c-Jun DNAzymes Inhibit Myocardial Inflammation, ROS Generation, Infarct Size, and Improve Cardiac Function After Ischemia-Reperfusion Injury

Xiao Luo, Hong Cai, Jun Ni, Ravinay Bhindi, Harry C. Lowe, Colin N. Chesterman, Levon M. Khachigian

Objectives—Coronary reperfusion has been the mainstay therapy for reduced infarct size after a heart attack. However, this intervention also results in myocardial injury by initiating a marked inflammatory reaction, and new treatments are keenly sought.

Methods and Results—The basic-region leucine zipper protein, c-Jun is poorly expressed in the normal myocardium and is induced within 24 hours after myocardial ischemia-reperfusion injury. Synthetic catalytic DNA molecules (DNAzymes) targeting c-Jun (Dz13) reduce infarct size in the area-at-risk (AAR) regardless of whether it is delivered intramyocardially at the initiation of ischemia or at the time of reperfusion. Dz13 attenuates neutrophil infiltration, c-Jun and ICAM-1 expression in vascular endothelium, cardiomyocyte apoptosis, and the generation of reactive oxygen species in the reperfused myocardium. It inhibits infiltration into the AAR of complement 3 (C3), C3a receptor (C3aR), membrane attack complex-1 (Mac-1), or matrix metalloproteinase-2 (MMP-2) positive inflammatory cells. Dz13 also improves cardiac function without influencing myocardial vascularity or fibrosis.

Conclusion—These findings demonstrate the regulatory role of c-Jun in the pathogenesis of myocardial inflammation and infarction following ischemia-reperfusion injury, and inhibition of this process using catalytic DNA. (Arterioscler Thromb Vasc Biol. 2009;29:1836-1842.)

Key Words: c-Jun ■ DNAzyme ■ myocardial ischemia-reperfusion injury

Outcomes after acute myocardial infarction (AMI) have improved markedly over the past decades, but unmet challenges remain. Although restoration of blood flow to the ischemic myocardium with primary percutaneous coronary intervention or thrombolytic therapy has been shown effective in reducing infarct size, these interventions can also result in cardiomyocyte injury by initiating marked inflammation. The pathophysiology of myocardial/ischemia-reperfusion (M/IR) injury is complex and involves the production and release of surface adhesion molecules and other inflammatory mediators, including intracellular adhesion molecule-1 (ICAM-1), reactive oxygen species (ROS), and activation of the complement system. For drugs to effectively attenuate this response, they would need to have broad-spectrum activity to inhibit the multiple pathways and limit their cross-amplification.

c-Jun, a 39-kDa basic-region leucine zipper protein and prototypic member of the activating protein-1 (AP1) family of transcription factors, is activated by multiple stimuli including inflammatory cytokines.1-2 Deletion of c-jun in mice results in embryonic lethality.3 Studies have shown the activation of AP1 as early as 15 minutes after reperfusion4 and that decreased c-Jun phosphorylation correlates with reduced infarct size.5 We have demonstrated that knockdown of c-Jun by RNA-cleaving catalytic agents, termed DNAzymes,6 suppress vascular permeability and transendothelial emigration of leukocytes in various rodent inflammatory models including cytokine-challenged mesenteric venules, endotoxin-induced lung sepsis, and collagen antibody-induced rheumatoid arthritis.2

Reperfusion injury after AMI involves degradation of extracellular matrix components by matrix metalloproteinases (MMPs), ROS production, apoptosis, and activation of the complement system.7 Complement activation is associated with neutrophil infiltration and increased myocardial infarct size as a consequence of ischemic cardiac damage after the reperfusion.8 Activation of the complement system produces tissue injury through formation of the membrane attack complex Mac-1 (C5b-9), which causes cell death through disruption of cellular integrity.9 The anaphylatoxin C3a is a proinflammatory mediator generated during complement activation10 and exerts its effects via the heptahelical receptor C3aR,11 which is expressed on leukocytes and endothelial cells.12,13 AP1 regulates the C3aR promoter in monomyeloblastic U937 cells.14 The C3b receptor, the CD11c leukocyte integrin, which mediates neutrophil adher-
ence,\textsuperscript{15} is also activated by AP1.\textsuperscript{16} C3b deposition in endothelial cells is ameliorated by ROS scavengers that interfere with the production of free radicals.\textsuperscript{17} M/IR injury is reduced in experimental models and certain clinical settings through blockade of the complement system by C1 esterase inhibition or sC1R and anti-C5a therapy.\textsuperscript{18} It is also well recognized that left ventricular remodeling and myocardial dysfunction after M/IR injury are mediated by MMPs, particularly MMP-2.\textsuperscript{19}

Other studies by our group have demonstrated that Dz13 suppresses solid tumor growth in part by inhibiting MMP-2 expression.\textsuperscript{20,21} These findings have made the complement system, MMPs, and cell adhesion molecules a focus of interventional approaches in the setting of AMI.

Here we hypothesized that molecular suppression of c-Jun, as a master regulator of these and other proinflammatory genes, may protect the myocardium from ischemia-reperfusion injury.

**Methods**

For details of cell culture and transfection, M/IR injury model, tissue collection, and measurement of infarct size and inflammatory cell infiltration, RT-PCR, neutrophil counts, immunohistochemistry and TUNEL staining, and statistical analysis, please see supplemental material (available online at http://atvb.ahajournals.org).

**Results**

**Dz13 Reduces Myocardial Infarct Size in the Area-at-Risk Regardless of Whether It Is Delivered Intramyocardially at the Initiation of Ischemia or at the Time of Reperfusion**

To determine the influence of c-Jun suppression on infarct size in the area-at-risk (AAR), Dz13 or Dz13scr was administered by intramyocardial (IM) injection at 4 sites within the AAR in the left ventricle (LV). Infarct size and the AAR were determined by Evans blue exclusion and triphenyl tetrazolium chloride (TTC) staining, respectively. These experiments involved DNAzyme delivery at 2 separate times relative to ischemia-reperfusion: the first, at the commencement of ischemia, and the second, at the time of reperfusion.

Representative LV slices from Dz13 or Dz13scr cohorts 24 hours after the drug was administered at the time the LCA was ligated are shown in Figure 1A. Ischemia-reperfusion with no injection (No ODN, No Veh) or IM injection of vehicle control (No ODN) resulted in a mean infarct size occupying approximately 32% of the AAR (Figure 1B). Administration of Dz13 at the start of ischemia inhibited infarct size by 55%. When Dz13 was delivered at the time of reperfusion, a 43% reduction in infarct size was achieved relative to the vehicle and scrambled groups (Figure 1C). No differences in AAR were observed between groups, indicating surgical consistency in both models (Figure 1D and 1E).

**Dz13 Attenuates Neutrophil Infiltration in the Reperfused Myocardium**

Twenty-four hours after ischemia-reperfusion, the infarcted myocardium loses its regular structure and will have had significant inflammatory cell infiltration. Previous studies have shown that many of these infiltrating cells are morphologically-distinct polymorphonuclear leukocytes (neutrophils).\textsuperscript{22} Compared with vehicle (No ODN) and Dz13scr groups, Dz13 caused a 50% reduction in neutrophil infiltration in the reperfused myocardium (Figure 1F). Immunofluorescence microscopy confirmed delivery of FITC-labeled DNAzyme (FITC-Dz) into cells of the AAR (data not shown).

**Dz13 Inhibits c-Jun and ICAM-1 Expression in Vascular Endothelium**

The preceding data implicate c-Jun, the primary target of Dz13 in the control of myocardial infarct size (Figure 1A through 1E) and inflammation (Figure 1F) in the AAR after M/IR. To provide evidence for the direct involvement of c-Jun in the injured myocardium, we determined the effect of Dz13 on levels of c-Jun protein. Immunohistochemical (IHC) analysis revealed that c-Jun is poorly expressed in the normal myocardium (Figure 2A). However, it is expressed in the microvasculature between cardiomyocytes in the AAR (Figure 2A) within 24 hours of ischemia-reperfusion (Figure 2A).

In contrast, c-Jun is not expressed in cardiomyocytes or inflammatory cells at this time point (Figure 2A). Dz13, but not Dz13scr, inhibited c-Jun expression in vascular endothelium, identified morphologically by CD31\textsuperscript{+} staining in the AAR (Figure 2B).

ICAM-1 is a well-recognized mediator of the early interaction and adhesion of neutrophils to coronary endothelial cells and myocytes after M/IR injury.\textsuperscript{23} Because Dz13 reduced neutrophil infiltration in the ischemia-reperfused myocardium, we examined the effect of Dz13 on ICAM-1 expression. IHC analysis revealed that Dz13 attenuated M/IR-inducible ICAM-1 expression in the blood vessels, whereas Dz13scr had no inhibitory effect (Figure 2B).

**Dz13 Inhibits Cardiomyocyte Apoptosis in the Injured Myocardium**

We next assessed the effect of Dz13 on apoptosis induced by M/IR in the AAR. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) 24 hours after M/IR identified apoptotic nuclei in cardiomyocytes (Figure 2C). Dz13 inhibited TUNEL\textsuperscript{+} staining in the AAR (Figure 2C), consistent with its ability to reduce infarct size (Figure 1A through 1E), whereas Dz13scr had no effect (Figure 2C).

**Dz13 Inhibits Cytokine-Inducible Components of the Complement System**

Although it is known that AP1 regulates transcription of the anaphylatoxin C3a receptor, C3aR,\textsuperscript{14} whether c-Jun/AP1 controls other components of the complement system is unclear. We overexpressed c-Jun using a RSV-based expression vector in human umbilical vein endothelial cells (HUVECs) and evaluated mRNA levels after 24 hours. c-Jun transcript levels, barely detectable in growth-quiescent cells, were increased following transfection with the c-Jun vector (supplemental Figure IA). c-Jun increased levels of C3 mRNA without affecting levels of βactin (supplemental Figure IA). Dz13 blocked both c-Jun expression in cells transfected with the plasmid and c-Jun–inducible C3 mRNA expression (supplemental Figure IA). In contrast, Dz13scr had no effect on c-Jun or C3 mRNA levels induced by the expression vector (supplemental Figure IA). To demonstrate
Figure 1. Dz13 reduces myocardial infarct size and neutrophil infiltration in AAR. A, Representative LV slices showing infarct (arrows) in AAR. Dz13 administered at start of ischemia. Quantitation of infarct size when DNAzyme administered at commencement of ischemia (B) or time of reperfusion (C). AAR (expressed as percent of LV) when DNAzyme administered at commencement of ischemia (D) or at time of reperfusion (E). M/IR indicates myocardial ischemia-reperfusion; No ODN/No Veh, M/IR with no injection; No ODN, M/IR with IM injection of vehicle. F, Quantitation of H&E-stained cross-sections showing neutrophils in AAR. Arrows indicate neutrophils. HPF indicates high-power field. *P<0.05.
the dependence on c-Jun of cytokine-inducible C3, C3aR, and C5, we exposed HUVECs to IL-1β for 2 hours with or without prior Dz13 or Dz13scr transfection. IL-1β stimulated c-Jun mRNA expression, as it did levels of C3 C3aR and C5, without affecting β-actin expression (supplemental Figure IB). Dz13 abolished the cytokine-inducible expression of these components of the complement system (supplemental Figure IB). C3 mRNA, like c-Jun, is poorly expressed in the AAR, but increased after M/IR (supplemental Figure IC). Dz13 reduced M/IR-inducible C3 and c-Jun expression, whereas Dz13scr had no effect (supplemental Figure IC).

**Dz13 Reduces Density of MMP-2–Positive Cells in the Injured Myocardium**

LV remodeling and myocardial dysfunction after M/IR injury are mediated by MMPs, particularly MMP-2.19,24–26 MMP-2 has also recently been shown to mediate M/IR injury by enhanced peroxynitrite biosynthesis during early reperfusion.27 MMP-2 is produced in the human heart after AML.28 Dz13 inhibited M/IR-inducible MMP-2 mRNA expression in the AAR (supplemental Figure IIC). IHC revealed that Dz13 reduced MMP-2 levels in endothelial cells, consistent with our previous findings in nonmyocardial tissue,2,20,21,29 and the density of MMP-2+ macrophages in the AAR (supplemental Figure IID).

**Dz13 Attenuates ROS Generation**

Nitrotyrosine is an established “footprint” of peroxynitrite,30 a toxic ROS produced by invading neutrophils. We reasoned that increased nitrotyrosine levels would coincide with neutrophil infiltration into the AAR of the LV and that this would be negatively regulated by Dz13. Nitrotyrosine levels, like those of c-Jun (Figure 2A), were negligible in sham-treated animals but increased 24 hours after M/IR injury (Figure 3). Nitrotyrosine was inducibly expressed in both macrophages and cardiomyocytes (Figure 3) in the AAR. Dz13 suppressed nitrotyrosine immunoreactivity compared with Dz13scr and vehicle groups (Figure 3).

**Dz13 Improves Function After M/IR Injury and Does Not Influence Myocardial Vascularity or Fibrosis in the AAR**

Finally, to determine the effect of Dz13 on cardiac function after M/IR injury and demonstrate the clinical relevance of our
findings, we measured ejection fraction (EF) and fractional shortening (FS) in DNazyme-treated and untreated rats using VisualSonics’ Vevo 770 high-resolution microultrasound imaging system. EF and FS were both reduced 24 hours after M/IR injury (Figure 4B). The decline in myocardial function was protected by Dz13 but not by Dz13scr (Figure 4B). Vascularity in the AAR was examined in animals subjected to M/IR injury 14 days after reperfusion by immunostaining and quantitating vWF positive vessels. No difference was observed in microvascular density between the 2 groups (Figure 4B and 4C). Moreover, comparison of Masson trichrome-stained sections of the AAR between Dz13 and Dz13scr-treated groups revealed no difference in the extent of fibrosis (Figure 4D and 4E).

Discussion

The data in this manuscript provide several novel findings. First, c-Jun regulates cytokine-inducible components of the complement system (supplemental Figure I). Second, a single dose of Dz13, delivered locally into the myocardium, reduces infarct size in the AAR irrespective of whether it is injected at the start of ischemia or at the time of reperfusion (Figure 1A through 1E). Third, Dz13 inhibits neutrophil infiltration.
into the myocardium and cardiomyocyte apoptosis (Figures 1F and 2C). Fourth, Dz13 inhibits c-Jun and ICAM-1 expression in microvessels in the AAR (Figure 2B). Fifth, Dz13 inhibits C3-, C3aR-, Mac-1−, or MMP-2−positive cells in the injured myocardium (supplemental Figure IIA through IID). Sixth, Dz13 inhibits nitrotyrosine expression (Figure 3). And finally, Dz13 does not differ from Dz13scr in its ability to influence myocardial vascularity or fibrosis in the AAR 14 days after M/IR injury (Figure 4B through 4E). The effects of Dz13 are therefore likely mediated by its knockdown of c-Jun (and c-Jun−dependent genes) in vascular endothelial cells and consequential inhibition of infiltration into the AAR of C3-, C3aR-, Mac-1−, or MMP-2−positive inflammatory cells. The clinical relevance of our findings is highlighted by improved myocardial function in the Dz13 group (Figure 4A), compared with the Dz13scr cohort after single administration.

Reduced myocardial infarct size after treatment with Dz13 is likely the consequence of DNAzyme blockade of a variety of c-Jun−dependent molecular and cellular processes. As this study shows, M/IR triggers the expression of numerous proinflammatory mediators including cell adhesion molecules, components of the complement system, and ROS. Dz13 clearly exerts its effects not through a single pathway, but rather multiple pathways, by targeting the “master regulator” c-Jun. Consistent with reduced neutrophil infiltration as a consequence of Dz13 treatment, we observed reduced ICAM-1, C3, and Mac-1 expression. All these molecules regulate neutrophil infiltration. ICAM-1 mediates neutrophil adhesion to vascular endothelium and transendothelial migration of leukocytes into the myocardial interstitium. Inhibition of neutrophil infiltration by reducing ICAM-1 expression by Dz13 is supported by our recent observations of Dz13 blockade of ICAM-1 expression in murine models of lung, joint, and venular inflammation. Components C3 and Mac-1 are thought to regulate neutrophil-mediated myocardial injury via several mechanisms. Mac-1 can upregulate CD62P on endothelial cells, resulting in increased neutrophil adherence. Sublytic amounts of Mac-1 have been shown to induce MCP-1 and IL-8 in endothelial cells. Increased ICAM-1 expression may be modulated by C3a expressed by leukocytes and endothelial cells. Moreover, C3b deposition on endothelial cells may mediate neutrophil adhesion via CD11b/CD18 interactions. Reduced MMP-2 mRNA and protein expression by Dz13 as demonstrated in this study may be an additional contributing factor to protect the myocardium from inflammatory cell injury. Inhibition of MMP-2 by Dz13 is consistent with previous studies, which indicate that MMP-2 is a c-Jun dependent gene and Jun proteins regulate MMP-2 transcription.

LV remodeling and myocardial dysfunction after M/IR injury are mediated by proinflammatory mediators including cell adhesion molecules, components of the complement system, and ROS. Dz13 inhibited the presence of C3-, C3aR-, and Mac-1−positive cells in the injured myocardium. The primary target of Dz13, c-Jun, is an upstream regulator of effector genes, such as ICAM-1, controlling the complex process of M/IR injury. Strategies targeting c-Jun therefore offer the advantage of blocking parallel gene expression cascades. The need for this in M/IR injury is underlined by the lack of measurable clinical effect of the C5 inhibitor, pexelizumab, on infarct size in patients with ST-elevation MI undergoing primary percutaneous coronary intervention. That said, whether Dz13 suppression of inflammatory cell infiltration is the cause of reduced infarct size, or that DNAzyme inhibits leukocyte emigration secondary to its inhibition of infarction, in definitive terms remains to be seen.

Apoptosis accounts for myocardial death after coronary occlusion. Neutrophil-mediated myocardial injury can induce necrotic as well as apoptotic cardiomyocyte death. That Dz13 inhibits TUNEL staining in the M/IR-injured myocardium is supported by previous reports that AP1 can serve as a proapoptotic transcription factor. Peroxynitrite, a highly reactive oxygen species, is a major cause of injury after M/IR and stimulates apoptosis. Nitrotyrosine is a marker of peroxynitrite. Dz13 inhibition of myocardial nitrotyrosine levels further demonstrates the utility of this DNAzyme for the prevention of apoptosis and reduction in infarct size.

That c-Jun is poorly expressed in the myocardium, but is inducible by M/IR, is consistent with its activation from a state of poor expression in other vascular settings such as normal carotid artery, retina, lung, joint, and venular microvasculature. This makes c-Jun an ideal drug target because it is expressed in pathological settings and targeted interventional approaches should minimize the possibility of interfering with normal cellular functions. In the present model, cell death and myocardial inflammation is apparent 24 hours post-M/IR, whereas fibrosis becomes apparent later. Dz13 did not reduce interstitial fibrosis despite its inhibition of infarct size and suppression of myocardial inflammation. Here we demonstrate Dz13-mediated reduction of infarct size after the DNAzyme was administered either at the commencement of ischemia or at the time of reperfusion. Although the physical size of the animals used here precluded intracoronary administration, in the clinical situation, Dz13 may be administered locally at the time of primary percutaneous coronary intervention or thrombolytic therapy.

In conclusion, this study demonstrates that strategies targeting c-Jun may be useful to reduce M/IR injury in a local delivery setting. A single dose of IM-injected Dz13 results in a significant reduction in infarct size and improved myocardial function. Dz13 suppressed c-Jun, ICAM-1, and MMP-2 expression in vascular endothelium and reduced infiltration of C3-, C3aR-, and Mac-1−positive cells in the injured myocardium.

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Disclosures
None.

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c-Jun DNAzymes Inhibit Myocardial Inflammation, ROS Generation, Infarct Size and Improve Cardiac Function After Ischemia-Reperfusion Injury

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2. Methods

2.1 Cell culture and transfection

Human umbilical vein endothelial cells (HUVECs) were prepared as described\(^1\) and grown in EGM2 complete medium (Clonetics). At 60-70\% confluence, cells in 100mm petri-dishes were transfected with 0.2\(\mu\)M DNAzymes (Dz13 or Dz13scr, Tri-Link) using FuGENE6 (Roche) 6h after changing the medium from 10\% to 0.05\% FBS. After 18h, cells were co-transfected again with the same concentration of DNAzyme (with or without 10\(\mu\)g pRSVjun, a c-Jun expression vector\(^2\). In separate experiments, HUVECs were transfected with DNAzyme and incubated with 10ng/ml IL-1\(\beta\) after the first transfection. This double-transfection protocol results in transfection efficiency of 30-50\%\(^2\). Two h after the second transfection and cytokine exposure, mRNA from each group was extracted for analysis of c-Jun, C3, C3aR and C5 by RT-PCR using methods described previously\(^2\). The effect of Dz13 or Dz13scr on pRSVjun-transfected cells was evaluated by RT-PCR after 24h.

2.2 M/IR injury model

Adult male Sprague-Dawley rats (250-300 g) were anesthetized with a mixture of ketamine (80 mg/kg body weight)/xylazine (10mg/kg). M/IR was produced in rats as recently described by us\(^3\). Briefly, the left coronary artery (LCA) was ligated 2-3 mm from its point of origin with 6-0 prolene
after a left thoracotomy under mechanical ventilation. The ligature was untied after 30 min to allow reperfusion of ischemic myocardium. Dz13 or its scrambled control (Dz13scr) at 500µg (in 200µl vehicle containing 60µl FuGENE6 and 1mM MgCl₂) was administrated by intramyocardial (IM) injection at four sites (50µl injections) within the area at risk (AAR) in the left ventricle (LV). Myocardial ischemia was assessed by myocardial blanching and later confirmed at the time of reperfusion. Dz13, Dz13scr or FuGENE6-containing vehicle was administered either within 2 min of the start of ischemia or within 1 min of the start of reperfusion. Two additional groups of rats were used to examine the effect of IM injection and the M/IR procedure on the infarct size. These included a no injection group in which rats underwent M/IR only, and a sham-operated group in which rats underwent surgery but without ligation of the LCA. All animal procedures were approved by UNSW Animal Ethics Committee.

Cardiac function was evaluated before and 24h after M/IR using the VisualSonics’ Vevo 770 high-resolution, micro-ultrasound imaging system. Briefly, mice were anesthetized with isofluorane and restrained on the platform. Chest hair was removed with Nair®. Once the clear view of both long axis and short axis was achieved by 710B detector, M mode analysis was performed to measure ejection fraction (EF) and fractional shortening (FS), which were calculated by Vevo cardiac measurements package. Each assessment was performed on 3 beats in different segments. Changes in EF and FS one day after surgery were analyzed by 2 independent observers and compared to baseline data.

2.3 Tissue collection and measurement of infarct size and inflammatory cell infiltration

Twenty-four h following reperfusion, hearts were again exposed under mechanical ventilation. The LCA was re-ligated at the same point and 1.5 ml 2% Evans blue was injected directly to the LV.
heart was excised and then cut transversely into four slices from the apex to the base. The slice from apex was snap frozen immediately and stored at -80°C for mRNA and protein extraction. The remaining slices were incubated with 4% TTC for 20 min and fixed in 10% formalin overnight. Slices were photographed with a digital camera (Canon). For each picture, the total LV area, the area lacking Evans blue staining (AAR) and the area lacking TTC staining (infarct area) were determined using Image J (1.31v). Infarct size was measured and verified by two blinded researchers and expressed as the percentage of infarct area/AAR. Hearts from a separate group of rats underwent a 14 d reperfusion protocol following the 30 min ischemia. The heart was sectioned and fixed, and evaluated for LV remodeling scar and blood vessel formation as indicated below. 5-6 rats were used per group. Inflammatory cell infiltration in the AAR was evaluated by examination of H&E-stained sections under high-power microscopy by three senior histopathologists blinded to the sections.

### 2.4 Reverse transcriptase–polymerase chain reaction

Total RNA was extracted from the AAR of each group using the total RNA Mini Kit (Bio-Rad) after homogenization with Lysing Matrix D (Qbiogene) using the BIO101 Thermo Savant FastPrep FP120 (Qbiogene) for 40 s at a power setting of 4. cDNA was generated using Superscript III and Oligo dT primer (Invitrogen) as per the manufacturer’s instructions. mRNA for c-Jun, C3, C3aR, and MMP-2 were measured by RT-PCR.

### 2.5 Neutrophil counts, immunohistochemistry and TUNEL staining

Following evaluation of infarct size, representative LV slices were paraffin-embedded and sectioned (2µm) for immunohistochemical (IHC) analysis, hematoxylin and eosin (H&E) staining and TUNEL analysis. Neutrophils (polymorphonuclear leukocytes, neutrophils) were identified in H&E-
stained sections and counted per high power field (HPF, 600x) within the AAR. Ten different fields per animal (n=3) in each section were randomly selected and analysed. IHC was performed using specific antibodies to c-Jun, MMP-2, CD31, C3aR (Santa Cruz Biotechnology), ICAM-1 (R&D Systems), C3 (AntiBodyShop), Mac-1, Mac-3 (Serotec), nitrotyrosine and von Willebrand factor (vWF) (Dako). Secondary antibodies and streptavidin were purchased from Dakocyotmation (Glostrup). These IHC procedures have been described elsewhere. Staining was quantified by counting the number of positive cells in 10 random HPF per animal (n=3). Apoptosis was determined by TUNEL staining using TACS XL Blue Label detection kit (R&D Systems). Cardiomyocyte nuclei staining positive for TUNEL were quantified in the infarct zone in 10 random HPF per animal (n=3). Separate sections were taken from animals underwent 14 d reperfusion. These sections were either stained with Masson’s trichrome to inspect for scar formation or subjected to IHC protocol for identification of blood vessels using antibodies to vWF. For quantitative purposes, vascular density was assessed in the AAR from the endocardium through the epicardium of the mid-portion of the LV free wall. Fibrosis was quantitated in Masson’s trichrome stained sections using Image J software and results were expressed as a percentage of the LV area.

2.6 Statistical analysis

All values in the text and figures are presented as mean ± SEM. All data were subjected to one-way ANOVA followed by Fisher’s protected least significant difference test. A value of $P<0.05$ was considered significant.

Supplementary References


**Supplementary Figure Legends**

**Supplementary Fig. I.** Dz13 inhibits c-Jun, C3, C3aR, C5 and MMP-2 expression. A, c-Jun overexpression induces C3 mRNA levels, which is blocked by Dz13. Growth-quiescent HUVEC were transfected with 10µg pRSVjun or the backbone (B/B) alone and c-Jun, C3 or beta-actin levels were assessed after 24h. Where DNAzymes were used, HUVEC were co-transfected with the c-Jun plasmid together with 0.2µM Dz13 or Dz13scr and mRNA expressed was determined after 24h. B, Blockade of cytokine-inducible c-Jun, C3, C3aR and C5 mRNA expression by Dz13. Growth-quiescent HUVEC transfected with 0.2µM Dz13 or Dz13scr were exposed to IL-1beta for 2h. C, RT-PCR showing changes in c-Jun, C3 and MMP-2 mRNA in the AAR 24h following M/IR injury.

**Supplementary Fig. II.** Dz13 reduces density of C3, C3aR, Mac-1 or MMP-2 positive cells in AAR. Quantitation and representative photomicrographs showing A, C3, B, C3aR, C, Mac-1 and D, MMP-2 immunostaining in the AAR 24h following M/IR. MAC-3 identifies macrophages. HPF denotes high power field. Black and white arrows indicate representative macrophages and blood vessels, respectively. Where indicated the y-axes represent inflammatory cells only, and do not include blood vessel counts (NIBV, not including blood vessels). *denotes P<0.05.