Cardiac RNA Induces Inflammatory Responses in Cardiomyocytes and Immune Cells via Toll-like Receptor 7 Signaling*

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Background: Necrotic extracellular RNA induces inflammation and may contribute to ischemic myocardial injury, but the underlying mechanism is unclear.

Results: Isolated cardiac RNA induced cytokine production in cardiomyocytes/immune cells as well as acute peritonitis. Genetic deletion of TLR7 or MyD88 markedly attenuates RNA-mediated cytokine production.

Conclusion: Cellular RNA induces cytokine production via TLR7-MyD88 signaling.

Significance: Cardiac RNA is a pro-inflammatory mediator sensed by TLR7.

We have recently reported that extracellular RNA (exRNA) released from necrotic cells induces cytokine production in cardiomyocytes and immune cells and contributes to myocardial ischemia/reperfusion injury. However, the signaling mechanism by which exRNA exhibits its pro-inflammatory effect is unknown. Here we hypothesize that exRNA directly induces inflammation through specific Toll-like receptors (TLRs). To test the hypothesis, we treated rat neonatal cardiomyocytes, mouse bone marrow-derived macrophages (BMDM), or mouse neutrophils with RNA (2.5–10 μg/ml) isolated from rat cardiomyocytes or the hearts from mouse, rat, and human. We found that cellular RNA induced production of several cytokines such as macrophage inflammatory protein-2 (MIP-2), ILs, TNFα, and the effect was completely diminished by RNase, but not DNase. The RNA-induced cytokine production was partially inhibited in cells treated with TLR7 antagonist or genetically deficient in TLR7. Deletion of myeloid differentiation primary response protein 88 (MyD88), a downstream adapter of TLRs including TLR7, abolishes the RNA-induced MIP-2 production. Surprisingly, genetic deletion of TLR3 had no impact on the RNA-induced MIP-2 response. Importantly, extracellular RNA released from damaged cardiomyocytes also induced cytokine production. Finally, mice treated with 50 μg of RNA intraperitoneal injection exhibited acute peritonitis as evidenced by marked neutrophil and monococyte migration into the peritoneal space. Together, these data demonstrate that exRNA of cardiac origin exhibits a potent pro-inflammatory property in vitro and in vivo and that exRNA induces cytokine production through TLR7-MyD88 signaling.

Recent studies have indicated that RNA molecules are actively secreted into the extracellular spaces or passively released from cells because of injury, chronic inflammation, and cell death (1). Extracellular RNA (exRNA),3 which include mRNA, tRNA, microRNA (miRNA), and long non-coding RNA, is protected against ubiquitous distribution of RNase and proposed to serve as sensitive biomarker in certain disease conditions. For instance, circulating mRNAs serve as biomarkers in various cancers (2–5). Circulating miRNAs, a family of short single-stranded non-coding RNAs, serve as biomarkers for different diseases such as cancers (6, 7), myocardial injury (8, 9), and liver damage (10, 11). While these and other studies may have established exRNA as sensitive biomarkers and potential intercellular communicators (12, 13), the biological function of exRNA and their contributions to the pathogenesis of diseases remain largely unknown.

We have recently reported that cellular RNAs are released from injured cardiomyocytes in culture and from ischemic myocardium in vivo (14). We demonstrate that RNase treatment attenuates necrotic cell-induced cytokine production in cardiomyocytes and protects animals against ischemia-reperfusion injury as evidenced by smaller infarct size, decreased myocardial inflammation, and apoptosis (14). A similar finding was subsequently reported by Cabrera-Fuentes et al. (15). These data suggest that exRNA mediates necrotic cell-induced inflammation in cardiomyocytes and may contribute to the pathogenesis of ischemic myocardial injury.

Toll-like receptors (TLRs) are a family of pattern recognition receptors in the innate immune system. They act as the first line of host defense against foreign pathogens and through pathogen-associated molecular pattern (16, 17). Four members of

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26688 JOURNAL OF BIOLOGICAL CHEMISTRY

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TLR family are reportedly involved in nucleic acid recognition: TLR3, TLR7, TLR8, and TLR9, all localized to endosomes (18, 19). TLR3 senses double-stranded RNA (dsRNA) of viral origin (20) and synthetic analog of dsRNA (e.g., polyinosinic-polycytidylic acid (poly(I:C))) (20). TLR7 and TLR8 recognize single-stranded RNA (ssRNA) of virus (21, 22), imidazoquinoline compounds such as imiquimod (R837) and resiquimod (R848) (23). TLR9 recognizes DNA sequence with unmethylated cytosine-guanosine (CpG) motif (24). Myeloid differentiation primary response protein 88 (MyD88) and Toll/IL-1 receptor domain-containing adapter-inducing interferon β (Trif) are two important adaptors in TLR signaling. TLR3 exclusively recruits Trif, while TLR7/8 and TLR9 are dependent on MyD88 signaling (24). The activation of TLR3, TLR7/8, and TLR9 leads to the production of proinflammatory cytokines and type I interferons with potent antiviral activity (24).

Although the innate immune system is capable of distinguishing self and non-self RNA, it has been reported that endogenous RNA released from necrotic cells induces inflammatory response and that in vitro synthesized mRNA elicits cytokine production via a TLR3-dependent mechanism in human dendritic cells (25). Moreover, in a stable-transfected HEK 293 cell line, in vitro transcribed mRNA can induce cytokine production via TLR7- and TLR8-dependent manner (26). These data suggest that non-pathogenic RNA can activate TLR signaling and may play a role in cellular inflammation.

In this study, we hypothesize that cellular RNA is an intracellular mediator that stimulates a potent inflammatory response in cardiomyocytes and immune cells specifically via a TLR-dependent mechanism. We treated cardiomyocytes and immune cells with RNA isolated from cardiac cells or tissues and measured cytokine response. Using specific TLR inhibitor and cells genetically deficient of TLRs or their adaptors, we determined the specific role of TLR3 and TLR7 signaling. Finally, we demonstrated the pro-inflammatory property of cellular RNA in a mouse model in vivo.

**Experimental Procedures**

**Materials**

Lipopolysaccharides (LPS; *Escherichia coli* 0111: B4, Cat. L4391), collagenase 2, polymyxin B sulfate (PMB), and RNase A of bovine pancreas (Cat. R6513) were from Sigma-Aldrich (St. Louis, MO). Poly(I:C), Pam3Cys (P3C) and CpG were purchased from Enzo Life (Plymouth Meeting, PA). DNase was bought from Thermo Scientific Inc. (Waltham, MA), while Benzonase was from Millipore (Billerica, MA). Imiquimod (R837, TLR7 ligand) and CL075 (TLR8 ligand) were provided by Invivogen (San Diego, CA). Specific immunoregulatory DNA sequences (IRSs) were synthesized as TLR antagonists by Integrated DNA Technologies (Coralville, IA) with phosphorothioate linkages as previously described (27). The following sequences were used: IRS661 (TLR7 inhibitor: 5′-TGTCTTGCAAGCTTGGCAAGCA-3′), IRS869 (TLR9 inhibitor: 5′-TCCTGGAGGTTTGT-3′), and control oligonucleotide (Con.: 5′-TCCTGGCAGGAAAGT-3′). All of the antibodies for Western blot were purchased from Cell Signaling Tech. (Danvers, MA).

**TLR7 Mediates Cellular RNA-induced Inflammation**

**Animals**

Wild-type (WT, C57BL/6), TLR3<sup>−/−</sup>, and TLR7<sup>−/−</sup> mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MyD88<sup>−/−</sup> mice were generated by Kawai and coworkers (28) and had been backcrossed > 10 generations into the C57BL/6 strain. Trif<sup>−/−</sup> mice were generated by Yamamoto *et al.* (29). All mice used in the study were gender and age matched, 8–12-week-old and weighed between 20–30 g. Mice were fed the same bacteria-free diet (Prolab Isopro RMH 3000, LabDiet, Brentwood, MO) and water. The animal protocols used in the study were approved by the Subcommittee on Research Animal Care of Massachusetts General Hospital (Boston, MA). The experiments were performed in compliance with the guideline from the National Institutes of Health (Bethesda, MD). Simple randomization method was used to assign animals to various experimental conditions.

**Human Hearts**

Human hearts were provided by the Cardiovascular Research Institute of Beth Israel Deaconess Medical Center in Boston and collected through the National Disease Research Interchange Program supported by the NIH. These donor hearts were found not suitable for transplantation due to lack of identification of a suitable recipient or lack of heart perfusion in donors in the emergency department or other medical reasons.

**Cell Isolation and Culture**

**Rat Neonatal Cardiomyocyte (CM) Culture—**Rat neonatal CMs were prepared as described previously with minor modifications (30). Briefly, the hearts were isolated, dissected from major vessels, and cut into small pieces. The heart tissues were then incubated in ADS buffer (pH 7.35, 116 mM NaCl, 20 mM HEPES, 0.8 mM Na<sub>2</sub>HPO<sub>4</sub>, 5.6 mM glucose, 5.4 mM KCl, and 0.8 mM MgSO<sub>4</sub>) containing 0.4 mg/ml collagenase 2 and 0.6 mg/ml pancratin (Worthington, Lakewood, NJ) at 37 °C for 8 min in a shaker. Cell suspension was slowly removed, and the remaining myocardial tissues were further incubated with the enzyme buffer for 9 more times. Cells in suspension were collected, spun, and resuspended in DMEM containing 20% FBS and 4.5% d-glucose. Fibroblasts were removed by plating cells on 10-cm dishes for 70 min. Neonatal CMs were then plated in a 96-well plate, pre-coated with 5 mg/ml fibronectin and 20 mg/ml gelatin (Sigma-Aldrich), (0.8 × 10<sup>5</sup> cells/well) and incubated in CO<sub>2</sub> incubator at 37 °C for 36 h before experiments.

**Bone marrow-derived Macrophage (BMDM) Culture**

Bone marrow cells were harvested from the tibias and femurs of mice, cultured, and differentiated into macrophages in the presence of 10 ng/ml macrophage colony-stimulating factor as described previously with minor modifications (31). Briefly, the cells were resuspended in RPMI 1640 medium supplemented with 10 ng/ml macrophage colony-stimulating factor (R&D systems, Minneapolis, MN), 10% FBS, and 5% horse serum, seeded in a 96-well plate (2 × 10<sup>5</sup> cells/well) and incubated in CO<sub>2</sub> incubator at 37 °C. Three days later, culture medium was changed, and the macrophages were ready for experiments at day 5.
**TLR7 Mediates Cellular RNA-induced Inflammation**

**Mouse Neutrophil Isolation**

Peritoneal neutrophils were elicited by intra-peritoneal injection of 1 ml of 4% thioglycollate as described previously (31). Twenty hours later, 5 ml of cold DPBS was injected into the peritoneum. Peritoneal lavage fluids were mixed thoroughly and collected. The cells were spun and re-suspended with pre-warmed serum-free RPMI 1640 medium containing 0.05% BSA. Cells were plated in a 96-well plate (1 × 10^5 cells/well for medium collecting) or 6-well plates (4 × 10^6 cells/well for RNA extraction).

**Treatments of Cell Cultures**

Medium of cultured beating neonatal CMs or BMDMs were changed to pre-warmed serum-free RPMI 1640 medium containing 0.05% BSA for 1 h before treatment. Purified RNA or poly(I:C) was complexed with Lipofectamine 3000 (Life Technologies, Grand Island, NY) following the instruction and incubated for 5 min at room temperature before applied to cell cultures. For additional nucleases pre-digestion experiments, 4 μg RNA was incubated with RNase (10 μg), DNase (1 unit) or Benzonase (25 units) at room temperature for 30 min before complexed with Lipofectamine 3000. For the IRS inhibition experiments, the cells were treated with 1 μM IRS or control sequence that had been complexed with Lipofectamine 3000, for 1 h before RNA treatment.

**Cytokine Protein Measurement**

Medium were prepared at 4 °C and stored at −80 °C. To measure MIP-2, ELISA kits were used (R&D systems, Minneapolis, MN). Other cytokine/chemokine concentrations were determined using a fluorescent bead-based multiplex immunoassay (Luminex Co., Austin, TX) (32, 33). Briefly, antibody for each cytokine was covalently immobilized to a set of fluorescent conjugates, the reporter fluorescent signal was measured with a Luminex 200® reader (Luminex Co., Austin, TX). Final cytokine concentrations were calculated based on a standard curve constructed in each experiment.

**RNA Extraction and Quantitation**

Total RNA was extracted from rat neonatal CMs, cardiac fibroblasts and hearts using TRI Reagent (Sigma-Aldrich) and was resolved in sterilized RNase-free water. The RNA concentration was determined by NanoDrop spectrophotometer (Thermo Scientific Inc., Waltham, MA) and the purified RNA was aliquot and stored at −80 °C. exRNA in culture media or mouse sera was extracted using Trizol LS and quantified using Quant-iT™ RNA assay kit (Life Technologies, Grand Island, NY).

**Quantitative RT-PCR**

Quantitative (q) RT-PCR was performed as described previously (34). Changes in relative gene expression normalized to GAPDH were determined using the relative C_t method (where C_t is the threshold cycle number). The sequences of the oligonucleotide primers were the same as reported previously (14).

**Western Blotting**

Cells were lysed in Nonidet P-40 buffer, and cell lysates were centrifuged at 12,000 × g at 4 °C for 30 min. Proteins in supernatants were separated in 4–12% gradient Bis-Tris SDS-PAGE and immunoblotted with antibodies (1:1000) against phospho-JNK, JNK, phospho-p38, p38, phospho-ERK, ERK, I-κBα, and GAPDH (all from Cell Signaling Tech, Danvers, MA) as reported previously (31).

**In Vivo RNA Administration**

After shaving the furs and sterilizing the skin with 70% ethanol, mice were administered with Lipofectamine-complexed RNA (50 μg/mouse) or Lipofectamine as the control through intra-peritoneal injection (intraperitoneal) using 31 Gauge insulin syringe. The injection site was covered by an adhesive 3 M Tegaderm film to prevent infection. Sixteen hours later, the peritoneal lavage was harvested as described previously (31). In brief, 2 ml of normal saline was injected into the peritoneal space and mixed thoroughly by gentle massage of the abdomen. About 1.2 ml of the peritoneal lavage was collected and centrifuged. The cell pellets were suspended. Total peritoneal cells were manually counted. Eight × 10^6 cells were incubated with 1:100 diluted specific anti-mouse Ly-6G (BD Biosciences, San Jose, CA) and anti-mouse F4/80 (eBiosciences, San Diego, CA) at 4 °C for 30 min. After washing, the neutrophil, macrophage, and monocyte percentage were determined by flow cytometry gating as Ly-6G^+^, F4/80^high^, or F4/80^low^, respectively.

**Statistical Analysis**

Statistical analysis was performed using Graphpad Prism 5 software (Graphpad, La Jolla, CA). Unless stated otherwise, the distributions of the continuous variables were expressed as the mean ± S.D. For those cytokine levels below detection limit, values input at the detection limit were used. The statistical significance of the difference between groups was measured by two-tailed unpaired Student t test. Of note, these specific comparisons were made based on a priori hypotheses rather than pure statistical considerations. The null hypothesis was rejected for p < 0.05 with the two-tailed test.

**Results**

Cardiac RNA Induces Cytokine Production in Cardiomyocytes and Neutrophils—Our recently published work demonstrates that necrotic cells induces cytokine production in cardiomyocytes and immune cells, which is specifically diminished by RNase treatment (14). These data imply that RNA mediates the necrotic cell-induced cytokine response. To further define the effect of RNA, we treated rat neonatal cardiomyocyte cultures or freshly isolated mouse neutrophils with RNA purified from rat neonatal cardiomyocytes. As shown in Fig. 1A, RNA induced a dose-dependent (12.5–50 μg/ml) increase in MIP-2 protein production at 24 h. To exclude the effect of any potential lipopolysaccharide (LPS) contamination during RNA purification or treatment, we pretreated RNA with PMB, a LPS neutralizer. While PMB pretreatment totally diminished LPS-
induced MIP-2, it did not affect RNA- or poly(I:C)-induced MIP-2 response, effectively ruling out the possibility of any potential LPS contamination (Fig. 1B). Moreover, RNA-induced cytokine production in cardiomyocytes was marked enhanced by Lipofectamine, a liposome transfection reagent that enhances cellular trans-membrane uptake of exRNA (Fig. 1C).

In the presence of Lipofectamine, RNA, isolated from rat cardiomyocytes (rCMs), induced MIP-2 response at much lower concentration range (2.5–10 μg/ml) in rCM cultures (Fig. 1D). A similar dose-dependent response was observed in rCMs treated with RNA isolated from rat heart tissue (rHeart) (Fig. 1D) or from rat cardiac fibroblasts (rCF) (Fig. 1E). Similar to rCMs, mouse neutrophils responded to RNA isolated from rCMs with an increased MIP-2 protein production (Fig. 1F). In all following experiments, we used 10 μg/ml of RNA complexed with Lipofectamine. qRT-PCR confirmed the up-regulation of MIP-2 and IL-1β transcripts in RNA-treated neutrophils (Fig. 1G–I). Finally, in separate experiments, RNA isolated from both rCMs and mouse heart tissues (mHeart) induced an increase in IL-1β and MIP-2 gene expression in neutrophils (Fig. 1I).

**Figures**

**FIGURE 1.** Cardiac RNA induces cytokine production. Rat neonatal cardiomyocytes (rCM, A–E) or mouse neutrophils (NE, F–I) were treated with purified cardiac RNA in the absence or presence of Lipofectamine 3000. Cells plated in 96-wells were treated for 18–24 h and the media were harvested for cytokine protein measurement. Cells cultured in 6-well plate were treated for 3 h and collected for RNA extraction and cytokine transcript detection. A, dose-response of RNA-induced MIP-2 production in rCM (in the absence of Lipofectamine). B, effect of polymyxin B sulfate (PMB, a LPS neutralizer) on RNA-induced MIP-2 production. RNA (50 μg/ml), poly(I:C) (IC, 25 μg/ml), or LPS (1 ng/ml) was incubated with 50 μg/ml PMB for 30 min at 4 °C before incubation with rCM. C, effect of Lipofectamine on RNA-induced MIP-2 production. rCMs were treated with RNA or poly(I:C) complexed with or without Lipofectamine 3000. D–F, dose-response of Lipofectamine-complexed RNA-induced MIP-2 production in media. G–H, dose-response of Lipofectamine-complexed RNA-induced cytokine transcript under different conditions in NE as shown. I, Lipofectamine-complexed RNA of rCM or mouse heart induced cytokine transcripts in NE. Each error bar represents mean ± S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001. n = 3 in each group. CF: cardiac fibroblast; lipo: Lipofectamine 3000.
of the cardiac RNA induced robust MIP-2 production in mouse macrophages, significant but modest responses in cardiomyocytes (Fig. 3B).

**RNase Pretreatment Diminishes RNA-induced Cytokine Production**—We incubated RNA purified from cardiomyocytes with various nucleases, including DNase, RNase, and Benzonase (hydrolyzing both DNA and RNA) prior to cell treatment. As illustrated in Fig. 4A, RNase and Benzonase, but not DNase, completely eliminated RNA-induced MIP-2 production in cardiomyocytes, whereas none of the nucleases had any effect on Pam3Cys (P3C, a TLR2 ligand)-induced MIP-2 response (Fig. 4A). Similar results were observed in RNA-treated neutrophils (Fig. 4B). Using a multiplex cytokine assay, we found that cellular RNA induced modest response in cardiomyocytes (Fig. 4C) but marked response in neutrophils (Fig. 4D) of TNFα and IL-6 productions. Consistently, RNase, but not DNase, abolished all of the cytokine responses induced by cardiac RNA (Fig. 4, C and D).

**RNA-induced Cytokine Response Is Attenuated by TLR7/8 Inhibitor**—We hypothesized that TLR signaling, including those via TLR3 and TLR7/8, were responsible for the exRNA-induced cytokine production. To test this, we synthesized the specific TLR7 or TLR9 inhibitory oligonucleotides (IRS661 and IRS869, respectively) (27). Cell cultures were incubated for 1 h with the TLR inhibitors before treated with RNA or specific TLR ligands. As shown in Fig. 5A, compared with the control oligonucleotide sequence (Con.), IRS661, but not IRS869, pretreatment markedly decreased R837 (TLR7 ligand)- or CI075 (TLR8 ligand)-induced MIP-2 production in cardiomyocytes (73.8% and 73.0% decrease, respectively), while P3C-induced MIP-2 expression remained unaffected. Importantly, IRS661, but not IRS869, significantly attenuated RNA-induced MIP-2 response in cardiomyocytes (77.3% reduction). Similarly, IRS661, but not IRS869, significantly decreased RNA-induced MIP-2 production in BMDM and neutrophils (Fig. 5, B and C). Of note, the specificity of IRS869 was confirmed by its selective blockade of CpG (TLR9 ligand) effect and the lack of effect on R837-induced cytokine response. We also tested other cytokine responses to exRNA. As shown in Fig. 5D, exRNA induced robust cytokine responses in macrophages. IRS661 significantly decreased the production of all four cytokines tested, IL-1β, IL-6, IL-10, and TNFα. In neutrophils, IRS661 inhibited exRNA-induced TNFα and IL-6 responses by 87 and 93%, respectively (Fig. 5E).

**Genetic Deletion of TLR7 or MyD88 Markedly Attenuates RNA-induced Cytokine Response**—To further test the impact of TLR7 signaling on RNA-induced cytokine responses, we isolated BMDM from WT, TLR7−/−, MyD88−/−, TLR3−/−, and Trif−/− mice and treated them with RNA or TLR ligands. As anticipated, TLR7 deletion totally blocked R837-induced MIP-2 response with no interference on P3C- or LPS-induced immune response (Fig. 6A). Similarly, TLR7 deletion markedly attenuated the RNA-induced MIP-2 production with 82% reduction compared with that in WT cells. Moreover, MyD88 deletion completely blocked RNA-induced MIP-2 production (Fig. 6A). In contrast, TLR3 and Trif deficiency had no effect on RNA-, R837-, or LPS-induced MIP-2 secretion. These data clearly demonstrated that the cellular RNA-induced MIP-2 production was in part mediated by TLR7 and completely dependent on MyD88 signaling pathway, while TLR3/Trif signaling was not involved in the RNA-induced MIP-2 expression.

Similarly, cellular RNA-induced TNFα production was significantly decreased in TLR7−/− BMDM (52% reduction), completely blocked in MyD88−/− BMDM, but remained the same in TLR3−/− and Trif−/− BMDM (Fig. 6B).

**Cardiac RNA Activates MAPKs and NF-κB**—To identify the downstream signaling of cellular RNA treatment, we measured the activation of MAPKs and NF-κBsignaling by detecting phosphorylation of MAPK and degradation of I-κBα, respectively. As shown in Fig. 7, A–C, treatment with cardiac RNA activated the phosphorylation of JNK, p38, and ERK with a peak at 30 min. The phosphorylation then decreased up to 90 min. Moreover, cardiac RNA also activated NF-κB signaling; as evidenced by degradation of I-κBα. I-κBα expression was decreased to 50% at 30 min following RNA treatment and increased to near basal level at 60 min (Fig. 7D).

**MyD88 Mediates Cardiac RNA-induced Peritoneal Neutrophil Migration in Vivo**—Next, we tested the ability of cellular RNA to induce inflammation in vivo. We administered RNA isolated from mouse hearts into mouse peritoneal space and harvested peritoneal lavage 16 h later. As shown in Fig. 8A, the total peritoneal cell was 4.1 ± 0.4 × 10⁶ in the WT mice without any injection. The cell number increased to 6.3 ± 0.5 × 10⁶...
after Lipofectamine injection ($p < 0.01$). Compared with the Lipofectamine-injected mice, mice treated with RNA intraperitoneal had significantly more peritoneal cells ($9.5 \pm 0.6 \times 10^6$, $p < 0.01$). In contrast, MyD88−/− mice injected with RNA or Lipofectamine had similar peritoneal cells ($4.5 \pm 0.6 \times 10^6$ versus $5.0 \pm 0.7 \times 10^6$).

Flow cytometry analysis (Fig. 8, B and C) revealed that in the control mice with no injection, $0.03\%$ of the gated peritoneal cells were Gr-1− neutrophils, $9.5\%$ F4/80high macrophages, and $6.4\%$ F4/80low monocytes. The remaining cells (Gr-1−/F4/80−) were presumably lymphocytes and other cell types. While Lipofectamine did increase total peritoneal cells, it did not signifi-
**TLR7 Mediates Cellular RNA-induced Inflammation**

**A**

![Graph showing cytokine production](image1)

**B**

![Graph showing cytokine production](image2)

FIGURE 6. cardiac RNA-induced cytokine production is dependent on TLR7-MyD88 signaling. Bone marrow-derived macrophages (BMDM) were isolated from wild-type (WT) and genetically modified mice, and treated with Lipofectamine-complexed RNA (10 µg/ml), R837 (TLR7 ligand, 0.25 µg/ml), Pam3Cys (P3C, a TLR2 ligand, 10 ng/ml), or LPS (10 ng/ml). Sixteen hours later, the culture medium was collected. MIP-2 (A) and TNF-α (B) expression in the medium was measured by ELISA and Luminex, respectively. Each bar represents mean ± S.D. **, p < 0.01; ***, p < 0.001. n = 3 in each group. Lipo: Lipofectamine 3000.

...cantly affect the percentage of neutrophils, macrophages, and monocytes among the gated peritoneal cells. In comparison, 16 h after cellular RNA injection, the peritoneal neutrophils increased from 1.1% to 2.6% (p < 0.05) and F4/80<sup>low</sup> monocytes from 9.5% to 32% (p < 0.01) (Fig. 8B), suggesting a robust inflammatory cell influx into the peritoneal space. In contrast, absence of systemic MyD88 effectively abolished the RNA-induced increase in neutrophil migration and partially blocked monocyte migration (48% reduction) (Fig. 8, B and C). Somewhat surprisingly, we found that systemic TLR7 deficiency did not affect the RNA-induced leukocyte migration into the peritoneal space. As illustrated in Fig. 9, RNA intraperitoneal injection led to a marked increase in the peritoneal neutrophils of both WT and TLR7<sup>−/−</sup> mice. There was no significant difference in leukocyte recruitment between WT and TLR7<sup>−/−</sup> groups (WT versus TLR7<sup>−/−</sup>-KO; neutrophils: 0.32 ± 0.09 × 10<sup>6</sup> versus 0.34 ± 0.06 × 10<sup>6</sup>, monocytes: 1.4 ± 0.3 × 10<sup>6</sup> versus 1.9 ± 0.3 × 10<sup>6</sup>).

**Discussion**

We and others have demonstrated that RNase pretreatment decreases immune response induced by necrotic cells in various immune and parenchymal cells (14, 25, 35–39). These studies conclude that endogenous exRNAs, released from or associated with injured cells, are capable of mediating inflammatory response and may contribute to tissue damage in various animal disease models. However, whether or not cellular RNA could function as a danger molecule and directly act on TLRs to initiate pro-inflammatory response has been unclear. In the current study, we made three main findings. First, we detected marked cytokine responses in cardiomyocytes and immune cells when they were treated with cellular RNA isolated from rat cardiomyocytes or from myocardial tissue of mouse, rat, and human. In addition, we found that extracellular RNA released from dying cardiomyocytes was capable of inducing a similar cytokine response. Second, employing specific TLR inhibitors and genetically modified mouse models, we identified that TLR7 was in part responsible for RNA-induced pro-inflammatory effect. The loss-of-function studies also demonstrated that cardiac RNA exhibited its effect entirely via a MyD88 signaling. In contrast, lack of TLR3/Trif signaling had no impact on the RNA-induced cytokine response. Finally, RNA administration into the peritoneal space induced significant acute peritonitis with marked neutrophil and monocyte peritoneal migration and in a MyD88-dependent manner.

In this study, mouse RNA, isolated from different cell types, e.g. cardiomyocytes, cardiac fibroblasts and the heart of different species, e.g. mouse, rat, and human, exhibits a pro-inflammatory property, especially in the presence of Lipofectamine, in cardiomyocytes, neutrophils, or macrophages. This is true with RNA isolated from the same species, such as the effect of rat RNA in rat cardiomyocytes, or from different species such as that of rat or human RNA in mouse neutrophils or macrophages. These data suggest that cardiac extracellular RNA, once gains access to the intracellular sensors, can trigger a potent inflammatory response even in cells of the same species. It is generally believed that pathogen RNA, such as those of viral origin, is recognized by mammalian host immune system and trigger robust immune response whereas host cellular RNA is relatively ineffective in activating a self-immune system. The molecular mechanisms by which the mammalian immune system distinguishes “self” from “non-self” RNA are incompletely understood. A few mechanisms have been proposed (24, 40). Eukaryotic RNA has different features from prokaryotic RNA. Kariko et al. report that bacterial RNA induces much stronger immune response than natural mammalian RNA that is rich in nucleoside modifications. Such RNA modifications are reportedly suppressive to their ability to activate dendritic cells (26). *In vitro* synthesized mRNA can activate transfected TLR3, TLR7 and TLR8, but its immune-stimulatory activity was abrogated after RNA modification, such as methylation and pseudouridine (26, 41). In addition, intracellular endosomal localization of the RNA sensors, such as TLR3 and TLR7/8, prevents the recognition of self RNA under normal conditions (24, 40). Heil et al. report that without transfection agent, free synthetic unmodified HIV RNA (RNA40) fails to induce cytokine production in human myeloid cells, demonstrating the importance of access of RNA to it intracellular sensor (22). In consistent with these, our data demonstrate that the cytokine-producing effect of exRNA is markedly enhanced in the presence of Lipofectamine. The liposome-mediated cellular uptake of exRNA may in fact simulate some *in vivo* situations, such as fusion of
FIGURE 7. Cardiac RNA activates MAPKs and NF-κB in BMDM. Bone marrow-derived macrophages (BMDM) were isolated from wild-type (WT) mice and treated with Lipofectamine-complexed cardiac RNA (10 μg/ml) for 30, 60, or 90 min. The control group was treated with Lipofectamine (Lipo) for 30 min. The cell lysates were harvested and tested for phosphorylation of JNK (A), ERK (B), p38 (C), and degradation of IκBα (D) using Western blot. The data were quantitated as the fold change of the phospho-JNK/JNK, phospho-p38/p38, phospho-ERK/ERK, and IκBα/GAPDH ratio as compared with that of the Lipo group. Each error bar represents mean ± S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus Lipo group. n = 3 in each group. Lipo: Lipofectamine 3000.

FIGURE 8. MyD88 mediates cardiac RNA-induced leukocyte migration in vivo. Wild-type (WT) or MyD88^−/− (KO) male mice were administered intraperitoneally with 50 μg of RNA isolated from mouse heart or Lipofectamine alone (Lipo). Sixteen hours later, the peritoneal lavage was harvested and centrifuged to spin down the peritoneal cells. Five WT mice without injection (None) were used as the control. The total peritoneal cells were counted and the neutrophils (NE), macrophages (MΦ), and monocytes (MO) in the peritoneal cavity were determined by flow cytometry as detailed in “Experimental Procedures.” A, total peritoneal cells in each group. B, percentage of NE (Ly-6G^−/−), MΦ (F4/80^high), and MO (F4/80^low) among total peritoneal cells. Each error bar represents mean ± S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001. n = 6 mice in WT-Lipo Group, n = 12 mice in WT-RNA group, n = 4 mice in KO-Lipo Group, n = 8 mice in KO-RNA group. C, representative flow cytometry pictures gated for Ly-6G^−/−, F4/80^high, and F4/80^low in each group.
Given the demonstrated role of mRNA as an endogenous ligand for TLR3 (25), several studies suggest that TLR3 may mediate necrotic cell RNA-induced immune activation (25, 35–38, 46, 47). It is worth noting that while these studies, including the one by our group (14), clearly demonstrate the role of TLR3 and RNA in necrotic cell-induced cytokine production in various cell types by using TLR3 blockade and RNase pretreatment, respectively, they provide no evidence that TLR3 mediates the cytokine response induced by RNA released from necrotic cells. To our surprise and quite different from the previous reports, we were unable to demonstrate the necessity of TLR3 in the exRNA-induced cytokine responses. We found that TLR3-deficient macrophages produced the same amount of cytokine as wild-type macrophages in response to exRNA treatments. Similarly, Trif deletion had no impact on exRNA-induced cytokine production. Taken together, these data suggest that TLR3/Trif signaling is not involved in the cardiac exRNA-induced cellular inflammation.

We examined the intracellular signaling event in macrophages treated with cellular RNA. exRNA treatment induced a rapid activation of the three major MAPks, JNK, ERK, and p38, and the degradation of I-κBα. These MAP kinases play a critical role in many cellular functions, such as growth and proliferation, cell apoptosis and survival, and metabolism. The increased I-κBα degradation in the RNA-treated cells demonstrates activation of the potent pro-inflammatory NF-κB signaling pathway.

While RNA, in particular ssRNA, has been shown to be immune-stimulatory in immune cells, including monocytes, macrophages and dendritic cells of human and mouse, whether exRNA activates immune system in vivo has been barely studied. In the current study, we demonstrated that cardiac RNA administration into the peritoneal space resulted in a strong innate immune response, as evidenced by recruitment of a large number of monocytes and a smaller number of neutrophils. Extending our in vitro studies, we found that MyD88−/− mice had much attenuated cellular innate immune response. However, unlike the in vitro cytokine response, TLR7 deficiency failed to impact on the RNA-induced cellular response. These data suggest that cellular RNA is capable of activating local innate immune response via a MyD88-dependent signaling mechanism.

We did not identify the specific type of exRNA that is responsible for the pro-inflammatory effect. Several types of exRNA are the potential candidates, which include mitochondrial RNA, mRNA, miRNA, and long non-coding RNA. It has been proposed that miRNAs, a group of small non-coding ssRNAs of 19–24 nt in length, can function as TLR7/8 ligands and activate pro-inflammatory response in cancer cells and neurons (48, 49). We have demonstrated that a number of muscle-specific miRNAs are released from necrotic cardiomyocytes and ischemic myocardium (14). Future study will focus on determining the identity of miRNAs that may contribute to the pro-inflammatory responses in cardiomyocytes and immune cells.

In summary, we demonstrated that cellular RNA isolated from cardiac cells or tissues or extracellular RNA released from injured cardiomyocytes acted as danger molecule provoking a profound inflammatory response not only in innate immune...
cells but also in cardiomyocytes. We identified the pivotal role of TLR7–MyD88 signaling in mediating this pro-inflammatory effect. Finally, we found that cellular RNA was capable of inducing acute cellular innate immune responses with robust monocyte migration when injected into the peritoneal space. These data suggest that cardiac RNA is a potent and specific innate immune stimulus and may play an important role in tissue inflammation.

Author Contributions—Y. F. conceived and designed the study, performed the experiments in Figs. 1–9, analyzed the data, and drafted the manuscript. H. C. and J. C. performed the experiments shown in Figs. 1, 4, and 6. L. Z. performed the experiments and analyzed the data shown in Fig. 8. D. Y. performed the experiments and analyzed the data shown in Figs. 7 and 9. G. X. and D. L. performed and analyzed the experiments shown in Fig. 5. W. C. is the principal investigator and responsible for all aspects of the project. All authors reviewed the results and approved the final version of the manuscript.

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