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The TIR/BB-loop mimetic AS-1 protects the myocardium from ischaemia/reperfusion injury

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Aims Innate immune and inflammatory responses are involved in myocardial ischaemia/reperfusion (I/R) injury. The interleukin-1 receptor (IL-1R)-mediated, MyD88-dependent nuclear factor kappa B (NF-κB) activation pathway plays an important role in the induction of innate immunity and inflammation. However, the role of the IL-1R–MyD88 pathway in myocardial I/R injury has not been thoroughly investigated. We hypothesized that inhibition of the interaction of IL-1R with MyD88 will attenuate myocardial ischaemic injury through reducing inflammatory responses.

Methods and results Male C57BL/6 mice were subjected to myocardial ischaemia (45 min) followed by reperfusion (4 h). In the treatment group, after mice were subjected to ischaemia (45 min), the TIR/BB-loop mimetic (AS-1), which inhibits the interaction of IL-1R with MyD88, was administered immediately before reperfusion. Hearts were harvested and cellular proteins were isolated for immunoprecipitation and immunoblotting. AS-1 administration significantly decreased infarct size by 32.92% compared with the untreated I/R group. Ejection fraction and fractional shortening in AS-1-treated mice were also significantly increased by 18.0 and 25.6%, respectively, compared with the untreated I/R group. AS-1 administration significantly decreased the I/R-increased interaction between IL-1R and MyD88, attenuated the I/R-increased NF-κB binding activity, and reduced levels of inflammatory cytokines and adhesion molecules in the myocardium compared with the untreated I/R group. In addition, AS-1 administration significantly decreased myocardial myeloperoxidase activity by 23.6% and neutrophil infiltration in the myocardium compared with the untreated I/R group.

Conclusion The results demonstrated an important role for the IL-1R-mediated MyD88-dependent signalling pathway in myocardial I/R injury. The data suggest that modulation of the IL-1R/MyD88 interaction could be a strategy for reducing myocardial ischaemic injury.

1. Introduction

Recent evidence suggests that innate immune and inflammatory responses play a critical role in the pathophysiology of myocardial ischaemia/reperfusion (I/R).[1–4] Repression of proinflammatory cytokine and chemokine synthesis is important for alleviation of myocardial ischaemic injury.[5,6] However, the cellular and molecular mechanisms by which the innate immune and inflammatory responses participate in the pathogenesis of myocardial I/R injury have not been entirely elucidated.

The interleukin-1 receptor (IL-1R)-mediated signalling pathway activates several transcription factors such as nuclear factor kappa B (NF-κB),[7] which is an important transcription factor controlling expression of multiple pro-inflammatory genes.[8,9] Recent studies have demonstrated that Toll-like receptors (TLRs) play a critical role in the induction of innate immune and inflammatory responses.[10,11] Mammalian TLRs are characterized by extracellular leucine-rich repeat motifs and a cytoplasmic Toll homology domain similar to that of the IL-1R family of proteins, designated as the Toll/IL-1R (TIR) homology domain. The IL-1R/TLR family shares a common signalling pathway leading to NF-κB activation.[12] Although the contribution of the TLR4-mediated NF-κB activation pathway to myocardial I/R injury has been well documented,[13–15] it has been demonstrated that IL-1R/MyD88 signalling plays an important role in the recruitment of neutrophils to the areas of

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both an infectious reaction and a sterile inflammatory response.[16,17] However, the role of the IL-1R-mediated MyD88 signalling pathway in the pathophysiology of myocardial I/R injury remains to be determined.

Davis and coworkers reported that disruption of the interaction of MyD88 and the IL-1RI/IL-1RAcP complex by a synthetic low molecular weight MyD88 mimetic hydrocinnamoyl-L-valyl pyrrolidine (AS-1) prevented development of the fever response induced by IL-1β in mice.[18,19] Since innate immune and inflammatory responses contribute to myocardial ischaemic injury, it is possible that disruption of the interaction between IL-1R and MyD88 could attenuate myocardial ischaemic injury through reducing inflammatory cytokine production.

In the present study, we examined whether inhibition of IL-1R/MyD88 interaction by AS-1 will attenuate myocardial I/R injury. We observed that administration of AS-1 to mice immediately before reperfusion following ischaemia significantly reduced myocardial ischaemic injury. AS-1 administration also significantly reduced the production of inflammatory cytokines and neutrophil infiltration in the myocardium. Our results suggested that the IL-1R-mediated MyD88-dependent signalling pathway contributes to myocardial ischaemic injury.

2. Methods

2.1 Synthesis of TIR/BB-loop mimetics

AS-1 was prepared as described previously.[18–20] AS-1 was obtained as slight yellow oil and dried *in vacuo* for 24 h. The structure of AS-1 was examined by nuclear magnetic resonance (1H NMR): (400 MHz, CDCl3)δ: 7.34–7.17(m, 5H), 6.24 (d, $J = 8.4$ Hz, 1H), 4.60, 4.58 (dd, $J = 6.8$ Hz, 7.2 Hz, 1H) 3.74–3.70(m, 1H), 3.49–3.40(m, 3H), 2.99–2.92(m, 3H), 2.53(t, $J = 8.0$ Hz, 1H), 1.97–1.83(m, 5H), 0.90 (d, $J = 6.8$ Hz, 3H), 0.82 (d, $J = 6.8$ Hz, 3H); LRMS (EI, 70 eV) m/z(%): 302 (M + 5), 260(2), 204(14), 154(5), 127(4), 105(8), 91 (14), 72(100). The crystallographic details of AS-1 coincided with those of published results.[18,19] The crystals of AS-1 were dissolved in DMSO. There was no significant-cytotoxicity of AS-1 determined by the MTT assay.[20]

2.2 Experimental animals

Male C57BL/6 mice, 6–7 weeks old, were obtained from the laboratory animal centre of The Academy of Military Medical Sciences (Beijing, China). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All aspects of the animal care and experimental protocols were approved by the Nanjing Medical University Committee on Animal Care.

2.3 Experimental model of myocardial I/R injury

Myocardial I/R injury was induced as described in our previous study.[21] Briefly, the mice were anaesthetized and ventilated with room air using a rodent ventilator. After left thoracotomy and exposure of the hearts, the left anterior descending coronary artery was ligated with a 6-0 silk ligature over a 1 mm polyethylene tube (PE-10). After completion of 45 min of occlusion, the coronary artery was reperfused for 4 h. On completion of the experiments, hearts were harvested, immediately frozen in liquid nitrogen, and then stored at -80°C. Sham surgically operated mice served as sham control. Mice that were assigned to the treated group that were received AS-1 (50 mg/kg body weight) by intraperitoneal injection immediately before reperfusion following 45 min of ischaemia. AS-1 was prepared by mixing one volume of AS-1 in DMSO with three volumes of saline to give a final AS-1concentration of 50 mg/kg body weight. Vehicle control was prepared by mixing one volume DMSO with three volumes of saline. There were four experimental groups: sham control (sham), untreated I/R, I/R + vehicle, and I/R + AS-1.

2.4 Assessment of myocardial infarct size

Infarct size was determined by triphenyltetrazolium chloride (TTC; Sigma-Aldrich) staining, as described in our previous study.[13] Briefly, the hearts were removed and perfused with saline on a Langendorff system to wash blood from the coronary vasculature, followed by staining with 1% Evans blue to determine the area at risk ($n = 8$ mice/group). Each heart was then sliced horizontally to yield four slices. The slices were incubated in 1% TTC prepared with 200 mM Tris buffer (pH 7.8) for 15 min at 37°C. Viable non-ischaemic myocardium stains blue with TTC. Ischaemic myocardium, which is still viable, stains red with TTC, whereas the necrotic myocardium does not stain and appears pale white. The infarct area (white) and the area at risk (red plus white) from each section were measured using an image analyser. Ratios of risk area vs. left ventricle area (RA/LV) and IA/RA were calculated and expressed as a percentage. A ratio of RA/LV was also calculated and expressed as a percentage which is used to indicate the consistent ligature placement on the left anterior descending coronary artery between groups of mice.

2.5 Echocardiography in mice

Transthoracic echocardiography of the LV using echocardiography (GE Vivid 7 equipped with a 14 MHz phase array linear transducer, S12, allowing a 150 maximal sweep rate) was performed as described in our previous study.[21] Mice ($n = 6$ in each group) were subjected to ischaemia (45 min) followed by reperfusion for 4 and 48 h, respectively. Cardiac function was measured at 4 and 48 h after reperfusion. All measurements were made by one observer who was blinded with respect to the identity of the tracings. All data were collected from 10 cardiac cycles.

2.6 Isolation of cellular protein from heart samples

Cytoplasmic and nuclear proteins were isolated as described in our previous studies.[21,22] Briefly, heart samples were homogenized in ice-cold hypotonic buffer (20 mM HEPES pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 12 mM DTT, 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 μM aprotinin, 14 μM leupeptin, and 25% glycerol) (pH 7.9) ($n = 6$ mice/group). The homogenates were incubated on ice for 20 min, vortexed for 10 s after adding 10% Nonidet P-40, and followed by centrifugation for 5 min. Supernatants containing cytoplasmic proteins were collected. The pellets were suspended in ice-cold hypotonic salt buffer (pH 7.9), incubated on ice for 30 min, mixed frequently, and centrifuged for 10 min at 9000 *g* at 4°C. The supernatants containing nuclear extracts were collected. The concentrations of proteins were determined with the Pierce reagent (Pierce).

2.7 Serum troponin I concentrations

Serum troponin I concentrations were measured as an index of cardiac myocyte damage using a commercially available ELISA kit (Life Diagnostics, West Chester, PA, USA).[23]

2.8 Immunoprecipitation and immunoblots

Cytoplasmic proteins were incubated with 3 μL of anti-MyD88 antibody (Santa Cruz) or 5 μL of anti-IL-1R antibody (Santa Cruz) for 2 h at 4°C on a rotator. Then, 20 μL protein A/G beads (Santa Cruz) was added to each sample followed by incubation overnight at 4°C on a rotator. The samples were centrifuged briefly in a microcentrifuge

and washed three times in the 1× lysis wash buffer (250 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, and 5 μg/mL aprotinin and 5 μg/mL leupeptin) (pH 8.0). Loading buffer (pH 6.8) (25 μL) was added to each sample and boiled for 5 min. The samples were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Amersham) using a Bio-Rad semidry transfer system (Bio-Rad) as described previously.[21] The membranes were incubated with the following primary antibodies: IL-1R (1:1000 dilution, Santa Cruz) antibody and MyD88 (1:1000 dilution, Santa Cruz) antibody, respectively. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000) according to the manufacturers' protocols. The signals were detected with an ECL Western Blot Detection Kit (Pierce), and band density was analysed using Image J software (NIH).

2.9 Electrophoretic mobility shift assay

The measurement of NF-κB binding activity in nuclear proteins was performed as described in our previous study.[22] Briefly, NF-κB binding activity was evaluated in 20 μL of reaction mixture according to the instructions of manufacturer (Pierce). For the competition assay, specific unlabeled NF-κB competitors (200-fold molar excess) were employed along with the binding reaction mixture. The reaction mixture was incubated and analysed by electrophoresis on 6% non-denaturing polyacrylamide gels and was transferred to a nylon membrane (Amersham). The membranes were subjected to UV light to cross-link proteins for 1 min followed by incubation with conjugate/blocking buffer containing stabilized streptavidin-horseradish peroxidase conjugate. The signal on the membranes was detected with the ECL system. The membranes were then exposed to X-ray film for 2–5 min. The NF-κB binding bands were scanned and the relative intensities were analysed with Image J software.

2.10 ELISA

Inflammatory cytokine levels (IL-1α, IL-1β, IL-6, TNF-alpha) were measured in cellular protein preparations from heart samples using commercial ELISA kits (IL-1α: Bender MedSystems; IL-1β, IL-6, TNF-alpha: R&D System). The protein samples were diluted 1:4 in diluent buffer, and measurement was performed according to the instructions of the manufacturer.

2.11 Quantitative assessment of myeloperoxidase activity

Myeloperoxidase (MPO) activity, which is a marker of neutrophil accumulation, in the myocardium was assessed as described previously.[24] Briefly, heart tissues were homogenized in saline ($n = 6$ mice/group). A 1:20 dilution of the homogenate (5% wt/vol) was centrifuged at 5000 *g* for 10 min at 4°C. One millilitre of the supernatant was then adjusted to a total volume of 5 mL saline. The supernatant was reacted in a mixture of 1.6 mM tetramethylbenzidine, 0.3 mM H_2O_2, 80 mM sodium phosphate buffer (pH 5.4), 8% *N*, *N*-dimethylformamide, and 40% PBS in a total volume of 500 μL. The mixture was incubated for 3 min at 37°C. The rate of change in absorbance was measured by spectrophotometry at 655 nm. MPO activity was defined as the change in absorbance of 1.0/min at 37°C and expressed in units per gram protein.

2.12 Histology

Heart samples were rapidly excised, cross-sectioned and fixed in 10% buffered formalin. Fixed tissue was embedded in paraffin and sectioned in a standard fashion ($n = 6$ mice/group).

Infiltrating neutrophils were identified using naphtol AS-D Chloroacetate Esterase (Sigma-Aldrich) on 5 μm thick paraffin embedded sections counterstained with Haematoxylin (magnification ×200 and ×400) according to the instructions of the manufacturer.[25] Four different areas of each section were observed.

2.13 Immunohistochemistry

Presence of adhesion molecules in the myocardial vasculature was examined by immunohistochemistry using the following primary antibodies as described previously:[26,27] anti-goat P-selectin (1:50 dilution, Santa Cruz) and anti-rabbit ICAM-1 (1:50 dilution, Santa Cruz). NF-κB translocation from the cytoplasm to the nucleus was evaluated by immunohistochemistry using anti-rabbit-NF-κB subunit p50 (1:40 dilution, Santa Cruz). The sections were then treated with the ABC staining system (Santa Cruz) according to the instructions of the manufacturer. For all sections, 3,3-diaminobenzidine was used as the indicator substrate, which appeared as a brown reaction product. Slides were counterstained with haematoxylin and examined microscopically. Four different areas of each section were observed.

2.14 Statistics

Data are presented as mean ± SEM. Comparisons between groups were performed using one-way ANOVA, and Tukey's procedure for multiple range tests was performed. Value of $P < 0.05$ was considered to be significant.

3. Results

3.1 AS-1 reduced myocardial infarct size and improved left ventricular function

To examine the effect of AS-1 in myocardial I/R injury, we administered AS-1 to mice immediately before reperfusion following 45 min of ischaemia and assessed myocardial infarction. *Figure 1* shows that AS-1 administration significantly decreased the ratio of infarct area/risk area by 32.92% compared with the untreated I/R group (32.93 ± 2.17% vs. 49.09 ± 2.26%, $n = 8$ mice/group, $P < 0.05$). There was no significant difference in risk area/left ventricle area, which reflects the position of coronary artery ligation, between the untreated or I/R group and the AS-1 treated group. The administration of vehicle control did not affect I/R-induced myocardial infarction.

We measured serum troponin I concentrations in mice. We observed that after 45 min ischaemia followed by 4 h of reperfusion, the troponin I concentrations were significantly increased by 2.99-fold compared with the sham group (5.45 ± 0.24 vs. 16.31 ± 1.12, $n = 6$ mice/group, $P < 0.05$). In the AS-1 treated group, the serum troponin I concentrations were decreased by 35.6% (10.51 ± 0.56, $n = 6$ mice/group, $P < 0.05$) compared with untreated I/R mice. Administration of vehicle did not affect I/R-increased serum troponin I levels.

We also examined cardiac function by echocardiography before and after I/R. As shown in *Figure 2*, after I/R, ejection fraction (EF) and fractional shortening (FS) were significantly decreased compared with before I/R. However, AS-1 administration immediately before reperfusion following ischaemia significantly attenuated I/R-induced cardiac dysfunction. EF was significantly improved by 18.0% (53.9 ± 2.07% vs. 44.2 ± 4.33%, $n = 6$ mice/group, $P < 0.05$) and FS by 25.6% (27.5 ± 1.92% vs. 20.7 ± 3.09%, $n = 6$ mice/group, $P < 0.05$), respectively, compared with the untreated I/R group. There was no significant difference between the vehicle treated I/R group and the untreated I/R group.

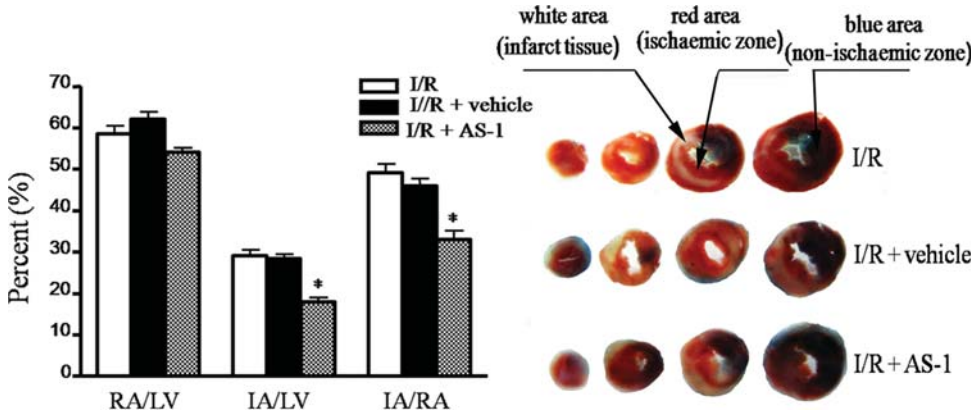

Figure 1 AS-1 reduced myocardial infarction. AS-1 was administered immediately before reperfusion following ischaemia. Hearts were harvested and infarct size was determined by TTC staining. Left ventricular, risk area, and infarct area were measured using an image analyser. The blue-stained areas represent non-ischaemic tissue, red-stained areas represent ischaemic LV area at risk, and white areas indicate necrotic areas. RA indicates risk area, expressed as red-stained areas plus white area. IA indicates infarct area, expressed as white area. LV indicates left ventricular area. $n = 8$ mice/group, $^*P < 0.05$.

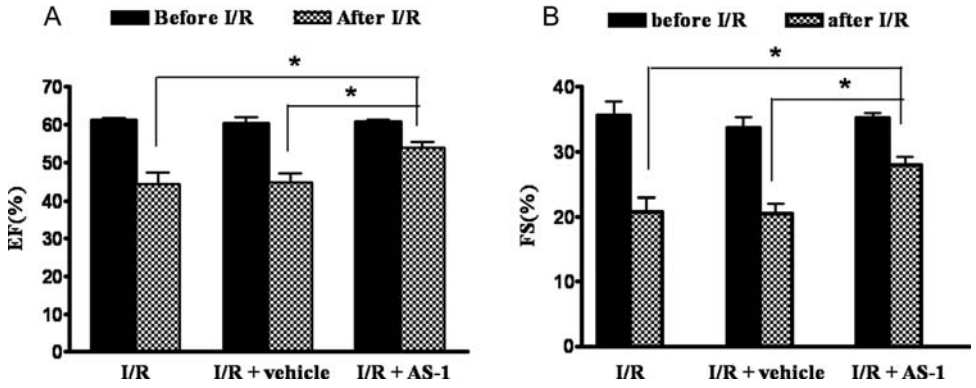

Figure 2 AS-1 administration improved cardiac function following I/R. AS-1 was administrated immediately before reperfusion following ischaemia (45 min). Cardiac function was examined by echocardiography before and after I/R. (*A*) Ejection fraction (%EF), (*B*) fractional shortening (%FS). $n = 6$ mice/group, $^*P < 0.05$.

Cardiac function was also examined by echocardiography after 48 h of reperfusion following ischaemia (45 min). The results showed that 48 h of reperfusion following ischaemia (45 min) significantly decreased EF by 25.91% ($60.64 \pm 1.01\%$ vs. $44.93 \pm 2.04\%$, $n = 6$ mice/group, $P < 0.05$) and FS by 29.47% ($32.64 \pm 1.07\%$ vs. $23.02 \pm 1.63\%$, $n = 6$ mice/group, $P < 0.05$) compared with before I/R. In AS-1 treated mice, EF was increased by 30.56% ($58.66 \pm 1.05\%$ vs. $44.93 \pm 2.04\%$, $n = 6$ mice/group, $P < 0.05$) and FS by 33.19% ($30.66 \pm 1.00\%$ vs. $23.02 \pm 1.63\%$, $n = 6$ mice/group, $P < 0.05$) compared with untreated I/R mice.

3.2 AS-1 administration decreased the association between IL-1R and MyD88 following I/R

AS-1 has been demonstrated to specifically block the association of IL-1R with MyD88 in cultured cells.[19] Therefore, we examined the effect of AS-1 on the interaction of IL-1R with MyD88 in the myocardium by immunoprecipitation with specific anti-MyD88 followed by immunoblot with specific anti-IL-1R. As shown in *Figure 3A*, I/R significantly increased the association of IL-1R with MyD88 compared with sham control. AS-1 administration significantly attenuated the increased interaction of IL-1R with MyD88 after I/R. The association between IL-1R and MyD88 in the AS-1 treated group was significantly reduced by 52.84% compared with the untreated I/R group. Administration of vehicle control did not affect the interaction of IL-1R and MyD88 after I/R compared with the untreated I/R group. *Figure 3B* shows the similar results obtained by immunoprecipitations with specific anti-IL-1R followed by immunoblot with specific anti-MyD88.

3.3 AS-1 decreased NF-κB binding activity in the myocardium following I/R

The IL-1R-mediated MyD88-dependent signalling pathway predominately activates NF-κB[28] which has been demonstrated to play an important role in myocardial ischaemic injury.[29] We examined the effect of AS-1 administration on myocardial NF-κB binding activity following I/R. As shown in *Figure 4A*, NF-κB binding activity was significantly increased 1.95-fold compared with the sham group ($P < 0.01$). In the AS-1 treated group, NF-κB binding activity was significantly reduced by 40.0% ($P < 0.05$) compared with the untreated I/R group. There is no significant difference between the vehicle treated I/R group and the untreated I/R group.

Immunohistochemistry examination showed that myocardial I/R increased NF-κB translocation from the cytoplasm

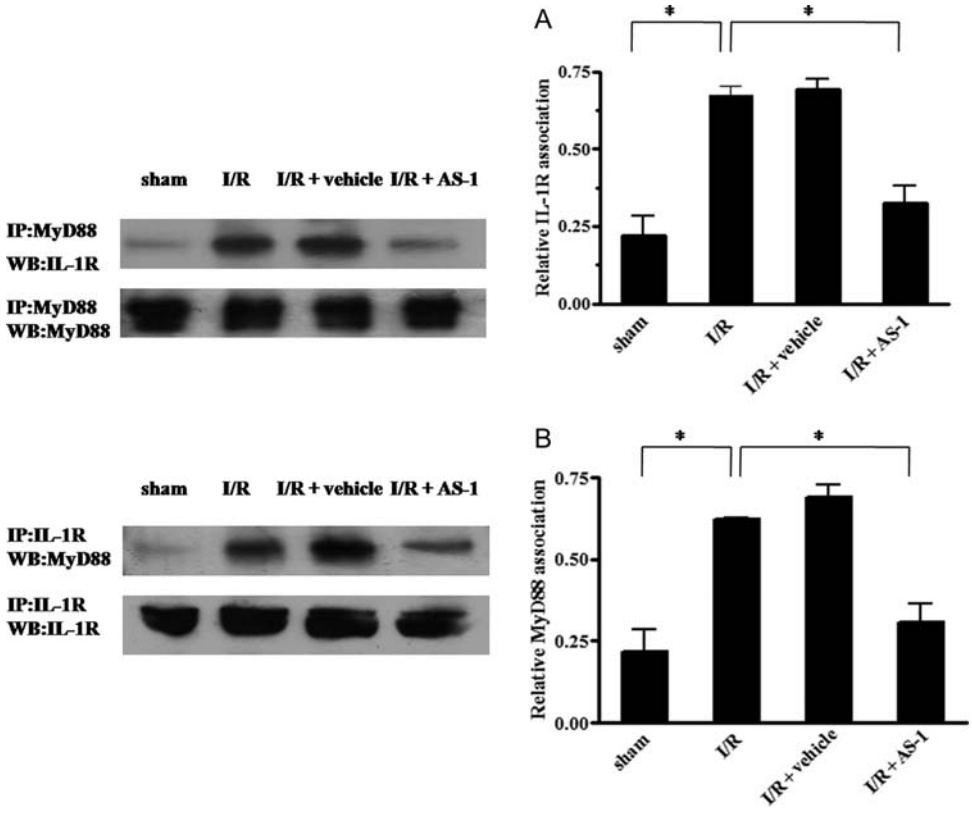

Figure 3 AS-1 decreased the association of IL-1R with MyD88 following myocardial I/R. AS-1 was administered immediately before reperfusion following 45 min of ischaemia. Hearts were harvested and cytoplasmic proteins were isolated. Immunoprecipitation was performed with anti-MyD88 Ab or anti-IL-1R Ab and assessed by western blot analysis with indicated antibodies. $n = 6$ mice/group, $^*P < 0.05$. IP, immunoprecipitation; WB, western blot.

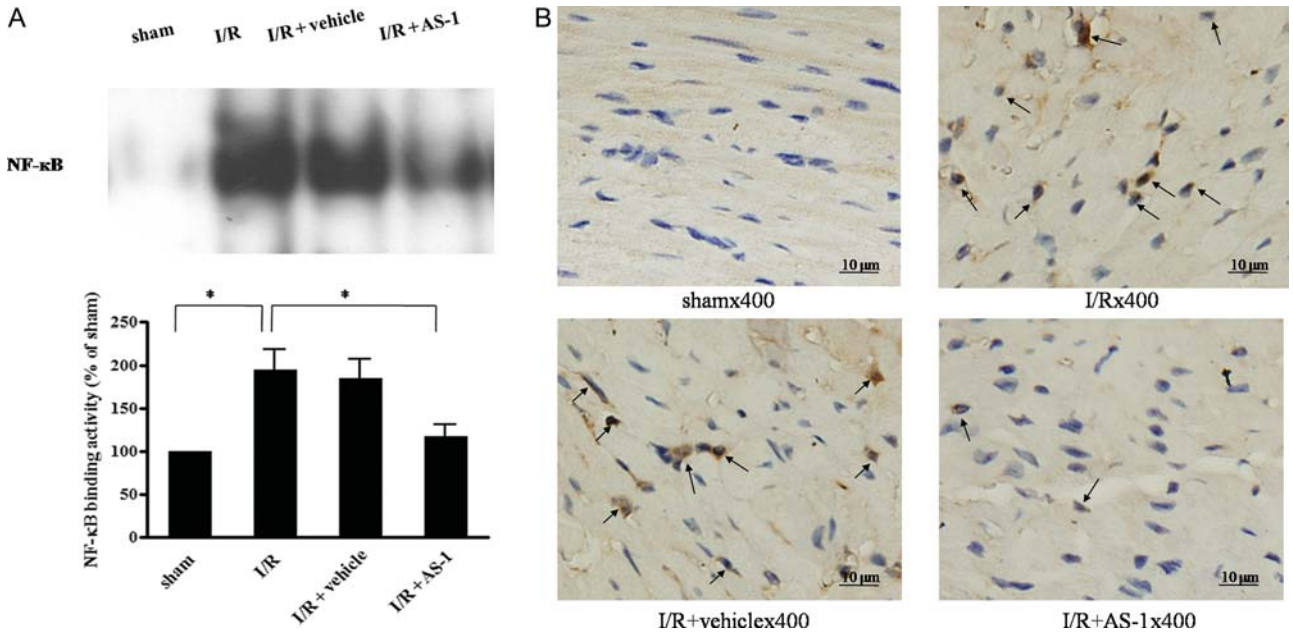

Figure 4 AS-1 administration decreased I/R-stimulated myocardial NF-κB binding activity. AS-1 was administered immediately before reperfusion following 45 min of ischaemia. Untreated I/R mice served as I/R control. (*A*) NF-κB binding activity was examined by electrophoretic mobility shift assay (EMSA). Representative EMSA results are shown on the left. The right panel is pixel values of the NF-κB binding activity. $n = 6$ mice/group, $^*P < 0.05$. (*B*) Immunohistochemistry evaluation of NF-κB nuclear translocation following myocardial I/R. Mice were subjected to I/R. AS-1 was administered immediately before reperfusion following 45 min of ischaemia. Hearts were harvested, sectioned, and subjected to immunohistochemistry for NF-κB translocation. Representative photomicrographs of sections stained for NF-κB translocation from the cytoplasm to the nucleus are shown (magnification ×400). Dark brown staining indicates the presence of NF-κB.

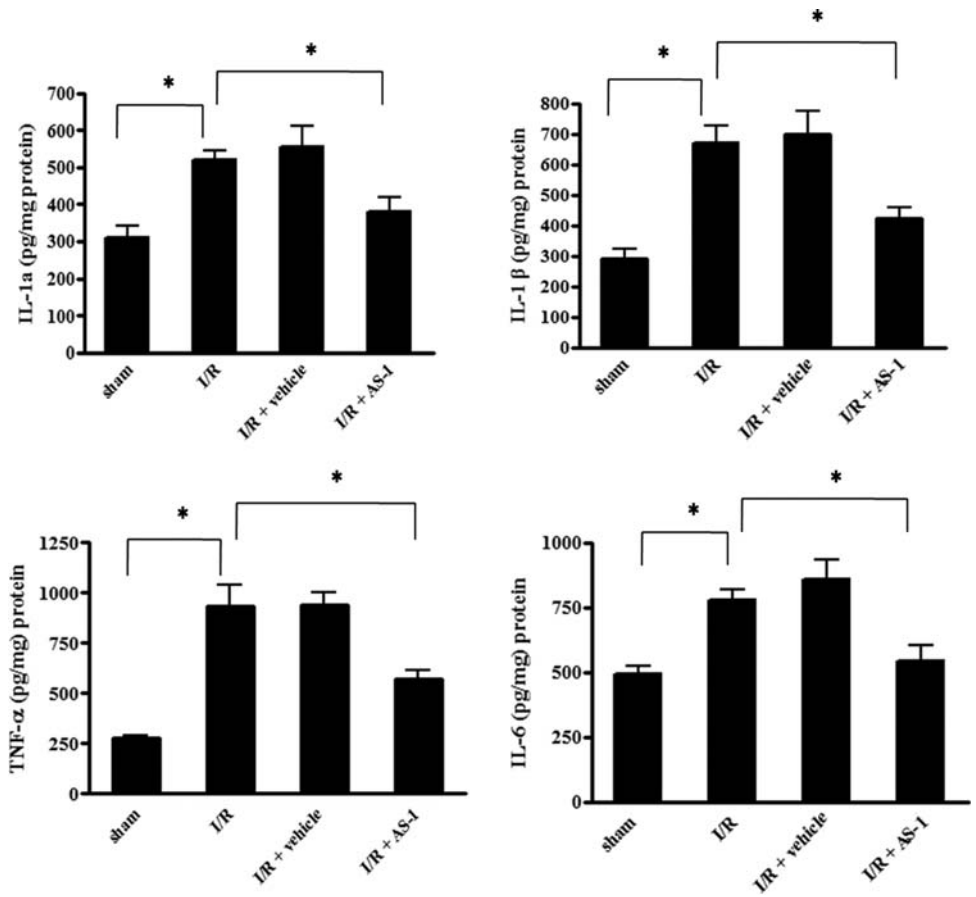

Figure 5 AS-1 decreased inflammatory cytokine expression in the myocardium following I/R. IL-1α, IL-1β, IL-6 and TNF-alpha were measured by ELISA, respectively, in the protein preparations of the hearts from sham, untreated I/R, vehicle treated, and AS-1-treated mice. $n = 6$ mice/group, $^*P < 0.05$.

to the nucleus compared with sham control. AS-1 treatment reduced the NF-κB nuclear translocation caused by I/R (*Figure 4B*).

3.4 AS-1administration reduced the levels of inflammatory cytokines in the myocardium following I/R

We analysed the levels of inflammatory cytokines in the myocardium following I/R by ELISA. *Figure 5* shows that I/R significantly increased the levels of IL-1α by 1.68-fold (519.3 ± 25.39 vs. 309.4 ± 31.36 pg/mg protein, $P < 0.05$), IL-1β by 2.31-fold, IL-6 by 1.58 fold, and TNF-alpha by 3.41-fold compared with sham control, respectively. In the AS-1 treated group, the levels of IL-1α (380.1 ± 37.54 vs. 519.3 ± 25.39 pg/mg protein), IL-1β (423.1 ± 37.43 vs. 671.2 ± 56.37 pg/mg protein), IL-6 (545.1 ± 56.61 vs. 519.3 ± 25.39 pg/mg protein), and TNF-alpha (572.7 ± 40.66 vs. 934.4 ± 103.2 pg/mg protein) were significantly reduced by 26.8, 37.0, 30.0, and 38.7%, respectively, compared with the untreated I/R group. Administration of vehicle did not affect I/R-increased levels of inflammatory cytokines in the myocardium.

3.5 AS-1 administration decreased neutrophil infiltration in the myocardium following I/R

MPO activity is an established marker of neutrophil infiltration,[30] therefore we examined MPO activity in the heart samples. As shown in *Figure 6*, MPO activity was

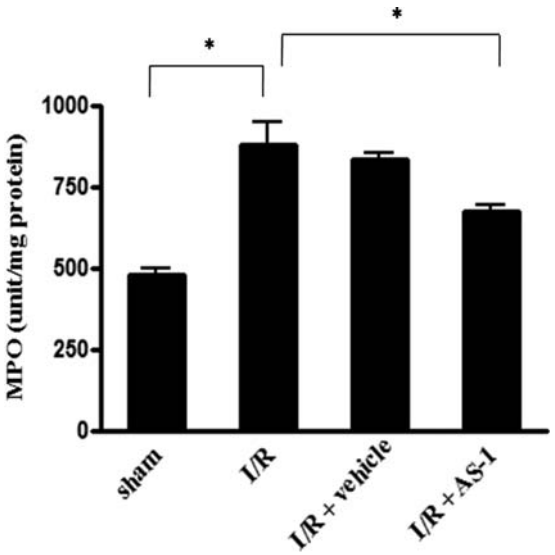

Figure 6 AS-1 reduced MPO activity in the myocardium following I/R. Mice were subjected to I/R and AS-1 was administered immediately before reperfusion following 45 min of ischaemia. MPO activity in the myocardial samples was examined as described in Methods. $n = 6$ mice/group, $^*P < 0.05$.

significantly increased by 84.6% (476.7 ± 50.67 vs. 879.9 ± 163.07 unit/mg protein; $n = 6$ mice/group, $P < 0.05$) following I/R compared with sham control. In the AS-1 treated group, MPO activity was significantly reduced by 24.9% (879.9 ± 163.07 vs. 672.2 ± 48.79 unit/mg protein;

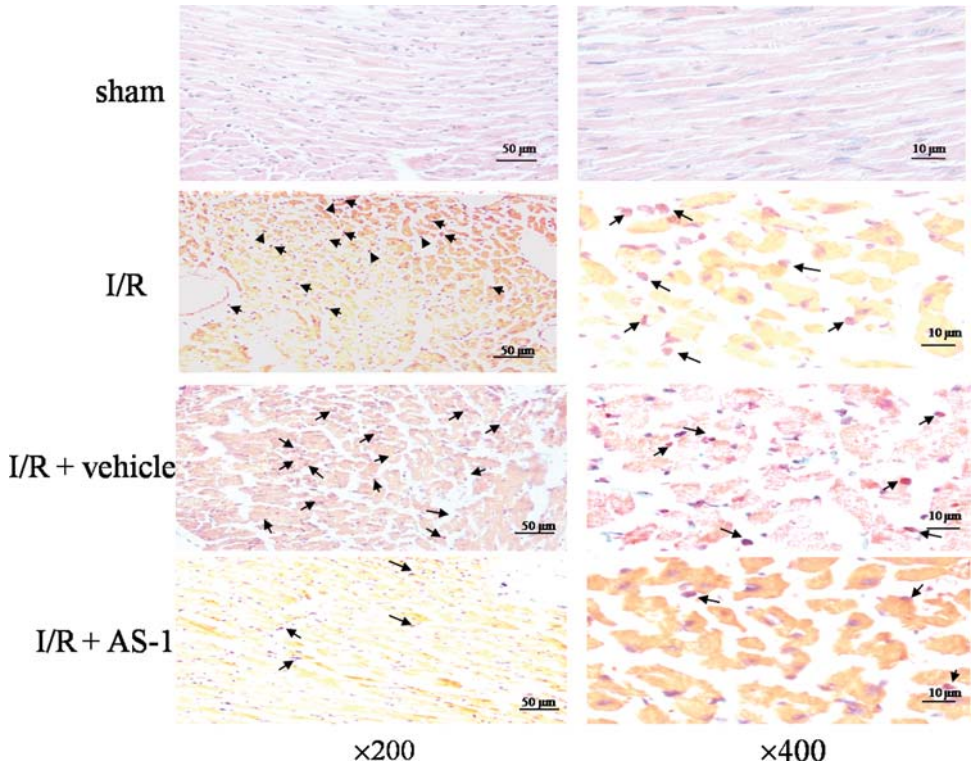

Figure 7 AS-1 reduced infiltrating neutrophils (PMN) in the myocardium following I/R. Mice were subject to I/R and AS-1 was administered immediately before reperfusion following 45 min of ischaemia. Hearts were harvested, sectioned, and subjected to histological examination of neutrophil infiltration. Representative naphtol AS-D chloroacetate esterase-stained histological images are shown (magnification ×200 and ×400).The red granulation showed infiltrating neutrophils.

$n = 6$ mice/group, $P < 0.05$) compared with the untreated I/R group. We also examined neutrophil infiltration in heart tissue sections by the naphtol AS-D chloroacetate esterase staining method. *Figure 7* shows that I/R increased the number of infiltrating neutrophils in the myocardium, while AS-1 administration reduced I/R-induced neutrophil infiltration in the myocardium.

Since adhesion molecules play an important role in the neutrophil infiltration during myocardial I/R, we examined the expression of P-selectin and ICAM-1 in the myocardial vasculature by immunohistochemistry. As shown in *Figures 8* and *9*, myocardial I/R significantly increased the expression of both P-selectin and ICAM-1 in the myocardial vasculature. AS-1 treatment inhibited P-selectin (*Figure 8*) and ICAM-1 (*Figure 9*) expression in the myocardial vasculature.

4. Discussion

In the present study, we observed that administration of a TIR/BB-loop mimetic AS-1 immediately before reperfusion, following ischaemia, protected the myocardium from I/R injury. AS-1 administration significantly attenuated cardiac dysfunction and decreased NF-κB binding activity as well as inflammatory cytokine levels in the myocardium following I/R. Our data suggests an important role of IL-1R-mediated MyD88-dependent NF-κB signalling in the pathophysiological response to myocardial I/R injury.

In our previous *in vitro* study,[20] we reported that AS-1 administration significantly blocked the interaction between IL-1R and MyD88 and inhibited IL-1β-stimulated activation of the MyD88-dependent signalling pathway. In the present study, we observed that I/R increased the association of IL-1R with MyD88 which is consistent with the NF-κB binding activity and nuclear translocation, the levels of inflammatory cytokines and adhesion molecules in the myocardium. In the IL-1R-mediated signalling pathway, stimulation of IL-1R will result in formation of a complex in which IL-1R interacts with IL-1R accessory protein.[31] This complex will recruit an adaptor protein called MyD88, which, in turn, interacts with the IL-1R-associated kinase (IRAK) through a death domain. Activated IRAK will stimulate NF-κB activation through tumour necrosis factor receptor-associated factor 6.[32] The role of NF-κB activation in myocardial ischaemic injury has been well demonstrated.[29,33] Activation of NF-κB will stimulate pro-inflammatory cytokine gene expression.[34] Therefore, it is possible that blockade of IL-1R-mediated NF-κB activation could reduce myocardial ischaemic injury. Indeed, Suzuki *et al.*[35] reported that transfection of an IL-1R antagonist into the myocardium significantly induced cardioprotection against I/R injury. Abbate *et al.*[36] reported that administration of an exogenous recombinant human IL-1R antagonist (anakinra) significantly ameliorated the remodelling process and inhibited cardiac myocyte apoptosis following myocardial I/R injury. In addition, Bujak *et al.*[37] reported that IL-1RI deficiency protected against the development of adverse remodelling following myocardial I/R. These investigators showed that IL-1RI-null mice exhibited decreased infiltration of neutrophils and macrophages in the infarcted myocardium and reduced chemokine and cytokine expression after reperfusion.[37] We observed in the present study that administration of AS-1 immediately before reperfusion following ischaemia significantly reduced the interaction between IL-1R and MyD88, resulting in decreased neutrophil infiltration and MPO activity in the myocardium. We also observed that AS-1 treatment inhibited

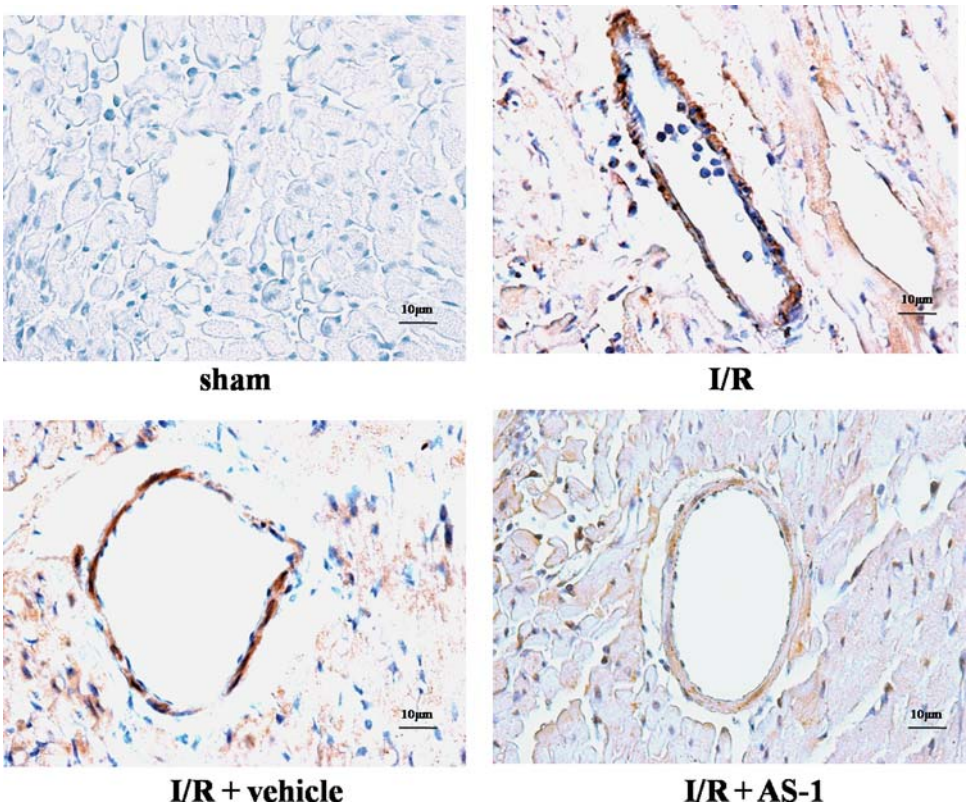

Figure 8 AS-1 treatment inhibits P-selectin protein expression in the myocardial vasculature following myocardial I/R. Mice were subjected to I/R. AS-1 was administered immediately before reperfusion following 45 min of ischaemia. Hearts were harvested, sectioned, and subjected to immunohistochemistry for P-selectin expression. Representative photomicrographs of sections stained for P-selectin are shown (magnification ×400). Brown staining indicates the presence of P-selectin.

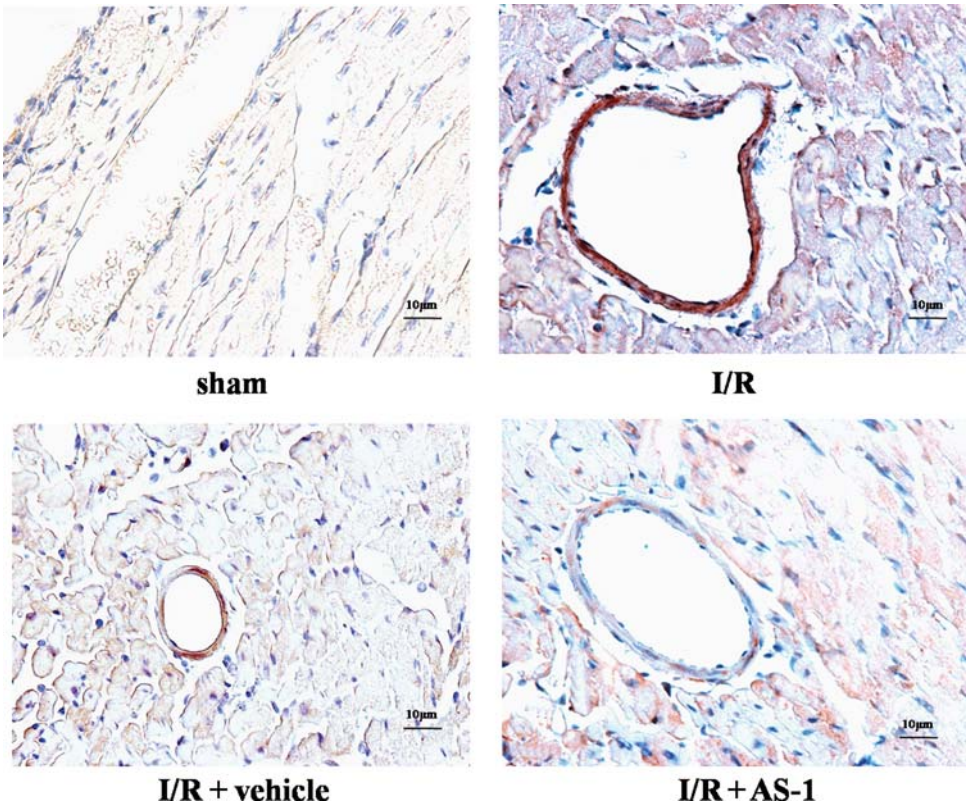

Figure 9 AS-1 treatment inhibits ICAM-1 protein expression in the myocardial vasculature. Mice were subjected to I/R. AS-1 was administered immediately before reperfusion following 45 min of ischaemia. Hearts were harvested, sectioned, and subjected to immunohistochemistry for ICAM-1 expression. Representative photomicrographs of sections stained for ICAM-1 are shown (magnification ×400). Brown staining indicates the presence of ICAM-1.

P-selectin and ICAM-1 expression in the myocardial vasculature. It is well known that adhesion molecules P-selectin and ICAM-1 play an important role in the neutrophil infiltration during myocardial I/R.[1] Both P-selectin and ICAM-1 mediate early interaction and adhesion of neutrophils to coronary endothelial cells and cardiomyocytes following myocardial I/R.[1] It has been reported that I/R-caused myocardial injury was significantly attenuated in ICAM-1 deficient mice.[38] Therefore, decreased neutrophil infiltration by AS-1 may be through downregulation of the expression of adhesion molecules in the myocardium following I/R. In addition, treatment of mice with AS-1 significantly reduced the levels of inflammatory cytokines in the myocardium. Collectively, these data suggest that the IL-1R-mediated NF-κB activation pathway contributes to myocardial ischaemic injury and inhibition of this signalling pathway will protect the myocardium against ischaemic injury.

MyD88 is a critical adaptor protein in signalling pathways inducing innate immune and inflammatory responses. Blocking the MyD88-mediated signalling pathway significantly reduced myocardial ischaemic injury.[39] Feng *et al.*[40] demonstrated an important role of MyD88 signalling in the pathogenesis of myocardial inflammation, infarction, and cardiac dysfunction after I/R. These investigators showed that systemic MyD88 deficiency attenuated neutrophil recruitment, diminished proinflammatory mediator production, reduced myocardial infarction, and dramatically improved LV contractility after I/R injury.[39] AS-1 is a TIR/BB-loop mimetic which will specifically disrupt the association between IL-1R and MyD88.[19] We observed that inhibition of the interaction of IL-1R with MyD88 by AS-1 significantly reduced myocardial infarction, improved cardiac dysfunction both early phage and 2 days after I/R and reduced the levels of myocardial inflammatory cytokines as well as neutrophil infiltration in the myocardium following I/R.

In summary, our results suggest that IL-1R-mediated MyD88-dependent signalling contributes to myocardial I/R injury and that inhibition of this signalling pathway could be a new therapeutic strategy for protection of myocardium against ischaemic injury.

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