs.

Acknowledgments: This study was supported by grants from Chinese Ministry of Science and Technology (2009CB522205) and National Science Foundation of China (30888004). Dr Xin is a visiting professor from Department of Molecular and Cellular Oncology, University of Texas, M.D. Anderson Cancer Center, Houston, TX. Dr J.C. is a visiting professor from Division of Nephrology, Baylor College of Medicine, Houston, TX.

#### Disclosure: The authors declared no conflict of interest.

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