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Original Paper

Cpg-ODN, a TLR9 Agonist, Aggravates Myocardial Ischemia/Reperfusion Injury by Activation of TLR9-P38 MAPK Signaling

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Key Words
Cpg-ODN • Ischemia/reperfusion injury• TLR9-p38 MAPK signaling

Abstract
Background/Aims: Toll-like receptors (TLRs) have been implicated in myocardial ischemia/reperfusion (I/R) injury. We examined the effect of CpG-oligodeoxynucleotide (ODN) on myocardial I/R injury. ***Methods:*** Male Sprague-Dawley rats were treated with either CpG-ODN or control ODN 1 h prior to myocardial ischemia (30 min) followed by reperfusion. Rats treated with phosphate-buffered saline (PBS) served as I/R controls (n = 8/group). Infarct size was determined by 2,3,5-triphenyltetrazolium chloride and Evans blue straining. Cardiac function was examined by echocardiography before and up to 14 days after myocardial I/R. ***Results:*** CpG-ODN administration significantly increased infarct size and reduced cardiac function and survival rate after myocardial I/R, compared to the PBS-treated I/R group. Control-ODN did not alter I/R-induced myocardial infarct size, cardiac dysfunction, and survival rate. Additionally, CpG-ODN promoted I/R-induced myocardial apoptosis and cleaved caspase-3 levels in the myocardium. CpG-ODN increased TLR9 activation and p38 phosphorylation in the myocardium. *In vitro* data also suggested that CpG-ODN treatment induced TLR9 activation and p38 phosphorylation. Importantly, p38 mitogen-activated protein kinase (MAPK) inhibition abolished CpG-ODN-induced cardiac injury. ***Conclusion:*** CpG-ODN, the TLR9 ligand, accelerates myocardial I/R injury. The mechanisms involve activation of the TLR9-p38 MAPK signaling pathway.

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Introduction

The Toll-like receptor (TLR) family now consists of 10 members in humans (TLR1–TLR10) and 12 members in mice (TLR1–TLR9, TLR11–TLR13), which function to recognize pathogen-associated molecular patterns (PAMPs) and trigger immune responses in the

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body. TLRs play a crucial role in inducing innate immune and inflammatory responses [1]. Upon PAMP ligand binding, TLRs trigger a cascade of molecular events, leading to activation of NF-κB signaling and expression of downstream targets. TLRs can induce p38 mitogen-activated protein kinase (MAPK) signaling as well [2]. A large body of evidence shows that p38 plays a central role in regulating many cellular processes, including inflammation, cell cycle progression, differentiation, and apoptosis. TLR9 is special in that it is localized in endolysosomes and has a unique ligand, the unmethylated CpG-DNA from bacteria and endogenous DNA [3, 4]. CpG motifs can activate the TLR9-mediated NF-κB signaling pathway and MAPKs [4]. Synthetic CpG-oligodeoxynucleotide (ODN) has been shown to activate TLR9 [4-6]. Recently, mitochondrial DNA (mtDNA), which is also unmethylated at CpG motifs, has been shown to be taken up by immune cells in which it induces an inflammatory response through activating TLR9 [7]. Innate immunity and inflammatory responses are also associated with myocardial ischemia/reperfusion (I/R) injuries [8-11].

Several studies have reported a benefit of CpG-ODN in sepsis and cardiac function after I/R [12-15]. However, recent studies have demonstrated that TLR9 could be a therapeutic target for reducing myocardial I/R injury. Zhou et al. have shown that inhibitory CpG-ODN (iCpG-ODN) induced protection against myocardial I/R via inhibiting inflammatory responses in a dose-dependent manner and may be useful in therapy for stroke patients [16]. Shaker et al. also reported that TLR9 signaling inhibition by COV08-0064 may be an effective approach in liver surgeries, including transplantation, to limit I/R injury [17]. Our previous study found that intravenous injection of mtDNA into rats aggravated I/R injury and increased infarct area through TLR9-p38 MAPK activation [18]. Similarly to mtDNA, CpG-ODN can also activate TLR9.

In the present study, we examined the effect of CpG-ODN on myocardial I/R injury. We hypothesized that CpG-ODN induces an innate immune response and aggravates I/R injury possibly via the TLR9-p38 MAPK pathway. To test this hypothesis, we examined the effect of CpG-ODN on rat H9c2s cells *in vitro* and on myocardium using the rat model of I/R injury.

Materials and Methods

Reagents

Mouse anti-TLR9 antibody was obtained from Abcam (Cambridge, MA). Antibodies against p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), and caspase-3 were purchased from Cell Signaling Technology (Danvers, MA). Goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP were obtained from Boster Biotechnology Co., Ltd. (Wuhan, China). The p38 MAPK inhibitor SB203580 was purchased from Calbiochem (San Diego, CA). The CpG-ODN (CpG-ODN 1826) and control ODN (control-ODN 1826) were purchased from InvivoGen (San Diego, CA).

Animals

Male Sprague-Dawley rats (aged ca. 4 months, 300–350 g) were used in these studies and were obtained from the Comparative Medicine Department of Jinling Hospital. The animal care and experimental procedures were performed in compliance with the Institutional Animal Care and User Guidelines and were approved by the Comparative Medicine Department of Jinling Hospital.

Establishment of I/R model

Myocardial I/R injury was induced as described previously [19-21]. Briefly, the rats were anesthetized by 5.0% isoflurane, intubated, and ventilated with room air using a rodent ventilator. Body temperature was maintained at 37°C by surface water heating. Following the skin incision, the hearts were exposed through a left thoracotomy in the fourth intercostal space. The left anterior descending (LAD) coronary artery was ligated with a 7-0 silk ligature over a 1-mm polyethylene tube (PE-10). After completion of 30 min of occlusion, the coronary artery was reperfused by pulling on the exteriorized suture to release the knot. The sham-operated animals underwent the same surgical procedures except that the suture around the LAD was not fastened. The skin was closed and the animals were allowed to recover in prewarmed cages.

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Rat experiment protocol

The rats were treated with CpG-ODN (n = 8, 3 mg/kg body weight), control-ODN (n = 8, 3 mg/kg body weight), or phosphate buffered saline (PBS; n = 8) by intraperitoneal (i.p.) injection 1 h before the hearts were subjected to I/R. We also examined the effect of CpG-ODN on cardiac function following myocardial I/R injury. The rats (n = 8 per group) were treated with CpG-ODN, control-ODN, or PBS 1 h before the hearts were subjected to 30 min of ischemia followed by reperfusion up to 14 days. Cardiac function was examined by echocardiography before I/R, and 7 and 14 days after reperfusion as described previously [22, 23]. To determine the role of the TLR9-p38 signaling pathway in TLR9-ligand-induced myocardial injury, the rats (n = 8) were injected with the p38 inhibitor SB203580 (10 mg/kg body weight) 20 min prior to CpG-ODN administration (3 mg/kg body weight).

Cell culture and treatment

The rat H9c2 cardiomyocyte cell line, obtained from American Type Culture Collection (ATCC; Rockville, MD), was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) at 37°C in an atmosphere of 95% air and 5% CO_2. The medium was replaced every 2 or 3 days and the cells were subjected to experimental procedures at 80–90% confluence. H9c2 cells were treated with CpG-ODN (100 nM) or control-ODN (100 nM), in the presence and absence of SB203580 (15 μM) for 20 min. Each group contained three replicates. The cells were harvested and cellular proteins were prepared for western blot analysis as described previously [22].

Echocardiography

Serial echocardiographic studies were performed as described under light anesthesia (pentobarbital, 25 mg/kg, i.p.) 1 day before surgery and at 7 days and 14 days after I/R treatment. The anterior chest was shaved and rats were placed in the left lateral decubitus position. Body temperature was maintained between 36.9°C and 37.3°C. Echocardiographic images were obtained using an HP SONOS 7500 ultrasound system equipped with a L12-5 linear broadband and a S12 phased array transducer fitted with a 0.3-cm standoff. The heart was imaged in the parasternal short axis view at the level of the papillary muscles to determine ejection fraction (EF). All measurements were averaged in three consecutive cardiac cycles and analyzed off-line by a single blinded observer using COMPACS image analysis software. All calculations were derived using standard formulas. Left ventricular end-diastolic diameters (LVDd) were measured from M-mode tracings obtained at the mid-papillary level and analyzed according to modified American Society for Echocardiography standards (posterior wall leading edge-to-leading edge and anterior wall trailing edge-to-trailing edge).

Western blot analysis

The H9c2s cells and the rat whole heart tissues in each group were disrupted in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na_3VO_4, 0.5 μg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride), and the cell lysates were centrifuged at 12, 000 × g at 4°C for 5 min. Protein concentrations of the supernatants were determined by a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). We performed a western blot analysis of the cellular and tissue lysates as previously described [24]. After denaturation at 100°C for 5 min in loading buffer (Bio-Rad), equivalent amounts of protein (10 μg) were separated by electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and electrophoretically transferred to a polyvinylidene difluoride membrane for 90 min at 100 V. After blocking membranes with 5% milk in TBST (50 mM Tris-HCl, pH 8; 154 mM NaCl and 0.1% Tween 20) for 2 h at room temperature, membranes were incubated overnight at 4°C with the respective antibodies. A chemiluminescence detection kit was used to detect the signals on the blots. Densitometry was performed on the X-ray films that were exposed to the blots using the ChemiDoc system (UVP, Upland, CA).

Measurement of infarct zone and risk area

The LAD coronary artery was re-occluded with a 7-0 Prolene suture that was used previously at the same place for rats assigned for measurement of infarct zone and risk area, and Evans blue dye (2 ml of a 1% solution) was injected via the external jugular vein to delineate the area at risk (AAR). The rats were sacrificed under deep pentobarbital anesthesia (60 mg/kg, i.p.) after blood sampling. The heart was then

rapidly excised and washed in 0.9% saline. After removal of the atrium, the ventricle was cut into transverse slices of equal thickness (3 mm) from the apex to the base. The slices were then incubated for 20 min in phosphate-buffered 1% 2, 3,5-triphenyltetrazolium chloride (TTC) at 37°C, and then fixed in 10% formalin solution. The AAR was defined as the area not stained with Evans blue dye. The area not stained by TTC was defined as the infarcted zone (AI). The border zones (Evans blue-stained area neighboring Evans blue-unstained area), infarcted zones (TTC and border zones [TTC-stained], infarcted zones [TTC and Evans blue-unstained]), and the nonischemic zones (Evans blue-unstained area remote from Evans blue-unstained area) were photographed and analyzed by the software program ImageJ 1.36 (National Institutes of Health [NIH], Bethesda, MD). The AAR, AI, and ventricle size were assessed by a technician who was blinded to the experimental protocol using computer-assisted planimetry (NIH ImageJ 1.57 software).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

We examined whether CpG-ODN would lead to increased apoptosis in LV myocardium, by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. TUNEL staining was performed on the heart sections using TdT-FragEL apoptosis detection kits (Calbiochem) according to the manufacturer's instructions. Counterstaining with methyl green was performed to distinguish between normal and apoptotic nuclei, in which normal nuclei were stained as blue and apoptotic nuclei as brown. The number of TUNEL-positive cells within a 2.5-mm^2 field in the LV free wall was counted. Eight randomly selected fields per section and five sections per heart were examined and the counts were averaged for statistical analysis. The observer was blinded to the treatment groups.

Statistical analysis

The study data are expressed as the mean ± standard deviation (SD) and were assessed for statistical significance using Student's *t* test. Results of the treatment groups were compared to those of the control group using one-way analysis of variance followed by Dunnett's test. All of the calculations and statistical analyses were performed using SPSS software for Windows (version 22.0; SPSS Corp., Chicago, IL). Rat survival curves were constructed using the Kaplan-Meier method within Prism (GraphPad Software, San Diego, CA).

Results

CpG-ODN administration increased myocardial infarction and reduced survival rate following I/R

To investigate the effect of the TLR9 ligand CpG-ODN on myocardial infarction following I/R, we administered CpG-ODN to rats 1 h prior to myocardial ischemia (30 min) followed by reperfusion (2 h). Fig. 1A shows that CpG-ODN treatment significantly increased infarct size compared with PBS-treated I/R hearts (50.66% ± 5.10% vs. 20.99% ± 4.11%). There was no significant difference in the ratio of risk area/LV area between CpG-ODN-treated rats and PBS-treated I/R rats, suggesting that the coronary artery was ligated in the same place in the groups (Fig. 1A). Administration of control-ODN to the rats did not alter I/R-induced myocardial infarction. We also evaluated the effect of CpG-ODN administration on survival rate following myocardial I/R. As shown in Fig. 2B, in PBS-treated rats, 93.3% of rats survived at 24 h and 70.9% survived 30 days after I/R. In CpG-ODN-treated rats, however, 13.3% of rats died in the first 24 h and 60.0% died 10 days following myocardial I/R. There was no significant difference in the survival rate between control-ODN-treated and PBS-treated I/R groups.

CpG-ODN administration significantly reduced cardiac function after myocardial I/R

We also examined the effect of CpG-ODN on cardiac function following myocardial I/R. Fig. 2A shows representative M-mode echocardiographic images from a PBS-treated and CpG-ODN-treated rat recorded 7 days after I/R. Fig. 2B and 2C show that EF and fractional shortening values in CpG-ODN-treated rats were significantly decreased by 62.3% and 34.6% at 7 days, and by 56.6% and 36.4% at 14 days after reperfusion, respectively, compared with

untreated I/R rats. LVDd in CpG-ODN-treated rats was significantly increased by 50.4% at 7 days and 72.9% at 14 days after reperfusion. Treatment of rats with control-ODN did not affect I/R-induced cardiac dysfunction. In contrast, CpG-ODN administration aggravated I/R-induced cardiac dysfunction. Fig. 2D shows that LVDd was significantly increased after myocardial ischemia followed by reperfusion up to 14 days.

CpG-ODN administration aggravated I/R-increased myocardial apoptosis

It is well known that myocardial apoptosis contributes to myocardial ischemic injury [25]. We examined the effect of CpG-ODN treatment on I/R-induced myocardial apoptosis.

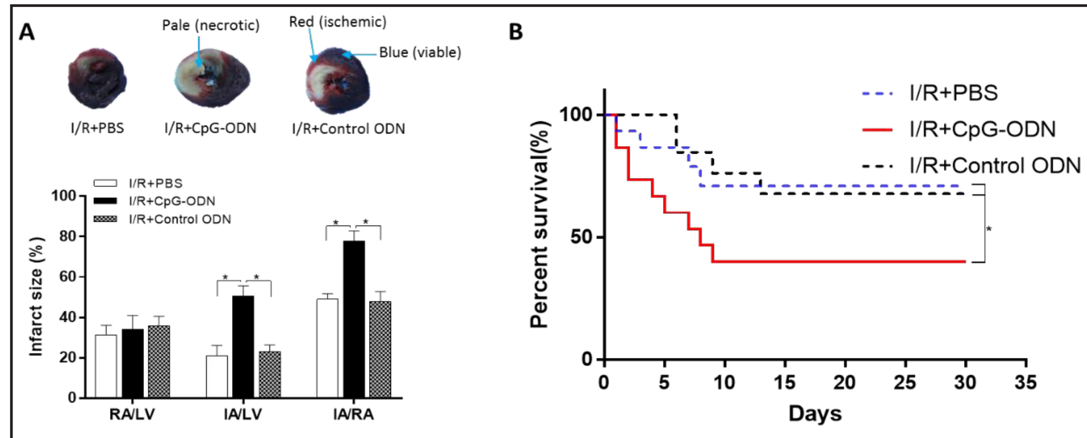

Fig. 1. (A) The TLR9 ligand CpG-ODN increased myocardial infarct size following I/R injury. The rats were treated with PBS, CpG-ODN, or control CpG-ODN (n = 8 rats per group) by i.p. injection 1 h prior to myocardial ischemia followed by reperfusion (2 h). The infarct area (white) and the area at risk (red+white) from each section were measured using an image analyzer. Ratios of risk area vs. left ventricle area (RA/LV) and infarct area vs. risk area (IA/RA) were calculated and are presented in the graph. *P<0.05 compared with the indicated group. (B) Survival rate was evaluated following myocardial I/R (n = 15 per group). *P<0.05 compared with the indicated group. I/R, ischemia/reperfusion; ODN, oligodeoxynucleotide; PBS, phosphate buffered saline.

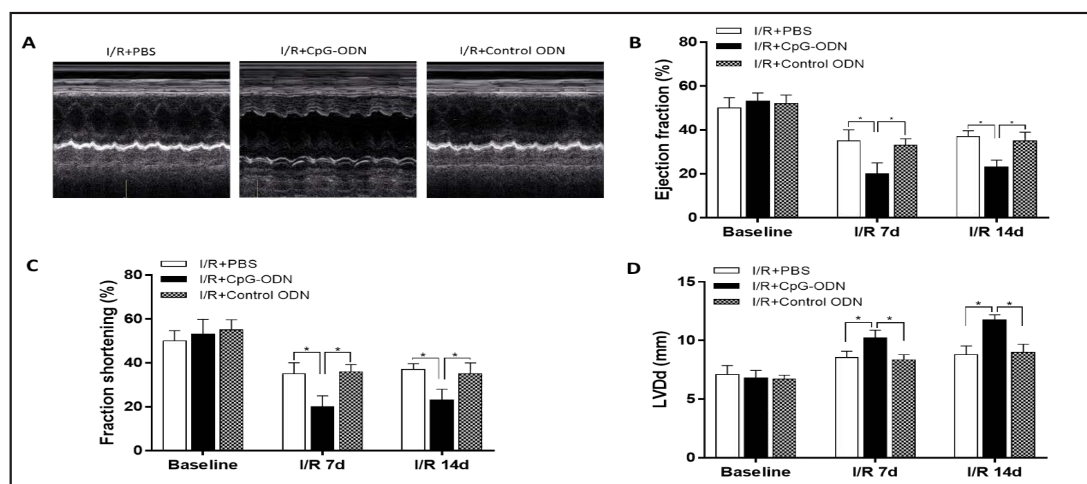

Fig. 2. Echocardiographic assessment of cardiac function. (A) Representative M-mode echocardiographic images from a PBS-, CpG-ODN-, and control-ODN-treated rat recorded 7 days after I/R. (B) Quantitative analysis of ejection fraction of each group recorded before I/R (baseline), and 7 days and 14 days after I/R. (C) Quantitative analysis of fractional shortening of each group. (D) Quantitative analysis of left ventricular end-diastolic diameter (LVDd) of each group. n = 8 rats per group. *P<0.05 compared with the indicated group. I/R, ischemia/reperfusion; ODN, oligodeoxynucleotide; PBS, phosphate buffered saline.

Fig. 3A and 3B show that I/R markedly induced myocardial apoptosis, as well as a 95% increase in the number of apoptotic cells in CpG-ODN-treated I/R rats compared with PBS-treated I/R hearts (*n* = 6 per group). Control-ODN administration did not affect I/R-induced apoptosis in the myocardium (Fig. 3A). We harvested the heart after 120 min of reperfusion. Fig. 3B shows an increased level of cleaved caspase-3 in CpG-ODN-treated I/R hearts (*n* = 6). The levels of the proteins were quantified using densitometry and normalized to GAPDH. Data are presented as the mean ± SD. These experiments were performed in quintuplicate with similar results.

CpG-ODN administration increases levels of TLR9 and p38 phosphorylation in the heart tissues following myocardial I/R

We examined whether CpG-ODN administration induces the activation of TLR9-p38 MAPK signaling using the myocardium at risk following I/R. Fig. 4 shows that CpG-ODN treatment significantly increased the levels of TLR9 and phospho-p38 compared with the untreated I/R group. Control-ODN administration did not significantly alter the levels of phospho-p38 or TLR9 in the myocardium of untreated sham and I/R rats. Treatment of rats with the specific p38 inhibitor SB203580 abolished CpG-ODN-increased levels of phospho-p38 in the myocardium.

CpG-ODN administration increases levels of TLR9 and p38 phosphorylation in H9c2 cells

To explore the mechanism of myocardial damage induced by CpG-ODN, we tested whether CpG-ODN can activate the TLR9-p38 MAPK pathway in cultured H9c2 cells. H9c2 cells were incubated with CpG-ODN with or without SB203580, followed by western blotting assay to examine the activation of TLR9-p38 MAPK signaling. As shown in Fig. 5A and 5B, CpG-ODN exposure induced significant upregulation of TLR9 protein and phosphorylated p38 MAPK, and the effect of CpG-ODN was weakened by SB203580 pretreatment. In these treatments, the levels of total p38 MAPK were not significantly changed. These results suggested that CpG-ODN activates the TLR9-p38 MAPK pathway in cultured H9c2 cells.

p38 MAPK inhibition abolished CpG-ODN-induced cardiac injury

To determine the role of the TLR9-p38 MAPK signaling pathway in CpG-ODN-induced cardiac injury, we treated the rats with the p38 inhibitor SB203580 20 min prior to CpG-ODN administration. The hearts were subjected to ischemia (30 min) followed by reperfusion (2 h). Fig. 6 shows that SB203580 administration abolished CpG-ODN-induced cardiac injury.

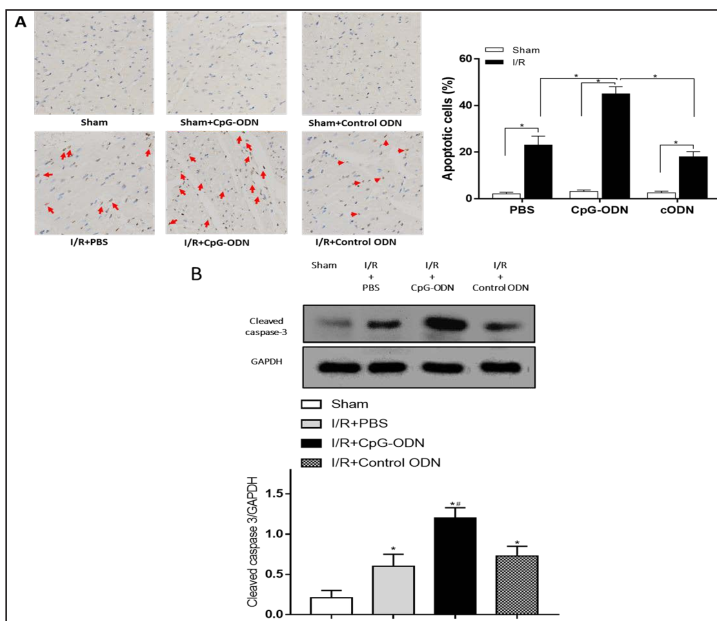

Fig. 3. Apoptosis in the myocardium. (A) Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining for apoptosis in the nonischemic and ischemic myocardium. Brown spots (arrows) in nuclei indicate cell death. (B) The level of cleaved caspase-3 protein in rat hearts treated with PBS, CpG-ODN, or control CpG-ODN. n = 6 rats per group. *P<0.05 compared with the indicated group. #P<0.05 vs. *. I/R, ischemia/reperfusion; ODN, oligodeoxynucleotide; PBS, phosphate buffered saline.

Infarct size in CpG-ODN-treated rats in the presence of SB203580 was significantly decreased compared with CpG-ODN-treated rats that did not receive SB203580 (21.71% ± 3.00% vs. 51.66% ± 5.12%). There was no significant difference in the infarct size between the CpG-ODN+SB203580 group and the PBS-treated I/R group.

Discussion

A major finding in this study is that administration of a TLR9 agonist, CpG-ODN, to rats increased infarct size and reduced cardiac function after myocardial I/R injury. Administration of control CpG-ODN did not affect I/R-induced myocardial infarction, suggesting that activation of TLR9 plays a traumatic role during myocardial I/R injury. More significantly, CpG-ODN treatment activated the TLR9-p38 MAPK signaling pathway. The data suggest that the TLR9 agonist CpG-ODN promotes myocardial injury involving activation of the TLR9-p38 MAPK signaling pathway. The data also indicate that the apoptosis-promoting effect of CpG-ODN may be one of the mechanisms by which CpG-ODN aggravates myocardial I/R injury.

Cardiac myocyte apoptosis plays a role in myocardial I/R injury. We have previously reported that mtDNA activates TLR9-p38 MAPK signaling and promotes myocardial I/R-induced myocardial injury and cardial apoptosis [18]. Both mtDNA and CpG-

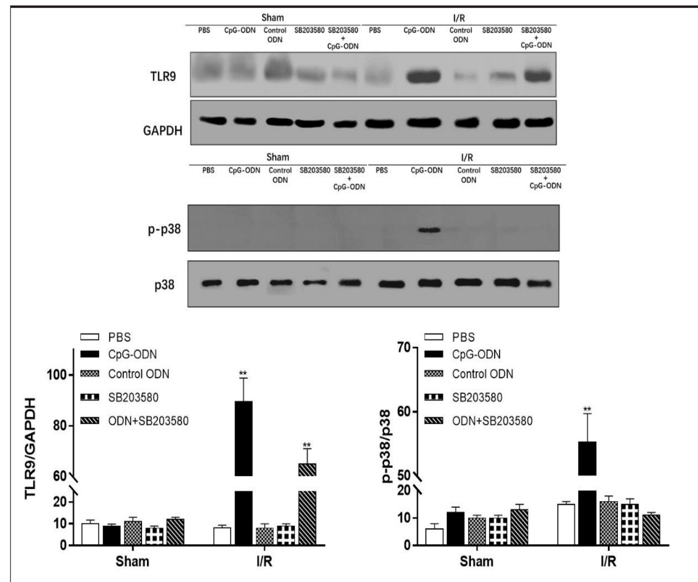

Fig. 4. CpG-ODN administration increases levels of TLR9 and p38 phosphorylation in heart tissues. The level of TLR9 protein in hearts treated with PBS, CpG-ODN, control CpG-ODN, SB203580, or CpG-ODN+SB203580 prior to I/R. Total heart proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotted for TLR9; GAPDH served as a loading control. **P<0.01, n = 3. The level of p38 and phosphorylated p38 (p-p38) protein in hearts treated with PBS, CpG-ODN, control CpG-ODN, SB203580, or CpG-ODN+SB203580 prior to I/R. **P<0.01, n = 3. I/R, ischemia/reperfusion; ODN, oligodeoxynucleotide; PBS, phosphate buffered saline.

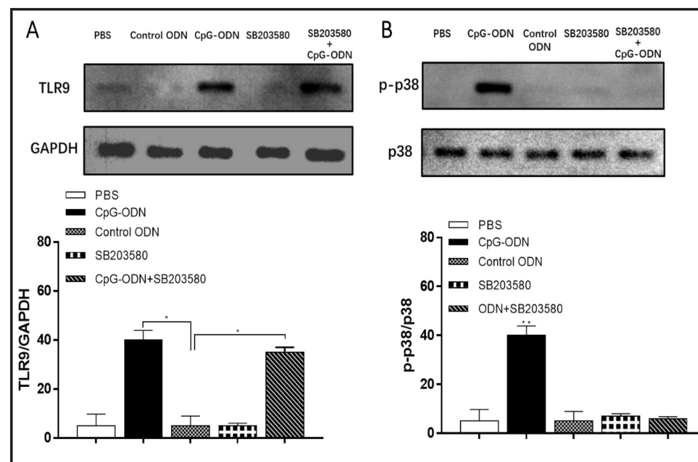

Fig. 5. CpG-ODN administration increases levels of TLR9 and p38 phosphorylation in cultured H9c2 cells. (A) Level of TLR9 protein in H9c2s treated with PBS, CpG-ODN, control CpG-ODN, SB203580, or CpG-ODN+SB203580 prior to I/R. GAPDH served as a loading control. *P<0.05, **P<0.01, n = 3. (B) Level of p38 and phosphorylated p38 (p-p38) protein in H9c2s treated with PBS, CpG-ODN, control CpG-ODN, SB203580, or CpG-ODN+SB203580 prior to I/R. *P<0.05, **P<0.01, n = 3. I/R, ischemia/reperfusion; ODN, oligodeoxynucleotide; PBS, phosphate buffered saline.

ODN contain unmethylated CpG motifs that activate TLR9 receptors. In the present study, we observed that administration of CpG-ODN increased the levels of phospho-p38 in the myocardium following myocardial I/R, indicating that CpG-ODN treatment activated the TLR9-p38 MAPK signaling pathway. Recently, two studies found that iCpG-ODN or TLR9 antagonist could reduce I/R damage in brain or liver [16, 17]. Therefore, activating the TLR9 receptor is not beneficial.

TLR9's role in I/R is controversial. Previous studies have found that TLR9 activates the downstream PI3K pathway, and numerous studies have found that this is a protective pathway in cardiac muscle [13, 26-30]. However, TLR9 can also activate other signaling pathways, such as those found to have a role in myocardial injury.

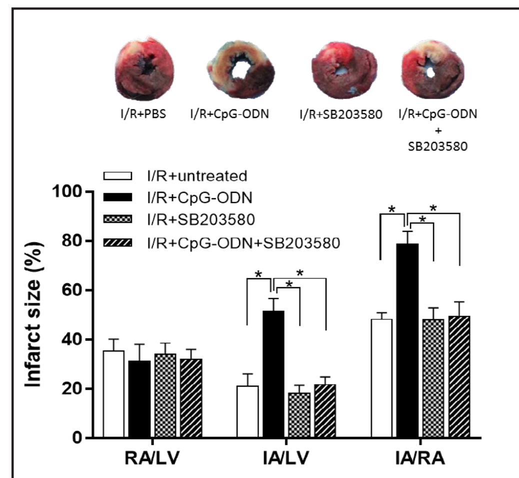

Fig. 6. Pharmacologic inhibition of p38 MAPK abrogates CpG-ODN-induced promotion of myocardial I/R injury. Infarct size was examined by 2,3,5-triphenyltetrazolium chloride and Evans blue staining. n = 8 rats per group. *P<0.05 compared with the indicated groups. IA, infarct area; I/R, ischemia/reperfusion; LV, left ventricle area; ODN, oligodeoxynucleotide; PBS, phosphate buffered saline; RA, risk area.

To determine the mechanisms by which CpG-ODN treatment activated TLR9-p38 MAPK signaling, we examined whether there is an association between TLR9 and p38 following CpG-ODN administration. We performed *in vitro* experiments using H9c2 cells and observed that CpG-ODN administration increased the levels of phospho-p38, while increased enzymatic activity of p38 was inhibited by SB203580. However, one limitation is that we did not further investigate p38 targets. Whether p38 phosphorylation was indeed reduced with SB203580 treatment or was unchanged is not clear. Despite these limitations, our data confirmed that CpG-ODN-induced cardiac injury is mediated through a TLR9-p38 MAPK-dependent mechanism.

In summary, administration of the TLR9 ligand CpG-ODN significantly increased I/R-induced infarct size and reduced cardiac function following myocardial I/R.

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Disclosure Statement

The author declare that they have no conflict of interests.

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