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Synergy between CD26/DPP-IV Inhibition and G-CSF Improves Cardiac Function after Acute Myocardial Infarction

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SUMMARY

Ischemic cardiomyopathy is one of the main causes of death, which may be prevented by stem cell-based therapies. SDF-1α is the major chemokine attracting stem cells to the heart. Since SDF-1α is cleaved and inactivated by CD26/dipeptidylpeptidase IV (DPP-IV), we established a therapeutic concept—applicable to ischemic disorders in general—by combining genetic and pharmacologic inhibition of DPP-IV with G-CSF-mediated stem cell mobilization after myocardial infarction in mice. This approach leads to (1) decreased myocardial DPP-IV activity, (2) increased myocardial homing of circulating CXCR-4$^+$ stem cells, (3) reduced cardiac remodeling, and (4) improved heart function and survival. Indeed, CD26 depletion promoted posttranslational stabilization of active SDF-1α in heart lysates and preserved the cardiac SDF-1-CXCR4 homing axis. Therefore, we propose pharmacological DPP-IV inhibition and G-CSF-based stem cell mobilization as a therapeutic concept for future stem cell trials after myocardial infarction.

INTRODUCTION

Ischemic disorders in general are the main cause of death in humankind. Among those, ischemic cardiomyopathy following acute myocardial infarction (MI) is the most important (Dickstein et al., 2008). Despite advances in medical treatment and interventional procedures, many patients are waiting for a transplant as their last resort. As loss of cardiac function is the most important prognostic factor, therapeutic approaches to improve myocardial function are warranted. While animal studies using BM-derived stem cells (CD45$^+$, CD34$^+$, c-kit$^+$, Sca-1$^+$, lin$^-$) and endothelial progenitors (CD45$^+$, CD34$^+$, CD31$^+$, CD133$^+$, Flk-1$^+$) showed increased cardiac function and survival after stem cell mobilization and direct myocardial injection (Deindl et al., 2006; Orlic et al., 2001a, 2001b), only some of the human trials reported beneficial effects (Ince et al., 2005; Schachinger et al., 2004; Wollert et al., 2004), whereas others failed to increase left ventricular ejection fraction (Engelmann et al., 2006; Zohlnhofer et al., 2006, 2008). Mechanistically, the original concept of cardiac regeneration by transdifferentiation of BM-derived stem cells to cardiomyocytes (CMs) (Orlic et al., 2001a) was questioned by the identification of paracrine repair mechanisms such as neovascularization and prevention of apoptosis (Balsam et al., 2004; Fazel et al., 2006; Murry et al., 2004; Zaruba et al., 2008). Of note, all of these mechanisms depend on an efficient homing and subsequent engraftment of these cells in the ischemic heart. Therefore, modern approaches have to focus on the process of cardiac homing to improve the clinical outcome of stem cell therapies.

Although several factors like hepatocyte growth factor (HGF) and stem cell factor (SCF) play an important role during stem cell engraftment into ischemic tissue in general, the main axis of homing is the interaction between myocardial SDF-1α and the homing receptor CXCR-4, which is expressed on many circulating progenitor cells (Askari et al., 2003; Franz et al., 2003). This homing axis represents a basic mechanism that is not only restricted to the heart. Generally, expression of SDF-1α is increased in ischemic tissue, as it also plays a prominent role, e.g., in apoplexy (Ceradini et al., 2004; Hill et al., 2004; Wang et al., 2008). Thus, SDF-1α is the essential target for any substantial improvement of stem cell homing: SDF-1α emerged in the mid-1990s as a biological ligand for the HIV-1 entry cofactor LESTR (Bleul et al., 1996). It is a 7.97 kDa chemokine, which is secreted from endothelial cells in ischemic tissue (Ceradini et al., 2004) and is a chemoattractant reported for human CD34$^+$ progenitor cells (Aiuti et al., 1997).

SDF-1 binds to CXCR-4 in its active form (1–68) (Crump et al., 1997) and is cleaved at its position 2 proline by CD26/dipeptidylpeptidase IV (DPP-IV), which is a membrane-bound extracellular peptidase (Christopherson et al., 2004). DPP-IV is expressed on many hematopoietic cell populations, including stimulated B and T lymphocytes, endothelial cells, fibroblasts, epithelial cells, and

$CD34^+$ stem cells (Huhn et al., 2000; Kahne et al., 1999; Ruiz et al., 1998; Vanham et al., 1993). Besides, DPP-IV is present in a catalytically active soluble form in plasma (Durinx et al., 2000). Other natural substrates of DPP-IV include the chemokines CCL3, CCL5, CCL11, and CCL22; the glucagon-like peptides; and neuropeptide Y (Christopherson et al., 2002). Previously, it was shown that intramyocardial injection of a modified, MMP-2 and CD26 protease-resistant SDF-1 protein may serve as a therapeutic tool to improve myocardial function and recruit progenitor cells to the heart (Segers et al., 2007). However, safety concerns and the need for invasive protocols limit SDF-1 protein delivery in the ischemic myocardium.

In order to retard the degradation of SDF-1α in a noninvasive manner, different small molecular weight inhibitors of DPP-IV are available, such as Diprotin A (Ile-Pro-Ile) or Val-Pyr. In experimental hematological settings, Diprotin A blocked the activity of DPP-IV and increased the capacity of transmigration of progenitor cells toward an SDF-1α gradient (Christopherson et al., 2004). We aimed to transfer this approach of DPP-IV inhibition to the treatment after acute MI representative for ischemic disorders in general. We hypothesized that DPP-IV inhibition (1) stabilizes active myocardial SDF-1α, (2) leads to enhanced recruitment of circulating CXCR-4-positive stem cells to the ischemic myocardium, (3) reduces cardiac remodeling, and, finally, (4) improves heart function and survival after MI.

Thereupon, we sought proof of concept on a genetic and pharmacological basis. We induced MI via surgical LAD ligation in CD26 knockout (KO) mice as well as Diprotin A-treated wild-type (WT) mice, both in combination with G-CSF-triggered stem cell mobilization. Then we examined DPP-IV activity, myocardial SDF-1 expression on (post-)translational levels, myocardial homing of stem cells, cardiac remodeling, left ventricular function, and finally survival.

RESULTS

Loss of CD26 Function Stabilizes Active SDF-1 Protein in Heart Lysates

Based on the fact that CD26 cleaves dipeptides from the N terminus of the homing factor SDF-1 (Busso et al., 2005), we addressed the question of whether depletion of CD26 increased posttranslational stabilization of intact SDF-1 protein. In the first step, we examined the proteolytic DPP-IV-activity in serum and myocardium 2 days after MI. In contrast to WT, CD26 KO mice revealed no DPP-IV activity in the heart (Figure 1A). Low levels of DPP-IV activity in the serum of KO mice are most likely related to the existence of distinct enzymes displaying DPP-IV-like proteolytic activity (Marguet et al., 2000). Combined application of G-CSF and Diprotin A in WT mice ("G-CSF-DipA mice") lead to a decreased DPP-IV activity after MI only in the myocardium, but not in the serum. In addition, we analyzed the quantity of $CD26^+$ cells in infarcted and noninfarcted hearts. WT animals revealed a significantly increased number of blood-derived $CD45^+/CD26^+$ cells after MI, whereas *CD26*$^{-/-}$ animals showed no detectable $CD26^+$ cell population in the heart (see Figure S1 available online).

Since SDF-1 mRNA is downregulated during G-CSF-induced mobilization in the BM (Semerad et al., 2005), we investigated the possibility of whether SDF-1 is regulated in the heart at a transcriptional level. Neither saline nor G-CSF ± Diprotin A treatment resulted in a significant change of SDF-1α mRNA 2 days after MI (data not shown). Next, protein levels of SDF-1α from tissue lysates of WT and KO hearts were analyzed. Due to oligomerization or different protein modifications, it was difficult to quantitate SDF-1α protein by western blot (Vergote et al., 2006). Therefore, we measured the amount of SDF-1α protein by quantitative ELISA. After MI, SDF-1α was upregulated in WT as well as in *CD26*$^{-/-}$ mice, with no significant differences between the treatment groups (Figure 1B). This may be due to the fact that an enzyme-linked polyclonal antibody was used to visualize SDF-1α, making it impossible to differentiate between the cleaved (3–68) and the intact active forms of SDF-1α (1–68) by this assay. To circumvent this limitation, we performed an experiment with mass spectrometry in which recombinant SDF-1 was incubated with lysates from WT and KO hearts. As shown in Figure 1C, extracts from CD26 KO hearts treated either with saline or with G-CSF after MI revealed one peak at 7.978 kDa representing the active form of 1–68 SDF-1α protein. In contrast, lysates derived from WT animals also showed high abundance of a second peak at 7.748 kDa corresponding to N-terminal cleavage of SDF-1α by DPP-IV. These data clearly show that CD26 depletion promoted posttranslational stabilization of active SDF-1 in heart lysates.

Enhanced Recruitment of $CXCR4^+$ Stem Cells to Myocardium after CD26 Inhibition and Treatment with G-CSF

To show the extent of stem cell mobilization, FACS analyses from peripheral blood (PB) samples of WT and CD26 KO mice were performed. G-CSF treatment of WT (± Diprotin A) and CD26 KO mice revealed a significantly increased mobilization of $CD45^+/CD34^+$ progenitors compared to saline-treated WT and KO controls (Figure S2A). Further analysis revealed high expression of CD26 on hematopoietic cells derived from blood and BM of WT mice (Figures S2B–S2D). Since active SDF-1 chemoattracts angiogenic $CD34^+$ progenitors (Askari et al., 2003; Naiyer et al., 1999), we analyzed the numbers of $CD34^+$ cells in the heart. The hematopoietic marker CD45 was used to track the fate of cells derived from PB. As shown in Figure 2A, sham-operated WT as well as KO animals revealed a small population of cardiac $CD45^+/CD34^+$ cells. After MI, genetic and pharmacological inhibition of CD26 in combination with G-CSF treatment significantly revealed the highest amount of $CD45^+/CD34^+$ progenitors in the heart as compared to other groups (Figure 2A). More than 80% of $CD45^+/CD34^+$ cells derived from hearts of G-CSF-treated *CD26*$^{-/-}$ mice expressed the myocardial homing factor receptor CXCR4 and revealed high coexpression of the stem cell markers c-kit or Sca-1 (Figure 2B). These expression patterns were similar in the other treatment groups after MI (data not shown). In contrast to the heart, $CD45^+/CD34^+$ progenitors obtained from PB revealed a significantly lower expression of CXCR4, suggesting that mainly the $CXCR4^+$ fraction of $CD34^+$ cells migrated from the PB to the heart (Figure 2B). Figures 2C and 2D show that genetic or pharmacological inhibition of CD26 in combination with G-CSF treatment significantly enhanced the recruitment of $CD45^+/CD34^+/c\text{-}kit^+$, $CD45^+/CD34^+/Sca\text{-}1^+$, $CD45^+/CD34^+/CXCR4^+$, and $CD45^+/CD34^+/Flk\text{-}1^+$ progenitor cells as well as $lin^-c\text{-}kit^+Sca\text{-}1^+$ hematopoietic stem cells into ischemic

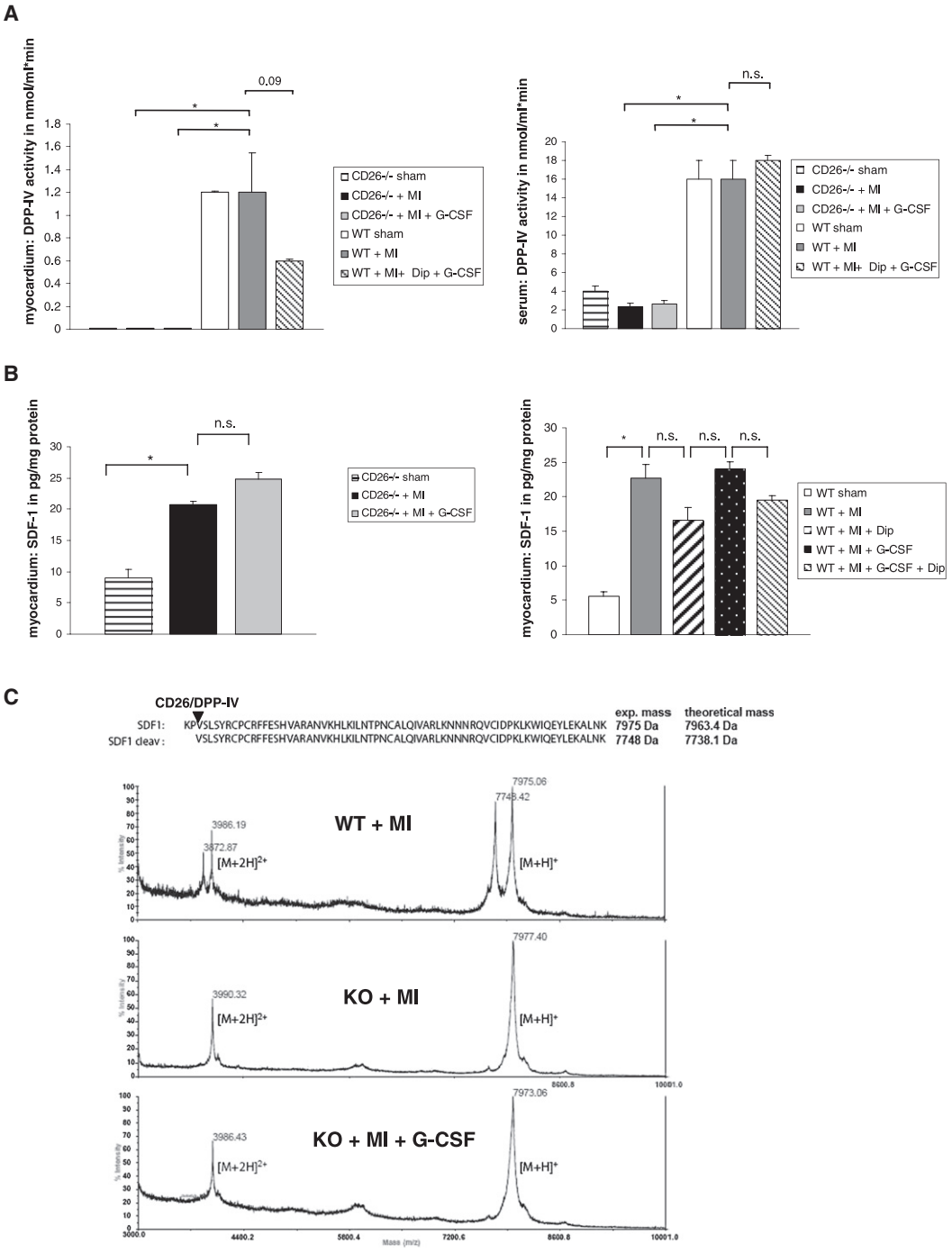

Figure 1. Loss of DPP-IV Activity in G-CSF-Treated CD26 KO Mice Is Associated with Stabilization of Active SDF-1α in Heart Lysates

(A) Diagrams show the activity of DPP-IV in hearts and serum of CD26 KO or WT ± Diprotin A mice 2 days after MI.

(B) Bar graph showing the increase of SDF-1α protein in the hearts of CD26 KO or WT animals after MI by ELISA. Data represent mean ± SEM (n = 3); *p < 0.05; n.s., not significant.

(C) Mass spectrometry demonstrates that full-length recombinant SFD-1 (7.97 kDa) is only cleaved in heart lysates of WT mice, but not in CD26 KO animals after MI.

myocardium. In order to address the question of whether the enhanced migration was regulated via an intact SDF-1-CXCR4 homing axis, we treated G-CSF-stimulated *CD26*$^{-/-}$, WT, or G-CSF-DipA mice with the CXCR4 antagonist AMD3100 (Broxmeyer et al., 2005). Notably, the migration of CD34^{+}/CXCR4^{+} stem cells after MI was only reversed after genetic or pharmacological inhibition of CD26, suggesting preservation of the cardiac SDF-1-CXCR4 homing axis (Figure 2E).

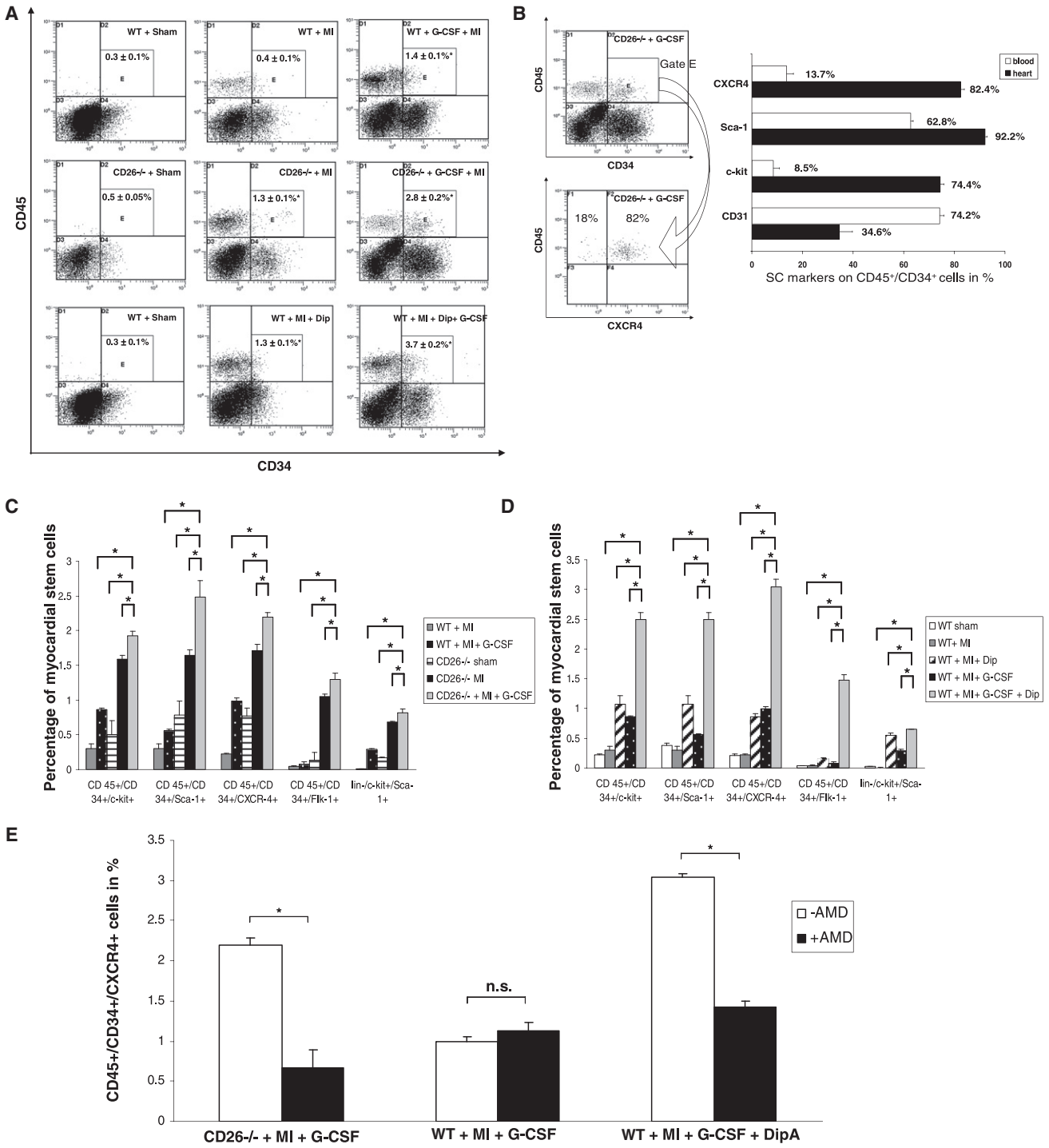

Figure 2. *CD26*$^{-/-}$ and Diprotin A-Treated Mice Reveal Enhanced Numbers of Stem Cells in the Ischemic Heart after G-CSF Application

(A) Representative cardiac FACS analyses showing the mean numbers of CD45$^+$/CD34$^+$ cells within the hearts of WT (upper row), *CD26*$^{-/-}$, or Diprotin A-treated WT mice (lower row) treated either with saline or G-CSF. Data represent mean ± SEM (n = 6); *p < 0.05 versus sham.

(B) (Left) Gating of CD45$^+$/CD34$^+$ cells (gate E) revealed high expression of the homing factor receptor CXCR4 (second row). (Right) Bar graph representing the antigen expression of stem and progenitor markers on CD45$^+$/CD34$^+$ cells obtained from the heart (black bars) compared to blood (white bars) showing that CXCR4 is highly expressed on CD34$^+$ cells in the heart.

(C and D) Histograms representing the percentage of myocardial CD45$^+$/CD34$^+$c-kit$^+$, CD45$^+$/CD34$^+$Sca-1$^+$, CD45$^+$/CD34$^+$CXCR-4$^+$, CD45$^+$/CD34$^+$Flk-1$^+$, and lin-c-kit$^+$Sca-1$^+$ cells 2 days after MI.

(E) In contrast to cytokine-treated WT animals, G-CSF treatment of CD26 KO and G-CSF-DipA animals with the CXCR4 antagonist AMD3100 reversed the recruitment of CD34$^+$/CXCR4$^+$ cells into the heart after MI. All data represent mean ± SEM (n = 6); *p < 0.05; n.s., not significant.

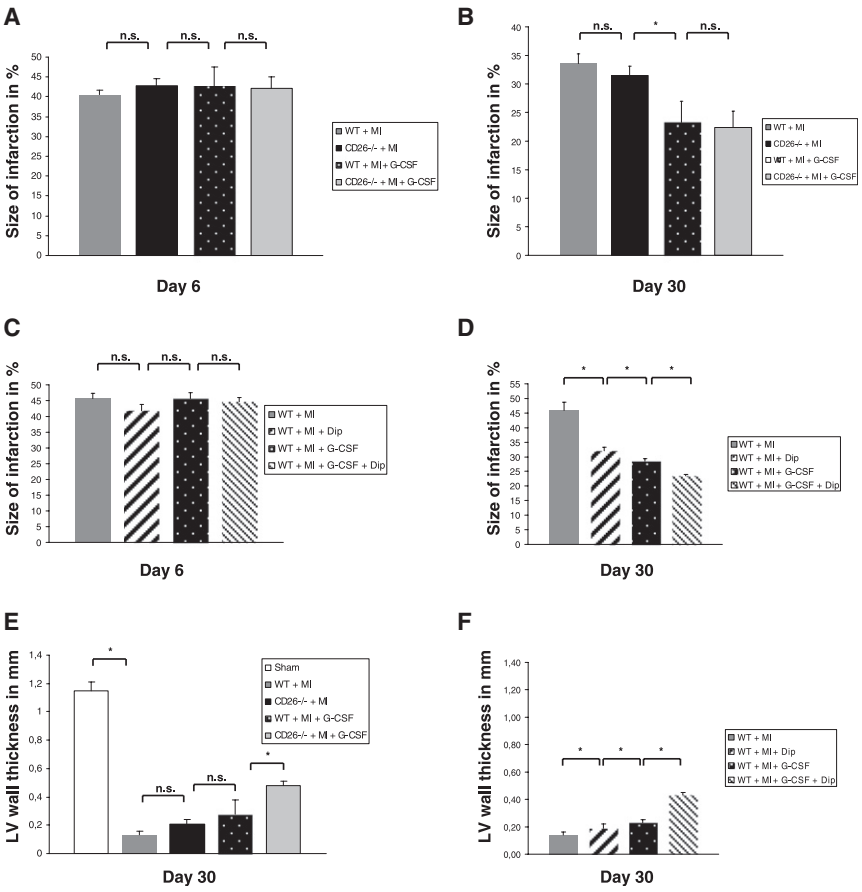

Figure 3. G-CSF-Treated CD26 KO and G-CSF-DipA Mice Show Attenuated Infarct Remodeling

(A–D) Bar graphs representing the size of infarction at day 6 (granulation tissue and cell necrosis) and at day 30 (scar tissue) after MI.

(E and F) Histograms showing that G-CSF-treated CD26 KO and G-CSF-DipA mice reveal significantly improved thickness of the LV wall at day 30 after MI. Data represent mean ± SEM (n = 6); *p < 0.05; n.s., not significant.

Loss or Inhibition of DPP-IV Function in Combination with G-CSF Treatment Attenuates Infarct Remodeling and Increases Neovascularization

At day 6 after LAD occlusion, LV-infarct sizes were comparable among the groups, which altered at day 30, when the sizes of LV infarction (scar tissue) were smaller in G-CSF-treated CD26 KO animals or G-CSF-DipA mice (Figures 3A–3D; Figure S3A). In contrast to untreated WT, G-CSF-treated CD26 KO animals or G-CSF-DipA mice significantly ameliorated the thickness of the left ventricular wall (Figures 3E and 3F). Since circulating $CD34^+$ cells are known carriers of angiogenic growth factors, we analyzed the amount of neovascularization. Consistent with the attenuated infarct remodeling, heart sections of G-CSF-treated CD26 KO animals or G-CSF-DipA mice revealed a significantly increased number of $CD31^+$ capillaries at the infarct borderzone (Figures 4A–4C). Costaining of $CD31^+$ endothelial cells with Ki67 antibodies demonstrated proliferation and sprouting of $CD31^+$ endothelial cells supporting neovascularization (Figure S3B).

G-CSF Treatment Reduces Apoptotic Cell Death in WT and *CD26*$^{-/-}$ Mice

Besides the beneficial effect of neovascularization on cardiac repair, early apoptosis of CMs is a major target for prevention of ischemic cardiomyopathy. Therefore, we analyzed apoptotic cell death in the border zone by TUNEL staining. In contrast to saline, G-CSF treatment of WT and *CD26*$^{-/-}$ animals reduced the number of TUNEL-positive CMs in the border zone, suggesting intrinsic anti-apoptotic effects of cytokine treatment (Figures 5A and 5B). Inhibition of CD26 alone did not show a significant effect on apoptosis of CMs.

Genetic or Pharmacological Inhibition of CD26/DPPIV Combined with G-CSF Treatment Improved Survival and Myocardial Function after MI

Four weeks after MI, pressure volume relations were measured in vivo from surviving sham-operated, saline, or G-CSF$^{+/-}$ Diprotin A-treated WT and CD26 KO mice (Figure S3C). Compared to nontreated WT, G-CSF treatment of CD26 KO or Diprotin A-treated mice revealed a significantly improved systolic function, reflected by an increased ejection fraction (Figures 6A and 6B), cardiac output and contractility (Table S1). Furthermore, G-CSF-treated CD26 KO and G-CSF-DipA mice revealed attenuated ventricular dilation, measured by enddiastolic volumes (Figures 6C and 6D), and improved diastolic heart function, calculated by the isovolumetric relaxation parameter Tau weiss (Table S1). Arterial afterload was markedly reduced in G-CSF-treated CD26 KO and G-CSF-DipA animals, reflected by a decreased arterial elastance (Table S1).

Finally, G-CSF-treated CD26 KO mice showed a significantly increased survival rate compared to cytokine-treated WT animals (75% versus 55%, $p < 0.05$) as well as to CD26 KO mice or WT mice (45% versus 40%, not significant) receiving saline (Figure 6E). In analogy, WT mice receiving combined treatment of G-CSF and the DPP-IV inhibitor Diprotin A revealed the highest survival rates (70%) (Figure 6F). Mortality was high within the first 7 days, in particular among saline-treated animals, but declined thereafter in all groups.

DISCUSSION

We present experimental evidence that genetic or pharmacological inhibition of DPP-IV in combination with G-CSF administration leads to (1) decreased myocardial DPP-IV activity, (2) stabilization of active SDF-1α in heart lysates, (3) enhanced myocardial homing of circulating CXCR-4^+ stem cells, (4)

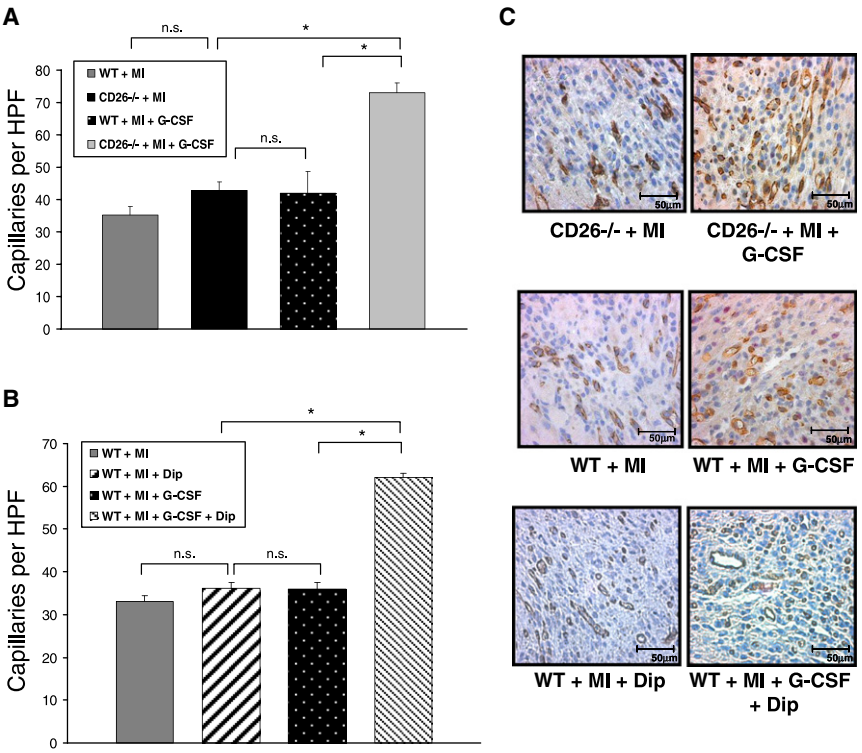

Figure 4. Increased Neovascularization in G-CSF-Treated CD26 KO and G-CSF-DipA Animals

(A and B) Histograms showing the numbers of $CD31^+$ capillaries at the infarct border zone in CD26 KO and WT animals after saline, G-CSF, Diprotin A, or G-CSF + Diprotin A treatment, respectively, 6 days after MI. Data represent mean ± SEM (n = 6); *p < 0.05; n.s., not significant.

(C) Representative immunohistochemical staining of CD31 (brown) in infarcted hearts 6 days after MI.

reduced cardiac remodeling, and (5) improved heart function and survival after MI (Figure 7).

In our approach, we achieved a reduced myocardial DPP-IV activity by genetic and pharmacological means. We concentrated on DPP-IV, although SDF-1α may also be cleaved by neutrophil elastase (NE), cathepsin G (CG), and various MMPs, including MMP-2 and MMP9 (Nervi et al., 2006). However, SDF-1α degradation by leukocyte-secreted MMPs may play a minor role in injured tissue due to predominant accumulation of inactive MMP-9 precursor forms and the presence of endogenous MMP inhibitors (Valenzuela-Fernandez et al., 2002). Moreover, studies in mice genetically lacking NE and CG showed that these proteases are not responsible for SDF-1 proteolysis during G-CSF-dependent stem cell mobilization (Levesque et al., 2004). The underlying hypothesis of our concept is the stabilizing impact of genetic and pharmacological DPP-IV inhibition on functional myocardial SDF-1α-protein. After MI, SDF-1α is upregulated for 24–72 hr (Askari et al., 2003; Hofmann et al., 2005), as confirmed by ELISA. Using a polyclonal antibody, we detected the whole amount of cleaved and intact SDF-1α without differentiating whether DPP-IV inhibition prevents cleavage of active SDF-1α protein in the ischemic heart. In order to address this key point of our hypothesis, we analyzed heart lysates by mass spectrometry. These analyses showed for the first time that recombinant SDF-1α (MW, 7.97 kDa) was DPP-IV dependent cleaved at the NH_2 terminus between Pro^2 and Val^3 (MW, 7.74 kDa) in heart lysates of WT, but not in CD26 KO mice, as was previously demonstrated in serum by others (Busso et al., 2005). The absence of MMP-2- or MMP-9-related cleavage products (7.56 kDa) between

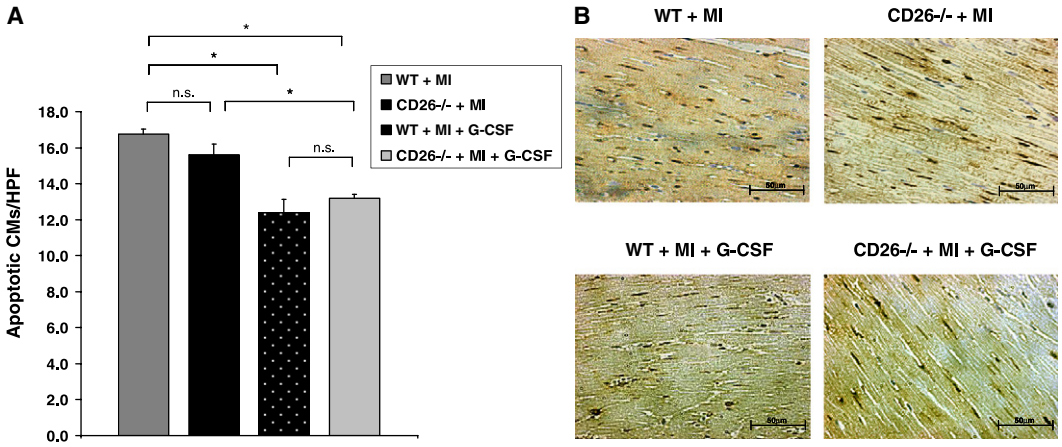

Figure 5. G-CSF Treatment Decreased Apoptotic Cell Death in WT and *CD26*$^{-/-}$ Animals

(A) Bar graph representing the number of TUNEL-positive CMs in the border zone 2 days after MI. Data represent mean ± SEM (n = 3); *p < 0.05; n.s., not significant.

(B) Representative TUNEL staining (brown nuclei) in WT or *CD26*$^{-/-}$ mice receiving either saline (upper row) or G-CSF (lower row) 2 days after MI.

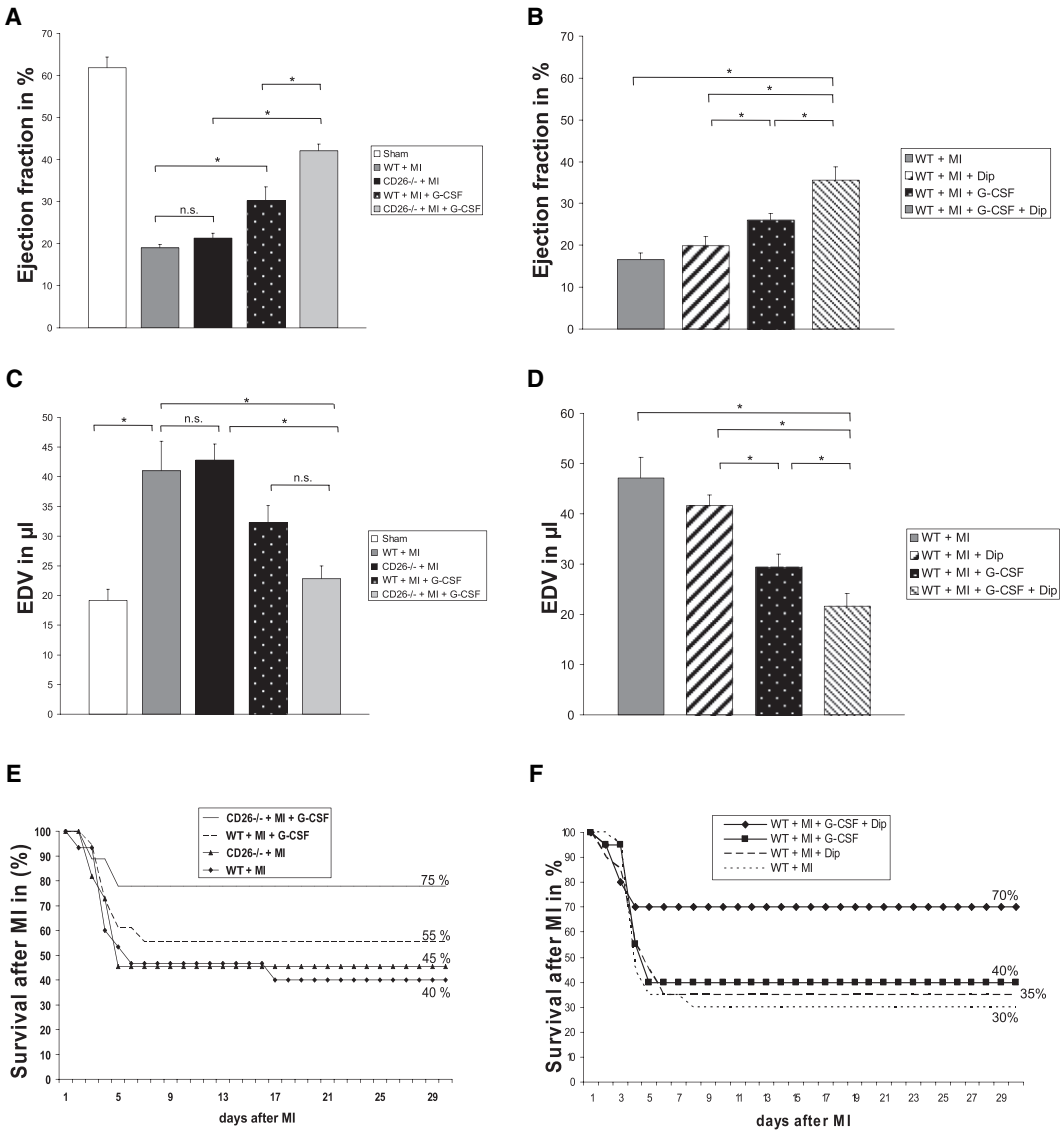

Figure 6. G-CSF-Treated CD26 KO and G-CSF-DipA Mice Reveal Improved Survival and Myocardial Function after MI

(A and B) Corresponding bar graphs representing the ejection fraction (EF) of CD26 KO or WT mice receiving saline, G-CSF, Diprotin A, or both at day 30 after LAD ligation.

(C and D) Diagrams show enddiastolic volume of CD26 KO or WT mice at day 30 after MI. Data represent mean ± SEM (n = 8); *$p < 0.05$; n.s., not significant.

(E and F) Kaplan-Meier curves showing survival rates of CD26 KO or WT mice treated either with saline, G-CSF, Diprotin A, or both after MI. All mice (n = 20 in each group) revealed histologically confirmed MIs.

residues Ser^4 and Leu^5 of SDF-1α (Valenzuela-Fernandez et al., 2002) suggests that proteolytic DPP-IV activity plays a critical role in SDF-1α degradation.

Although we could proof posttranslational stabilization of active SDF-1α only in heart lysates, our data provide indirect evidence that implicates CD26 inhibition in preservation of functional SDF-1α in the heart in vivo. (1) Proteolytic DPP-IV activity in the myocardium was either absent or markedly decreased after CD26 depletion or inhibition, respectively. (2) After MI, SDF-1α protein was equally upregulated in WT and in KO mice. Nevertheless, depletion or inhibition of CD26 significantly increased the amount of $CXCR4^+$ cells in the ischemic myocardium, most likely by an enhanced response to its active ligand. (3) Antagonization via the CXCR4 antagonist AMD3100 diminished the number of $CXCR4^+$ progenitors only in KO or Diprotin A-treated mice, emphasizing the essential role of an intact SDF-1-CXCR4 homing axis. However, whereas SDF-1α-CXCR4 interactions play important roles in homing of bone marrow-derived stem cells, they are not the only players. It is possible that CD26 is acting to cleave other chemokines or factors, which may also have effects on the homing ability of the cells.

Since SDF-1α has an outstanding status as the major chemokine for initiating stem cell migration and homing to the site of ischemia (Smart and Riley, 2008), several studies were targeted on enhancement of myocardial SDF-1α levels by invasive means. They performed either transplantation of

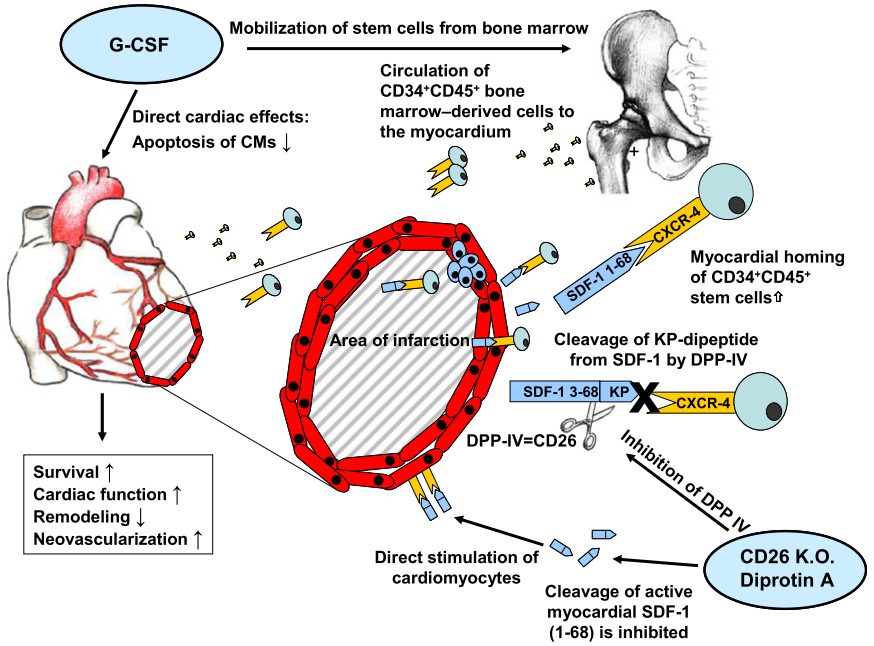

Figure 7. Therapeutic Concept of CD26/DPP-IV Inhibition Combined with G-CSF

After MI, G-CSF application exerts direct anti-apoptotic effects on ischemic myocardium and mobilizes stem cells from bone marrow. These stem cells circulate to the damaged heart, where they are incorporated by interaction of intact myocardial SDF-1α (1–68) and the homing receptor CXCR-4. Genetic or pharmacological inhibition (by Diprotin A) prevents the degradation of intact SDF-1α by DPP-IV. Thus, an increased amount of SDF-1α (1–68) improves homing of mobilized stem cells and directly stimulates pre-apoptotic CMs. Altogether, G-CSF and CD26/DPP-IV inhibition reduce cardiac remodeling after MI, enhance cardiac function, and finally increase survival by attenuating the development of ischemic cardiomyopathy.

SDF-1α-expressing fibroblasts (Askari et al., 2003) or adenoviral SDF-1α gene delivery (Abbott et al., 2004). More recently, Segers et al. showed that intramyocardial transplantation of a modified, MMP-2 and DPP-IV protease-resistant SDF-1α protein may serve as a therapeutic tool to improve heart function and recruit progenitor cells to the heart (Segers et al., 2007). However, the invasive nature of these strategies limits exogenous delivery of SDF-1α to the ischemic myocardium. Therefore, we intended to establish a noninvasive pharmacological strategy for SDF-1α stabilization to minimize the risk for patients treated after MI.

The beneficial effects of combined DPP-IV inhibition and G-CSF treatment most probably are due to two different pathways. On the one hand, CD26 depletion most likely stabilized intact SDF-1α, which consecutively increased the amount of $CD45^+CD34^+CXCR4^+$ progenitors after mobilization with G-CSF. These progenitors are known to express elevated levels of angiogenic growth factors and cytokines (Majka et al., 2001), which may contribute to increased neovascularization. Consequently, G-CSF-treated CD26 KO and G-CSF-DipA mice demonstrated decreased scar expansion and concurrent increase in microvasculature. This scenario is supported by others showing that intramyocardial transplantation of a SDF-1α protein, which is DPP-IV protease resistant (Segers et al., 2007), revealed elevated numbers of angiogenic $CD34^+/CXCR4^+$ cells in the ischemic heart, associated with increased neovascularization and improved left ventricular function.

On the other hand, our data revealed G-CSF-dependent reduction of apoptosis in WT as well as in $CD26^{-/-}$ animals, suggesting additional intrinsic cytokine-mediated mechanisms like Jak/Stat-dependent reduction of apoptosis (Harada et al., 2005; Ohtsuka et al., 2004). Reduced cardiac remodeling and increased neovascularization finally led to improved left ventricular function in G-CSF-treated CD26 KO and G-CSF-DipA animals. Although only the combination of DPP-IV inhibition and G-CSF treatment markedly reduced postinfarct remodeling, we can not rule out the possibility that SDF-1α alone—at least in part—acts in a direct manner to prevent apoptosis of CMs and enhance angiogenesis via upregulated phosphorylation of Akt (Hu et al., 2007; Sexana et al., 2008).

Previous reports presumed an impaired chemotaxis of stem cells after G-CSF treatment via N-terminal cleavage of the chemokine receptor CXCR4 in vitro (Levesque et al., 2003). Additionally, G-CSF-dependent upregulation of DPP-IV on $CD34^+$ progenitors (Christopherson et al., 2006), which we reversed by genetic or pharmacological DPP IV inhibition, may also contribute to a diminished chemotactic response after cytokine treatment. Thus, our data suggest a pivotal role of DPP-IV in disrupting the SDF-1α-CXCR4 homing axis after MI especially in combination with G-CSF.

Interestingly, beneficial effects concerning heart function and survival only occurred in G-CSF-treated CD26 KO and G-CSF-DipA mice. Our data suggest that the combination of homing of progenitor cells, enhancement of neovascularization, and direct effects of G-CSF and SDF-1α on preapoptotic CMs has to pass a certain threshold until it translates into significant improvement of cardiac function and survival. It seems that DPP-IV inhibition by itself is not strong enough to sufficiently attenuate cardiac remodeling and enhance cardiac function; sole G-CSF treatment was not able to increase global cardiac function in human studies (Engelmann et al., 2006; Zohlnhofer et al., 2006, 2008).

These findings may contribute essential aspects for design of future stem cell trials, since the key issue of all therapeutic stem cell approaches emerges to be the process of cardiac homing. Therefore, we propose the use of combined DPP-IV inhibition and G-CSF application as a therapeutic concept for future stem cell trials.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). CD26 KO mice (on a C57BL/6 background) were kindly obtained from Dr. N. Wagtmann (Novo Dordisk, Blagsvaerd, Denmark) with approval from Dr. D. Marguet (Centre d'Immunologie de Marseille Luminy-INSERM, Marseille Luminy, France) (Marguet et al., 2000).

Animal Model

MI was induced in 10-week-old male CD26 KO or C57BL/6 mice by surgical occlusion of the left anterior descending artery (LAD), as described previously (Deindl et al., 2006). Experiments concerning survival and cardiac function were performed by two different operators ([1] Figures 6A, 6C, and 6E, Table S1A; [2] Figures 6B, 6D, 6F, Table S1B). Animal care and all experimental procedures were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication no. 85-23, revised 1996).

Administration of G-CSF and Diprotin A

Experimental design is shown in Figure S4. Mice were randomly divided into the following groups: sham-operated animals (n = 20); infarcted wild-type (n = 20) (WT) receiving either saline (0.9% NaCl), G-CSF (100μg/kg/d i.p.), Diprotin A (70 μg/kg/twice per day), or G-CSF + Diprotin A; and CD26 KO animals (n = 20) receiving either saline (0.9% NaCl) or G-CSF (100 μg/kg/d) for up to 6 days. G-CSF and/or Diprotin A treatment was initiated immediately after the surgical procedure.

Functional Parameters

For evaluation of pressure-volume relationships in vivo, mice were anesthetised with thiopental (100 mg/kg, i.p.), intubated, and ventilated (MiniVent, HUGO SACHS, Freiburg, Germany). After catheterization via the right carotid artery, an impedance micromanometer catheter (Millar Instruments, Houston, Texas) was introduced into the left ventricle. Raw conductance volumes were corrected for parallel conductance by the hypertonic saline dilution method as described previously (Zaruba et al., 2008). Hemodynamic measurements as well as data analyses were performed by a blinded person using PVAN analysis software (HUGO SACHS, March, Germany).

Histology and Immunohistochemistry

At days 6 (n = 6) and 30 (n = 6), hearts were excised. After fixation in 4% phosphate-buffered formalin, the hearts were cut transversally into 2 mm thick slices and embedded in paraffin. Sections 4 μm thick were cut and mounted on positively charged glass slides. Standard histological procedures (hematoxylin and eosin and Masson's trichrome) and immunostaining were performed. Infarct size and wall thickness were determined according to Deindl et al. (2006).

For immunostaining, the following primary antibodies were used: CD45 (rat anti-mouse, BD Pharmingen), CD31 (goat anti-mouse, Santa Cruz), and Ki67 (goat anti-mouse, Santa Cruz). AEC was used as chromogen. Double staining for CD31 and Ki67 was performed using DAB as additional chromogen (all from Dako). Apoptotic cells were detected according to Harada et al. (2005) using the TUNEL assay (ApopTag, MP Biomedicals). Digital photographs were taken at a magnification of 400×, and ten random high-power fields (HPFs) from the border zone of each heart sample (n = 3) were analyzed. Quantification of blood vessels was assessed by $CD31^+$ immunohistochemistry in the granulation tissue at the border zone. The numbers of $CD31^+$ capillaries were quantified from ten random HPFs with 400× magnification.

Flow Cytometry of Peripheral Blood and Nonmyocyte Cardiac Cells

Ten-week-old CD26 KOs (n = 6) were either treated with G-CSF (100 μg/kg/d) or saline daily for 6 days, and/or C57BL/6 mice (n = 6) were treated with saline, G-CSF, Diprotin A, or both daily for 6 days. Cells were separated as described previously (Deindl et al., 2006). The following monoclonal antibodies were used: CD45-PerCP, CD34-FITC, CD31-PE, c-kit-PE, Sca-1-PE, CXCR4-PE, Flk-PE, CD3-biotin, CD45R/B220-biotin, CD11b-biotin, TER-119-biotin, and Ly-6G-biotin (all from BD Pharmingen). Matching isotype antibodies (BD Pharmingen) served as controls. Cells were analyzed by three-color flow cytometry using a Coulter Epics XL-MCLTM flow cytometer (Beckman Coulter). Each analysis included 50,000 events.

Cardiac cells from sham-operated and infarcted hearts of WT and CD26 KO mice were analyzed 48 hr after MI (n = 6). Therefore, a "myocyte-depleted" cardiac cell population was prepared, incubating minced myocardium in 0.1% collagenase IV (GIBCO BrL) 30 min at 37°C, lethal to most adult mouse CMs (Zhou et al., 2000). Cells were then filtered through a 70 μm mesh. To exclude spurious effects of enzymatic digestion, BM cells with or without collagenase treatment were stained, revealing no significantly changed staining of labeled cell antigens (data not shown). Cells were stained with CD45-PerCP, CD34-FITC, CD26-FITC, c-kit-PE, Sca-1-PE, CXCR4-PE Flk-1, Flk-PE, CD3-biotin, CD45R/B220-biotin, CD11b-biotin, TER-119-biotin, and Ly-6G-biotin Abs (all from BD Pharmingen) and subjected to flow cytometry using EPICS XLMCL flow cytometer and Expo32 ADC Xa software (Beckman Coulter). For evaluation of SDF-1-CXCR4-dependent homing, G-CSF-stimulated CD26 KO mice were treated i.p. with the CXCR4 antagonist AMD3100 (1.25 mg/kg). Cardiac cells were analyzed 8 hr after the last AMD3100 application. Each analysis included 50,000 events.

ELISA/DPP-IV Activity Assay

Hearts were extracted from C57/Bl6 WT mice and CD26 KOs on day 2 after MI. After digestion in 0.1% collagenase for 45 min, cells were lysed by ultrasonic pulse echo instrument. SDF-1α protein was determined using a commercially available Quantikine kit (R & D Systems, MCX 120) according to the manufacturer's instructions. Enzyme activity of DPP-IV was measured according to Scharpé et al. (1988) with the following modifications: the activity was determined as substrate rate-time curve (H-Gly-Pro-AMC; 353 nm_{em}, 442 nm_{ex}), and one reaction well contained 5 mM H-Gly-Pro-AMC with 10 μL sample in 100 mM Tris-HCL (pH 8). From this kinetic curve, the increase was defined as the activity. The fluorescence signal was converted into amount of product via conversion of the maximal fluorescence signal after complete substrate turnover.

Mass Spectrometry

Samples were purified from recombinant SDF-1α that has been incubated with various amounts of heart extract, as indicated in a total volume of 40 μl PBS. After incubation, the cleavage products were incubated for at least 3 hr with 5 μl of an anti-SDF1 antibody (Torrey Pines Biolabs) and 10 μl of a 1:1 slurry of protein G Sepharose (Sigma) in PBS (Busso et al., 2005). After incubation, beads were collected by centrifugation and washed three times with 100 μl of IP buffer (140 mM NaCl, 0.1%N-octyle glycopyranoside 10 mM Tris-HCl [pH 8.0], 5 mM EDTA) and two times with 100 μl of H_2O. Finally, all the buffer solution was aspirated from the beads, and the semidried beads were incubated with 10 μl of a 50% ACN, 0.6% TFA solution for 5 min to elute the bound peptide. Of the eluted sample, 4 μl was mixed with a saturated CHCN solution, spotted completely to a stainless steel target plate, and analyzed in a Voyager-STR MALDI-TOF mass spectrometer. For the acquisition of the spectra, 500 laser shots were collected using a mass window of m/z values between 3,000 and 10,000. Spectra were calibrated using an external calibration standard, manually inspected, and quantified using Data Explorer (Applied Biosystems, Framingham, MA).

Statistical Analyses

Results were expressed as mean ± SEM. Multiple group comparisons were performed by one-way analysis of variance (ANOVA) followed by the Bonferroni procedure for comparison of means. Comparisons between two groups were performed using the unpaired Student's t test. Data were considered statistically significant at a value of $p \leq 0.05$. Mortality was analyzed by the Kaplan-Meier method.

SUPPLEMENTAL DATA

The Supplemental Data include four figures and one table and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00068-X.

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