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SR-A deficiency reduces myocardial ischemia/reperfusion injury; involvement of increased microRNA-125b expression in macrophages

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ABSTRACT

The macrophage scavenger receptor class A (SR-A) participates in the innate immune and inflammatory responses. This study examined the role of macrophage SR-A in myocardial ischemia/reperfusion (I/R) injury and hypoxia/reoxygenation (H/R)-induced cell damage. SR-A$^{-/-}$ and WT mice were subjected to ischemia (45 min) followed by reperfusion for up to 7 days. SR-A$^{-/-}$ mice showed smaller myocardial infarct size and better cardiac function than did WT I/R mice. SR-A deficiency attenuated I/R-induced myocardial apoptosis by preventing p53-mediated Bak-1 apoptotic signaling. The levels of microRNA-125b in SR-A$^{-/-}$ heart were significantly greater than in WT myocardium. SR-A is predominantly expressed on macrophages. To investigate the role of SR-A macrophages in H/R-induced injury, we isolated peritoneal macrophages from SR-A deficient (SR-A$^{-/-}$) and wild type (WT) mice. Macrophages were subjected to hypoxia followed by reoxygenation. H/R markedly increased NF-κB binding activity as well as KC and MCP-1 production in WT macrophages but not in SR-A$^{-/-}$ macrophages. H/R induced caspase-3/7 and -8 activities and cell death in WT macrophages, but not in SR-A$^{-/-}$ macrophages. The levels of miR-125b in SR-A$^{-/-}$ macrophages were significantly higher than in WT macrophages. Transfection of WT macrophages with miR-125b mimics attenuated H/R-induced caspase-3/7 and -8 activities and H/R-decreased viability, and prevented H/R-increased p-53, Bak-1 and Bax expression. The data suggest that SR-A deficiency attenuates myocardial I/R injury by targeting p53-mediated apoptotic signaling. SR-A$^{-/-}$ macrophages contain high levels of miR-125b which may play a role in the protective effect of SR-A deficiency on myocardial I/R injury and H/R-induced cell damage.

1. Introduction

Innate immune and inflammatory responses mediated by Toll-like receptors (TLRs) [26] are involved in myocardial ischemia/reperfusion (I/R) injury [24]. The contribution of TLR-mediated NF-κB activation to myocardial I/R injury has been well documented [15,22,23,27,28]. We and others have shown that modulation of the TLR4-mediated signaling pathway or TLR4 deficiency protects against myocardial I/R injury [15,23,28]. Recently, we have reported that the macrophage scavenger receptor class A type I/II (SR-A aka CD204) is required for lipopolysaccharide, a TLR4 ligand, induced activation of NF-κB signaling pathway [42].

SR-A was initially discovered as a receptor for recognition of modified low-density lipoprotein [11]. Subsequently, SR-A has been reported to participate in clearance of modified host components, apoptotic cells, and pathogens [12]. Recent evidence suggests that SR-A contributes to induction of the innate immune and inflammatory responses by cooperating with TLRs in the recognition of exogenous pathogen-associated molecular patterns and endogenous ligands [1,31,39]. For example, TLR ligands synergize with SR-A to mediate bacterial phagocytosis [1], induce SR-A expression [39], and promote SR-A recognition of LPS [39]. On the other hand, SR-A ligands trigger apoptosis in endoplasmic reticulum (ER)-stressed macrophages by cooperating with TLR4 [31]. SR-A may also serve as a negative regulator of TLR4 in mediating immune responses [41].

SR-A is principally expressed on macrophages and dendritic cells [17,41]. The heart contains resident macrophages located in the perivascular space surrounding medium to large arteries [8]. Macrophages play a role in myocardial repair and remodeling after myocardial infarction [18,35]. Recent studies have reported that ischemia

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alone causes rapid recruitment of circulating macrophages into the myocardium [7,16]. Both resident and recruited macrophages release inflammatory cytokines and chemokines that attract neutrophil infiltration and promote inflammatory responses [10], indicating that macrophages participate in the initial innate immune and inflammatory responses [43] during early stages of acute ischemia.

MicroRNAs (miRs) are 21 to 23 nucleotide non-protein-coding RNA molecules which have been identified as a novel regulators of gene expression at the post-transcriptional level by binding to target messenger RNAs [36]. Several miRs have been reported to play a role in ischemic heart disease [2,36,38]. For example, miR-21 protects cells from oxidative stress-induced damage [3] and the myocardium from ischemic injury [3,5]. miR-320 is involved in I/R-induced cardiac injury and dysfunction via regulation of Hsp20 [30].

In the present study, we found that SR-A deficiency (SR-A$^{-/-}$) attenuated myocardial I/R injury. miR-125b expression in SR-A$^{-/-}$ hearts and macrophages is significantly greater than in wild type (WT) heart and macrophages. SR-A deficiency protects the macrophages from hypoxia/reoxygenation (H/R) induced damage and the myocardium from I/R injury. We demonstrated that miR-125b exerts a protective role in SR-A$^{-/-}$ macrophages by targeting p53-mediated apoptotic signaling.

2. Materials and methods

2.1. Animals

SR-A$^{-/-}$ mice on the C57BL/6J background were provided by Dr. Siamon Gordon at Oxford University. Male WT C57BL/6J mice were obtained from Jackson Laboratory. Mice were maintained in the Division of Laboratory Animal Resources, East Tennessee State University (ETSU). The experiments conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). The experimental protocols were approved by the ETSU Committee on Animal Care.

2.2. Induction of myocardial I/R injury

Myocardial I/R injury was induced as described previously [13,15,23]. Briefly, mice were anesthetized by isoflurane inhalation before the left anterior descending coronary artery (LAD) was ligated with a 7–0 silk ligature over a 1-mm polyethylene tube (PE-10). After completion of 45 min of occlusion, the coronary artery was reperfused by pulling on the exteriorized suture to release the knot. After reperfusion for indicated time points, cardiac function was measured by echocardiography as described previously [13,14]. The hearts were harvested for evaluation of infarct size and for cellular protein preparations.

2.3. Determination of myocardial infarct size

Infarct size was evaluated by triphenyltetrazolium chloride (TTC, Sigma-Aldrich) staining as described previously [13,15,23]. Briefly, the hearts were removed, perfused with saline on a Langendorff system and stained with 1% Evans Blue. Each heart was then sliced horizontally to yield five slices. The slices were incubated in 1% TTC for 15 min at 37 °C. Ratios of risk area vs. left ventricle (RA/LV) and infarct area vs. risk area (IA/RA) were calculated and expressed as a percentage.

2.4. In vitro experiments

Peritoneal macrophages were isolated from WT and SR-A$^{-/-}$ mice as described previously [42]. Briefly, elicited peritoneal macrophages were collected and suspended in RPMI 1640 medium supplemented

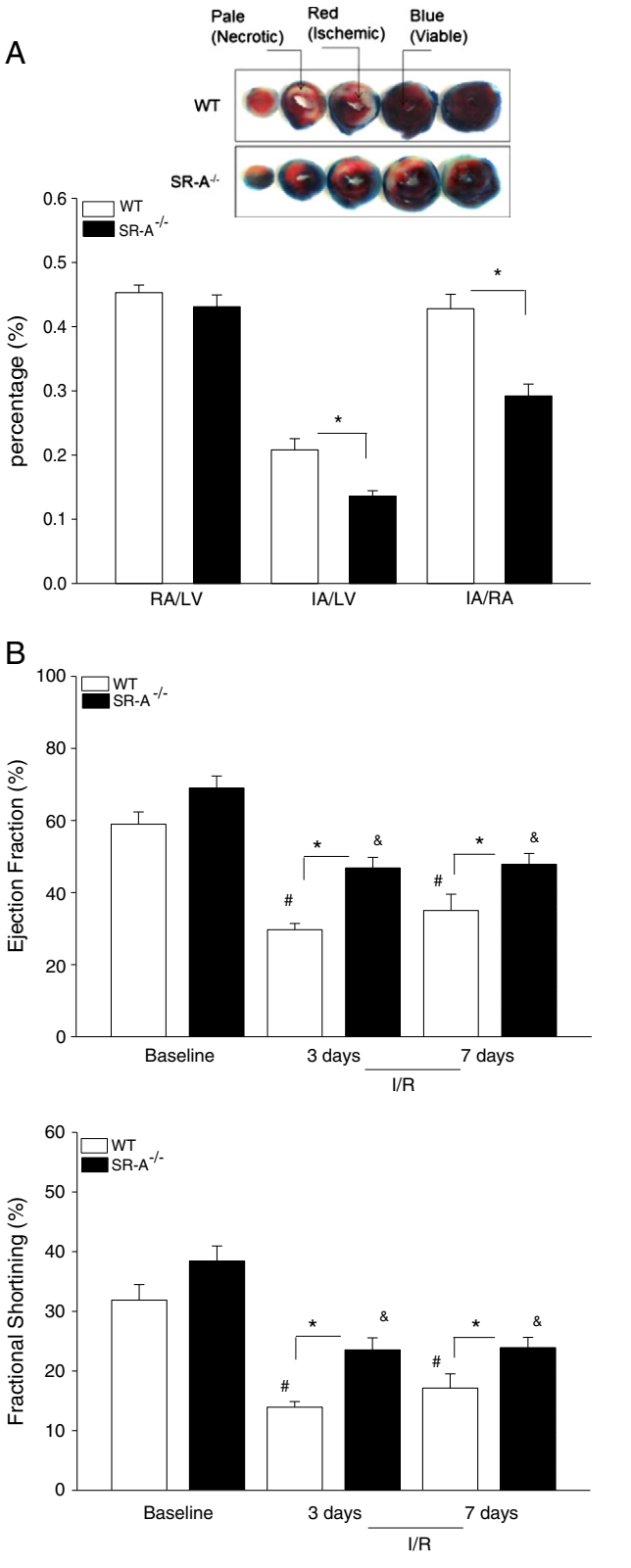
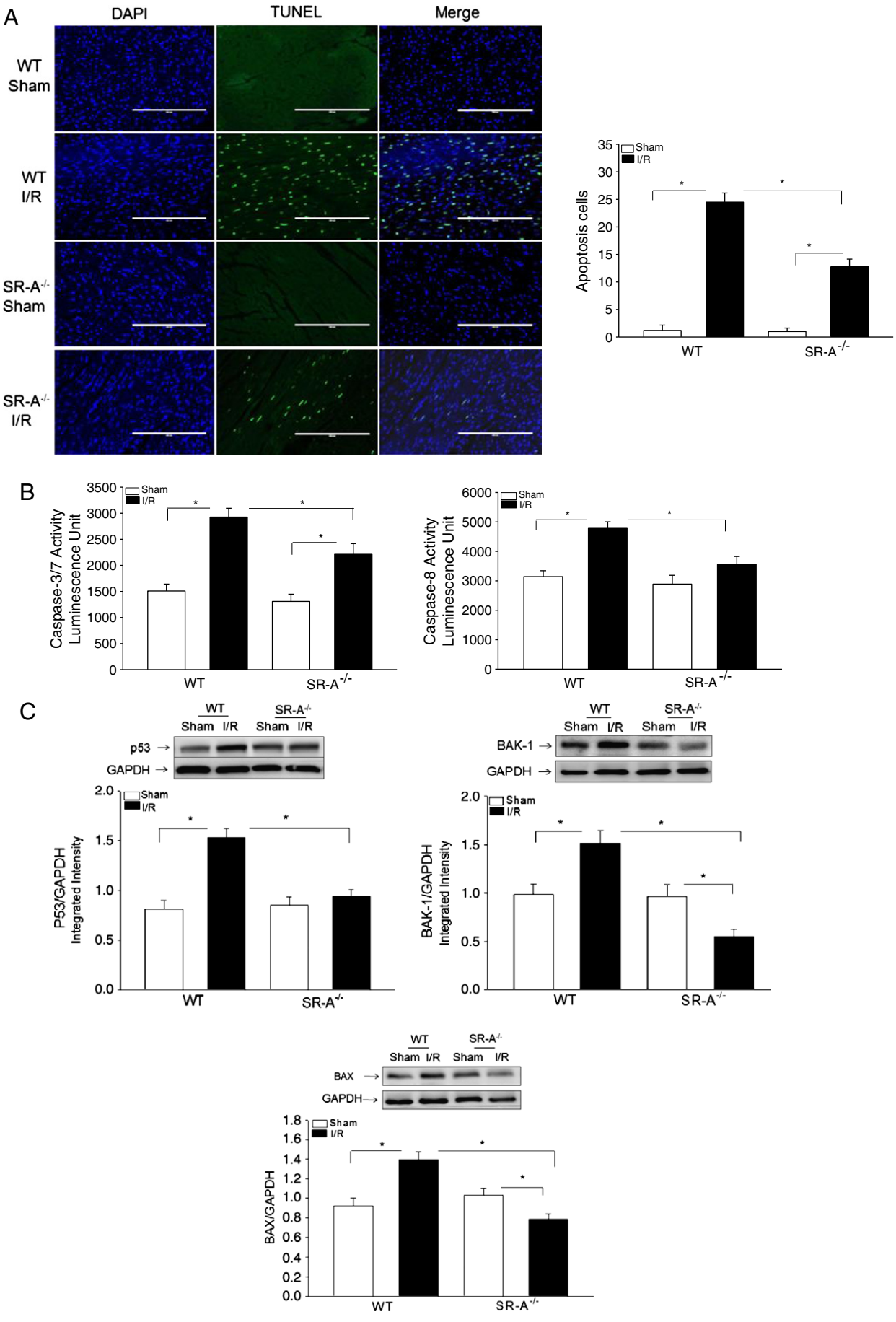


Fig. 1. SR-A deficiency attenuated I/R-induced myocardial infarction and cardiac dysfunction. (A) SR-A$^{-/-}$ ($n=10$) and WT mice ($n=9$) were subjected to myocardial ischemia (45 min) followed by reperfusion (4 hrs). Infarct size was determined by TTC staining. Ratios of risk area vs. left ventricle area (RA/LV) and infarct area vs. risk area (IA/RA) were calculated and are presented in the graphs. Photographs of representative heart sections are shown above. (B) SR-A$^{-/-}$ and WT mice were subjected to myocardial I/R ($n=6$/group). Cardiac function was examined by echocardiography before (Baseline), 3 and 7 days after I/R. *$p<0.01$ compared with indicated groups. #, &$p<0.05$ compared with baseline.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Caspase-3/7 Activity Luminescence Unit
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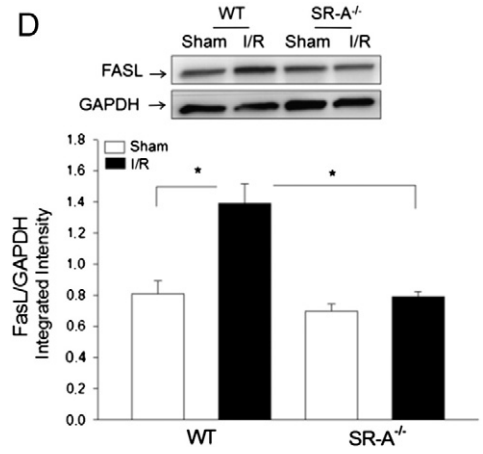

Fig. 2. I/R-induced myocardial apoptosis was attenuated by SR-A deficiency. SR-$A^{-/-}$ and WT mice were subjected to myocardial I/R ($n=6$/group). Sham operation served as sham controls ($n=4$/group). The hearts were harvested and sectioned for analysis of apoptosis by the TUNEL assay (A). DAPI stains nucleus (blue color) and TUNEL positive cells show green fluorescence. The bar graph shows the percent apoptotic cells. (B) SR-A deficiency attenuated I/R-induced caspase-3/7 and -8 activities. (C, D) SR-A deficiency prevents I/R-increased p53, Bak-1, Bax, and FasL levels in the myocardium. Cellular proteins prepared from heart tissues were subjected to Western blot examination with specific antibodies. *$p<0.05$ compared with indicated groups.

with 10% fetal calf serum, 0.1 mg/ml streptomycin, and 100 U/ml penicillin. The cells (2×10^6/ml) were cultured in 6-well tissue culture plates (Corning, Inc, Corning, NY) for 2 hrs at 37 °C with 5% CO_2. After washing sufficiently with PBS, adherent macrophages were incubated at 37 °C with 5% CO_2 overnight. The medium was then changed to hypoxia-equilibrated medium (5% CO_2 and 0.1% O_2) and the cells were immediately incubated at 37 °C with 5% CO_2 and 0.1% O_2 in a hypoxia chamber (Pro-Ox Model C21, BioSpherix Ltd., Redfield NY) for 2 hrs followed by reoxygenation in an incubator with 5% CO_2. The control groups were incubated at 37 °C with 5% CO_2 for the same time periods. There were 4 replicates in each group. The supernatants and cells were harvested for analysis of chemokine production, NF-κB binding activity, and for Western blot [13,23].

2.5. Transfection of macrophages with miR-125b mimics

Peritoneal macrophages were isolated from WT mice and cultured in 6-well and 48 well plates, respectively. Twenty-four hours after incubation, the cells were transfected with miR-125b mimics (40 nM) or mimic control with Dy547 (Dharmacon) by Lipofectamine 2000 reagent (Invitrogen), respectively, according to the manufacturer's protocol. To examine the role of "loss-of-function" of miR-125b in hypoxia/reoxygenation induced cell injury, the cells were transfected with anti-miR-125b (40 nM, Cat. No: 444084, Applied Biosystems). AntimiR-negative control (40 nM, Cat. No: AM17011, Applied Biosystems). Six hours after transfection, the medium was replaced with fresh medium supplemented with 10% FBS and antibiotics. Two days after transfection, the cells were subjected to hypoxia (2 hrs) followed by reoxygenation at indicated time points. The cells and supernatants were harvested for analysis of cell viability, LDH activity and caspase-3/7 and -8 activities. There were 6 replicates in each group.

2.6. Real time PCR assay of microRNAs (miRs)

The miRs were isolated from cultured cells and heart tissues using a mirVana™ miR isolation kit (Ambion) according to the manufacturer's protocol. Quantitative real-time PCR (qPCR) was conducted using a 4800 Real time PCR machine (Bio-Red). miR levels were quantified by qPCR using specific Taqman assays for miR (Applied Biosystems, USA). Specific primers for miR-125b were obtained from Applied Biosystems (Primer identification numbers: 000449 for hsa-miR-125b and 001973 for snRU6). miRNA expression was quantified with the 2(-ΔΔct) relative quantification method that was normalized to the U6 small nucleolar RNA (snRU6).

2.7. Measurement of cell viability and LDH activity

Cell viability was assessed by measuring mitochondrial dehydrogenase activity using the MTT assay kit (Sigma). Cell injury was assessed by measurement of lactate dehydrogenase (LDH) activity in culture medium using a commercial kit (Cytotoxicity Detection Kit, Sigma).

2.8. Western blot

Western blot was performed as described previously [13,15,23]. Briefly, the cellular proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ). The ECL membranes were incubated with the appropriate primary antibody [anti-p53, anti-Bax, anti-Bak-1 (Cell Signaling Technology, Inc, Beverly, MA) and anti-FasL (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)] respectively, followed by incubation with peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc.). The signals were detected with the ECL system (Amersham Pharmacia). The signals were quantified using the G:Box gel imaging system by Syngene (Syngene, USA, Fredrick, MD).

2.9. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were isolated from heart samples as previously described [13,15,23]. NF-κB binding activity was measured using a LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific, Waltham, MA) according to the instructions from the manufacturer.

2.10. Caspase activity assay

Caspase-3/7 and -8 activities in heart tissues were measured as described previously [25] using a Caspase-Glo assay kit (Promega) according to the manufacturer's protocol.

2.11. In situ apoptosis assay

Cardiac myocyte apoptosis was examined by the TUNEL assay (Roche Applied Science, Indianapolis, IN) in the heart sections according to the instructions provided by the manufacturer as described previously [14]. Three slides from each block were evaluated for percentage of apoptotic cells. Four fields of each slide were randomly examined using a defined rectangular field area with a magnification of 40×. Myocardial apoptotic cells are presented as the percentage of field.

2.12. Chemokine ELISA

The levels of monocyte chemoattractant protein-1 (MCP-1), keratinocyte chemoattractant (KC), TNF-α, and IL-1β were measured [42] using commercially available ELISA kits (PeproTech, Rocky Hill, NJ) according to instructions provided by the manufacturer.

2.13. Statistical analysis

Data are expressed as mean ± SE. Comparisons of data between groups were made using one-way analysis of variance (ANOVA) and Tukey's procedure for multiple range tests was performed. A $p<0.05$ was considered significant.

3. Results

3.1. Reduced myocardial infarct size and improved cardiac function in SR-A$^{-/-}$ mice following I/R

We examined the effect of SR-A deficiency on myocardial I/R injury. Fig. 1A shows that I/R induced large infarct size in WT mice. However, SR-A deficiency significantly attenuated I/R-induced infarct size by 32% compared with WT I/R mice.

Fig. 1B shows that after myocardial I/R, ejection fraction (EF%) and fractional shortening (%FS) in WT mice were significantly decreased on day 3 (49.6% and 40.6%) and on day 7 (56.4% and 46.4%). In contrast, SR-A deficiency attenuated I/R-induced cardiac dysfunction. SR-A$^{-/-}$ mice showed EF% and %FS values that were markedly greater on day 3 (33.2% and 38.8%) and on day 7 (30.7% and 46.4%), respectively, when compared with WT I/R mice.

3.2. SR-A deficiency attenuated I/R-induced myocardial apoptosis

Cardiac myocyte apoptosis contributes to myocardial I/R injury. We evaluated the effect of SR-A deficiency on I/R-induced myocardial apoptosis. Fig. 2A shows that I/R induced myocardial apoptosis by 19.4 fold in WT mice and by 11.7 fold in SR-A$^{-/-}$ mice, compared with respective sham controls. However, the numbers of apoptotic cells in SR-A$^{-/-}$ I/R mice ($12.7 \pm 1.40\%$) were 40% lower compared with WT I/R mice ($24.5 \pm 1.66\%$). Fig. 2B shows that I/R-increased caspase-3/7 and -8 activities were significantly attenuated in SR-A$^{-/-}$ I/R mice.

3.3. I/R increases myocardial p53, Bak-1, Bax and FasL levels in WT mice, but not in SR-A$^{-/-}$ mice

We examined the effect of SR-A deficiency on p53-mediated apoptotic signaling during myocardial I/R injury. I/R increased the levels of p53 (125%), Bak-1 (50%), and Bax (64%) in WT myocardium, respectively, compared with WT sham control (Fig. 2C). FasL levels in WT myocardium were also significantly increased by 55.2% following I/R compared with sham control (Fig. 2D). In contrast, SR-A deficiency prevented I/R-induced increases in the expression of p53, Bak-1, Bax and FasL in the myocardium, respectively, compared with WT I/R group (Fig. 2C–D).

3.4. SR-A deficiency prevents I/R-induced myocardial NF-κB binding activity and cytokine production in the serum

Activation of NF-κB contributes to myocardial I/R injury [22,27]. Fig. 3A shows that I/R significantly increased NF-κB binding activity in the WT myocardium by 95% compared with sham control. However, SR-A deficiency prevented I/R-induced NF-κB binding activity in the myocardium.

Activation of NF-κB stimulates inflammatory cytokine expression [26]. Fig. 3B shows that I/R increased the levels of TNF-α by 1.8 fold and IL-1β by 8.6 fold in serum of WT mice compared with WT sham control. I/R also increased the serum levels of TNF-α by 47% and IL-1β by 125% in SR-A$^{-/-}$ mice compared with SR-A$^{-/-}$ sham control. However, both TNF-α and IL-1β levels in serum of SR-A$^{-/-}$ I/R mice were significantly lower than that in WT I/R mice.

3.5. Increased expression of miR-125b in the myocardium of SR-A$^{-/-}$ mice

microRNA-125b has been shown to target p53 expression [19,20]. We examined the expression of miR-125b in the myocardium. As shown in Fig. 4A, the levels of miR-125b in SR-A$^{-/-}$ sham hearts were significantly greater than in WT sham group. Ischemia (45 min) followed by reperfusion (4 hrs) further increased the levels of miR-125b in SR-A$^{-/-}$ hearts but not in WT hearts.

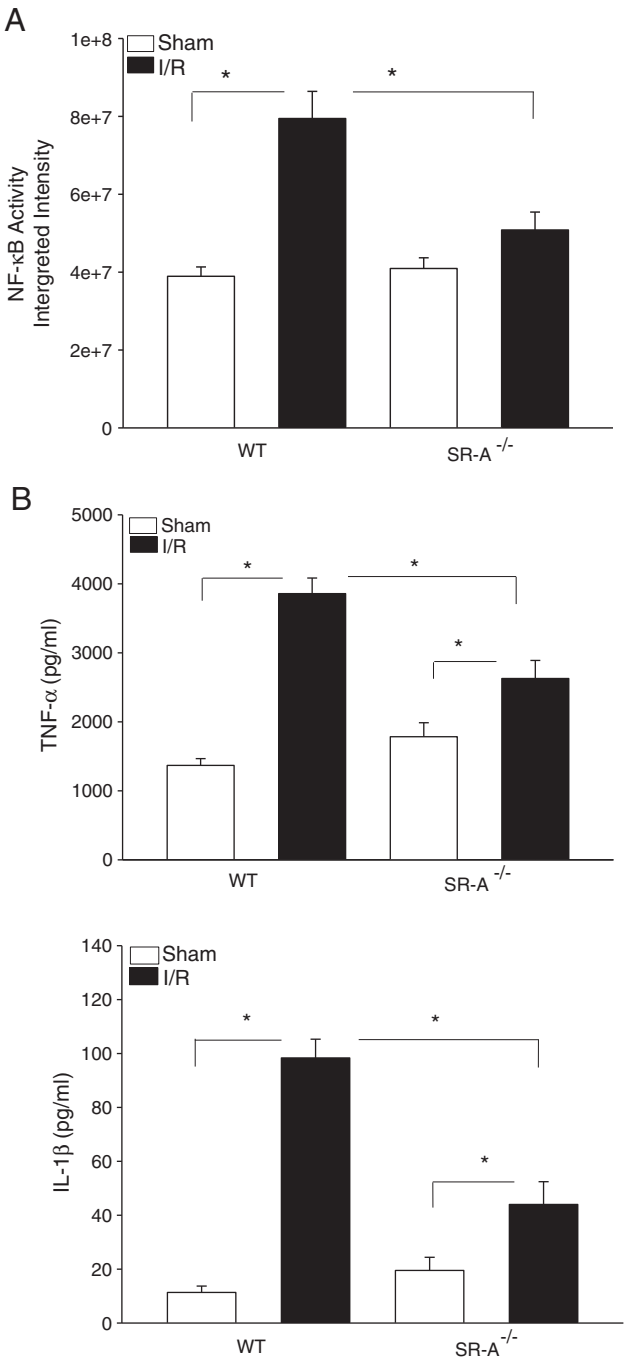

Fig. 3. SR-A deficiency prevented I/R-induced NF-κB binding activity and cytokine production. SR-A$^{-/-}$ and WT mice ($n = 6$/group) were subjected to myocardial I/R. Sham operation served as sham control ($n = 4$/group). Hearts were harvested and nuclear proteins were prepared for analysis of NF-κB binding activity by EMSA. (A) SR-A deficiency prevented I/R-induced NF-κB binding activity. (B) SR-A deficiency attenuated I/R-increased the levels of TNFα and IL-1β in the serum. *$p < 0.05$ compared with indicated groups.

3.6. SR-A$^{-/-}$ macrophages show increased levels of miR-125b in the presence and absence of H/R

SR-A is predominantly expressed on macrophages [17,41]. We examined the expression of miR-125b in SR-A$^{-/-}$ macrophages. As shown in Fig. 4B, the levels of miR-125b in SR-A$^{-/-}$ macrophages are significantly greater than in WT macrophages. Hypoxia alone further increased miR-125b expression in SR-A$^{-/-}$ macrophages but not in WT macrophages. Hypoxia followed by reoxygenation markedly

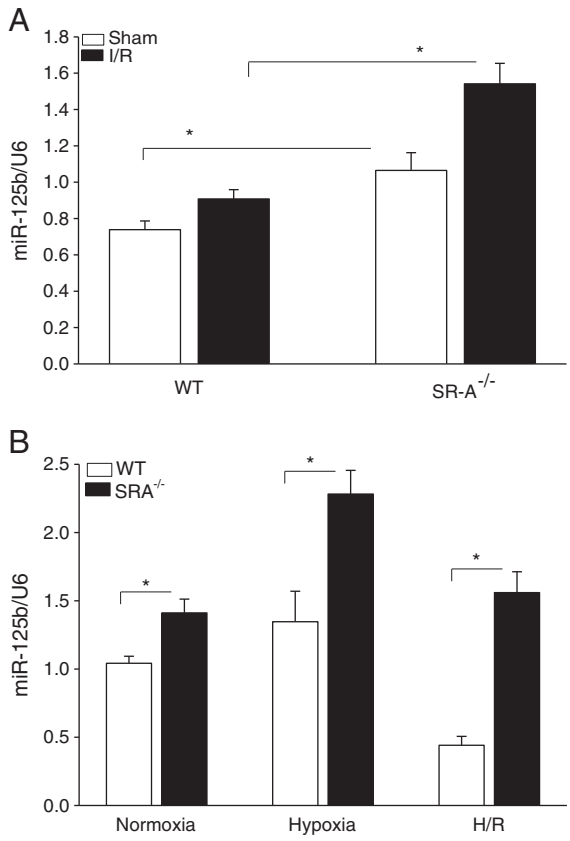

Fig. 4. Increased miR-125b expression in SR-A$^{-/-}$ heart and SR-A$^{-/-}$ macrophages. (A) WT and SR-A$^{-/-}$ mice were subjected to ischemia (45 min) followed by reperfusion (4 hrs) ($n=4$/group). Sham operation served as sham control ($n=3$/group). Hearts were harvested and miR-125b levels were determined by qPCR. (B) Peritoneal macrophages were isolated from WT and SR-A$^{-/-}$ mice and were subjected to hypoxia (2 hrs) followed by reoxygenation (24 hrs). miR-125b expression ($n=4$/group) was examined by qPCR. *$p<0.05$ compared with indicated groups.

decreased miR-125b expression in WT macrophages. In contrast, the levels of miR-125b in SR-A$^{-/-}$ macrophages following H/R were comparable with the levels in SR-A$^{-/-}$ normal macrophages.

3.7. SR-A deficiency prevents hypoxia/reoxygenation-induced NF-κB activation and chemokine production in macrophages

We examined the role of SR-A in NF-κB activation following hypoxia/reoxygenation (H/R) challenge in macrophages. SR-A is predominantly expressed on macrophages [17,41]. Peritoneal macrophages were isolated from WT and SR-A$^{-/-}$ mice, respectively and subjected to hypoxia (2 hrs) followed by reoxygenation (4 hrs). Fig. 5A shows that H/R induced NF-κB binding activity in WT macrophages but not in SR-A$^{-/-}$ macrophages. We also examined the effect of SR-A on macrophage secretion of chemokines following hypoxia (2 hrs) followed by reoxygenation (24 hrs). H/R significantly increased the levels of KC by 61% and MCP-1 by 41% in WT macrophages compared with WT control (Fig. 5B). In contrast, H/R did not increase MCP-1 and KC levels in SR-A$^{-/-}$ macrophages.

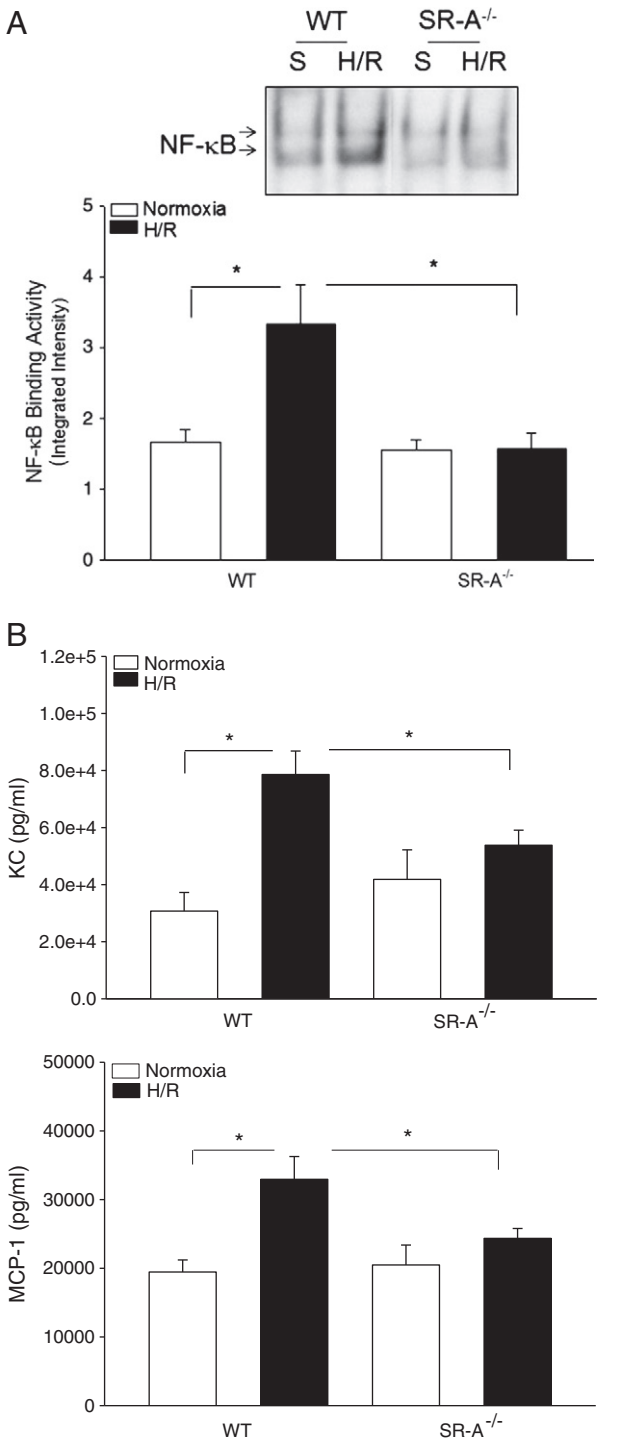

Fig. 5. SR-A deficiency prevents H/R-induced NF-κB activity and chemokine production in macrophages. Peritoneal macrophages were isolated from WT and SR-A$^{-/-}$ mice and subjected to hypoxia followed by reoxygenation. H/R induced NF-κB activation (A) and stimulated KC and MCP-1 secretion (B) in WT macrophages but not in SR-A$^{-/-}$ macrophages. There were four replicates in each group. *$p<0.05$ compared with indicated groups.

3.8. Macrophage SR-A deficiency attenuated hypoxia/reoxygenation-induced cellular injury and decreased viability

We examined the role of SR-A in H/R-induced cell injury. Fig. 6 shows that H/R significantly increased LDH activity by 173% (A) and decreased cell viability by 52.7% (B) in WT macrophages compared with WT control group. H/R also increased LDH activity by 83% and decreased cell viability by 31.2% in SR-A$^{-/-}$ macrophages compared with the SR-A control group. However, SR-A deficiency significantly attenuated H/R-induced cell injury by 52% and H/R-decreased viability by 40.7%. SR-A deficiency also significantly attenuated H/R-increased caspase-3/7 and -8 activities (Fig. 6C). To determine

whether increased miR-125b in SR-A$^{-/-}$ macrophages plays a protective role in H/R induced cell injury, we transfected cells with anti-miR-125 mimics before the cells were subjected to H/R. As shown in Fig. 6D, attenuation of cell viability in SR-A$^{-/-}$ macrophages was lost after inhibition of miR-125b.

3.9. Transfection of wild type macrophages with miR-125b prevented H/R-induced cell injury and caspase-3/7 and -8 activities

To determine the role of miR-125b in the protection against H/R-induced cell injury, we transfected WT macrophages with miR-125b mimics. Scrambled mimics served as miR-control. The cells were subjected to hypoxia (2 hrs) followed by reoxygenation for 12, 24, and 36 hrs, respectively. Fig. 7A and B show that transfection of miR-125b mimics significantly attenuated H/R-induced cell injury and H/R-decreased viability. miR-125b transfection also prevented H/R-induced caspase-3/7 and -8 activities in macrophages (Fig. 7C). Transfection of control mimics did not alter H/R-induced cell injury and caspase-3/ and -8 activities.

3.10. miR-125b transfection suppresses p53 and Bak-1 expression and prevents H/R-induced increases in Bax levels in macrophages

We examined the effect of miR-125b transfection on p53-mediated apoptotic signaling in macrophages following H/R. Fig. 7D shows that H/R markedly increased the levels of p53, Bak-1 and Bax

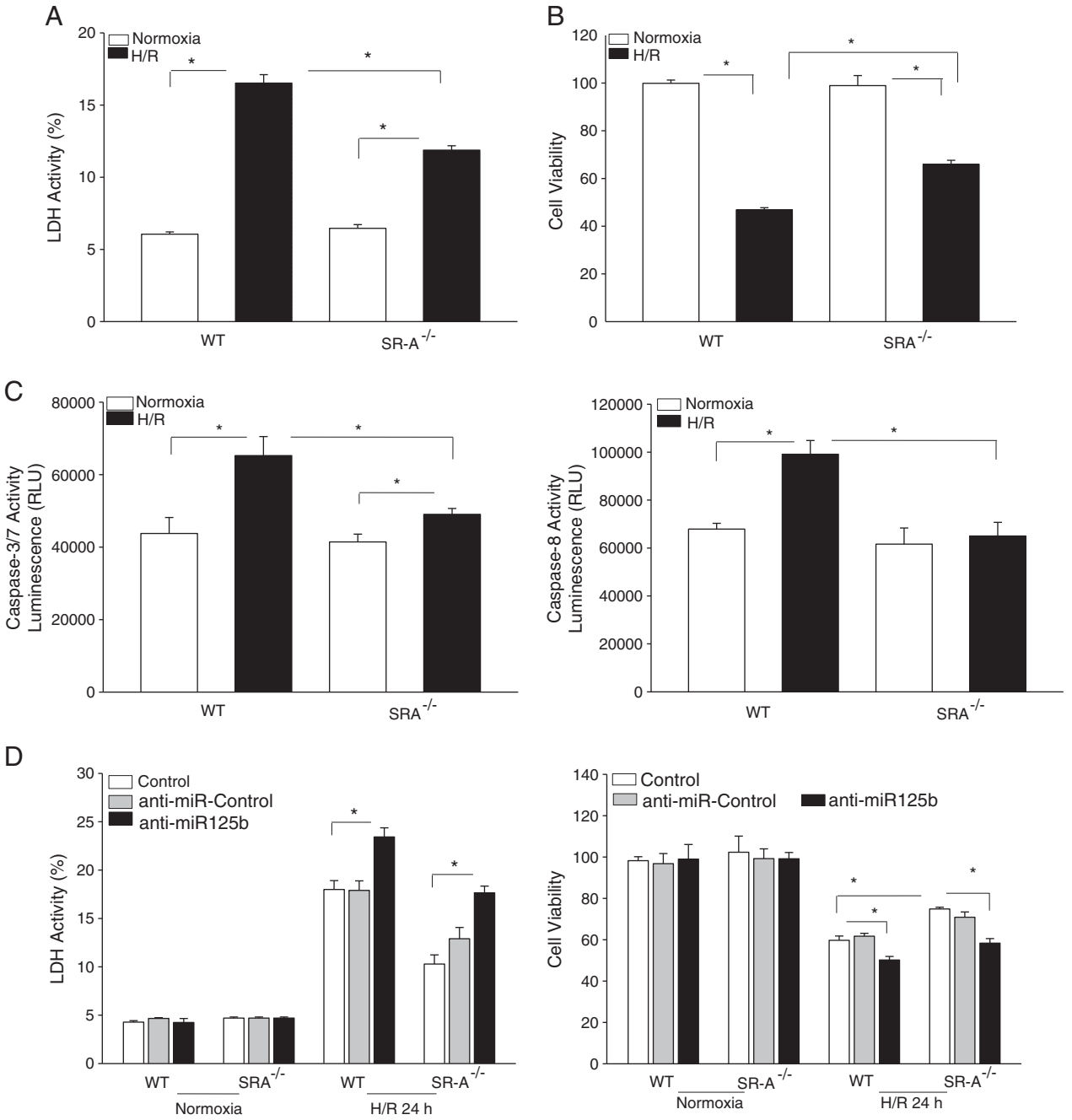

Fig. 6. H/R-induced macrophage injury and decreased viability is attenuated by SR-A deficiency. Peritoneal macrophages isolated from WT and SR-A$^{-/-}$ mice were subjected to hypoxia (2 hrs) followed by reoxygenation (24 hrs). SR-A deficiency attenuated H/R-induced LDH activity (A), decreased viability (B), and increased caspase-3/7 and -8 activities (C). Inhibition of miR-125b abolished protective effect of SRA deficiency on H/R-induced cell injury (D). There were 6 replicates in each group. *$p<0.05$ compared with indicated groups.

compared with control group. However, transfection of macrophages with miR-125b suppresses both p-53 and Bak-1 expression in macrophages in the presence and absence of H/R. miR-125b transfection also prevented H/R-induced increases in Bax levels.

4. Discussion

In the present study, we have observed that SR-A$^{-/-}$ mice showed significantly smaller infarct size and better cardiac function following acute myocardial I/R injury compared with WT I/R mice. The mechanisms involve attenuation of I/R-activated p53-mediated apoptotic signaling and prevention of I/R-induced NF-κB activation in the myocardium. Importantly, we found that miR-125b levels in SR-A$^{-/-}$ hearts are significantly greater than in WT hearts. In addition to miR-125b, the levels of miR-21 and miR-146a were also significantly increased in SR-A$^{-/-}$ heart. We have also observed that miR-125b expression is significantly increased in macrophages of SR-A$^{-/-}$ mice. SR-A$^{-/-}$ macrophages showed prevention of H/R-induced NF-κB activity and chemokine production and attenuation of H/R-induced cell injury. Transfection of miR-125b mimics into WT macrophages markedly attenuated H/R-induced cell injury and caspase-3/7 and -8 activities through inhibition of p53-mediated apoptotic signaling. These data suggest that increased miR-125b in SR-A$^{-/-}$ macrophages may play a role in protection against acute myocardial I/R injury.

Historically, SR-A is known to be responsible for the uptake of modified LDL [11]. Recent evidence indicates that SR-A participates in the induction of innate and immune responses [4]. Recent studies have reported that SR-A can recognize and clear modified host components, apoptotic cells, and pathogens [12]. SR-A has been reported to cooperate with TLR4 in response to LPS stimulation [31]. Recently, we have reported that deficiency of SR-A decreases cerebral I/R injury [25] and increases survival in polymicrobial sepsis [29]. Unfortunately, we still do not know endogenous ligands for SR-A during I/R or infectious challenge. However, SR-A$^{-/-}$ mice showed significant attenuation of I/R-increased myocardial apoptosis and prevention of I/R-induced increases in the levels of p53, Bak-1, and Bax in the myocardium. The data is consistent with *in vitro* data showing that the levels of p53 and Bak-1 were markedly lower in SR-A$^{-/-}$ macrophages than in WT macrophages following H/R. p53 is a well-known transcription factor which mediates apoptosis by stimulating the expression of pro-apoptotic genes, including Bax, apaf-1 and caspase-3 [37]. p53 also interacts with the proapoptotic mitochondrial membrane protein Bak-1 which causes oligomerization of Bak-1 and release of cytochrome c from mitochondria, leading to apoptosis [37,44]. Recent studies have demonstrated that miR-125b targets both p53, Bak-1 and TNF-α [19,20,32,44]. We have observed that miR-125b expression is significantly increased in SR-A-deficient hearts. We speculate that the mechanisms by which SR-A deficiency attenuates I/R-induced myocardial apoptosis may be due, in part, to increased levels of miR-125b in SR-A$^{-/-}$ hearts.

We have observed that increased levels of miR-125b were present in SR-A$^{-/-}$ macrophages and that SR-A$^{-/-}$ macrophages showed attenuation of H/R-induced NF-κB activation, chemokine production, and cell injury. MicroRNA-125b is a homolog of lin-4, which is the first miR discovered and an important regulator of developmental timing in *C. elegans* [21]. miR-125b has been proposed to regulate both cell proliferation and apoptosis by repressing p53 and Bak-1 expression [19,20]. In the present study, we observed that the increased

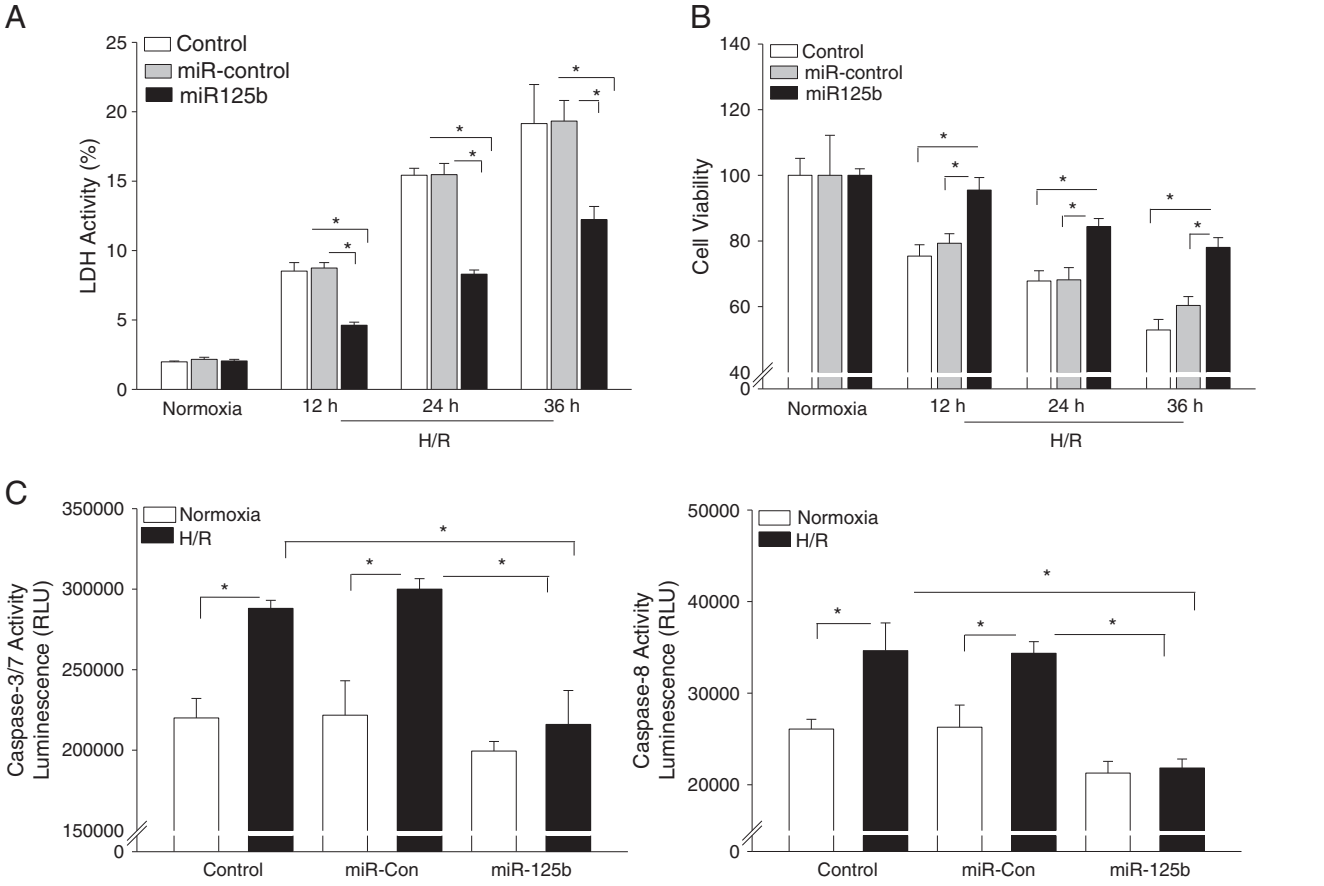

Fig. 7. Overexpression of miR-125b attenuated H/R-induced cell injury. WT macrophages were transfected with miR-125b mimics. Scrambled miR-mimics served as control mimics. Forty-eight hours after transfection, the cells were subjected to hypoxia followed by reoxygenation. miR-125b mimics attenuated H/R-increased LDH activity (A), decreased viability (B), and increased caspase-3/7 and caspase-8 activities (C). Overexpression of miR-125b prevents H/R-increased p53, Bak-1 and Bax levels in macrophages (D). There were 6 replicates in each group. *$p<0.05$ compared with indicated groups.

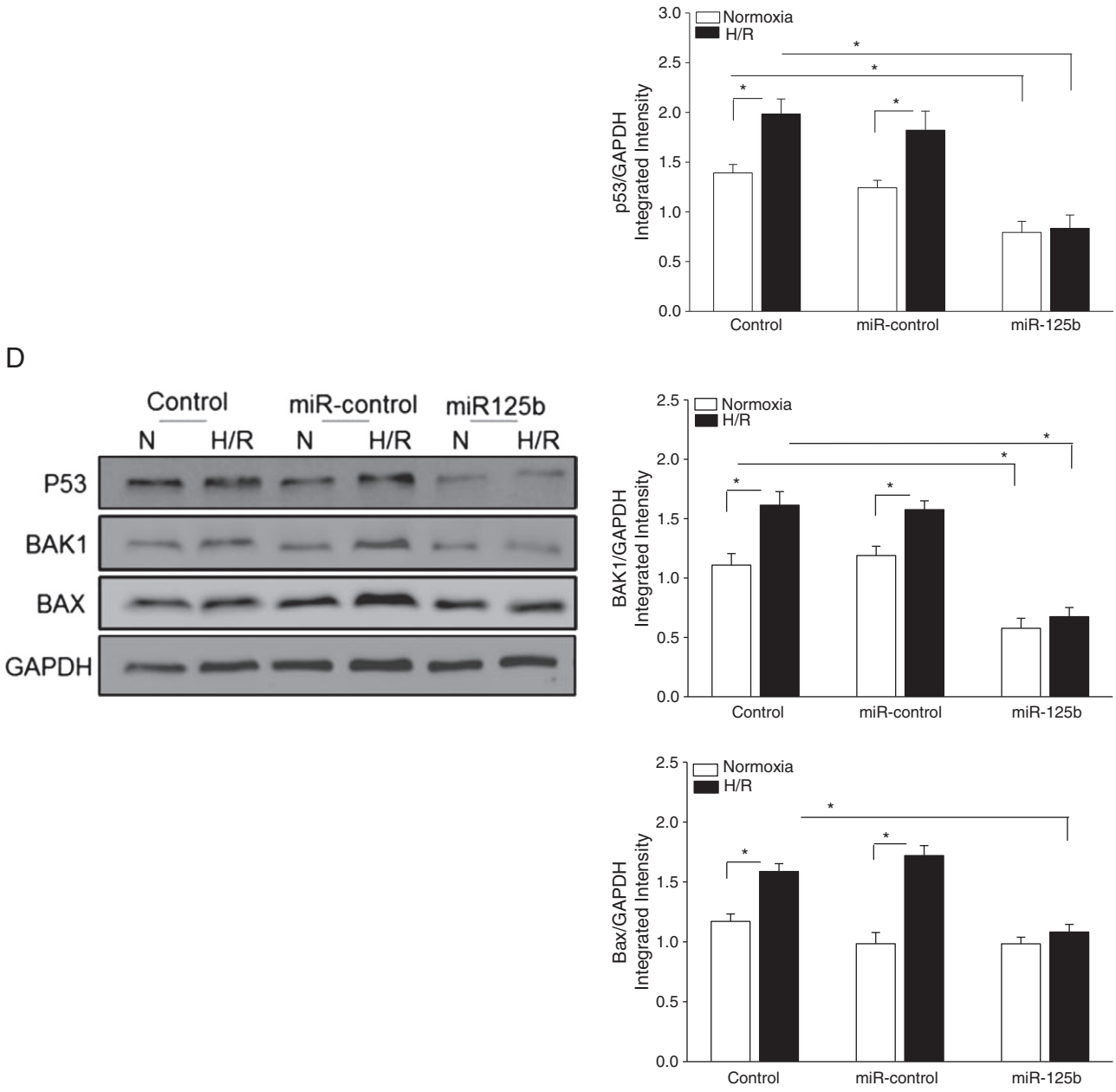

Fig. 7 (*continued*).

levels of miR-125b in SR-A$^{-/-}$ macrophages positively correlated protection against H/R-induced cell injury while inhibition of miR-125b expression abolished the protective effect of SR-A$^{-/-}$ macrophages on H/R-induced cell injury. To further confirm the role of miR-125b in SR-A$^{-/-}$ macrophages in the protection against H/R-induced cell injury, we transfected WT macrophages with miR-125b mimics and observed that overexpression of miR-125b significantly attenuated H/R-induced cell injury, caspase-3/7 and -8 activities, and prevented H/R-activated p-53-mediated apoptotic signaling. Our observation is consistent with previous reports that miR-125b targets p53 expression [19,20]. At present, we do not understand the mechanisms by which SR-A deficiency results in increased expression of miR-125b in macrophages. Recent studies have demonstrated that SR-A could serve as a co-receptor for TLR4 in response to LPS stimulation [6,39]. We have reported that SR-A is required for LPS-induced TLR4 mediated NF-κB activation in macrophage [42]. Tili et al. [32] have reported that LPS stimulation significantly down regulates the expression of miR-125b which is reversely correlated with NF-κB activity. The data indicates that activation of NF-κB suppresses the expression of miR-125b [32]. Therefore, it is possible that presence of SR-A suppresses the expression of miR-125 via TLR4-mediated NF-κB dependent mechanism. Our data showed that miR-125b plays a protective role in H/R-induced cell injury in macrophages. We have observed that increased expression of miR-125b in the myocardium protects against myocardial I/R injury in vivo (unpublished data).

SR-A expression is primarily on macrophages [17,41]. The normal heart contains resident macrophages [8] and I/R stimulates circulating macrophage infiltration into the myocardium [7,16]. We have observed that miR-125b levels in SR-A$^{-/-}$ hearts are significantly greater than in WT hearts. I/R further increased miR-125b levels in SR-A$^{-/-}$ hearts, but not in WT hearts. Based on the data obtained from *in vitro* and *in vivo* experiments, we speculated that SR-A deficiency reduces myocardial injury following I/R, in part, through miR-125b-dependent mechanisms. Indeed, *in vivo* data showed I/R-induced myocardial infarct size and cardiac dysfunction were significantly attenuated in SR-A$^{-/-}$ mice compared with WT I/R mice.

Our observation indicates two important concepts. First, increased miR-125b levels in macrophage may contribute to cardioprotection in SR-A deficiency. Recent studies have shown that several cell types, including macrophages and dendritic cell can secrete exosomes containing miRs [34,45]. The exosomes can transfer genetic message from cell to cell [34,45]. We have observed using *in vitro* co-cultured macrophages and cardiomyoblasts, that miR-125b mimics in macrophages can be transferred to cardiomyoblasts through an undetermined mechanism (unpublished data). At present, we do not know if a similar transfer occurs *in vivo* through exosomes. Second, macrophages play an important role in myocardial I/R injury. It is well known that both resident and infiltrating macrophages play a critical role in myocardial remodeling and repair, by ingesting necrotic cardiac myocytes and apoptotic neutrophils, secreting inflammatory cytokines and growth factors, and modulating angiogenic responses after myocardial infarction [18]. Recent studies have shown that macrophages contribute to induction of the innate immune and inflammatory responses during the early stage of cardiac ischemia [7,16,18]. Kakio et al. have reported that ischemia alone rapidly promoted macrophage infiltration into the myocardium [16] and activated macrophages secrete cytokines and chemokines that recruit neutrophils into the tissues causing further tissue damage [16,18]. We have observed that *in vitro* H/R increased the secretion of MCP-1 and KC by WT macrophages but not by SR-A$^{-/-}$ macrophages. MCP-1 and KC are CC chemokines that are associated with the recruitment of macrophages and neutrophils into tissues [9]. Collectively, these data indicate that SR-A positive macrophages responded to I/R challenge by secreting chemokines which attract neutrophils and macrophages into the myocardium, which may contribute to myocardial I/R injury.

Our observation suggests that SR-A macrophages may play a critical role in the pathophysiology of acute myocardial I/R injury. Tsujita et al. [33] reported that SR-A deficiency might cause impairment of infarct remodeling that results in cardiac rupture following permanent occlusion of left coronary artery for 4 weeks. Tsujita et al. employed the model of permanent occlusion of the left coronary artery to examine the role of SR-A in remodeling after myocardial infarction, whereas, we employed an acute myocardial I/R model to investigate the role of SR-A in initial innate immune and inflammatory responses during early stage of myocardial I/R. Therefore, the results of Tsujita et al. may not be comparable to our results due to the significant differences in the experimental models employed. Indeed, we have previously reported that SR-A deficiency protects against cerebral I/R injury [25]. Our observation is supported by a recent study showing SR-A promotes cerebral I/R injury [40].

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Disclosure

None.

References

[1] E. Amiel, A. Alonso, S. Uematsu, S. Akira, M.E. Poynter, B. Berwin, Toll-like receptor regulation of scavenger receptor-A-mediated phagocytosis, J. Leukocyte Biol. 85 (2009) 000.
[2] A. Bonauer, G. Carmona, M. Iwasaki, M. Mione, M. Koyanagi, A. Fischer, J. Burchfield, H. Fox, C. Doebele, K. Ohtani, E. Chavakis, M. Potente, M. Tjwa, C. Urbich, A.M. Zeiher, S. Dimmeler, MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice, Science 324 (2009) 1710–1713.
[3] Y. Cheng, X. Liu, S. Zhang, Y. Lin, J. Yang, C. Zhang, MicroRNA-21 protects against the H(2)O(2)-induced injury on cardiac myocytes via its target gene PDCD4, J. Mol. Cell. Cardiol. 47 (2009) 5–14.
[4] M.P. De Winther, K.W. van Dijk, L.M. Havekes, M.H. Hofker, Macrophage scavenger receptor class A: a multifunctional receptor in atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 290–297.
[5] S. Dong, Y. Cheng, J. Yang, J. Li, X. Liu, X. Wang, D. Wang, T.J. Kralll, E.S. Delphin, C. Zhang, MicroRNA expression signature and the role of microRNA-21 in the early phase of acute myocardial infarction, J. Biol. Chem. 284 (2009) 29514–29525.
[6] R.D. Feigin, W.T. Shearer, Opportunistic infection in children. I. In the compromised host, J. Pediatr. 87 (1975) 507–514.
[7] L. Formigli, L.I. Manneschi, C. Nediani, E. Marcelli, G. Fratini, S.Z. Orlandini, A.M. Perna, Are macrophages involved in early myocardial reperfusion injury? Ann. Thorac. Surg. 71 (2001) 1596–1602.
[8] N.G. Frangogiannis, A.R. Burns, L.H. Michael, M.L. Entman, Histochemical and morphological characteristics of canine cardiac mast cells, Histochem. J. 31 (1999) 221–229.
[9] N.G. Frangogiannis, M.L. Entman, Chemokines in myocardial Ischemia, Trends Cardiovasc. Med. 15 (2005) 163–169.
[10] N.G. Frangogiannis, C.W. Smith, M.L. Entman, The inflammatory response in myocardial infarction, Cardiovasc. Res. 53 (2002) 31–47.
[11] J.L. Goldstein, Y.K. Ho, S.K. Basu, M.S. Brown, Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition, Proc. Natl. Acad. Sci. 76 (1979) 333–337.
[12] D.R. Greaves, S. Gordon, The macrophage scavenger receptor at 30 years of age: current knowledge and future challenges, J. Lipid Res. (2009) S282–S286 (April Supplement).
[13] T. Ha, Y. Hu, L. Liu, C. Lu, J.R. McMullen, T. Shioi, S. Isumo, J. Kelley, R.L. Kao, D.L. Williams, X. Gao, C. Li, TLR2 ligands induce cardioprotection against ischemia/reperfusion injury through a PI3K/Akt-dependent mechanism, Cardiovasc. Res. 87 (2010) 694–703.
[14] T. Ha, C. Lu, L. Liu, F. Hua, Y. Hu, J. Kelley, K. Singh, R.L. Kao, J. Kalbfleisch, D.L. Williams, X. Gao, C. Li, TLR2 ligands attenuate cardiac dysfunction in polymicrobial sepsis via a phosphoinositide-3-kinase dependent mechanism, Am. J. Physiol. Heart Circ. Physiol. 298 (2010) H984–H991.
[15] F. Hua, T. Ha, J. Ma, Y. Li, J. Kelley, X. Gao, I.W. Browder, R.L. Kao, D.L. Williams, C. Li, Protection against myocardial ischemia/reperfusion Injury in TLR4 deficient mice is mediated through a phosphoinositide 3-kinase dependent mechanism, J. Immunol. 178 (2007) 7317–7324.
[16] T. Kakio, A. Matsumori, K. Ono, H. Ito, K. Matsushima, S. Sasayama, Roles and relationship of macrophages and monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 in the ischemic and reperfused rat heart, Lab. Invest. 80 (2000) 1127–1136.
[17] M. Krieger, The other side of scavenger receptors: pattern recognition for host defense, Curr. Opin. Lipidol. 8 (1997) 275–280.
[18] J.M. Lambert, E.F. Lopez, M.L. Lindsey, Macrophage roles following myocardial infarction, Int. J. Cardiol. 130 (2008) 147–158.
[19] M.T.N. Le, N. Shyh-Chang, S.L. Khaw, L. Chin, C. Teh, J. Tay, E. O'Day, V. Korzh, H. Yang, A. Lal, J. Lieberman, H.F. Lodish, B. Lim, Conserved regulation of p53 network dosage by MicroRNA-125b occurs through evolving miRNA-target gene pairs, PLoS Genet. 7 (2011) e1002242.
[20] M.T.N. Le, C. Teh, N. Shyh-Chang, H. Xie, B. Zhou, V. Korzh, H.F. Lodish, B. Lim, MicroRNA-125b is a novel negative regulator of p53, Genes Dev. 23 (2009) 862–876.
[21] R.C. Lee, R.L. Feinbaum, V. Ambros, The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14, Cell 75 (1993) 843–854.
[22] C. Li, W. Browder, R.L. Kao, Early activation of transcription factor NF-κB during ischemia in perfused rat heart, Am. J. Physiol. 276 (1999) H543–H552.
[23] C. Li, T. Ha, J. Kelley, X. Gao, Y. Qiu, R.L. Kao, W. Browder, D.L. Williams, Modulating Toll-like receptor mediated signaling by (1–>3)-β-D-glucan rapidly induces cardioprotection, Cardiovasc. Res. 61 (2003) 538–547.
[24] A. Linde, D. Mosier, F. Blecha, T. Melgarejo, Innate immunity and inflammation – New frontiers in comparative cardiovascular pathology, Cardiovasc. Res. 73 (2007) 26–36.
[25] C. Lu, F. Hua, L. Liu, T. Ha, J. Kalbfleisch, J. Schweitzer, J. Kelley, R. Kao, D. Williams, C. Li, Scavenger receptor class-A has a central role in cerebral ischemia/reperfusion injury, J. Cereb. Blood Flow Metab. 30 (2010) 1972–1981.
[26] R. Medzhitov, P. Preston-Hurlburt, C.A. Janeway Jr., A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity, Nature 388 (1997) 394–397.
[27] R. Morishita, T. Sugimoto, M. Aoki, I. Kida, N. Tomita, A. Moriguchi, K. Maeda, Y. Sawa, Y. Kaneda, J. Higaki, T. Ogihara, In vivo transfection of cis element "decoy" against nuclear factor-kappaB binding site prevents myocardial infarction, Nat. Med. 3 (1997) 894–899.
[28] J. Oyama, C. Blais Jr., X. Liu, M. Pu, L. Kobzik, R.A. Kelly, T. Bourcier, Reduced myocardial ischemia–reperfusion injury in toll-like receptor 4-deficient mice, Circulation 109 (2004) 784–789.
[29] T.R. Ozment, T. Ha, K.F. Breuel, T.R. Ford, D.A. Ferguson, J. Kalbfleisch, J.B. Schweitzer, J.L. Kelley, C. Li, D.L. Williams, Scavenger receptor class a plays a central role in mediating mortality and the development of the pro-inflammatory phenotype in polymicrobial sepsis, PloS Pathog. 8 (10) (Oct 2012) e1002967, http://dx.doi.org/10.1371/journal.ppat.1002967 (Epub 2012 Oct 11).

Ref Type: Abstract

[30] X.P. Ren, J. Wu, X. Wang, M.A. Sartor, J. Qian, K. Jones, P. Nicolaou, T.J. Pritchard, G.C. Fan, MicroRNA-320 is involved in the regulation of cardiac ischemia/reperfusion injury by targeting heat-shock protein 20, Circulation 119 (2009) 2357–2366.

[31] T.A. Seimon, A. Obstfeld, K.J. Moore, D.T. Golenbock, I. Tabas, Combinatorial pattern recognition receptor signaling alters the balance of life and death in macrophages, PNAS 103 (2006) 19794–19799.
[32] E. Tili, J.J. Michaille, A. Cimino, S. Costinean, C.D. Dumitru, B. Adair, M. Fabbri, H. Alder, C.G. Liu, G.A. Calin, C.M. Croce, Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock, J. Immunol. 179 (2007) 5082–5089.
[33] K. Tsujita, K. Kaikita, T. Hayasaki, T. Honda, H. Kobayashi, N. Sakashita, H. Suzuki, T. Kadoma, H. Ogawa, M. Takeya, Targeted deletion of class A macrophage scavenger receptor increases the risk of cardiac rupture after experimental myocardial infarction, Circulation 115 (2007) 1904–1911.
[34] H. Valadi, K. Ekstrom, A. Bossios, M. Sjostrand, J.J. Lee, J.O. Lotvall, Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells, Nat. Cell Biol. 9 (2007) 654–659.
[35] M.J. van Amerongen, M.C. Harmsen, N. van Rooijen, A.H. Petersen, M.J.A. van Luyn, Macrophage depletion impairs wound healing and increases left ventricular remodeling after myocardial injury in mice, Am. J. Pathol. 170 (2007) 818–829.
[36] E. van Rooij, W.S. Marshall, E.N. Olson, Toward microRNA-based therapeutics for heart disease: the sense in antisense, Circ. Res. 103 (2008) 919–928.
[37] A.V. Vaseva, U.M. Moll, The mitochondrial p53 pathway, Biochim. Biophys. Acta 1787 (2009) 414–420.
[38] X. Wang, X. Zhang, X.P. Ren, J. Chen, H. Liu, J. Yang, M. Medvedovic, Z. Hu, G.C. Fan, MicroRNA-494 targeting both proapoptotic and antiapoptotic proteins protects against ischemia/reperfusion-induced cardial injury, Circulation 122 (2010) 1308–1318.
[39] W.Y. Xu, L. Wang, H.M. Wang, Y.Q. Wang, Y.F. Liang, T.T. Zhao, Y.Z. Wu, TLR2 and TLR4 agonists synergistically up-regulate SR-A in RAW264.7 through p38, Mol. Immunol. 44 (2007) 2315–2323.
[40] Y. Xu, L. Qian, G. Zhong, K. Ma, X. Zhu, H. Zhang, N. Li, Z. Yang, H. Bai, J. Ben, X. Li, Y. Xu, Q. Chen, Class A scavenger receptor promotes cerebral ischemic injury by pivoting microglia/macrophage polarization, Neuroscience 218 (2012) 35–48. (Epub ahead of print).
[41] H. Yi, X. Yu, P. Gao, Y. Wang, S.-H. Baek, X. Chen, H.L. Kim, J.R. Subjeck, X.-Y. Wang, Pattern recognition scavenger receptor SRA/CD204 down-regulates Toll-like receptor 4 signaling-dependent CD8 T-cell activation, Blood 113 (2009) 5819–5828.
[42] H. Yu, T. Ha, L. Liu, X. Wang, M. Gao, J. Kelley, R. Kao, D. Williams, C. Li, Scavenger receptor A (SR-A) is required for LPS-induced TLR4 mediated NF-κB activation in macrophages, Biochim. Biophys. Acta, Mol. Cell Res. 1823 (2012) 1192–1198.
[43] X. Zhang, D.M. Mosser, Macrophage activation by endogenous danger signals, J. Pathol. 214 (2008) 161–178.
[44] M. Zhou, Z. Liu, Y. Zhao, Y. Ding, H. Liu, Y. Xi, W. Xiong, G. Li, J. Lu, O. Fodstad, A.I. Riker, M. Tan, MicroRNA-125b confers the resistance of breast cancer cells to paclitaxel through suppression of pro-apoptotic Bcl-2 antagonist killer 1 (BAK1) expression, J. Biol. Chem. 285 (2010) 21496–21507.
[45] H. Zhu, G.C. Fan, Extracellular/circulating microRNAs and their potential role in cardiovascular disease, Am. J. Cardiovasc. Dis. 1 (2011) 138–149.