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Extracellular MicroRNAs Induce Potent Innate Immune Responses via TLR7/MyD88-Dependent Mechanisms

Yan Feng,*† Lin Zou,*† Dan Yan,* Hongliang Chen,* Ganqiong Xu,* Wenling Jian,* Ping Cui,* and Wei Chao*†

Tissue ischemia, such as transient myocardial ischemia, leads to release of cellular RNA including microRNA (miRNA) into the circulation and extracellular (ex-) space, but the biological function of the ex-RNA is poorly understood. We recently reported that cardiac RNA of both human and rodent origins induced cytokine production and immune cell activation. However, the identity of the ex-RNA responsible for the proinflammatory effect remains unclear. In the current study, using an miRNA array, we profiled the plasma miRNAs 4 h after transient myocardial ischemia (45 min) or sham procedure. Among 38 plasma miRNAs that were elevated following ischemia, eight were tested for their ability to induce cytokine response in macrophages and cardiomyocytes. We found that six miRNA mimics (miR-34a, -122, -133a, -142, -146a, and -208a) induced cytokine production in a dose-dependent manner. The effects of miRNAs (miR-133a, -146a, and -208a) were diminished by uridine-adenosine mutation and by RNase pretreatment. The miRNA-induced cytokine (MIP-2, TNF-α, and IL-6) production was abolished in cells deficient of TLR7 or MyD88, or by a TLR7 antagonist, but remained the same in TLR3- or Trif-deficient cells. In vivo, mice i.p. injected with miR-133a or miR-146a had marked peritneal neutrophil and monocyte migration, which was significantly attenuated in TLR7−/− mice. Moreover, locked nucleic acid anti-miRNA inhibitors of these six miRNAs markedly reduced cardiac RNA-induced cytokine production. Taken together, these data demonstrate that ex-miRNA mimics (miR-34a, -122, -133a, -142, -146a, and -208a) are potent innate immune activators and that the miRNAs most likely induce cytokine production and leukocyte migration through TLR7 signaling. The Journal of Immunology, 2017, 199: 2106–2117.

We have previously demonstrated that within 30–45 min of transient myocardial ischemia, there is an increase in the plasma cell–free RNA concentration (1). To determine the potential role of these cellular RNA in inflammation and tissue injury, we and others have demonstrated that administration of RNase attenuates necrotic cell–induced cytokine production in cardiomyocytes (CMs) and immune cells in vitro (1), and reduces myocardial infarction following transient ischemia in vivo (1, 2). These data suggest that extracellular (ex-) RNA may mediate necrosis-induced inflammation and contribute to myocardial ischemic injury. Moreover, cellular RNA, either purified from rodent and human hearts, or released from hypoxia-injured CMs, induces multiple cytokine production in both CMs and immune cells (3). We further show that the ex-RNA–induced cytokine effect is significantly attenuated by TLR7 inhibitor or in TLR7-deficient cells, and was completely abolished by MyD88 deficiency (3). However, the nature and identity of the ex-RNA responsible for cytokine production is unclear.

MicroRNAs (miRNAs) are short, highly evolutionarily conserved, single-stranded noncoding RNA (17–25 nucleotides) (4–6). miRNAs bind to the 3′ untranslated region of target mRNA and regulate gene expression either by inhibiting mRNA translation or inducing its degradation. A variety of cardiac miRNAs are modulated following acute myocardial infarction (7, 8), and some are released from the ischemic myocardium into the circulation (9–11). More than 200 miRNAs exist in the heart (12). Some are reportedly expressed in a tissue-specific manner (13), such as miR-208a in the heart (14). In addition, other muscle-enriched miRNAs, such as miR-1, miR-133, and miR-499, are highly expressed in CMs and skeletal muscle cells (13). Several studies indicate that the circulating levels of miR-208a, miR-499, miR-1, and miR-133 are markedly elevated following acute myocardial infarction in patients and animals (15, 16), and may serve as sensitive biomarkers (11, 13). However, the specific biological function of these circulating miRNAs following cardiac ischemia and whether they play any particular role in myocardial inflammation or injury remains unclear.

In the current study, we hypothesize that multiple miRNAs are released into the circulation following transient myocardial ischemia, and that certain ex-miRNAs are capable of activating innate immunity and inducing cellular and tissue inflammation via specific TLR signaling. To test this, we profiled the circulating miRNAs in the plasma following a short period of coronary artery ligation and tested a set of eight miRNAs for their abilities to induce inflammation in macrophages and CMs, and in a mouse model of...
peritonitis. Moreover, using specific TLR1/TLR2 agonists and knockout (KO) mice, we tested the role of TLRs in mediating miRNA’s effects.

Materials and Methods

**Materials**

miRNAs with phosphorothioate linkages were synthesized by Integrated DNA Technologies (Coralville, IA). The RNA sequences were purchased from miRBase 21 (http://www.mirbase.org/) (Table I). All miRNA mutant derivatives were generated by replacing uridines (U) with adenosines (A). Locked nucleic acid (LNA) miRNA inhibitors and the control oligonucleotide (Negative Control A) were purchased from Exiqon (Vedbaek, Denmark). Collagenase 2 and RNase A were from Sigma-Aldrich (St. Louis, MO). DNase was from Thermo Scientific (Waltham, MA). Imiquimod (R837, TLR7 ligand) was provided by InvivoGen (San Diego, CA). Pam3Cys (P3C) was purchased from Enzo Life (Plymouth Meeting, PA). Specific immunoregulatory DNA sequences (IRs) with phosphorothioate linkages were synthesized by Integrated DNA Technologies as previously described (17). The following sequences were synthesized by Integrated DNA Technologies (Coralville, IA) and tested: IRS641 (TLR7 inhibitor: 5'-TCTCTGGCAAGCTTGCAAGCA-3'), IRS869 (TLR9 inhibitor: 5'-CTCTGAGCGGGTTGTG-3'), and the control oligonucleotide (control: 5'-TCTTGCCGGAAGT-3'). Abs were purchased from Cell Signaling Technology (Danvers, MA).

**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 colleagues (18). Trif mice were generated by Yamamoto et al. (19). Mice were 8–12 wk-old, weighed between 20 and 30 g, and were gender and age matched. Mice were fed the same bacteria-free diet (Prolab Isopro RMH 3000; LabDiet, Brentwood, MO) and water. The animal protocols were approved by the Subcommittee on Research Animal Care of Massachusetts General Hospital (Boston, MA). The experiments were performed in compliance with the guidelines of the National Institutes of Health (Bethesda, MD). A simple randomization method was used to assign animals to various experimental conditions.

**Mouse myocardial ischemia reperfusion model**

The surgical procedures of myocardial ischemic injury were performed as previously described (1, 20, 21). In brief, male mice were anesthetized by i.p. injection of ketamine (120 mg/kg) and xylazine (4 mg/kg), intubated, and ventilated. Body temperature was maintained at 36.5–37.5°C on a heating pad. A left intercostal thoracotomy was performed to expose the heart. The left anterior descending coronary artery was visualized and ligated with 7-0 silk sutures under a surgical microscope. Myocardial ischemia was confirmed by myocardial Blanching and electrocardiogram (ECG) change on a Lead II. Then 45 min after LAD occlusion, the ligature was released and reperfusion was visually confirmed. Sham-operated mice underwent the same procedure with a suture passed under the LAD but without ligation. Four hours after reperfusion, the blood was collected from the heart in an EDTA-containing tube and the plasma samples were subsequently used for miRNA measurements.

**RNA extraction and quantitation**

RNA in the plasma was extracted using Trizol LS (Life Technologies, Grand Island, NY) and quantified using Quant-IT RNA assay kit (Life Technologies) as reported previously (1, 3).

**Circulating miRNA array**

EDTA-anticoagulated serum samples were prepared for the miRNA array assay (Firefly microRNA Assay, Abcam, MA; http://www.abcam.com/content/firefly1-discovery-engine). The assay system was based on encoded hydrogel particles and used a unique posthybridization ligation-based scheme to fluorescently label bound miRNA targets. After hybridizing the miRNA targets to the target-specific probes attached to the hydrogel Firefly Particles, a universal biotinylated adapter was ligated to the captured targets. A fluorescent reporter binds to the universal adapter and is ultimately used for miRNA detection in a flow cytometer. Because RNA precipitation was no longer needed, handling of the miRNA with this technology, we were able to detect the miRNA array in a tiny volume of plasma (100 μl) without the risk of losing RNA. This is particularly advantageous for mouse studies.

Circulating miRNA detection by quantitative RT-PCR

Total RNA was isolated from 0.2 ml mouse plasma using Trizol LS using the extraction procedure as described above. The *Caenorhabditis elegans* miRNA (cel-miR-39) was added before RNA extraction following the instruction of miScripti Real-Time PCR Accessory Kit (Qiagen, Ven#encia, CA). RNA pellets were suspended in 14 μl of RNA-free water and 12 μl samples were used for reverse transcription according to the protocol of the miScript II reverse transcription kit (Qiagen). Mouse miRNAs and cel-miR-39 were quantified by using miScript SYBR Green PCR kit and corresponding primers following the protocol of the manufacturer (Qiagen). Relative expression was calculated using the comparative cycle threshold method (2^-ΔΔCT) normalized to the expression of cel-miR-39.

**Cell isolation and culture**

**Bone marrow–derived macrophage 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 recombinant mouse M-CSF (R&D Systems) as described previously with minor modifications (21). Briefly, mice were resuspended in RPMI 1640 culture medium and seeded in a 96-well plate (2 x 10^3 cells per well) in a CO2 incubator at 37°C. After 3 d, the culture media were changed and the macrophages were ready for experiments at day 5.

**Rat neonatal CM culture.** Rat neonatal CMs (rCMs) were prepared as described previously with minor modifications (22). Briefly, the hearts were isolated, dissociated from cardiac muscle cells, and cultured in adipocytes. The heart tissue was then incubated in buffer (pH 7.35, 116 mM NaCl, 20 mM HEPES, 0.8 mM Na2HPO4, 5.6 mM glucose, 5.4 mM KCl, and 0.8 mM MgSO4) containing 0.4 mg/ml collagenase 2 and 0.6 mg/ml pancreatic (Worthington, Lakewood, NJ) at 37°C for 8 min in a shaker. The cell suspension was slowly removed, and the remaining myocardial tissues were further incubated with the enzyme buffer nine more times. Cell suspensions were collected and resuspended in DMEM containing 20% FBS and 4.5% n-gelatine. Fibroblasts were removed by plating cells on 10 cm dishes for 70 min. Neonatal CMs were then plated in a 96-well plate, precoated with 5 μg/ml fibronectin and 0.2 mg/ml gelatine (Sigma-Aldrich), (0.8 x 10^5 cells per well) and incubated in CO2 incubator at 37°C for 36 h before experiments.

**Cell culture of rCMs.** Cell culture medium was changed to pre-warmed serum-free RPMI 1640 medium containing 0.05% BSA for 1 h before treatment. rCMs were treated with lipofectamine (Life Technologies) following the manufacturer’s instructions, and incubated for 5 min at room temperature before being applied to cell cultures. For nuclease-digestion experiments, miRNA (50 nM for the treatment of bone marrow–derived macrophages [BMDMs] and 150 nM for the treatment of rCM) was incubated with RNase (10 μg) or DNase (1 U) at room temperature for 30 min before being packed with Lipofectamine 3000. For the NOS inhibition experiments, CMs were treated with 300 mM L-NAME or a control sequence that had been packed with Lipofectamin 3000, for 1 h before miRNA treatment.

**Cytokines.** Media were prepared at 4°C, stored at −80°C, and subsequently measured for MIP-2, TNF-α, and IL-6 using ELISA kits (R&D Systems, Minneapolis, MN). Final cytokine concentrations were calculated based on a standard curve constructed in each experiment.

**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 immuno-blotted with Abs (1:1000) against phospho-JNK, INK, phospho-p38, p38, phospho-ERK, ERK, IκBα, and GAPDH (all from Cell Signaling Tech) as reported previously (21).

**In vivo miRNA administration.** After shaving the fur and disinfecting the skin with 70% ethanol, mice were administered with or without Lipofectamine-packet miR-133a-3p, miR-146a-5p, or miR-146a mutant (20 μg per mouse), or Lipofectamine alone through i.p. injection using a 31 G insulin syringe. The injection site was subsequently covered by an adhesive 3M Tegaderm film to prevent infection. After 20 h, the peritoneal lavage was harvested as described previously (21). 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. A total of 8 x 10^5 cells were incubated with 1:100 diluted specific anti-mouse Ly-6G (BD Biosciences, San Jose, CA) anti-mouse F4/80 (BD Biosciences, San Diego, CA) at 4°C for 30 min. After washing, the neutrophils, resident macrophages, and recruited monocytes were determined by flow cytometry gating as Ly-6G<sup>+</sup>, F4/80<sup>high</sup>, or F4/80<sup>low</sup>, respectively.

**miRNA inhibition.** BMDMs or rCMs were treated with 10 μg/ml RNA purified from the heart. Six miRNA inhibitors (specifically for miRNAs
FIGURE 1. Multiple circulating miRNAs were increased following myocardial ischemia/reperfusion. Mice were subjected to sham or myocardial ischemia/reperfusion (I/R) procedures. I/R mice had a left anterior descending coronary artery (LAD) ligation for 45 min followed by 4 h reperfusion, whereas sham mice only underwent left thoracotomy without coronary artery ligation. In separate experiments, EDTA-treated plasma or serum was harvested for RNA quantitation, miRNA profiling by miRNA array, and qRT-PCR (as detailed in Materials and Methods). (A) Blood RNA quantitation following myocardial I/R. Each error bar represents mean ± SD. n = 4 in each group. (B) Heatmap of circulating miRNA array. (C) Quantitative data of increased circulating miRNA following myocardial I/R compared with sham group. Each error bar represents mean ± SE. n = 6 in sham group, n = 9 in I/R group. (D) Mean fluorescent intensity of selected eight circulating miRNAs in sham and I/R mice. Each error bar represents (Figure legend continues)
miR-133a, -208a, -146, -122, and -134a) were mixed together at the indicated concentration as anti-miR combo. The anti-miR combo (final concentration = 100 nM) was added instead.

The uptake of miRNA. BMDMs isolated from WT or TLR7 KO mice were treated with fluorescent 6-FAM–conjugated miR-133a-3p (50 nM, packed with Lipofectamine). After 4 h, fluorescent images were taken using confocal microscopy (Nikon). The cells were detached by trypsin digestion and the intracellular fluorescence was determined by flow cytometry.

Colocalization of miRNA and TLR7. BMDMs or rCMs were treated with Lipofectamine-packed 6-FAM–conjugated miR-133a-3p (50 or 100 nM, respectively). After 4 h, the cells were fixed and costained with specific Abs to TLR7 (Novus, Littleton, CO) and early endosome Ag 1 (EEA1) (Santa Cruz, Dallas, TX). The fluorescent imaging was analyzed by confocal microscopy (Nikon).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad, La Jolla, CA). Unless stated otherwise, the Statistical analysis was performed using GraphPad Prism 5 software (GraphPad, La Jolla, CA). Statistical analysis was performed using GraphPad Prism 5 software (GraphPad, La Jolla, CA).

Results

Transient myocardial ischemia leads to an increase in circulating miRNAs

Briefly, 4 h after transient myocardial ischemia (45 min of coronary artery ligation) or sham procedures, mouse plasma RNA was extracted and quantified. As shown in Fig. 1A, there was a significant increase in the plasma RNA concentration in mice subjected to ischemic injury (ischemia-reperfusion [I/R]) as compared with sham mice (465 ± 164 versus 248 ± 62, p < 0.05). The RNA measurement with a fluorescent dye was validated as the purified RNA was completely degraded by RNase, but not DNase, treatment (Fig. 1A).

We profiled the circulating plasma miRNAs following the transient myocardial ischemia protocol noted above. Among the panel of 65 miRNAs reportedly related to cardiac diseases (Fig. 1B) (http://www.abcam.com/fireplexreg-mirna-panel-cardiology-v2-2ab218368.html), 38 were significantly elevated in the I/R compared with those in the sham group (Fig. 1C). The complete miRNA array data (accession number GSE74951) are available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE74951. There are 31 miRNAs that exhibited >2-fold increase in the plasma of the I/R mice compared with that of the sham mice.

To test the function of the miRNAs, we selected eight miRNAs with the I/R: sham ratio >2 and with fluorescence intensity >100 as indicated by the arrows in Fig. 1B, 1C. These were miR-146a, miR-133a, miR-122, miR-34a, miR-208a, miR-192, and miR-210. Fig. 1D illustrates the mean fluorescent intensity of these eight miRNAs in both sham and I/R groups. Moreover, we validated, using quantitative RT-PCR (qRT-PCR), the change of seven miRNAs in the plasma samples from a separate set of sham and I/R animals. As shown in Fig. 1E, with the exception of miR-142a, the other seven miRNAs, including miR-146a, miR-133a, miR-122, miR-34a, miR-208a, miR-192, and miR-210, were all significantly increased in the plasma samples isolated from I/R mice compared with those from sham mice.

miRNA mimics induce dose-dependent cytokine production in macrophages and CMs

We tested the eight miRNA mimics (Table I) for their ability to induce cytokine production. As shown in Fig. 2A, 2B, of the eight miRNA mimics, six miRNAs (i.e., miR-146a, miR-133a, miR-142a, miR-122, miR-34a, and miR-208a) induced a dose-dependent MIP-2 response in both BMDM and rCM. miR-142a, miR-146a, and miR-133 seemed the most potent whereas miR-34a and miR-122 were the weakest. In contrast, miR-192 and miR-210 induced no MIP-2 production at the same concentrations. When tested in a combination, the six miRNAs (miR-146a, -133a, -142a, -122, -34a, and -208a) mixture induced a dose-dependent MIP-2 production with EC50 of 18 nM and 44 nM in macrophages and CMs, respectively (Fig. 2C, 2D).

U→ A miRNA mutation and RNase diminish miRNA-induced cytokine response

To test the specificity of miRNA-induced cytokine production, we mutated miR-133a, miR-208a, and miR-146a by replacing U with A. As shown in Fig. 2E, 2F, the miRNA_{U→A} mutants failed to induce MIP-2 production in both BMDMs and CMs. Moreover, RNase, but not DNase, pretreatment of the miRNA mimics prior to Lipofectamine packaging completely abolished the cytokine response induced by miRNAs in BMDMs and CMs, respectively.

Table I. Sequences of miRNAs and anti-miRNAs

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Anti-miRNA

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miRNA mimics induce cytokine response in a TLR7/MyD88-dependent manner

Early studies have established TLR3 and TLR7 as the sensors for viral RNA and TLR9 for DNA (23–26). Thus, we hypothesize that miRNA mimics induce cytokine production through the intracellular RNA sensors, TLR3 or TLR7, and their signaling molecule Toll/IL-1 receptor domain-containing adapter inducing IFN-β (Trif) or MyD88. We isolated BMDMs from WT, TLR7^−/−, MyD88^−/−, TLR3^−/−, and Trif^−/− mice and treated them with specific TLR3 or TLR7 ligand or various miRNA mimics. As shown in Fig. 3A, the six miRNA mimics, R837 (TLR7 ligand), and P3C (TLR2 ligand) induced various degrees of MIP-2 response in macrophages. TLR7 deletion completely blocked the R837-induced MIP-2 response, but had no effect on the P3C-induced response. Importantly, TLR7 deficiency blocked all six miRNA-induced MIP-2 production (Fig. 3A). Moreover, genetic deletion of MyD88, the key adaptor of several TLRs including TLR7, blocked the MIP-2 response induced by the miRNAs and the TLR7/TLR2 ligands (Fig. 3A). In contrast, TLR3 or Trif deficiency had no impact on the miRNA-induced cytokine response.

We also measured both TNF-α and IL-6 production in response to the miRNA treatments in BMDMs. As shown in Fig. 3, similar to MIP-2 response, miR-146a and miR-142 induced strong TNF-α and IL-6 responses, which were completely abolished in TLR7- and MyD88-deficient cells (Fig. 3B, 3C).

Next, we demonstrated the critical role of TLR7 in the miRNA-induced MIP-2 production in rCM. As shown in Fig. 3D, compared with the control oligonucleotide or the TLR9 antagonist (IRS869), IRS661, a specific TLR7 inhibitory oligonucleotide, markedly decreased R837 (TLR7 ligand)-induced MIP-2 production (50% decrease), but had no impact on P3C-induced effect. Most importantly, IRS661, but not IRS869 (17), significantly attenuated the six miRNA mixture (miR-133a, -146a, -142a, -122, -34a, and -208a)–induced MIP-2 response (88.1% reduction). Collectively, these data suggest that TLR7 → MyD88 signaling likely mediates the miRNA-elicited cytokine production.

miRNA mimics activate intracellular MAPKs and NF-κB pathways

To determine the intracellular signaling pathways of these miRNA mimics, we tested the phosphorylation of the three MAP kinases, JNK, ERK, and p38, at 30, 60, and 90 min following the miRNA mixture treatment. As shown in Fig. 4A–C, phosphorylation of all
miRNA mimics (50 nM), R837 (TLR7 ligand, 0.25 μg/ml), or P3C (TLR2 ligand, 10 ng/ml). (A) The deficiency of TLR7 and MyD88, but not TLR3 and Trif, completely abolished miRNA mimic-induced MIP-2 production in BMDMs. BMDMs were isolated from WT and genetically modified mice and treated with various Lipofectamine-complexed miRNA mimics (50 nM), R837 (TLR7 ligand, 0.25 μg/ml), or P3C (TLR2 ligand, 10 ng/ml). (B) Specific TLR7 antagonist (IRS661) attenuated miRNA mixture–induced cytokine response in rCM. Neonatal CMs were cultured and treated with 300 nM Lipofectamine-complexed IRSs or a control oligo for 1 h before treatment of miRNA (150 nM of mixture of six miRNA mimics: miR-133a, -146a, -142a, -122, -34a, and -208a), R837 (0.5 μg/ml), or P3C (30 ng/ml). Then 16 h after treatment, the culture media were collected and cytokine expression in the media was measured by MIP-2 ELISA. Each error bar represents mean ± SD. n = 3 in each group. *p < 0.05, **p < 0.01, ***p < 0.001. Con, control.

miRNA-133a-3p promotes peritoneal leukocyte migration in vivo

To examine whether miRNA induces inflammation in vivo, we administered miR-133a-3p mimic into the mouse peritoneal space (20 μg per mouse) and harvested the peritoneal lavage 20 h later. As shown in Fig. 5A, the total peritoneal cells were 5.8 ± 0.5 × 10^6 in the WT mice injected with Lipofectamine (lipo control). Flow cytometry analysis revealed that among the gated peritoneal cells, 0.6% was Ly-6G+ neutrophils, 8.9% F4/80low recruited monocytes, and 16.6% F4/80high resident macrophages (Fig. 5B, 5C). The remaining cells (Ly-6G /F4/80−) were mostly B and T lymphocytes, and other immune cells (NK and dendritic cells) (27, 28). In WT mice injected with miR-133a-3p, there were significantly more peritoneal cells (9.2 ± 0.6 × 10^6 versus 5.8 ± 0.5 × 10^6, p < 0.01), with a significant increase in neutrophils (15.0 ± 3.1%), F4/80low infiltrated monocytes (19.8 ± 3.5%), but decreased F4/80high resident macrophages (4.1 ± 1.4%, p < 0.01) (Fig. 5) compared with the Lipofectamine control group. Compared with WT mice, TLR7−/− mice had a reduced number of peritoneal cells (6.7 ± 0.5 × 10^6 versus 9.2 ± 0.6 × 10^6, neutrophils (4.6 ± 1.3%, p < 0.05), and infiltrated monocytes (11.9 ± 2.4%), but sustained F4/80high resident macrophages (11.9 ± 2.0%) (Fig. 5) following miR-133a administration. MyD88 deficiency completely abolished miR-133a–induced peritoneal leukocyte migration (Fig. 5). Surprisingly, Trif−/− mice had even more total peritoneal leukocytes than WT mice after miR-133a injection (14.5 ± 1.2 × 10^6, p < 0.01). However, there was no significant difference in the percentage of the leukocyte population between miRNA-treated WT and Trif−/− mice (Fig. 5). Of note, the absolute number of the three cell types are summarized in Table II. Taken together, these data suggest that miRNA mimics are capable of activating the intracellular MAPKs and NF-κB pathways.

miR-146a-5p induces peritoneal leukocyte migration in vivo

To further test the ability of ex-miRNAs to induce acute inflammation in vivo, we injected another miRNA mimic, miR-146a-5p, into WT and TLR7−/− mice. For these experiments, we used the miR-146a (U→A) mutant as the control. As shown in Fig. 6, compared with the mice without any injection, the miR-146aU→A mutant-injected WT mice only had mild neutrophil recruitment (2.8 ± 0.7% versus none). The total peritoneal cell numbers and the populations of monocytes and macrophages were the same (Fig. 6). Similar to miR-133a, miR-146a-5p i.p. injection led to a marked increase in total peritoneal cells (9.1 ± 3.1 × 10^6 versus 3.2 ± 1.2 × 10^6, p < 0.001), neutrophils (15.8 ± 7.6% versus 2.8 ± 0.7%, p < 0.01), monocytes (31.1 ± 9.9% versus 9.2 ± 1.6%, p < 0.001), and significant reduction in the peritoneal resident macrophages (2.8 ± 1.2% versus 12.02 ± 6.1%, p < 0.05). Of note, the absolute numbers of the three cell types are summarized in Table III. In comparison, systemic deletion of TLR7 almost completely abolished miR-146a–induced peritoneal neutrophil and monocyte recruitment and macrophage efflux (Fig. 6, Table III). These data further demonstrate that certain miRNA mimics are capable of inducing acute inflammation.
by promoting leukocytes migration in vivo through a local TLR7-sensing mechanism.

Endogenous miRNAs play a role in cardiac RNA–induced cytokine response

To test the role of endogenous miRNAs in cytokine production, we employed an anti-miR combo containing LNA anti-miRNA inhibitors against the selected six miRNAs (miR-133, -208, -146, -142, -122, and -34) or a negative control nucleotide (Table I), aiming to block the endogenous miRNAs in purified cellular RNA. We first determined the efficacy of the anti-miR inhibitor combo in blocking the six miRNA mixture–induced cytokine production in both BMDM and CM cultures. The anti-miR combo, at concentration of 100 nM and added to cell cultures one hour prior to the miRNA mixture, almost completely abolished MIP-2 and TNF-α production (Fig. 7A, 7B). Importantly, cardiac RNA–elicited MIP-2 production (3) was also markedly inhibited by the anti-miR inhibitor combo with 82.4% or 44.6% reduction in MIP-2 production in BMDM or CMs, respectively, and 76.6% reduction in TNF-α production in BMDM. Similar results were obtained in IL-6 and TNF-α production. These data suggest that miRNAs contribute significantly to the endogenous cardiac RNA–induced cytokine production.

miRNA is colocalized with endosomes and TLR7 in macrophages and CMs

To determine whether miRNA mimics are taken up by cells and reside with endosomes, we first incubated BMDM with fluorescence-labeled miR-133a [FAM (6-FAM)-miR-133a] for 4 h and imaged cell-associated 6-FAM-miR-133a by flow cytometry and fluorescent microscopy. As indicated in Fig. 8A, 8B, there was a strong fluorescence intensity associated with WT BMDMs but no significant difference between TLR7<sup>−/−</sup> and WT BMDMs in the fluorescence intensity as determined by flow cytometry. Microscopic imaging showed cytosolic localization of fluorescent FAM-133a in both WT and TLR7<sup>−/−</sup> BMDMs (Fig. 8C). Using confocal microscopy, we found that TLR7 (purple in BMDM, Fig. 8D and red in CMs, Fig. 8E) is largely costained with the EEA1 (red in BMDM and purple in CMs, respectively) in both macrophages and CMs (Fig. 8D, 8E). The green fluorescence-labeled miRNA-133 was taken up into the endosome (middle panel of Fig. 8D, 8E) and partially colocalized with TLR7 (lower panels of Fig. 8D, 8E). These data suggest that miRNA could reach TLR7 within the endosome after a period of incubation with macrophages and CMs.

Discussion

In the current study, we tested the hypothesis that multiple cellular miRNAs are released into the circulation following myocardial I/R and that certain ex-miRNAs are capable of inducing cellular and tissue inflammation via specific TLRs. First, we profiled circulating miRNAs in a mouse model of transient myocardial ischemic injury and found 38 out of 65 miRNAs were significantly increased following I/R compared with sham animals. Out of 38 miRNAs, eight single-stranded miRNAs were synthesized and tested for their abilities to induce inflammation. Six miRNA mimics (miR-34a, miR-122, miR-133a, miR-142, miR-146a, and miR-208a) induced dose-dependent cytokine production in macrophages and CMs. Of interest, each of these six miRNA mimics exhibits a different potency in inducing MIP-2 production, with miR-142, miR-146a, and miR-133 being the most potent. Second, the miRNA mimic-induced cytokine response appeared to be dependent on TLR7→MyD88 signaling as either TLR7 or MyD88 deficiency blocked the effect.

FIGURE 4. miRNA mimic activates MAPKs and NF-κB in BMDMs. Mouse BMDMs were treated with a mixture of the six miRNAs (50 nM) for 0, 30, 60, or 90 min. The cell lysates were harvested and tested for phosphorylation of JNK (A), ERK (B), p38 (C), and degradation of I-κBα (D) using immunoblotting. The data were quantitated as the fold change of the phospho-JNK/JNK, phospho-p38/p38, phospho-ERK/ERK, and I-κBα/GAPDH ratio as compared with that of the time 0. Each error bar represents mean ± SD. n = 3 in each group. *p < 0.05, **p < 0.01, ***p < 0.001 versus time 0 group.
of the miRNA mimics. Third, the administration of miR-133a or miR-146a into the peritoneal space induced significant neutrophil and monocyte recruitment, which was also dependent on TLR7→MyD88 signaling. Fourth, we found that inhibition of endogenous miRNAs (miR-34a, miR-122, miR-133a, miR-142, miR-146a, and miR-208a) markedly attenuated the cardiac RNA–induced cytokine response, demonstrating an important role of endogenous cardiac miRNAs in promoting cellular inflammation. Finally, the confocal microscope imaging data demonstrate that miR-133a-3p resides closely with TLR7 within the endosomes in macrophages and CMs.

To identify the possible miRNA candidates that may play a critical role in myocardial I/R injury and inflammation, we profiled the circulating levels of 65 miRNAs that were reportedly related to cellular and tissue responses to various cardiovascular diseases, including myocardial ischemic infarction (29, 30), coronary artery diseases (31), heart failure (32), and peripheral artery diseases (33). We found that 38 plasma cell–free miRNAs were significantly increased following myocardial I/R. Of importance, some of these miRNAs, such as miR-208-1, -34, -142, and -499, are reportedly associated with patients with acute myocardial infarct and have been proposed as sensitive biomarkers for myocardial ischemic injury (11, 13). To test their proinflammatory effect, we selected eight miRNAs based on the following criteria: 1) the I/R: shamt ratio ≥ 2; 2) fluorescence intensity > 100, seven of which were independently validated using qRT-PCR in a separate set of experiments. Moreover, these miRNAs were reportedly related to host immunological functions (i.e., miR-192-5p, -122, -34a-5p, -210-3p, and -146a-5p) and expressed at relatively high levels in myocardial and skeletal muscles (i.e., miR-208a-3p and -133a-3p). Six miRNAs (miR-34a, miR-122, miR-133a, miR-142, miR-146a, and miR-208a) exhibited a remarkable ability to induce cytokine production, e.g., MIP-2, TNF-α, and IL-6. The MIP-2–inducing effect of these miRNA mimics was dose dependent with an EC_{50} at nM range, sensitive to RNase (but not DNase) digestion, U-dependent as the U → A mutation led to a complete loss of the activities of miR-133a, miR-208a, and miR-146a. The miRNA-induced MIP-2 response was also mediated via TLR7→MyD88 signaling, not TLR3-Trif, as both TLR7- and MyD88-deficient macrophages failed to respond to these miRNA mimics. This finding was further supported by the observation that the specific TLR7 antagonist, IRS661, blocked the effect of the miRNA mixtures in CMs.

To determine whether endogenous miRNAs possess similar proinflammatory activities, we employed six anti-miRNAs to block endogenous target miRNAs and found that the cardiac RNA–elicited cytokine response was significantly decreased by the

![Image](http://www.jimmunol.org/)

**FIGURE 5.** TLR7→MyD88 signaling mediates miR-133a-3p mimic–induced leukocyte migration in vivo. WT, TLR7^{−/−}, MyD88^{−/−}, or Trif^{−/−} male mice were administered i.p. with 20 μg of miR-133a-3p mimic or Lipofectamine alone (lipo). Then 20 h later, the peritoneal lavage was harvested and the peritoneal cells were spun down. The total peritoneal cells were counted and the neutrophils (NE), resident macrophages (Mφ), and recruited monocytes (MO) in the peritoneal cavity were determined by flow cytometry as detailed in Materials and Methods. (A) The total peritoneal cells in each group. (B) The percentage of NE (Ly-6G^+), Mφ (F4/80^{high}), and MO (F4/80^{low}) among total peritoneal cells. Each error bar represents mean ± SE. n = 6 mice in MyD88^{−/−}-lipo group, n = 4 mice in other lipo group, n = 6 mice in all miRNA groups. (C) Representative flow cytometry pictures gated for Ly-6G^+, F4/80^{high}, and F4/80^{low} in each group. *p < 0.05, **p < 0.01, ***p < 0.001. Lipo, Lipofectamine; miR, miR-133a-3p.

### Table II. Peritoneal leukocyte numbers following Lipofectamine or miR-133a-3p mimic i.p. injection in WT and KO mice

<table>
<thead>
<tr>
<th></th>
<th>Neutrophils (×10^6)</th>
<th>Monocytes (×10^5)</th>
<th>Macrophages (×10^5)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipo</td>
<td>0.4 ± 0.2</td>
<td>5.3 ± 1.2</td>
<td>9.9 ± 2.3</td>
</tr>
<tr>
<td>miR-133a</td>
<td>14.1 ± 3.2***</td>
<td>19.0 ± 4.1**</td>
<td>3.6 ± 1.0</td>
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<tr>
<td>TLR7^{−/−}Lipo</td>
<td>0.1 ± 0.0</td>
<td>3.4 ± 1.2</td>
<td>12.4 ± 3.9</td>
</tr>
<tr>
<td>miR-133a</td>
<td>3.2 ± 1.1†††</td>
<td>7.8 ± 1.5†††</td>
<td>7.6 ± 1.1</td>
</tr>
<tr>
<td>MyD88^{−/−}Lipo</td>
<td>0.0 ± 0.0</td>
<td>1.4 ± 0.6</td>
<td>5.0 ± 2.2</td>
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<tr>
<td>miR-133a</td>
<td>0.0 ± 0.0†††</td>
<td>2.9 ± 0.8†††</td>
<td>10.8 ± 3.2</td>
</tr>
<tr>
<td>Trif^{−/−}</td>
<td>0.0 ± 0.0</td>
<td>3.3 ± 1.3</td>
<td>8.5 ± 1.8</td>
</tr>
<tr>
<td>miR-133a</td>
<td>11.9 ± 1.3***</td>
<td>21.3 ± 3.6***</td>
<td>4.1 ± 0.9</td>
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</table>

* †††p < 0.01, **p < 0.001 versus individual Lipo group.
††‡‡p < 0.001 versus WT/miR-133a group.
Lipo, Lipofectamine.
FIGURE 6. miR-146a-5p mimic, but not its A→U mutant, elicited peritoneal leukocyte migration in vivo via TLR7. WT and TLR7−/− male mice were administered i.p. with 20 µg of synthetic miR-146a-5p or mutant (Mut). Then 20 h later, the peritoneal lavage was harvested and spun down. The total peritoneal cells were counted and the neutrophils (NE), resident macrophages (MΦ), and recruited monocytes (MO) in the peritoneal cavity were determined by flow cytometry as detailed in Materials and Methods. (A) The total peritoneal cells in each group. (B–D) The percentage of NE [Ly-6G+, F4/80 high], and MO [F4/80 low] among total peritoneal cells. Each error bar represents mean ± SD. n = 4 in each group. (E) Representative flow cytometry pictures gated for Ly-6G+, F4/80 high, and F4/80 low in each group. *p < 0.05, **p < 0.01, ***p < 0.001. None, without injection; miR, miR-146a-5p.

anti-miRNA treatment as compared with an oligonucleotide control. Of note, in our studies the control oligo exhibits nonspecific inhibition on the miRNA-induced MIP-2 production; this effect seems dose dependent. At concentrations at or below 25 nM, the control oligo had minimal inhibition on the MIP-2 production induced by 50 nM of miRNA mixture (data not shown). Nevertheless, these data support the notion that ex-miRNAs of cardiac origin may contribute to the RNA-induced inflammation. Because other unidentified cellular miRNAs or noncoding RNAs may possess similar proinflammatory properties, it is not surprising that the blocking of these six miRNAs only leads to partial attenuation of the RNA-induced cytokine production. It is worth noting that in vivo, most circulating miRNAs are enveloped in some type of carrier, such as ex-vesicles (e.g., exosomes and microvesicles) or proteins (e.g., HDL or Ago-2). These carriers protect RNA from degradation induced by circulating RNase. Indeed, we found that although in the absence of exosome or Lipofectamine, the “naked” miRNA-induced cytokine production is sensitive to RNase digestion as illustrated in Fig. 2, our recent pilot study suggests that exosome and exosome-associated miRNA-induced cytokine production is resistant to RNase treatment (data not shown).

Of the eight miRNA mimics tested, miR-192 and miR-210 failed to induce a cytokine response. We wondered whether there was any sequence consensus that determines the activity of the miRNAs. Several sequence motifs have been reported important for ssRNA effects. For example, it has been reported that the GUUGUGU motif is important for the neuron activity of let-7b and miR-599 because let-7a, which contains GUUGAU, failed to elicit an inward current (34, 35). Others have reported the importance of the GU-rich element (24, 36, 37) and the amount of U ribonucleotides (38) for ssRNA activities. In our study, we could not identify the GUUGUGU motif among the six miRNAs that induces robust MIP-2 response in CMs and macrophages. However, five of the six active miRNAs contain seven or more U ribonucleotides (Table I). These and the miRNA U→A mutant data demonstrate the specificity of the observed ex-miRNA effects and seem to suggest the importance of the U nucleotide for the miRNA-induced innate immune responses including cytokine response and peritoneal leukocyte migration in vivo. This notion is further supported by a recent study where Zhang et al. (39) generated crystal structures of TLR7 complexes and identified two ligand binding sites. One of the binding sites is specific for U moieties in ssRNA and the other site is for guanosine. TLR7 is synergistically activated by guanosine and U-containing ssRNA.

Table III. Peritoneal leukocyte numbers following i.p. injection of Lipofectamine, miR-146a<sub>U→A</sub> mutant, or miR-146a in WT and KO mice

<table>
<thead>
<tr>
<th></th>
<th>Neutrophils (×10⁶)</th>
<th>Monocytes (×10⁶)</th>
<th>Macrophages (×10⁶)</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipo</td>
<td>0.0 ± 0.0</td>
<td>1.9 ± 0.4</td>
<td>5.0 ± 1.0</td>
</tr>
<tr>
<td>Mut</td>
<td>0.9 ± 0.2</td>
<td>3.0 ± 0.7</td>
<td>3.6 ± 0.9</td>
</tr>
<tr>
<td>miR-146a</td>
<td>16.0 ± 6.3&lt;sup&gt;**&lt;/sup&gt;&lt;sup&gt;†††&lt;/sup&gt;</td>
<td>30.3 ± 9.0&lt;sup&gt;**&lt;/sup&gt;&lt;sup&gt;†††&lt;/sup&gt;</td>
<td>2.6 ± 0.7</td>
</tr>
<tr>
<td>TLR7&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipo</td>
<td>0.1 ± 0.0</td>
<td>4.3 ± 0.4</td>
<td>11.7 ± 2.1</td>
</tr>
<tr>
<td>Mut</td>
<td>4.2 ± 1.9</td>
<td>4.8 ± 1.1</td>
<td>9.2 ± 2.5</td>
</tr>
<tr>
<td>miR-146a</td>
<td>0.8 ± 0.3&lt;sup&gt;††&lt;/sup&gt;</td>
<td>4.5 ± 0.9&lt;sup&gt;††&lt;/sup&gt;</td>
<td>14.6 ± 4.5&lt;sup&gt;††&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>**p < 0.01, ***p < 0.001 versus individual Lipo group</sup>,<sup>††p < 0.01, †††p < 0.001 versus individual Mut group</sup>,<sup>*p < 0.01, †p < 0.001 versus WT/miR-146a group</sup>, Lipo, Lipofectamine; Mut, miR-146a<sub>U→A</sub> mutant.
We found that miR-133a and miR-146a mimics were capable of inducing ly-6G+ neutrophil and F4/80 low monocyte infiltration when injected into the peritoneal space. In the case of miR-146a, the U→A mutant failed to induce the effect, a finding consistent with the in vitro cytokine data. Moreover, the in vivo effects of both miR-133a and miR-146a appeared through TLR7 signaling as they were significantly attenuated by the deletion of TLR7. Deletion of MyD88, but not Trif, completely blocked miR-133a–induced leukocyte recruitment. These data suggest that the miRNA-elicited leukocyte migration is in part dependent on TLR7 and completely dependent on MyD88. Because MyD88 is the key adaptor for all TLR signaling with exception of TLR3, we speculate that other TLRs, such as TLR8, may sense miRNAs and have contributed to miR133- and miR146a-induced leukocyte migration.

We found that both cardiac RNA (3) and synthetic miRNA significantly decreased the number of the peritoneal F4/80 high macrophage after i.p. administration. Consistent with this finding is the observed rapid disappearance of resident macrophages (F4/80high) following i.p. administration of bacterial Ags (40, 41), thioglycollate (27, 42), or LPS (27). Geoffrey et al. report that during inflammation resolution, macrophages migrate rapidly into the draining lymphatics instead of dying locally (43). The macrophage efflux induced by diverse peritoneal inflammatory mediators may...
play an essential role in the Ag presentation and the development of adaptive immunity (44–46). In this context, our data clearly demonstrate the ability of miRNAs to accelerate macrophage efflux via TLR7 signaling, although the exact mechanism remains to be investigated.

We tested the possibility that TLR7 mediates miRNA transport in macrophages and that the lack of cytokine response in TLR7−/− cells might be due to disrupted miRNA transport in these cells. Using flow cytometry, we found that TLR7−/− macrophages had a similar level of miRNA uptake as those isolated from WT macrophages. Confocal imaging data demonstrate that miRNA is localized to the endosome inside the cells and colocalized with TLR7 within the endosome, a necessary prerequisite to ligand binding to TLR7. Together, these data suggest that TLR7 deficiency does not interfere with the uptake of miRNA and the absence of cytokine production in TLR7−/− BMDMs is most likely due to the lack of miRNA sensing. We did not test the efficacy of LNA anti-miRNA inhibitors to block circulating and myocardial target ex-miRNAs in vivo and their abilities to inhibit myocardial inflammation after I/R, which is considered a limitation of this study. Instead, we used peritonitis as an intermediate model to establish the proinflammatory property of these miRNAs in vivo. We are currently testing the efficacy of various protocols of delivering LNA anti-miRNAs in vivo. We anticipate that demonstrating the efficacy of anti-miRNA inhibitors to neutralize circulating/myocardial target miRNAs would be essential to determine the critical role of endogenous miRNAs in the development of myocardial inflammation and injury following transient ischemia.

In summary, we found that multiple miRNAs were increased in the blood circulation 4 h after transient myocardial ischemia. We demonstrated that a group of miRNAs, e.g., miR-34a, miR-122, miR-133a, miR-142, miR-146a, and miR-208a, were capable of inducing a profound inflammatory response in innate immune cells as well as in CMs. In vivo, miRNA133 and miR-146a induce neutrophil and monocyte periportal recruitment, and resident macrophage efflux after i.p. administration. We further identified the pivotal role of TLR7→MyD88 signaling in mediating these proinflammatory effects. Together, our data suggest that certain miRNAs are potent TLR7 ligands and may contribute to cellular and tissue innate immune response following release from damaged cells.

Disclosures

The authors have no financial conflicts of interest.

References