

IL-18 binding protein-expressing mesenchymal stem cells improve myocardial protection after ischemia or infarction

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IL-18 is a proinflammatory cytokine known to cause tissue injury by inducing inflammation and cell death. Increased levels of IL-18 are associated with myocardial injury after ischemia or infarction. IL-18-binding protein (IL-18BP), the naturally occurring inhibitor of IL-18 activity, decreases the severity of inflammation in response to injury. In the present study, mesenchymal stem cells (MSCs) derived from mice transgenic for over expression of human IL-18BP were tested in rat models of global myocardial ischemia and acute myocardial infarction. Improved myocardial function is associated with production of VEGF, and in vitro, IL-18BP MSCs secreted higher levels of constitutive VEGF compared to wild-type MSCs. Whereas IL-18 increased cell death and reduced VEGF in wild-type MSCs, IL-18BP MSCs were protected. In an isolated heart model, intracoronary infusion of IL-18BP MSCs before ischemia increased postischemic left ventricular (LV) developed pressure to 79.5 ± 9.47 mmHg compared to 59.3 ± 7.8 mmHg in wild-type MSCs and 37.8 ± 5 mmHg in the vehicle group. Similarly, using a coronary artery ligation model, intramyocardial injection of IL-18BP MSCs improved LV ejection fraction to $67.8 \pm 1.76\%$ versus wild-type MSCs ($57.4 \pm 1.33\%$) and vehicle ($39.2 \pm 2.07\%$), increased LV fractional shortening 1.25-fold over wild-type MSCs and 1.95-fold over vehicle, decreased infarct size to $38.8 \pm 2.16\%$ compared to $46.4 \pm 1.92\%$ in wild-type MSCs and $60.7 \pm 2.2\%$ in vehicle, reduced adverse ventricular remodeling, increased myocardial VEGF production, and decreased IL-6 levels. This study provides the concept that IL-18BP genetically modified stem cells improve cardioprotection over that observed with unmodified stem cells.

IL-1 family | inflammation | vascular endothelial growth factor

Stem cells are a promising therapeutic modality for myocardial ischemia (1). Mesenchymal stem cells (MSCs) in particular have been shown to protect the heart from ischemic injury (2). It is unclear how much the benefit of MSCs is due to stem cell differentiation into cardiomyocytes or due to the paracrine signaling associated with stem cells (3, 4). However, a growing body of evidence indicates a relatively low rate of stem cell differentiation following transplantation, suggesting that the paracrine action of stem cells may be of greater importance to stem cell mediated cardioprotection (5, 6). Indeed, we have reported that preischemic infusion of MSCs into isolated rodent hearts improved myocardial function after 25 min of ischemia followed by 40 min of reperfusion, indicating that MSC cardiac protection is achieved acutely without cell differentiation (7).

The function and survival of transplanted MSCs are likely limited due to the inflammatory environment of the injured heart. However, genetic modification of MSCs may partially mitigate this problem. For example, MSCs engineered to overexpress myocardial protective factors such as VEGF and protein kinase B (Akt) exhibit significantly improved survival and enhanced cardiac protection (8, 9). Genetic modification of stem cells, therefore, may offer a means by which the protective characteristics of the stem cell can be optimized for clinical use.

Although recognized for its role with IL-12 in the induction of IFN gamma (IFN γ), in the absence of IL-12 and other immunostimulatory cytokines, IL-18 is itself a uniquely proinflammatory cytokine (10) and participates in myocardial suppression and the atherosclerotic process (11, 12). In addition, IL-18 induces the Fas ligand causing cell death (13). Furthermore, increased IL-18 expression has been observed in the hearts subjected to ischemia (11, 14). Therefore, elevated levels of IL-18 following myocardial infarction (MI) may attenuate the protective effects of MSCs.

IL-18-binding protein (IL-18BP) is the naturally occurring inhibitor of IL-18 that exhibits a higher affinity for IL-18 than that of the IL-18 cell surface receptor (15). In fact, the balance between free IL-18 and IL-18BP affects the severity of some inflammatory diseases (16). Transgenic mice overexpressing human IL-18BP (IL-18BP Tg) produce high levels of bioactive IL-18BP in the circulation providing protection against inflammatory stimuli (17). However, it remains unknown whether stem cells engineered to overexpress IL-18 BP (IL-18BP MSCs) provide greater cardiac protection against ischemia compared to wild-type (WT) MSCs. Therefore, the purposes of this study were to determine whether overexpression of IL-18BP improves MSC function and enhances MSC-mediated cardiac protection after acute ischemia/reperfusion (I/R) or infarction.

Results

IL-18BP Counteracted the Down-Regulation of IL-18 on MSC VEGF Production. Supernatants from WT and IL-18BP MSCs were analyzed for IL-18BP secretion. Levels of IL-18BP were undetectable in the WT MSC supernatants but high in IL-18BP MSC medium ($13,611 \pm 1,159$ pg/mL). During acute myocardial ischemia, substantial amount of IFN γ is produced (18, 19). Therefore, we examined the effects of IFN γ on the induction of IL-18BP production in IL-18BP MSCs. In WT MSCs exposed to recombinant IFN γ , levels of IL-18BP secretion were still undetectable. In IL-18BP MSCs, however, 10 and 100 ng/mL, but not 1 ng/mL recombinant IFN γ increased IL-18BP production 1.28-fold ($17,230 \pm 1,035$ pg/ml, $P = 0.006$) and 1.35-fold ($18,134 \pm 455$ pg/mL, $P < 0.001$) over constitutive levels ($13,450 \pm 293$ pg/mL), respectively. With respect to importance of VEGF in MSC-mediated protection, constitutive VEGF production was measured in WT and IL-18BP MSCs. Basal levels of VEGF

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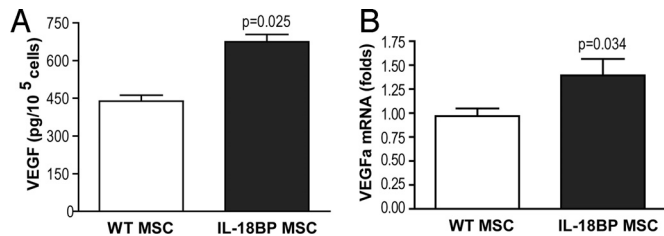


Fig. 1. Constitutive VEGF expression in wild-type and IL-18BP MSCs. (A) Constitutive levels of VEGF secretion. After 24-h incubation, cell supernatants from wild-type and IL-18BP MSCs were collected and measured for VEGF levels by ELISA (Mean \pm SEM, $n = 3$ individual experiments). (B) Steady-state mRNA levels of VEGF. Total RNA was isolated from wild-type and IL-18BP MSCs after 24-h incubation and analyzed for VEGF mRNA by RT real-time PCR. (Mean \pm SEM, $n = 4$ individual experiments).

production and VEGF mRNA were higher in IL-18BP MSCs compared to WT cells (Fig. 1).

Although similar levels of basal IL-18 were measured in both lysates and supernatants from the WT and IL-18BP MSCs, incubation with exogenous IL-18 decreased VEGF production in WT MSCs (Table S1). In contrast to WT MSCs, VEGF production was unchanged in IL-18BP MSCs exposed to recombinant IL-18.

Effects of TNF α , LPS or Hypoxia on MSC VEGF Production. Transplanted stem cells face a hostile environment to local TNF α and hypoxic conditions in ischemic tissues as well as to LPS during endotoxemia. Thus, we investigated the role of naturally produced IL-18 and these stresses with regard to MSC VEGF production. In WT MSCs, recombinant TNF α increased VEGF production 1.7-fold over constitutive levels ($P = 0.004$). In IL-18BP MSCs, constitutive production of VEGF was elevated compared to WT MSCs and when stimulated with TNF α , VEGF production increased further. These data suggest that endogenous IL-18 may be a negative regulator of TNF α -induced VEGF. Indeed, when recombinant IL-18 was added to TNF α -stimulated WT MSCs, there was a reduction in VEGF production. This reduction was not observed in IL-18BP MSCs (Fig. 2A). As shown in Fig. 2B and C, similar differences were observed in LPS-treated MSCs as well as MSCs exposed to hypoxia. Thus, endogenous IL-18 appears to exert an inhibitory influence on VEGF production and constitutive production of IL-18BP by IL-18BP MSCs provides for neutralization of IL-18 and reversal of this inhibition.

IL-18 Increased Apoptosis and Decreased MSC Proliferation. Increased cytoplasmic levels of mono- and oligonucleosomes,

indicators of apoptosis, were observed in WT MSCs after exposure to IL-18 for 24 and 48 h (Fig. S1A). In contrast, these levels did not change in IL-18-treated IL-18BP MSCs. A TUNEL assay also indicated that DNA fragments were more than two-fold higher in the WT MSCs after exposure to IL-18 but not in IL-18BP MSCs (Fig. S1B and C). Similarly, decreased MSC proliferation was noted after 24 and 48 h incubation with IL-18 in WT MSCs but not in IL-18BP MSCs (Fig. S1D).

Effects of MAPKs on MSC Function after Exposure to IL-18. In this study, activation of p38 MAPK (phosphorylated-p38 [p -p38]) was observed 2 h after incubation with IL-18 and was maintained until 6 h in WT MSCs (Fig. S2A Upper). However, phosphorylated p38 did not change in IL-18BP MSCs (Fig. S2A Lower). Adding a p38 inhibitor (SB 203580) to WT MSCs decreased IL-18-induced apoptosis (Fig. S2B). However, cell proliferation and VEGF production were not affected (Fig. S2C and D).

ERK1/2 activation was decreased after 6 h of incubation with IL-18 in WT MSCs (Fig. 3A Upper). However, increased ERK1/2 activation at 2 h until 6 h was observed in IL-18BP MSCs in the presence of IL-18 (Fig. 3A Lower). Addition of an ERK1/2 inhibitor (328006) increased apoptosis by 44% and decreased cell proliferation, but did not affect VEGF production in IL-18BP MSCs (Fig. S3B–D).

Increased IL-18 Levels after Myocardial Ischemia. Global I/R increased myocardial IL-18 production 1.7-fold (14.3 pg/mg protein, $P = 0.022$) over normal hearts. In addition, higher levels of IL-18 production were noted in the at-risk area of heart tissue in the group of ischemia + vehicle (13.8 ± 0.95 pg/mg protein, $P = 0.016$) at 28 days post left anterior descending coronary artery (LAD) ligation compared to sham group (8.1 ± 0.58 pg/mg protein).

IL-18BP Expression Increased Stem Cell-Mediated Cardioprotection after Global I/R. We next tested the hypothesis that endogenous IL-18 exerts a detrimental effect on myocardial function. Using the Landendorff isolated heart perfusion model, intracoronary infusion of WT MSCs before global I/R significantly increased post-ischemic recovery of myocardial function as exhibited by improved left ventricular developed pressure (LVDP), the maximal positive and negative values of the first derivative of pressure (\pm dP/dt) at end reperfusion (Fig. 3). Preischemic infusion of IL-18BP MSCs resulted in improved cardiac contractility and compliance following I/R compared to WT MSCs ($P < 0.01$). These protective effects of IL-18BP MSCs on myocardial function were observed during the early period of reperfusion as shown by increased recovery of \pm dP/dt at 10 min of reperfusion compared to WT MSCs ($P < 0.01$) (Fig. 3C

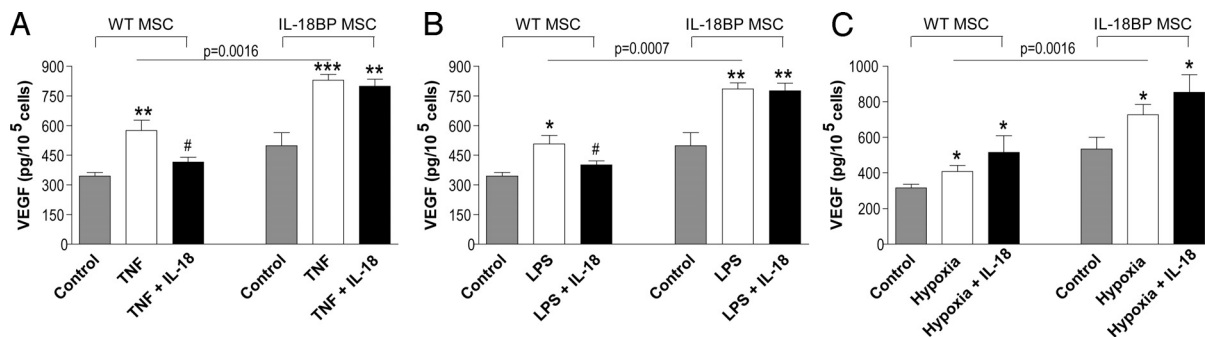


Fig. 2. VEGF production in wild-type and IL-18BP MSCs exposed to TNF α , LPS, or hypoxia. MSCs from wild-type and IL-18BP transgenic mice were stimulated with 50 ng/mL TNF α (A), 100 ng/mL LPS (B), or 1% O $_2$ -hypoxia (C) in the absence or presence of IL-18 (100 ng/mL). After 24 h of incubation, cell supernatants were collected for the measurement of VEGF production by ELISA. (Mean \pm SEM, $n = 3$ individual experiments, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. control; #, $P < 0.05$ vs. TNF or LPS)

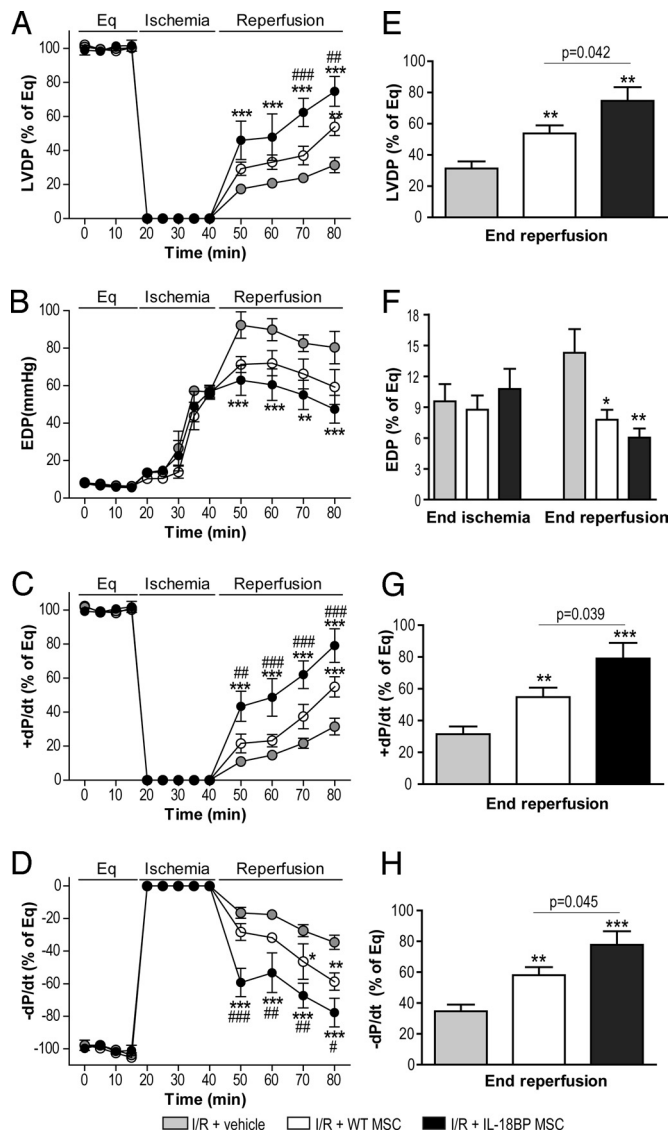


Fig. 3. Effect of IL-18BP MSCs on posts ischemic myocardial function following acute global I/R. 1×10^6 MSCs from WT and IL-18BP Tg mice were infused over 1 min immediately before inducing global ischemia. The left ventricular function in vehicle (shaded, $n = 6$ rat hearts), WT MSCs (open, $n = 5$), and IL-18BP MSCs (solid, $n = 6$) is presented. Left ventricular function parameters over time included LVDP (% of equilibration [Eq], A), EDP (B), +dP/dt (% of Eq, C), and -dP/dt (% of Eq, D). Recovery at end reperfusion is indicated in bar graph form: LVDP (E), EDP (folds of Eq; F), +dP/dt (G), and -dP/dt (H). All results are reported as the mean \pm SEM. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. I/R + vehicle; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ vs. I/R + WT MSCs).

and D). These results demonstrated that IL-18BP MSCs provided a faster and greater protection of the myocardium from I/R. Notably, the value of end-diastolic pressure (EDP), an index of myocardial injury, progressively increased following ischemia and was similar in each of the three groups at the end of ischemia (Fig. 3F), indicating similar ischemia-induced injury. IL-18BP MSC pretreatment decreased the value of EDP during the reperfusion compared to vehicle group. This suggests that IL-18BP MSCs did not reduce ischemic injury, but rather protected the heart after endogenous IL-18 produced by the induction of ischemia or alternatively, the mechanism of protection may require more time to attain a measurable effect.

I/R induced proinflammatory cytokines such as $TNF\alpha$, IL-1 β , and IL-6 play an important role in worsening myocardial func-

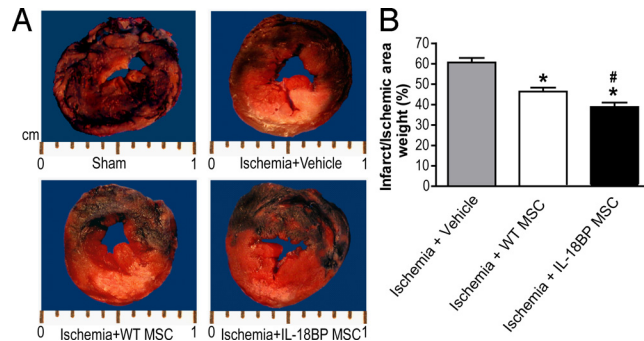


Fig. 4. Effect of IL-18BP MSC on left ventricular infarct size after LAD ligation. Ten minutes after ligation, a solution of 100 μ L PBS (vehicle), WT MSCs, or IL-18BP MSCs (1×10^7 cells/mL) was injected into the myocardium at two sites around the infarct border zone. (A) Representative transverse sections of rat hearts at 28 days post-LAD ligation in sham, ischemia + vehicle, ischemia + WT MSC and ischemia + IL-18BP MSC groups. The non-ischemic area (blue region) was stained with 2% Evans Blue Dye. The at-risk area (red region) was stained with 1% TTC. The infarct area (pale/white zone) did not stain with Evans or TTC. (B) The percentage of the infarct area weight to the ischemic area weight is represented. The results are the mean \pm SEM. ($n = 6$ rats/group; *, $P < 0.05$ vs. ischemia + vehicle; #, $P < 0.05$ vs. ischemia + WT MSC).

tion, and therefore we measured myocardial production of these cytokines. Compared to I/R + vehicle, hearts infused with MSCs demonstrated lower levels of $TNF\alpha$ (Fig. S4A). However, infusion of IL-18BP MSC significantly decreased myocardial levels of $TNF\alpha$, IL-1 β , and IL-6, and increased VEGF production following acute I/R (Fig. S4).

IL-18BP MSCs Enhanced Cardioprotection after Myocardial Infarction.

We next turned to a model of acute coronary occlusion and infarction. The effects of IL-18BP MSCs on cardioprotection were measured using an in vivo model in which the LAD was ligated to induce an MI. Twenty-eight days postoperatively, a visible difference in infarct size was noted among the vehicle, WT MSC and IL-18BP MSC groups (Fig. 4A), whereas the ischemic area (risk area + infarct area) caused by LAD was similar in these groups. Myocardial injection of WT MSCs decreased myocardial infarct size to 46.4% compared to vehicle 60.7%, with significantly further reduction to 38.8% in infarct size noted in the IL-18BP MSC group (Fig. 4B).

Ventricular function as measured by left ventricular (LV) ejection fraction (EF) and fractional shortening (FS) was significantly improved in the MSC groups with greater protection provided by IL-18BP MSCs (Table 1). Improved myocardial function was also observed using the Langendorff model in which findings demonstrated greater cardiac function (LVDP and +/- dP/dt) in the IL-18BP MSC treatment group (Fig. 5).

Ventricular remodeling was evident by the increased LV dimensions (end-diastolic/systolic diameter [LVEDD/LVESD]), decreased LV wall thickness (anterior wall thickness at end diastole/systole [AWTd/AWTs]), and decreased relative wall thickness (RWT). Although direct injection of WT MSCs improved primarily the LVESD and RWT parameters, all indices of detrimental LV remodeling were reduced in the IL-18BP MSC group (Table 1).

As shown in Fig. 6, LAD ligation increased myocardial $TNF\alpha$, IL-1 β , and IL-6 levels 28 days after the procedure. WT MSC treatment decreased levels of myocardial $TNF\alpha$ and IL-1 β (Fig. 6A and B); but injection of IL-18BP MSCs also reduced myocardial IL-6 levels (Fig. 6C). Similarly, myocardial VEGF levels were significantly higher in MSC-treated groups but further increased in the IL-18BP MSC group (Fig. 6D). Of note, following global I/R or LAD ligation, treatment with MSCs did

Table 1. Echocardiographic assessment of heart function, ventricular dimension, and wall thickness

	Sham (n = 5)	Ischemia + Vehicle (n = 13)	Ischemia + WT MSC (n = 12)	Ischemia + IL-18BP MSC (n = 12)
EF, %	85.63 ± 2.10	39.19 ± 2.07***	57.43 ± 1.33****	67.84 ± 1.76****#†††
FS, %	56.00 ± 2.60	20.22 ± 1.11***	31.51 ± 1.74****	39.29 ± 1.70****#††
LVEDD, mm	2.34 ± 0.08	6.28 ± 0.20***	5.08 ± 0.23****	3.88 ± 0.24****#††
LVEDD, mm	6.08 ± 0.38	7.95 ± 0.22***	7.55 ± 0.33*	6.74 ± 0.34#
AWTs, mm	2.46 ± 0.11	1.08 ± 0.06***	1.25 ± 0.07***	1.47 ± 0.07****#†
AWTd, mm	1.50 ± 0.12	0.80 ± 0.03***	0.86 ± 0.04***	1.03 ± 0.04****#††
RWT	0.46 ± 0.05	0.25 ± 0.01***	0.32 ± 0.01***	0.38 ± 0.02##†

Data are mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. Sham; #, $P < 0.01$; ##, $P < 0.001$ vs. Ischemia + Vehicle; †, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.001$ vs. Ischemia + WT MSC

not affect myocardial IL-10 production (Fig. S5), which has been shown to inhibit synthesis of $TNF\alpha$, IL-1 β , and IL-6. This suggested that IL-10 is not responsible for the decreased production of proinflammatory cytokines observed in the IL-18BP MSC group.

Discussion

There is a growing recognition that ex vivo genetic modification of stem cells before transplant into myocardium may enhance survival of the donor cells as well affect host myocytes associated with increased neovascularization and improved cardiac function (8, 9, 20). Since IL-18 is a proinflammatory cytokine that induces Fas ligand (13), it would expect that endogenous IL-18 decreases the function and survival of MSCs and cardiomyocytes. IL-18BP is a naturally occurring inhibitor of IL-18 activity with an affinity greater than that of its receptor (15, 21). IL-18BP Tg mice were generated to overexpress human IL-18BP to study the biological and physiological effects of endogenous IL-18 in models of inflammation and autoimmunity (15, 17, 21). In the present study, we compared MSCs from WT and IL-18BP Tg mice to determine possible benefits of these genetically modified MSCs in the setting of myocardial I/R and infarction. The data demonstrated that: 1) IL-18BP MSCs constitutively secrete high levels of IL-18BP (13,611 pg/mL) associated with higher basal levels of VEGF compared to WT MSCs; 2) IL-18BP MSCs provided greater cardiac protection compared to WT MSCs in rodent models of global I/R and MI.

The IL-18BP modified stem cells may have provided increased cardioprotective effects via paracrine signaling. As previously reported, VEGF is a paracrine mediator of MSC-mediated cardiac protection (5, 7, 22). Delivery of MSCs into the ischemic myocardium increases local VEGF levels and thereby improves myocardial function (23). Bone marrow cells genetically modified to overexpress VEGF have also been reported to provide greater repair of cardiac function (8). Conversely, silencing VEGF in MSCs using siRNA abolished MSC-mediated cardioprotection in ischemic hearts (7).

In the present study, higher levels of constitutive as well as $TNF\alpha$ - and LPS-inducible VEGF production were observed in IL-18BP MSCs, indicating that these MSCs may provide greater protection against myocardial ischemia compared to WT MSCs. Indeed, improved myocardial function was observed in the IL-18BP MSC-treated group following acute I/R. In addition, enhanced cardioprotection was also observed in the MI model with myocardial implantation of IL-18BP MSCs compared to the WT MSC group. From previous studies, locally produced VEGF not only improves implanted stem cell survival but mediates cardiac protection by reducing apoptosis and decreasing proinflammatory cytokine production (8, 23, 24). Therefore, it was not unexpected that intramyocardial IL-18BP MSC injection improved MSC survival and provided protection in the ischemic hearts to a greater extent than WT MSCs.

After delivery/transplantation into ischemic myocardium, stem cells face a foreign, inflammatory environment that is detrimental toward their survival. IL-18 is constitutively expressed in various types of cells and is involved induction of apoptosis (13, 25). Elevated IL-18 levels have been observed in ischemic myocardium (11, 14). Indeed, our current study also indicated that ischemic injury was able to induce more myocardial IL-18 secretion. In addition, decreased cell proliferation and reduced VEGF production were observed in WT MSCs after exposing the cells to exogenous IL-18. Hence, MSCs modified to overexpress IL-18BP have greater resistance to the inflammatory/ischemic environment, confirmed by the findings that IL-18BP MSCs conferred greater benefit after global I/R. In addition, markedly increased LV function in the setting of MI and decreased infarct size were also observed.

During and after MI, the myocardium produces substantial amounts of proinflammatory cytokines including $TNF\alpha$, IL-1 β , and IL-6. These proinflammatory cytokines lead to cardiomyocyte apoptosis, negative inotropy, and ventricular remodeling (26–29). In this study, myocardial ischemia increased levels of these cytokines while MSC treatment decreased myocardial $TNF\alpha$ and IL-1 β levels. However, only IL-18BP MSCs significantly decreased myocardial IL-6, which has been demonstrated

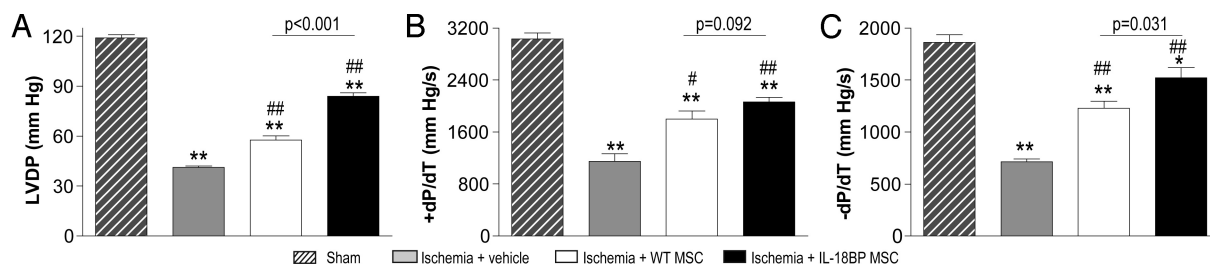


Fig. 5. Effect of IL-18BP MSCs on left ventricular function at 28 days post-LAD ligation. The left ventricular function was measured in the sham (n = 5), ischemia + vehicle (n = 6), ischemia + WT MSC (n = 6), and ischemia + IL-18BP MSC (n = 6) groups using isolated heart perfusion system. (A) LVDP, (B) +dP/dt, and (C) -dP/dt. The results are the mean ± SEM. (*, $P < 0.05$; **, $P < 0.001$ vs. sham; #, $P < 0.01$; ##, $P < 0.001$ vs. ischemia + vehicle).

PowerPC computer (Apple Computer Inc.). For details and MSC infusion, please see the [SI Text](#).

Myocardial Infarction Model. Myocardial infarction was induced as previously described in the literature by ligating the LAD between the main pulmonary artery trunk and left atrial appendage (34). Please see the [SI Text](#) for details of procedures.

Myocardial Function and Dimensions. Parasternal short-axis two-dimensional M-mode echocardiograms (VisualSonics Vevo-770) of the LV at the level of the papillary muscle were obtained in all animals on post-operative day 28. FS, EF, LVEDD/LVESD, AWTd/AWTs, and posterior wall thickness at end diastole (PWTd) were measured over three adjacent cardiac cycles. RWT was calculated according to the following formula: $RWT = 2 \times PWTd/LVEDD$ (35).

Isolated heart function was also evaluated using the Langendorff perfusion system as previously described (7).

Measurement of Infarct and At-Risk Areas. The measurement of infarct size and risk area was performed as described previously (36). Intravenous injection of 1 mL of 2% Evans Blue Dye (Sigma) was used to identify the viable area of the myocardium. The heart was sectioned and incubated in 1% triphenyltetrazo-

lium chloride (TTC, Sigma). TTC stained the at-risk area of the myocardium red. The infarct size was calculated as the percentage of the infarct area weight to the ischemic area weight using the following formula: $\text{Infarct area weight/Ischemic area weight (\%)} = 100\% \times \frac{\text{infarct area weight of each slice}}{[\sum (\text{risk area} + \text{infarct area weight of each slice})]}$ (36).

Myocardial Cytokine Production. Myocardial levels of rat IL-18, VEGF, $TNF\alpha$, IL-1 β , IL-6, and IL-10 after acute I/R and in the at-risk area at post-operative day 28 were determined by ELISA (R&D Systems; Biosciences).

Presentation of Data and Statistical Analysis. Data were compared using one-way analysis of variance (ANOVA) with post-hoc Tukey test or Student's *t* test.

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