

## Statin Administration Does Not Improve the Mobilization of Very Small Embryonic-Like Stem Cells (VSELs) in Contrast to Resveratrol Treatment in a Murine Model of Acute Myocardial Infarction

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### Summary

We have found that short-term statin treatment plus stem cell transplantation in acutely infarcted hearts improves cardiac function because statins promote the efficacy of cellular cardiomyoplasty. Autologous Sca-1<sup>+</sup>Lin<sup>-</sup>CD45<sup>+</sup>(CXCR<sup>+</sup>) very small embryonic-like stem cell (VSEL) mobilization in acute myocardial infarction (AMI) correlates with the preservation of cardiac function. Whether short-term atorvastatin (Ato) can enhance the mobilization or recruitment of VSELs in AMI is still unclear. We divided mice into 4 groups: 1) sham; 2) AMI; 3) AMI+resveratrol (RSV) as a positive control; and 4) AMI+Ato. There was an increase in the circulating VSEL/full population of leukocytes (FPL) ratio 48 hours after AMI, and AMI+RSV increased it further. Ato administration did not increase the VSEL/FPL ratio. The cardiac stromal cell-derived factor-1 (SDF-1) and SDF-1 $\alpha$  levels were in agreement with the results of VSEL mobilization. One week after AMI, more Sca-1<sup>+</sup>CXCR<sup>+</sup> cells were recruited to the myocardium of AMI+RSV mice but not AMI+Ato mice. Short-term Ato administration failed to upregulate cardiac SDF-1 and could not enhance the recruitment of VSELs early after AMI.

### Key words

Very small embryonic-like stem cells • Acute myocardial infarction  
• Atorvastatin • Stromal cell-derived factor-1

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Stem cell-based therapies are promising procedures for myocardium regeneration (Singh *et al.* 2009) after acute myocardial infarction (AMI), and these therapies include stem cell mobilization and transplantation. The mobilized cells can participate in organ repair naturally or can be concentrated *in vitro* for transplantation. Although the transplantation of bone marrow-derived stem cells (BM-SCs) has been proven to be safe and effective, improvement of the left ventricular ejection fraction (LVEF) is still limited. We have confirmed that one week of statin treatment in AMI improves the cardiac micro-environment created by AMI, facilitates the survival and differentiation of implanted stem cells, and increases LVEF compared with stem cell transplantation models (Yang *et al.* 2008). However, whether the beneficial effects of statins on the cardiac function improvement in these experiments are partially mediated by mobilizing myogenic stem cells is still unclear.

The BM-SCs released into peripheral blood after AMI consist of hematopoietic stem cells (HSCs, approximately 40 % of CD34<sup>+</sup> HSCs are CXCR4<sup>+</sup>) (Wu *et al.* 2009), endothelial progenitor cells (EPCs,

approximately 20 % of which are CXCR4<sup>+</sup>) (Garolla *et al.* 2009), and very small embryonic-like stem cells (VSELs) (Wojakowski *et al.* 2009, Zuba-Surma *et al.* 2008). HSCs and EPCs are possibly progenitor cells that arise from VSELs (Bhartiya *et al.* 2012), which have recently been identified in mice and humans as populations of very small Sca-1<sup>+</sup>Lin<sup>-</sup>CD45<sup>-</sup> cells (82 % of which are CXCR4<sup>+</sup>) and very small CD34<sup>+</sup>CD133<sup>+</sup>Lin<sup>-</sup>CD45<sup>-</sup>(CXCR4<sup>+</sup>) cells, respectively. VSELs exhibit better myocardium differentiation and beneficial effects on improving cardiac function after AMI (Dawn *et al.* 2008, Wojakowski *et al.* 2009, Wojakowski *et al.* 2010, Zuba-Surma *et al.* 2008).

The stromal cell-derived factor-1 (SDF-1)/CXCR4 (the receptor of SDF-1) axis may play an essential role in the mobilization of VSELs from the BM. SDF-1 attracts VSELs in direct chemotactic studies, and the number of circulating VSELs in MI patients positively correlates with the plasma level of SDF-1 (Kucia *et al.* 2006, Wojakowski *et al.* 2009). We have found that resveratrol (RSV) enhances VSEL mobilization in the early stage of AMI *via* upregulating cardiac SDF-1 (Wang *et al.* 2011). Whether statins have similar effects as RSV is unknown. To clarify the effect of statins on VSEL mobilization in AMI, we used AMI+RSV mice as a positive control and examined the effect of atorvastatin (Ator).

The experimental design was approved by the Ethics Committee of the Chinese Academy of Medical Sciences and Peking Union Medical College. Animal care and experimental procedures were conducted in accordance with the European Guidelines on Laboratory Animal Care. Male C57BL/6 mice that were 8-10 weeks old were randomly divided into sham; AMI; AMI+RSV (Sigma, 25 mg/kg/day in drinking water, one week); and AMI+Ator (Pfizer, 25 mg/kg/day in diet, one week) groups. Because VSEL mobilization peaks at 48 hours after AMI and Sca-1<sup>+</sup> cell recruitment in the infarcted area is obvious at one week after AMI (Zuba-Surma *et al.* 2008, Zhang *et al.* 2007), mice were sacrificed at these two time points.

After five days of drug loading, mice were anesthetized with an intraperitoneal injection of chloral hydrate (20 ml/kg), endotracheally intubated by tracheotomy, and mechanically ventilated using the Inspira-Advanced Safety Ventilator (Harvard Corp.). After exposing the heart *via* a left thoracotomy and removing the pericardium, the left anterior descending coronary artery (LAD, 2 mm below the tip of the left

auricle) was occluded with an 8.0 Prolene suture (ETHICON, Inc.). Occlusion was confirmed by observing the LV pallor immediately after ligation or the measurement of serum creatine kinase MB isoenzyme (CK-MB) 48 hours after AMI. Serum cholesterol was evaluated together with CK-MB (Beckman Coulter automatic biochemical analyzer, n=4-5 in each group).

Flow cytometry analysis and VSEL sorting were performed as described previously (Zuba-Surma *et al.* 2008), with minor modifications. The full population of leukocytes (FPLs, n=6-8 in each group) in the arterial blood (500  $\mu$ l) was obtained using erythrocyte lysis buffer (Becton Dickinson Pharmingen) and stained for lineage cocktail (APC), CD45 (PE), and Sca-1 (FITC) according to the manufacturer's instructions (Miltenyi Biotec.). The cell sizes of the FPLs were determined by the AccuCount Blank particles ACBP-20-10 (2  $\mu$ m) and ACBP-100-10 (10  $\mu$ m) (Spherotech, Inc.) using flow cytometry (Becton Dickinson).

LVs were isolated after drawing blood and kept at -80 °C until use. Protein (n=4 in each group) was extracted from LVs through homogenization in RIPA buffer. Western blotting was performed with an anti-SDF-1 (Cell Signaling Technology) antibody. Signals were quantified with Scion Image Software and standardized to the expression of  $\beta$ -actin (Sigma). SDF-1 $\alpha$  is a predominant isoform of SDF-1 (Wen *et al.* 2012) and was measured with a mouse ELISA kit (RayBiotech Inc.). Approximately 0.05 g of infarct (approximately 0.02 g) and peri-infarct tissues each were cut out of the LVs, washed, homogenized in ice-cold 0.9 % NaCl solution, and centrifuged. The clear supernatant was kept on ice for the assay (n=8 in each group) according to the manufacturer's instructions.

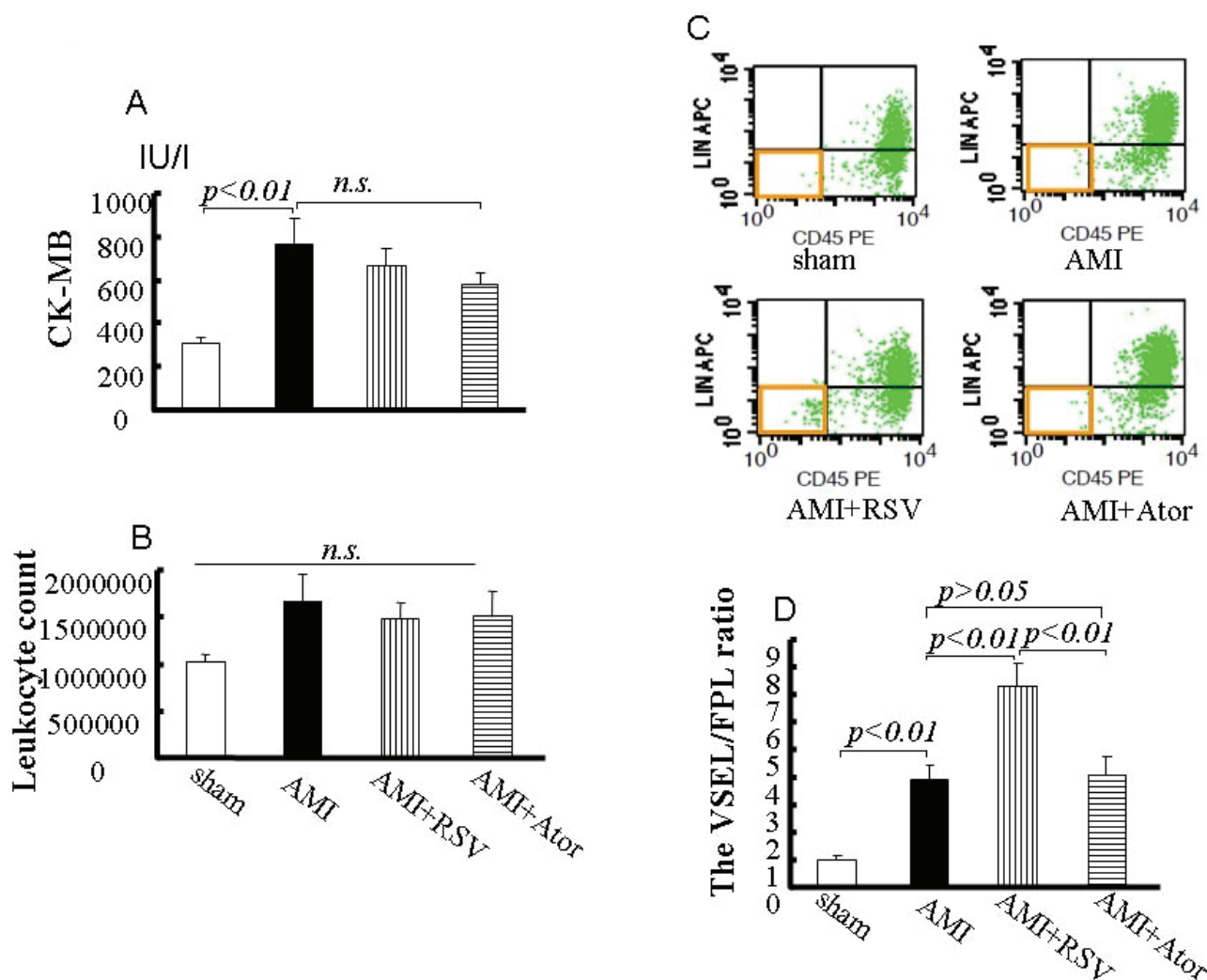
One week after AMI, frozen sections of the heart (n=3-4 in each group) were immunostained with anti-Sca-1 (1:10, Miltenyi Biotec.) and anti-CXCR4 (1:100, Abcam) antibodies. Cells that were double positive for CXCR4 and Sca-1 proteins were counted in 15 high-power fields (HPF,  $\times$ 400) in the infarct and peri-infarct regions.

All values were expressed as the means  $\pm$  SEMs. The statistical analysis was carried out with an ANOVA followed by Fisher's method. A value of  $p < 0.05$  was considered significant.

After AMI, CK-MB increased (sham: 306.00 $\pm$ 30.13 IU/l; AMI: 766.60 $\pm$ 116.97; AMI+RSV: 664.20 $\pm$ 84.73; AMI+Ator: 579.00 $\pm$ 52.54, Fig. 1A), but the cholesterol levels showed no difference (sham:

2.22±0.17 mmol/l; AMI: 2.19±0.06; AMI+RSV: 2.47±0.23; AMI+Ator: 2.09±0.15). FPLs were counted during flow cytometry analysis. The number of leukocytes increased after AMI, although not significantly (Fig. 1B). To avoid the influence of transient leukocytosis, the VSEL/FPL ratio was analyzed, and it was increased approximately 4 (3.89±0.53) times in the AMI group and nearly 7 (7.35±0.84) times in the AMI+RSV group. However, AMI+Ator (VSEL/FPL: 4.07±0.65) did not mobilize more VSEs (Fig. 1C, D). Compared with sham mice, the cardiac SDF-1 expression

in AMI mice increased. AMI+RSV mice had the highest cardiac SDF-1, and AMI+Ator mice failed to show increased levels (Fig. 2A, B). The levels of SDF-1 $\alpha$  in infarct and peri-infarct tissues of each group (sham: 187.43±5.32 pg SDF-1 $\alpha$ /mg tissue; AMI: 249.83±16.13; AMI+RSV: 318.39±15.24; AMI+Ator: 251.66±27.85, Fig. 2C) were consistent with the results of western blotting. Compared with AMI mice (0.53±0.19/HPF), Sca-1<sup>+</sup>CXCR4<sup>+</sup> cells in the myocardium of AMI+RSV mice (1.47±0.32) but not AMI+Ator (0.40±0.19) mice were increased (Fig. 2D).



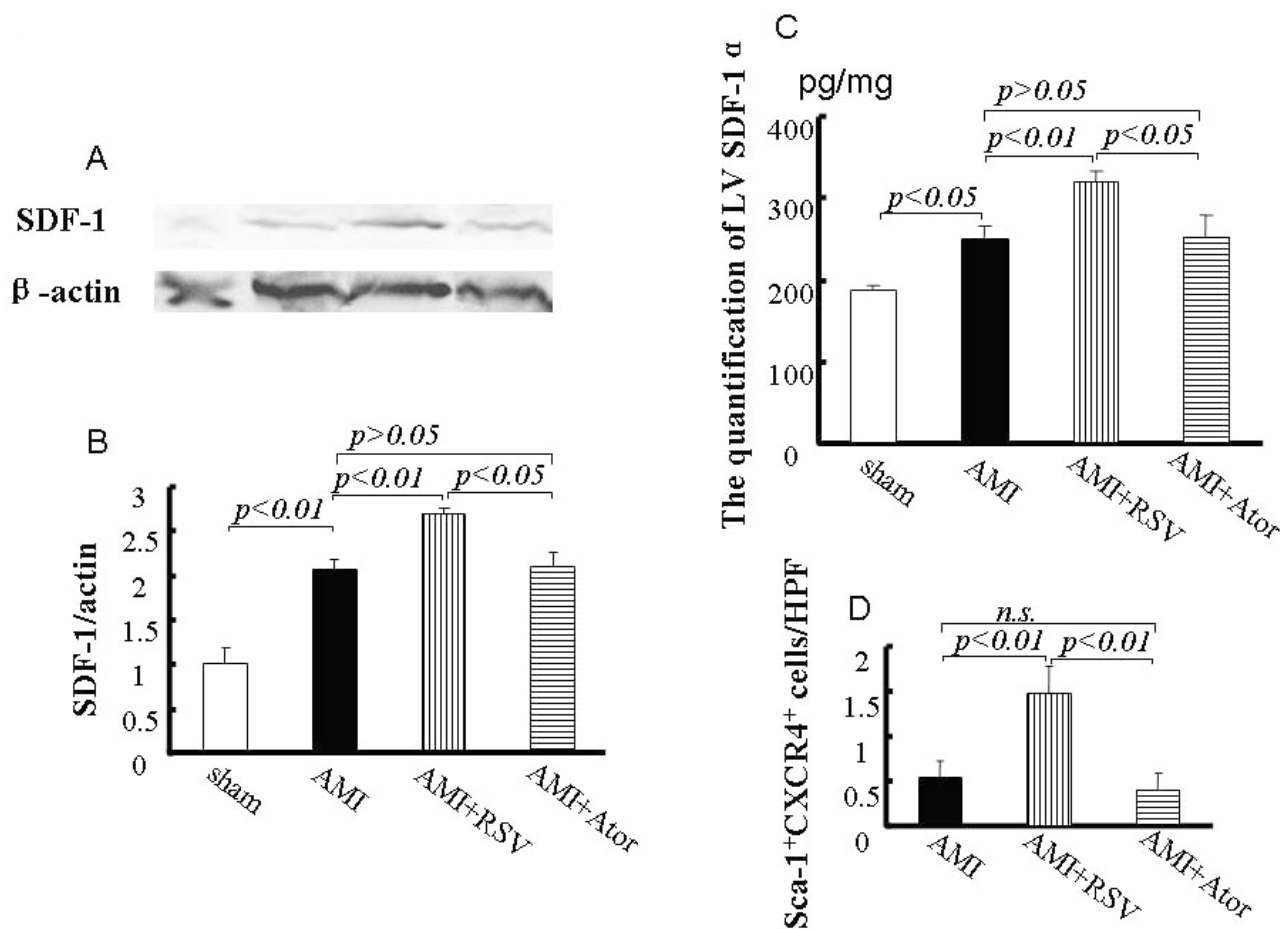
**Fig. 1.** Isolation and count of circulating VSEs after AMI. **A.** CK-MB increased in the mice with LAD ligation. **B.** FPL count during flow cytometry analysis. **C.** Representative flow cytometry images of the sham, AMI, AMI+RSV, and AMI+Ator groups. Flow cytometry analysis was based on the size and immunophenotype of the VSEs. The leukocytes sized 2-10  $\mu$ m and positive for Sca-1 were identified first, then Lin<sup>+</sup>CD45<sup>+</sup> VSEs (orange squares) were selected. **D.** Circulating VSEs standardized by the number of FPLs. n.s.: no significant difference among all groups.

VSEs mobilize to peripheral blood after AMI as a self-repair approach, and the number of circulating VSEs correlates positively with LVEF (Wojakowski *et al.* 2009). For various reasons, SDF-1 expression is

increased in cardiac tissue soon after myocardial infarction and sustained for 5-7 days following AMI (Wang *et al.* 2006, Wen *et al.* 2012). Consistently, VSEL mobilization reaches a peak at 48 hours and returns to

control levels at 7 days after AMI (Kucia *et al.* 2004, Zuba-Surma *et al.* 2008). Statins can mobilize EPCs under different conditions, including normal conditions, acute vascular injuries, cardiac ischemia, obesity and chronic heart failure. Moreover, short-term statin loading is sufficient to increase EPC mobilization (Hibbert *et al.* 2011, Hong *et al.* 2010, Shao *et al.* 2008, Thum *et al.* 2006, Tousoulis *et al.* 2011, Westerweel *et al.* 2008). Although statins can influence the mobilization of SCs, a recent report on VSEL mobilization did not perform a

subgroup analysis of whether statins were taken (Wojakowski *et al.* 2009). These results motivated us to determine whether one week of statin treatment mobilized VSELs at the same time as it improved cardiac micro-environments created by AMI (Yang *et al.* 2008). Unfortunately, in the current study, we found that short-term Ator failed to enhance the mobilization of VSELs early after AMI (5 mg/kg/day Ator to AMI mice showed similar result).



**Fig. 2.** SDF-1 evaluation and the detection of Sca-1 $^{+}$ CXCR4 $^{+}$  cell recruit in cardiac tissue. **A.** Representative immunoblots of SDF-1 in LV. **B.** The bar graph showed the quantification of the immunoblots. **C.** The quantification of SDF-1 $\alpha$  (infarct and peri-infarct areas) by ELISA. **D.** Sca-1 $^{+}$ CXCR4 $^{+}$  cells in the infarct and peri-infarct regions/HPF.

Consistent with the previous report (Hsu *et al.* 2010), we found that SIRT1 expression in infarcted LV was decreased (Wang *et al.* 2011). p53 acts upstream of SDF-1. SIRT1 inhibition activates p53 and thus hinders the upregulation of SDF-1 (Addadi *et al.* 2010, Wang *et al.* 2011 and 2012, Olive *et al.* 2008) after AMI. Through SIRT1 activation, RSV increases SDF-1 and the mobilization of VSELs. However, short-term Ator administration in AMI did not improve the expression or

secretion of SDF-1 in the infarcted heart.

VSELs are usually quiescent in the adult BM. A hypomethylation of paternally methylated imprints and a hypermethylation of maternally methylated ones are observed at some crucial developmentally genes in isolated BM VSELs. This modification leads to the upregulation of growth-repressive genes and the repression of growth-promoting genes (Shin *et al.* 2009). Therefore, the rapid expansion of VSELs is still difficult.

In contrast, the expansion of EPCs is much easier. Many studies suggest that statins promote the proliferation/differentiation and inhibit the death of EPCs (Shao *et al.* 2008, Thum *et al.* 2006). A step-forward multivariate linear regression analysis found that EPC number was inversely related to plasma SDF-1 $\alpha$  (Xiao *et al.* 2007). More interestingly, a study in pulmonary artery injury showed that statins downregulated SDF-1 (Satoh *et al.* 2009). All of these results support that the mechanism of EPC mobilization is potentially different from VSEL mobilization.

The result of the current study is in accordance with our previous report that one week of Ator treatment (0.25 mg/kg/day without stem cell transplantation in swine) had little effect on cardiac regeneration (Yang *et al.* 2008). However, the beneficial activities of statins on chronic systolic cardiac insufficiency (Zhang *et al.* 2011) are undoubted. The pleiotropic effects of statins, such as

the protection of endothelial function, antioxidant effect, anti-inflammatory reaction and the prevention of apoptosis, are important for cardiac function recovery.

### Conflict of Interest

There is no conflict of interest.

### Acknowledgements

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