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Original Article

Knockdown of *EMMPRIN* improves adverse remodeling mediated by IL-18 in the post-infarcted heart

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Abstract: Interleukin-18 (IL-18) exacerbates cardiac dysfunction following myocardial infarction (MI). Extracellular matrix metalloproteinase inducer (EMMPRIN) has been shown to exacerbate ventricular remodeling via induction of extracellular matrix metalloproteinase (MMP) synthesis. While up-regulation of EMMPRIN expression by IL-18 has been demonstrated *in vitro*, little is known regarding its *in vivo* effects. Here, we investigated the role of EMMPRIN in progressive post-infarct ventricular remodeling induced by IL-18. Cardiac function was impaired on echocardiography and organ weight was increased in mice receiving daily intraperitoneal injection of IL-18 following MI. Accompanying these adverse functional effect were increased EMMPRIN levels. Gene silencing of cardiac EMMPRIN by intramyocardial RNA interference rescued IL-18 mediated adverse effects on post-infarct cardiac function. Finally, EMMPRIN silencing reduced MMP-9 expression in the post-infarcted left ventricular myocardium. In conclusion, progressive post-infarct left ventricular remodeling induced by IL-18 can be reversed by gene silencing of EMMPRIN. Knock down of EMMPRIN may be a potential therapeutic strategy to abrogate the adverse effects of IL-18 on post-infarct left ventricular remodeling likely via MMP-9 inhibition.

Keywords: Interleukin-18, extracellular matrix metalloproteinase inducer, myocardial infarction, ventricular remodeling

Introduction

Myocardial infarction (MI) is a major cause of death and disability worldwide. A plethora of studies reveal the importance of excessive extracellular matrix (ECM) degradation as well as inflammatory responses in post-infarct ventricular remodeling.

As a proinflammatory cytokine, interleukin (IL)-18 participates in the pathophysiology of various cardiovascular diseases, including MI, atherosclerosis and myocarditis. Serum and myocardial levels of IL-18 are increased following MI [1-3]. Daily administration of IL-18 causes cardiac dysfunction, *in vivo* [4]. Furthermore, treatment with anti-IL-18 neutralizing antibody improves ischemia/reperfusion injury [5]. An epidemiological study indicated that IL-18 can predict future events in patients with acute coronary syndromes [6]. However, mechanisms of IL-18 in post-infarct ventricular remodeling remain unclear.

Enhanced expression of matrix metalloproteinases (MMPs) such as MMP-9 following MI [7], enhances the rate of ECM degradation, which in turn, exacerbates ECM remodeling. Extracellular matrix metalloproteinase inducer (EMMPRIN), an integral membrane protein, induces the secretion of various MMPs [8, 9]. We and others have demonstrated increased expression of EMMPRIN in various settings, including ischemia, inflammation, and immune responses [10-12]. Moreover, up-regulation of EMMPRIN levels under pathological conditions is involved in adverse tissue remodeling including post-infarct ventricular remodeling [10, 13].

Recently, studies have shown that IL-18 can up-regulate EMMPRIN expression in monocytes, cardiomyocytes, and vascular smooth muscle cells under physiological and pathological conditions [14-17]. Here, we investigated the potential role of EMMPRIN in progressive post-infarct ventricular remodeling induced by IL-18.

Materials and methods

Experimental animals

Three-month-old specific pathogen-free male C57BL/6 mice, weighing 20-23 g, were purchased from the Laboratory Animal Center of Sun Yat-Sen University. Animals were maintained according to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 1.0.0. Revised 2011), and experiments were approved by the Animal Ethics Committee of Sun Yat-sen University.

All mice were housed in a controlled environment, with a 12-hour light/12-hour dark cycle at 23°C. Mice were allowed access to food and water ad libitum.

Left anterior descending (LAD) coronary artery ligation, intramyocardial injection of adeno-associated virus (AAV), daily intraperitoneal injections of IL-18 and echocardiography

MI was produced in mouse as previously described [18]. Briefly, mice were anesthetized, intubated and ventilated using a rodent ventilator. Thoracotomy was performed, and the proximal LAD was ligated using a 6-0 prolene suture. Ligation was confirmed by blanching and dysfunction of the anterior wall. After LAD ligation, EMMPRIN mRNA (Genebank accession number: D00611) interference in the infarcted zone, was performed immediately using AAV-based vector expressing short hairpin RNAs (shRNA) (sequences 5'-CAGCTTACCTGCTCTTTGA-3'). 1×10^{11} viral genome particles of AAV with or without EMMPRIN shRNA expression in 50 μl phosphate-buffered saline were injected at five sites around the infarct border using an insulin syringe with incorporated 30-gauge needle. After surgery, mice were received daily intraperitoneal injection of a volume of 1 ml 0.9% NaCl or 1 ml NaCl containing 0.5 μg of mouse IL-18 (R&D Systems) during a time period of 28 days. Left ventricular (LV) function indexed by the ejection faction (EF), LV end diastolic diameter (LVEDD), LV end systolic diameter (LVESD) were evaluated four weeks after surgery using a high-resolution echocardiographic imaging system (Vevo2100, Visualsonics, Canada). The animals were euthanized after echocardiography for heart collection and staining.

Experimental groups

Male mice that underwent LAD ligation were randomly divided into 4 groups: the untreated MI group (n=12), the MI plus IL-18 (MI+IL-18) group (n=15), the MI receiving treatment of IL-18 plus GFP-AAV (MI+IL-18+GFP) group (n=13), and the MI receiving treatment of IL-18 plus EMMPRIN shRNA-AAV (MI+IL-18+shRNA EMMPRIN) group (n=13).

Immunohistochemistry

Left ventricles were fixed and embedded in paraffin. 5-10 μm sections were used for immunohistochemical staining as described previously [10]. In brief, slides were deparaffinized in xylene bath, rehydrated through graded ethanol, and target-retrieved by heat-induced epitope retrieval in citrate buffer (pH=6). The slides were incubated in 10% goat serum with 1% bovine serum albumin (BSA) in TBS to block non-specific binding. Slides were subsequently incubated with rabbit anti-EMMPRIN antibody (1:250 diluted, Abcam) overnight at 4°C and immersed in 3% hydrogen peroxide to reduce endogenous peroxidase activity. Finally, slides were sequentially incubated with goat polyhorseradish peroxidase conjugated secondary antibody (Dako), developed with 3,3-diaminobenzidine (DAB), and counterstained with Mayer's hematoxylin. A negative control reagent containing normal rabbit IgG was used to evaluate nonspecific or undesired staining. This facilitated interpretation of specific staining at the antigen site. The percentage of positive area was analyzed by Image-Pro Plus software (Media Cybernetics, MD).

Western blotting

LV infarct area was lysed with radioimmunoprecipitation assay buffer (CST) containing 1% phenylmethanesulfonyl fluoride (CST) for total protein extraction. Protein concentrations were measured using bicinchoninic acid protein Kit (Pierce). Equal protein lysates were separated via 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Merck millipore). After blocking in 5% w/v BSA in TBS supplemented with 0.1% Tween-20 (TBST) for 1 hour, membranes were incubated in anti-EMMPRIN antibody (1:1000, Abcam), anti-MMP 9 antibody (1:1000, Abcam), and anti-actin

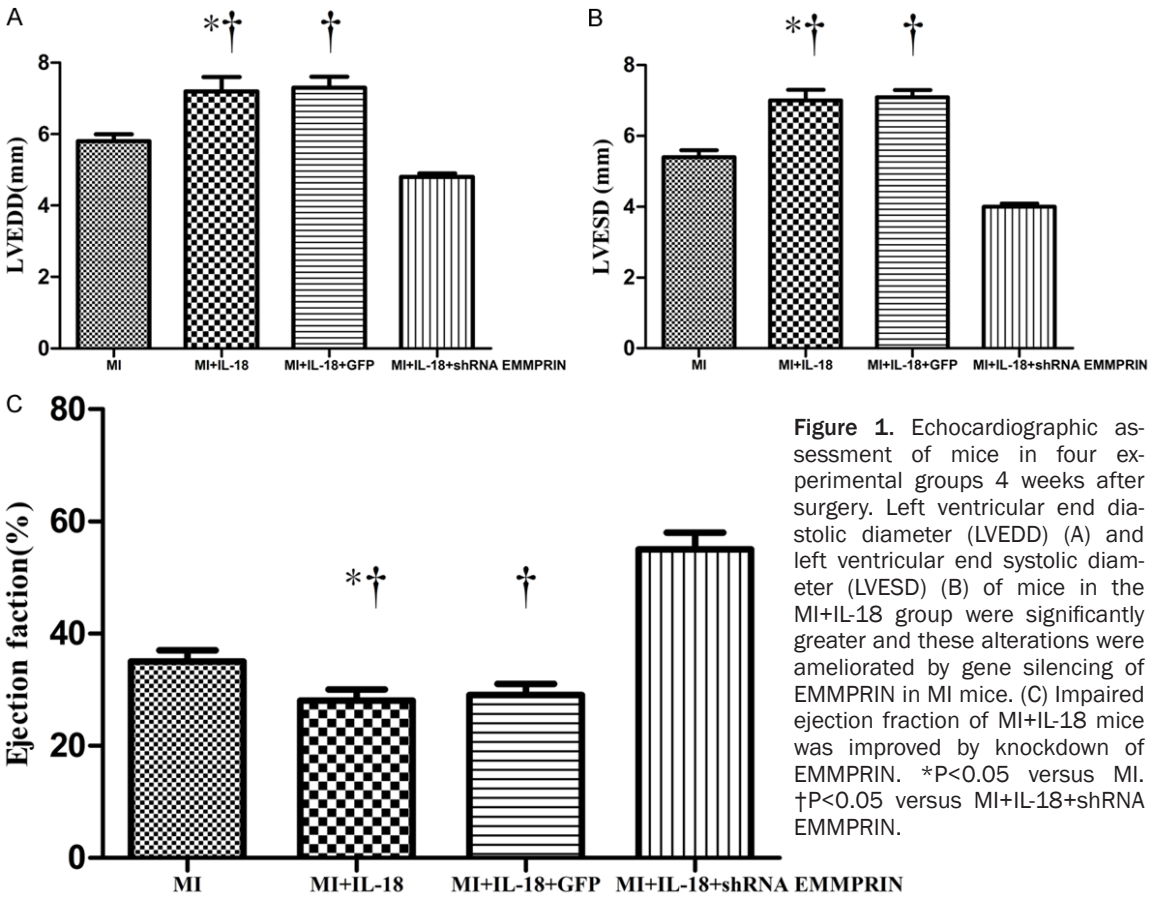

Figure 1. Echocardiographic assessment of mice in four experimental groups 4 weeks after surgery. Left ventricular end diastolic diameter (LVEDD) (A) and left ventricular end systolic diameter (LVESD) (B) of mice in the MI+IL-18 group were significantly greater and these alterations were ameliorated by gene silencing of EMMPRIN in MI mice. (C) Impaired ejection fraction of MI+IL-18 mice was improved by knockdown of EMMPRIN. *P<0.05 versus MI. †P<0.05 versus MI+IL-18+shRNA EMMPRIN.

antibody (1:1000, Abcam) overnight at 4°C. Membranes were rinsed 3 times in TBST and subsequently incubated with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) for 1 hour at room temperature. Blots were visualized using an enhanced chemiluminescence detection kit (Bio-rad). Quantification of band density was performed using *Image J* software (NIH).

Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was purified from homogenizing LV infarct area using Trizol reagent (Life Technologies). Extracted mRNA (1 μg) was reverse-transcribed to complementary DNA using PrimeScript RT Reagent Kit (Takara) with oligo dT primer and random 6 mers. Quantitative real-time PCR was performed using SYBR ® Premix Ex Taq II (Takara) according to the manufacturer's instructions. The reference gene actin served as an internal normalizing control. The following primers were used: 1) EMMPRIN (Genbank accession number: D00611): forward 5'-GGGAAACCATCTCACTGCGT-3' and reverse 5'-TAGATAAAGATGATGGTAACCAACACCA-3'; 2) MMP-9 (Genbank accession number: NM_013599.3): forward 5'-ACCCGAAGCGGACATTGTC-3'; and reverse 5'-CGAAGGGATACCCGTCTCC-3'; 3) actin (Genbank accession number: NM_007393.3) forward 5'-TCACCCACACTGTGCCCATCTACGA-3' and reverse 5'-GGATGCCACAGGATTCCATACCCA-3'.

Other parameters

Lung weight, heart weight, lung-to-body weight ratio and heart-to-body weight ratio were documented.

Statistical analysis

Values are presented as mean ± SEM. Normally-distributed quantitative data were analyzed using one-way ANOVA followed by LSD post hoc test. Probability of survival was analyzed using the Kaplan-Meier method, and grouped differences in survival were performed using the log-rank test. Analyses were performed with SPSS 16.0 (IBM, USA). P<0.05 was considered statistically significant.

Table 1. Organ weight of mice in Experiment

	MI (n=6)	MI+IL-18 (n=5)	MI+IL-18+GFP (n=5)	MI+IL-18+shRNA EMMPRIN (n=10)
Body weight (g)	21.81±0.87	21.71±0.79	21.73±1.33	21.55±1.1
Lung-to-Body weight ratio (mg/g)	7.14±0.30	9.75±0.56*,†	9.76±0.54†	5.57±0.26
Heart-to-Body weight ratio (mg/g)	6.71±0.22	9.39±0.53*,†	9.41±0.3†	5.16±0.28

*P<0.05 versus MI. †P<0.05 versus MI+IL-18+shRNA EMMPRIN.

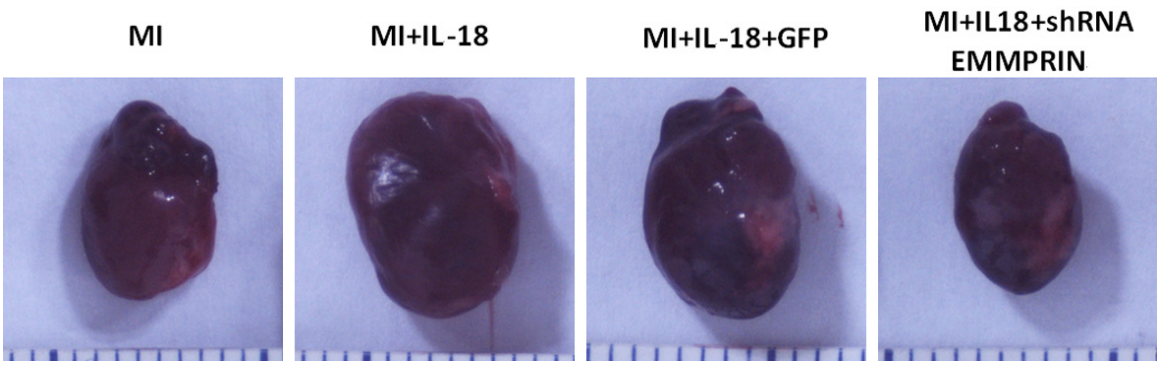

Figure 2. Representative photographs of hearts in four groups 4 weeks after surgery. Size of hearts harvested from the MI+IL-18 group was significantly augmented; silencing EMMPRIN markedly reduced size of hearts collected from MI+IL-18+shRNA EMMRPIN group .

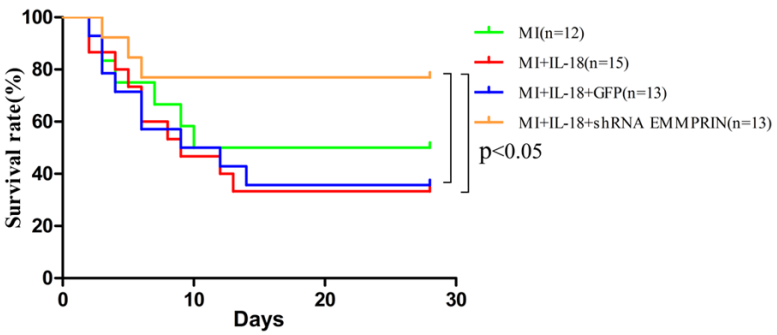

Figure 3. 28-day survival. Percent survival of mice in the four groups displayed in Kaplan-Meier survival curves. Statistical analyses were performed using the Log-Rank test. P<0.05 versus MI+IL-18+shRNA EMMPRIN.

Results

Administration of IL-18 worsens post-MI left ventricular dysfunction in mice

We initially investigated the role of IL-18 in post-MI ventricular remodeling by daily intraperitoneal injections of IL-18 after LAD ligation. 4 weeks after LAD ligation, animals in the MI+IL-18 group exhibited more adverse cardiac remodeling as documented by echocardiography (EF, 28±2% vs. 35±2%, P<0.05; LVEDD, 7.2±0.4 vs. 5.8±0.2, P<0.05; LVESD, 7.0±0.3 vs. 5.4±0.2, P<0.05) in comparison with the untreated MI group (**Figure 1**). Impaired cardiac function in IL-18 treated post-MI animals was associated with increased heart-to-body weight (9.39±0.53 vs. 6.71±0.22, P<0.05) (**Table 1**, **Figure 2**) and lung-to-body weight (9.75±0.56 vs. 7.14±0.3, **Table 1**; P<0.05) compared to the untreated MI group. Furthermore, the MI+IL-18 group exhibited a trend towards a lower survival rate during the course of 28 days compared to MI only animals (33.3% vs. 50%) although differences were not statistically significant (**Figure 3**). These data show that additional administration of IL-18 further impair post-infarct cardiac function.

Increased cardiac EMMPRIN levels in mice receiving IL-18 administration

IL-18 induces cardiomyocyte EMMPRIN expression *in vitro* [15]. We therefore explored the in vivo role of IL-18 in cardiac EMMPRIN expression following MI. A significant increase in

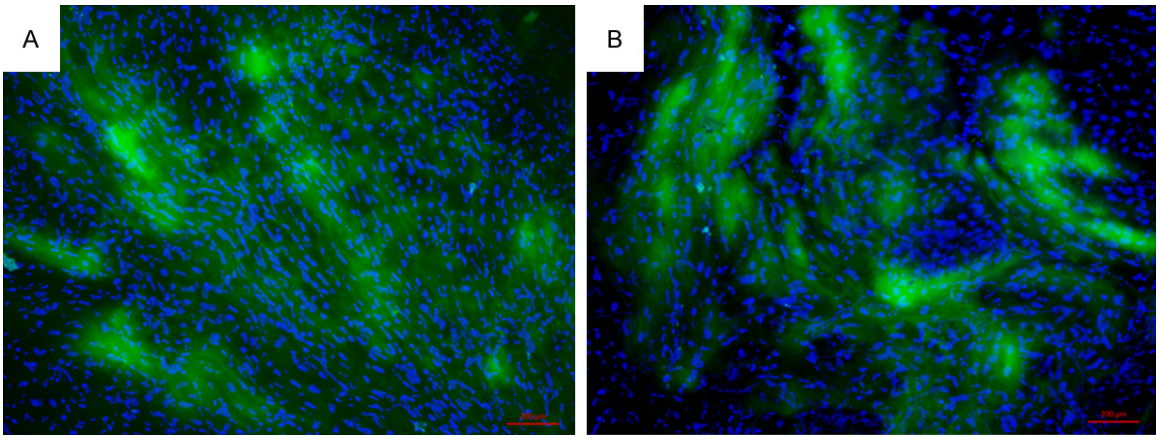

Figure 4. Expression of AAV-based vector in left ventricles after 4 weeks' transfection. A. Transfection of GFP labeled AAV-based vector into the infarct border. B. Transfection of GFP labeled AAV-based vector expressing EMMPRIN shRNA into the infarct border.

EMMPRIN expression was observed in the IL-18 injected MI animals compared to their uninjected counterparts, both the mRNA and protein levels using RT-qPCR (**Figure 5A**), western blotting (**Figure 5B**), and immunohistochemistry analysis (**Figure 6**). These results emphasize the notion that IL-18 acts as an inducer of EMMPRIN expression in myocardium.

Improved cardiac function in mice with transfection of EMMPRIN shRNA

Up-regulation of EMMPRIN accelerates ECM remodeling following MI. We further investigated the potential role of EMMPRIN in progressive post-infarct ventricular remodeling induced by IL-18. Indeed, intramyocardial injection of an AAV-based vector expressing EMMPRIN shRNA (**Figures 4**, **5A**, **5B** and **6**) caused a significant decrease in EMMPRIN expression. Subsequently, we evaluated whether knock down of EMMPRIN would rescue the adverse effects of IL-18 on cardiac function. Interestingly, mice infected with EMMPRIN shRNA displayed robust improvements in EF (55±3% vs. 28±2%, $P<0.05$; 55±3% vs. 29±2%, $P<0.05$) LVEDD (4.8±0.1 vs. 7.2±0.4, $P<0.05$; 4.8±0.1 vs. 7.3±0.3, $P<0.05$) and LVESD (4±0.1 vs. 7±0.3, $P<0.05$; 4±0.1 vs. 7.1±0.2, $P<0.05$), as compared with the MI+IL-18 or the MI+IL-18+GFP groups (**Table 1**, **Figure 1**). In accordance with improved cardiac function, heart-to-body weight (5.16±0.28 vs. 9.39±0.53, $P<0.05$; 5.16±0.28 vs. 9.41±0.3, $P<0.05$) (**Table 1**, **Figure 2**) and lung-to-body weight (5.57±0.26 vs. 9.75±0.56, $P<0.05$; 5.57±0.26 vs. 9.76±0.54, $P<0.05$) (**Table 1**) ratios were markedly decreased in the MI+IL-18+shRNA EMMPRIN group compared to the MI+IL-18 group or the MI+IL-18+GFP group. Furthermore, mice in the MI+IL-18+shRNA EMMPRIN group exhibited a 28-day survival rate of 76.9%, which was higher than that of MI+IL-18+GFP or MI+IL-18 groups (**Figure 3**). These data reveal that EMMPRIN gene silencing reverses the detrimental effects of daily administration of IL-18 on post-infarct ventricular remodeling.

Decreased cardiac MMP-9 expression in mice with treatment of RNA interference of EMMPRIN

EMMPRIN has been shown to increase the synthesis of various MMPs [13]. In particular, MMP-9 is now believed to play a major role in the regulation of ECM remodeling. We found a marked reduction in left ventricular MMP-9 expression as assessed by RT-qPCR (**Figure 5A**) and western blot analysis (**Figure 5B**) following EMMPRIN gene silencing. These findings highlight the importance of EMMPRIN in the induction of myocardial MMP-9 expression following MI.

Discussion

The present study emphasizes the adverse effects of IL-18 on post-infarct ventricular remodeling. We demonstrated that chronic exposure to IL-18 induced progressive post-infarct ventricular remodeling concomitant with elevated myocardial expression of EMMPRIN.

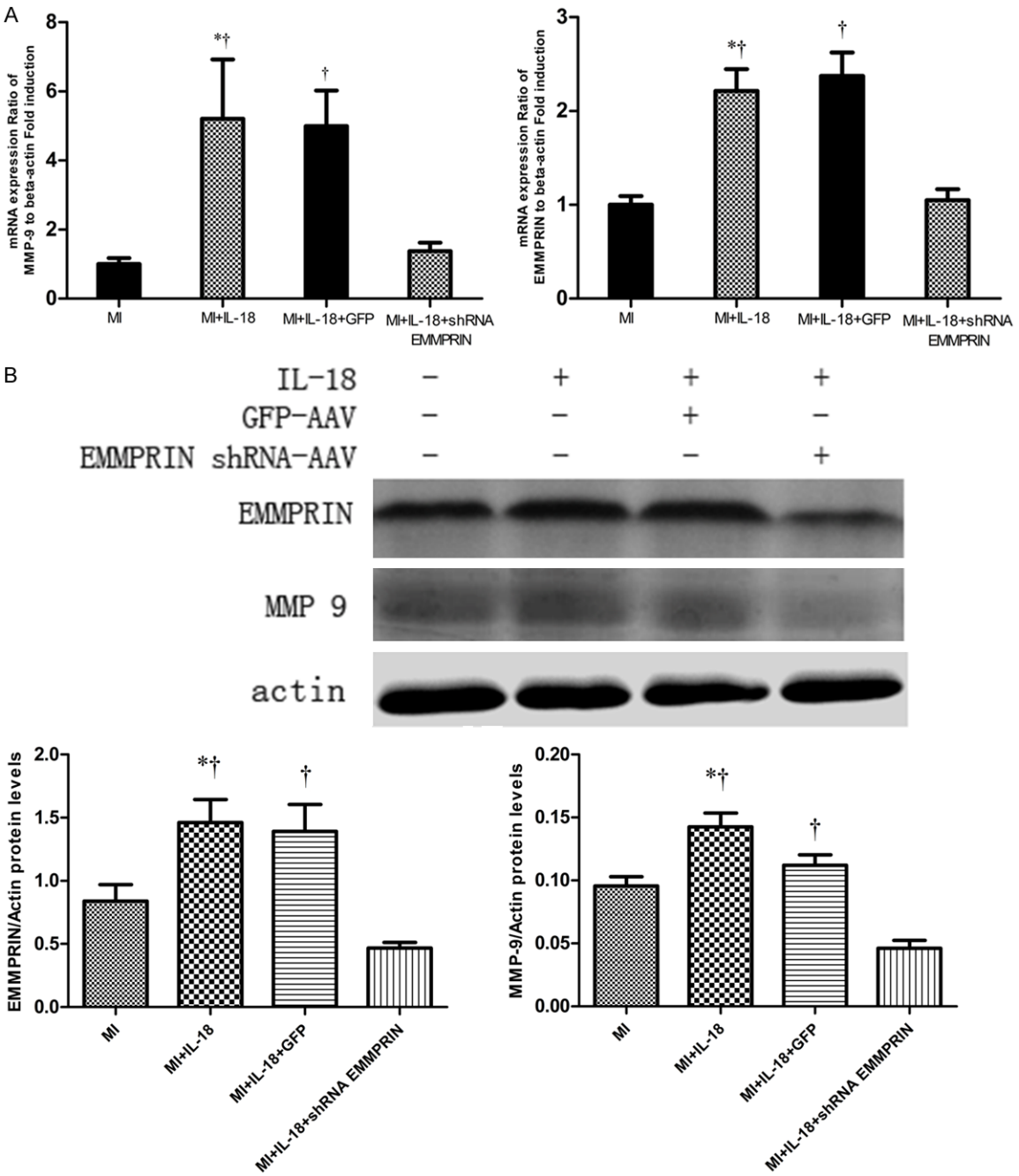

Figure 5. EMMPRIN and MMP-9 levels in left ventricular infarct area of the four experimental groups 4 weeks after left anterior descending coronary artery ligation. A. Mean mRNA expression of EMMPRIN and MMP-9 measured by RT-qPCR on total RNA abstracted from heart. B. Protein levels of EMMPRIN and MMP-9 in lysates determined by western blot analysis. *P<0.05 versus MI. †P<0.05 versus MI+IL-18+shRNA EMMPRIN.

Moreover, we found that knock down of EMMPRIN which improved post-infarct cardiac dysfunction caused by IL-18, markedly decreased the secretion of MMP-9. Our data strongly suggest that EMMPRIN may act as a downstream signaling molecule that actively participates in IL-18-mdiated post-infarct ventricular remodeling.

Cardiomyocytes death in MI triggers the rapid secretion of diverse cytokines leading to an intense inflammatory response. Elevated serum levels of IL-18 have been observed in patients and mice following acute MI [1-3]. In addition increased cardiac pro-IL-18 levels have been confirmed in mice post-MI [3]. Previous studies have shown that chronically

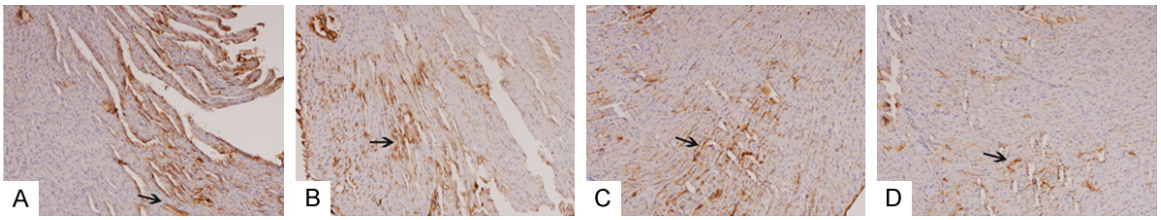

Figure 6. Immunohistochemical detection of EMMPRIN in post-MI left ventricles harvested from the four experimental groups 4 weeks after surgery. (A) Brown staining (arrow) indicates positive staining of EMMPRIN in left ventricular samples harvested from the MI group. Similarly, brown staining (arrows) in (B-D) represents positive staining for EMMPRIN in hearts harvested from the MI+IL-18, MI+IL-18+GFP, and MI+IL-18+shRNA EMMPRIN groups, respectively.

increased circulating IL-18 caused cardiac dysfunction even in healthy mice and that transplantation of IL-18 binding protein-expressing mesenchymal stem cells or neutralization of IL-18 improves post-infarct cardiac dysfunction [4, 5, 19]. In our study, we found that daily administration of IL-18 exacerbated post-MI cardiac dysfunction. Our data and that of others indicate that IL-18 promotes cardiac dysfunction under both physiological and pathological conditions.

There is increasing evidence that EMMPRIN plays an important role in ECM remodeling following MI. Indeed, mice that underwent genetic silencing of EMMPRIN or were treated with an EMMPRIN neutralizing antibody exhibited milder cardiac dysfunction following MI [20, 21]. *In vitro* studies have demonstrated reciprocal modulation of IL-18 and EMMPRIN expression in cardiomyocytes via different signaling pathways [15, 16]. Similarly, we found that IL-18 enhanced the expression of EMMPRIN in vivo. Up-regulation of EMMPRIN exacerbates ECM remodeling by stimulating excessive ECM breakdown following MI. In our present study, AAV-mediated expression of EMMPRIN shRNA markedly down-regulated EMMPRIN expression *in vivo*. In addition, we found that silencing of EMMPRIN significantly ameliorated progressive post-MI cardiac dysfunction induced by IL-18. Taken together, our findings suggest that IL-18 exacerbates progression of post-infarct ventricular remodeling mediated by EMMPRIN.

MMP-9 is one of the most important MMPs involved in ECM remodeling following MI. Inhibition of MMP-9 activation or knockdown of the MMP-9 gene improves post-MI ventricular remodeling [20, 22]. Expression of MMP-9 is regulated by EMMPRIN [13]. In this study, silencing of EMMPRIN markedly inhibited cardiac MMP-9 expression. These data highlight the importance of EMMPRIN in the secretion of cardiac MMP-9 following MI. Our current findings suggest that down-regulation of MMP-9 expression may be mechanistically involved in the cardioprotective effects of EMMPRIN silencing against adverse IL-18 mediated remodeling in the post-infarcted heart.

In conclusions, progressive post-infarct left ventricular remodeling induced by daily administration of IL-18 is mediated by EMMPRIN. Silencing of EMMPRIN may be a novel therapeutic strategy in the post-infarcted heart likely via inhibition of MMP-9 secretion.

Acknowledgements

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Disclosure of conflict of interest

None.

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