The Bispecific SDF1-GPVI Fusion Protein Preserves Myocardial Function after Transient Ischemia in Mice

Running title: Ziegler et al.; Bispecific SDF1-GPVI protein and myocardial repair

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Abstract:

**Background** - CXCR4-positive bone marrow cells (BMCs) are critically involved in cardiac repair mechanisms contributing to preserved cardiac function. “Stromal cell-derived factor-1” (SDF-1) is the most prominent BMC homing factor known to augment BMC engraftment which is a limiting step of stem cell-based therapy. Following myocardial infarction (MI), SDF-1 expression is rapidly upregulated and promotes myocardial repair.

**Methods and Results** - We have established a bifunctional protein consisting of a SDF-1-domain and a GPVI-domain with high binding affinity to the SDF-1 receptor CXCR4 and extracellular matrix proteins that become exposed after tissue injury. SDF1-GPVI triggers chemotaxis of CXCR4-positive cells, preserves cell survival, enhances endothelial differentiation of BMCs *in vitro* and reveals pro-angiogenic effects *in ovo*. In a mouse model of MI, administration of the bifunctional protein leads to enhanced recruitment of BMCs, increased capillary density, reduces infarct size and preserves cardiac function.

**Conclusions** - These results indicate that administration of SDF1-GPVI may be a promising strategy to treat MI to promote myocardial repair and to preserve cardiac function.

**Key words:** SDF-1, Myocardial Infarction, GPVI, Tissue Repair, Progenitor Cells
In recent years, extensive efforts have been made to identify mechanisms to promote myocardial repair and regeneration\textsuperscript{1,2}. It has been increasingly recognized that bone-marrow (BM)-derived progenitor cells (BMCs) participate in cardiac repair mechanisms and regeneration and contribute to function recovery after myocardial infarction (MI). Elevated levels of BMCs in patients with cardiovascular disease are associated with a reduced risk of death from cardiovascular complications\textsuperscript{3,4}.

In the late nineties, the isolation of putative BMCs was firstly described\textsuperscript{5} and it has become evident that circulating BMCs significantly contribute to the regeneration of vascular integrity and support locally induced repair mechanisms of resident endothelial cells\textsuperscript{6}. \textit{In vivo} studies in mice and rats provide evidence that vascular homing of BMCs improves endothelial regeneration and preserves myocardial function after transient coronary ischemia\textsuperscript{7-9}. Myocardial BMC transplantation into immunodeficient mice induces sustained humoral effects and leads to increased mobilization of endogenous BM-derived or progenitor cells that are incorporated into sites of neovascularization and myocardial repair\textsuperscript{10}.

Progressive adverse left ventricular (LV) remodeling after MI is the pathomorphological substrate of post-infarction heart failure and reduced survival\textsuperscript{11}. Cell therapy for patients with acute MI has been evaluated in the past by various clinical studies. In some studies intracoronary administration of BMC has been found to improve recovery of left ventricular contractile function in patients with acute MI\textsuperscript{12-14}. Other clinical studies did not show a beneficial effect of intracoronary administration of BMCs on left ventricular ejection fraction (LVEF) in patients after ST-elevation myocardial infarction (STEMI)\textsuperscript{15,16}.

Regulation of homing of BMCs plays an important role in mobilization of BMCs from the bone marrow to the ischemic myocardium. The most prominent BMC homing factor is the chemokine
SDF-1/CXCL12\textsuperscript{17}. SDF-1 has been shown to be upregulated in experimental MI\textsuperscript{18}. Platelet-derived SDF-1 regulates BMC function and differentiation\textsuperscript{19;20}. Moreover, platelet-SDF-1 is enhanced in MI in humans and correlates with the number of circulating BMCs\textsuperscript{21}. Experimental data provide evidence that SDF-1 augments BMC engraftment in ischemic myocardium and preserves myocardial function\textsuperscript{22}. \textit{In vivo} administration of recombinant SDF-1 protects mice against myocardial ischemia and leads to reduced infarct size after coronary occlusion\textsuperscript{1;18}.

Augmentation of myocardial repair mechanisms through recombinant SDF-1 is limited by its short half-life and lack of lesion-directed accumulation leading to low enrichment in the injured myocardium. The purpose of the present study was to develop a bispecific, recombinant SDF-1 molecule that preferentially binds to tissue lesions with destroyed vascular integrity. We constructed a recombinant, bispecific molecule consisting of SDF-1 and a recombinant form of the soluble platelet collagen receptor glycoprotein VI (GPVI)\textsuperscript{23} serving as an anchor structure to direct BMCs to sites of vascular and myocardial damage preserving cardiac function after MI.

**Methods**

**Generation of SDF1-GPVI**

SDF1-GPVI is a fusion protein consisting of the human chemokine SDF-1, the platelet collagen receptor GPVI\textsuperscript{23} and the Fc part of human IgG2. This fragment was produced by gene synthesis and cloned into the expression vector pcDNA5-FRT (GENEART, Regensburg, Germany). The Flp-In\textsuperscript{TM} CHO cell line (Invitrogen, Carlsbad, CA, USA) was stably transfected, produced and purified as described previously\textsuperscript{24}. 
Binding and adhesion studies

Binding capacity of SDF1-GPVI to collagen was tested by a collagen binding ELISA. Plate was coated with collagen, incubated with SDF1-GPVI, GPVI-SDF1, GPVI or FcIgG2, detected with TMB and measured using the ELISA 550 plate reader as described previously23.

CXCR4 binding capacity of SDF1-GPVI was analysed by a competition assay. 2 x 10⁵ human monocytes were freshly isolated as described previously25 and were analysed by FACS after incubation with SDF1-GPVI, GPVI-SDF1, GPVI or FcIgG2 and human anti-CXCR4 PE-conjugated antibody (BD Biosciences, CA, USA).

Adhesion experiments under flow conditions (flow rate 15ml/h and shear rate 2000 s⁻¹) were performed with 1.5x10⁵ cells/ml CD34⁺ cells diluted in IMDM medium. Rectangular coverslips were coated with collagen type I and SDF1-GPVI, GPVI or hSDF-1 as described previously20.

Colony forming unit assay (CFU)

CD34⁺ cells (5 x 10⁴/cm²) were seeded in precoated 96-well plates and cultivated at 37°C and 5% CO₂ in VascuLife medium (CELLSystems®, Troisorf, Germany). Endothelial colony-forming units were counted at day 1, 3, 5, 7, 10 and 14 using light microscopy (Axiovert, Carl Zeiss, Oberkochen, Germany).

Additional characterization experiments like chemotaxis, survival assay and chorioallantoic membrane (CAM) assay are provided in the online-only Data Supplement.

Myocardial ischemia and reperfusion in mice

All animal studies were approved by the district government of Tübingen. Myocardial ischemia was induced in 10 to 12 weeks old C57BL/6J mice by ligation of the left anterior descending artery (LAD) for 45 min26. Mice were randomly divided into the SDF1-GPVI group
(n=7) or the FcIgG2 control group (n=6). SDF1-GPVI (10 µg/g body weight) and FcIgG2 (4.2 µg/g body weight) control protein was injected intravenously directly after LAD ligation and repeated 48 hrs later. Both groups were treated intraperitoneally with G-CSF (100 µg/kg body weight) after surgical procedure for three consecutive days. Further, mice were treated after LAD ligation with SDF1-GPVI (n=4) group or FcIgG2 control group (n=6) in the absence of G-CSF. 28 days after MI echocardiography was performed as described previously. Immunohistochemistry and pharmacokinetics are described in the online-only Data Supplement.

**Preparation of murine BMCs and specific detection of injected BMCs in the injured myocardium**

BMCs were freshly isolated from the tibiae and femurs of transgenic green fluorescent protein (GFP) positive mice (kindly provided by Dr. Bischof). The bone marrow was ejected using a 26 gauge needle and filtered through a 70 µm cell strainer (BD Bioscience, CA, USA). 1x10^6 bone marrow cells were resuspended in 250 µl PBS and were incubated for 30 min with 20 µg/ml SDF1-GPVI or with 8.4 µg/ml FcIgG2 control protein. Prepared BMCs were injected i.v. directly after LAD ligation. Twenty-four hrs after ischemia and reperfusion, hearts were removed, cut in cryosections, stained with DAPI and analysed by immunofluorescence. The numbers of GFP-positive BMCs were quantified from ten random cryosections of each heart sample (n=3).

**Statistics**

All experiments were carried out at least 3 times in duplicates. Results are depicted throughout as means ± standard error (s.e.m.). Statistical analysis was performed using SPSS Statistics software for Windows Version 19.0 (IBM SPSS Inc., Chigaco, IL, USA). Statistical
significance was set at p < 0.05. Statistical comparison of more than 2 experimental conditions was performed using the Kruskal-Wallis Test. To compare specific pairs of outcome one-way ANOVA with adjustment for multiple testing (Bonferroni) was performed in case the global test was significant. Measurements taken at different times from the same experimental units were compared using repeated measures ANOVA.

Results

Soluble SDF1-GPVI fusion protein

We designed, constructed and cloned two proteins, the bifunctional protein GPVI-SDF1-FcIgG1 (termed GPVI-SDF1) and SDF1-GPVI-FcIgG2 (termed SDF1-GPVI). We followed these two concepts to provide different strategies because it is well known that the direction of different domains of the fusion protein is important for proper protein function. Both proteins (Figure 1A) contain the platelet collagen receptor GPVI, which is able to bind to collagen at the exposed extracellular matrix of disrupted endothelium or myocardium, or to activated endothelium via fibronectin or vitronectin. Furthermore, the proteins exhibit a human SDF-1-domain to attract and bind CXCR4-positive BMCs for enhanced accumulation of these cells at sites of vascular and/or myocardial injury. Only SDF1-GPVI offers an amino acid linker ((G4S)3) to separate the N-terminal SDF-1-domain from other domains, whereas the SDF-1-domain of the GPVI-SDF1 is located at the C-terminal site without any additional linker sequence (Figure 1A).

Furthermore, we generated two corresponding control proteins, GPVI-FcIgG2 (termed GPVI) lacking SDF-1-domain and FcIgG2 lacking SDF-1- and GPVI-domains.

A predicted molecular 3D model of both proteins demonstrates a free and separate SDF-1-domain by the SDF1-GPVI molecule in contrast to the GPVI-SDF1 protein (Figure 1A).
predicted binding model of the SDF-1-domain to its CXCR4 receptor revealed a high calculated binding affinity with a dissociation constant (K_D) of 105 nM in contrast to the low binding affinity of the GPVI-SDF1 construct with a K_D of 188 μM, reflecting a high-affinity interaction only between the SDF1-GPVI protein and CXCR4.

Stable cell lines of all proteins were prepared and expressed as secreted soluble proteins using the Flp-In™ CHO cell line. The proteins were produced and purified from cell supernatants. Using silver staining (Figure 1B) we showed, that SDF1-GPVI was isolated with high purity. The molecular mass of SDF1-GPVI was confirmed by Western blotting at 82 kDa under reducing conditions (Figure 1C). A ~130 kDa protein and a ~250 kDa protein were identified under non-reducing conditions, which confirms that SDF1-GPVI is present as a homodimer as well as an oligomer.

**SDF1-GPVI binding to collagen and CXCR4**

Next, we determined the binding activity of both proteins to their binding partners collagen and CXCR4. The specific binding of the GPVI domain to collagen was verified by a collagen-GPVI binding ELISA (Figure 2A, left) using different concentrations of the proteins. Both proteins, GPVI-SDF1 and SDF1-GPVI, display a similar dose-dependent binding capacity to immobilized collagen whereas the control FcIgG2 protein without GPVI-domain showed no collagen binding as expected. A competition experiment with solubilized collagen demonstrated that a concentration of 200 μg/ml collagen was sufficient to completely inhibit GPVI binding to immobilized collagen (Figure 2A, right). Taken together, these data demonstrated high specific binding of the GPVI domain of both proteins (GPVI-SDF1 and SDF1-GPVI) to its ligand collagen.
To explore sensitivity and specificity of the SDF-1-binding site we used a CXCR4-competition based binding assay. Isolated monocytes were incubated with different concentrations of the bispecific proteins to compete with PE-labeled anti-CXCR4 antibody that was determined by flow cytometry analysis. Incubation of monocytes with the SDF1-GPVI protein displayed a clear concentration dependent decrease of detectable CXCR4 (Figure 2B). In contrast, the GPVI-SDF1 protein revealed only low binding activity (Figure 2B). No binding to CXCR4 was detected for the control proteins GPVI or FcIgG2, respectively (Figure 2B). These results demonstrate that the SDF-1-domain of the SDF1-GPVI protein binds its receptor CXCR4 with high sensitivity and specificity in contrast to the GPVI-SDF1 protein that only shows low binding affinity. These results were in line with the calculated dissociation constant (Kd) as shown in Figure 1A.

The bispecific properties of the SDF1-GPVI construct were also shown by a dynamic adhesion assay. Collagen coated cover slips were incubated with SDF1-GPVI, native SDF-1 or control protein. CXCR4-positive BMCs were perfused through the flow chamber under high shear rates (2000 sec⁻¹). A strong increase in firm adhesion of BMCs was detected when collagen coated surfaces were preincubated with SDF1-GPVI protein before perfusion (Figure 2C). In contrast, native SDF-1 or control protein showed marginal adhesion of BMCs. Taken together, these results demonstrate high sensitivity and specificity of SDF1-GPVI binding to collagen and CXCR4 and that SDF1-GPVI augments cell adhesion under high shear rates.

**SDF1-GPVI triggered chemotaxis and preserved cell survival**

To study the functional consequences of SDF1-GPVI binding to CXCR4 we used a transwell system to test if both proteins, GPVI-SDF1 and SDF1-GPVI, are able to trigger chemotactic activity of CXCR4-positive cells (Figure 3A). SDF1-GPVI was able to induce...
strong chemotactic responses of BMCs in a concentration-dependent manner. In contrast, the GPVI-SDF1 protein displayed only low chemotactic activity as shown by low number of migrated cells (SDF1-GPVI versus GPVI-SDF1; 4170 ± 75 versus 248 ± 23, n=3, p<0.001). Furthermore, we tested the ability of a neutralizing CXCR4 antibody to block the chemotactic effects. A concentration of 10 μg/ml of CXCR4 antibody was sufficient to decrease the amount of migrated cells by more than 60% (anti-CXCR4 Ab versus IgG isotype control; 1635 ± 354 vs 4065 ± 867, p=0.04) confirming that the specific interaction of SDF-1 and CXCR4 is responsible for the chemotactic effects of the SDF1-GPVI protein. To validate that our human SDF-1 fusion protein also triggers chemotaxis of murine CXCR4-positive cells, we performed comparable experiments confirming that SDF1-GPVI stimulates migration of mouse-derived CXCR4-positive cells as well (data not shown).

SDF-1 regulates hematopoiesis by suppressing apoptosis. Because of this, we performed an apoptosis assay using BMCs to investigate the potential of SDF1-GPVI as a survival factor. As shown in Figure 3B, the presence of SDF1-GPVI (177 pM) enhanced survival of living cells compared to FcIgG2 control (48.3 ± 1.4% versus 23.5 ± 4.8% live cells, p<0.001) to a similar extent as the positive control proteins TGFβ1 (43.3 ± 0.1%) or SDF-1 (56.6 ± 2.1%).

**SDF1-GPVI enhanced endothelial differentiation**

Following homing, BMCs must undergo integration into the endothelial monolayer before they can contribute to restoration of vascular integrity. We performed a colony forming unit assay to test the potential of SDF1-GPVI to induce maturation of CD34+ cells into endothelial like cells (Figure 4A). Cultivation with SDF1-GPVI propagated and accelerated colony forming units (CFU) of CD34+ cells (Figure 4). Within day 1-5 *in vitro* (DIV1-5) we...
detected 3 times more colonies on SDF1-GPVI coated surfaces compared to uncoated or control GPVI coated surfaces (22 ± 5.6 colonies/10^6 CD34^+ cells versus 8 ± 2.3 colonies/10^6 CD34^+ cells versus 9 ± 2.8 colonies/10^6 CD34^+ cells). This positive effect on CFU efficiency was consistent up to DIV14 when progenitor cells cultivated on SDF1-GPVI were completely differentiated into a confluent mature endothelial monolayer whereas cells cultivated on control coating surfaces still displayed individual colonies (control: 9.7 ± 3.5 colonies/10^6 CD34^+ cells; GPVI: 20 ± 6 colonies/10^6 CD34^+ cells), indicating a clear benefit for progenitor cells cultivated on SDF1-GPVI.

These findings were supported by the analysis of Dil-labeled acetylated low density lipoprotein (Dil-Ac-LDL) uptake (Figure 4B, red fluorescence). CD34^+ cells cultivated on SDF1-GPVI coated surfaces displayed enhanced attachment of living cells (green Calcein staining, Figure 4B, d compared to a - c) and more rapid (DIV5) and more efficient uptake of Dil-Ac-LDL (red fluorescence in Figure 4B, l compared to i - k). Moreover, these cells develop an endothelial-like morphology (Figure 4B, t compared to q - s).

The endothelium is essential to provide an intact and selective barrier between the vessel lumen and surrounding tissue. Integrity of intercellular junctions is a major determinant of permeability of the endothelium and essential in endothelial differentiation. Therefore we examined the formation of the adherens junction molecule vascular endothelial cadherin (VE-cadherin, red fluorescence, Figure 4C) known to be important for the formation of a restrictive endothelial barrier. We were able to detect first intercellular junction formation within DIV5 when BMCs were cultured in the presence of SDF1-GPVI (see arrow, Figure 4C, l). These cells displayed strong and early cell proliferation and differentiation with early intercellular VE-cadherin expression between adjacent cells (see arrow, Figure 4C, l and p) resulting in a
confluent endothelial monolayer at DIV14 (Figure 4C, x). In contrast, BMCs cultivated on control coating surfaces displayed first intercellular junction formation at DIV14 (Figure 4C, u-w) indicating a retarded endothelial differentiation potential. These results indicate that SDF1-GPVI augments and accelerates endothelial differentiation of BMCs.

Consequently, we examined typical endothelial marker expression at DIV14 when confluent monolayers were formed. Within DIV14, monolayers on SDF1-GPVI displayed expression pattern of typical endothelial markers comparable to naïve human coronary artery endothelial cells (hCAECs) (Figure 4D). We were able to detect the characteristic von Willebrand factor staining (compare Figure 4D, a and g), the adherens junction proteins VE-cadherin (compare Figure 4D, d and j) and PECAM-1 (compare Figure 4D, f and l) as well as the occludens junction protein claudin 5 (compare Figure 4D, c and i) were exclusively located at the intercellular borders as it is known for endothelial cells29. Moreover, differentiated progenitor cells showed VEGFR-2 (compare Figure 4D, b and h) and angiopoetin receptor Tie-2 (compare Figure 4D, e and k) expression demonstrating distinct endothelial differentiation of cultured progenitor cells after SDF1-GPVI administration.

**SDF1-GPVI mediated pro-angiogenic effects**

Testing angiogenic characteristics of implants or substances, the “Chorioallantoic membrane” (CAM) assay is a well accepted and widely used in ovo model (Figure 5A). Application of SDF1-GPVI (10 μg) resulted in a 200% a/b ratio increase caused by enhanced number of blood vessels orientated towards the applied SDF1-GPVI (reflected by 2.7 ± 0.2 a/b ratio, Figure 5B). This pro-angiogenic effect was comparable to the effect of soluble hSDF-1 that was used as a positive control (2.66 ± 0.2 a/b-ratio). In comparison, addition of PBS or GPVI resulted in 1.2 ± 0.1 and 1.16 ± 0.2 a/b ratio, respectively. Furthermore, we used recombinant

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**Figure 4C**

**Figure 4D**

**Figure 5A**

**Figure 5B**
VEGF as an internal control, which strongly induced pro-angiogenic conditions indicated by an a/b-ratio of 3.75 ± 0.2. These data clearly demonstrate pro-angiogenic effects induced by SDF1-GPVI.

**SDF1-GPVI improved cardiac function after MI**

To assess the effects of SDF1-GPVI administration on myocardial function in vivo, we used a mouse model of myocardial ischemia. In this model myocardial damage is assessed after LAD ligation for 45 min followed by reperfusion. C57BL/6J mice were treated with two times administration of SDF1-GPVI as well as G-CSF for three days. The pharmacokinetics of SDF1-GPVI in mouse serum was detected by ELISA and Western Blot analysis. It demonstrates a slow and time dependent elimination of SDF1-GPVI with an estimated half-life of > 48 hrs as shown for other Fc-immunoadhesins.

In SDF1-GPVI treated mice, infarct areas 28 days after reperfusion, as assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining to differentiate between metabolically active and inactive tissue, were reduced by 24.4% in comparison to mice treated with FcIgG2 control protein (34.3 ± 4.2% I/Aar versus 58.7 ± 8.3% I/Aar, Figure 6C). Next, we determined if treatment with SDF1-GPVI shows an enhanced recruitment of CXCR4-positive BMCs to the infarcted myocardium. Freshly isolated GFP-positive BMCs from GFP-mice, incubated with SDF1-GPVI, control FcIgG2 or hSDF-1, were specifically detected in the myocardium after 24 hrs. We found enhanced accumulation of GFP-positive BMCs in the presence of SDF1-GPVI (57.3 ± 0.66 BMCs/10 cryosections, Figure 6D) compared to control FcIgG2 (13.0 ± 2.0 BMCs/10 cryosections), hSDF-1 (11.67 ± 1.2 BMCs/10 cryosections) or control hearts (13.3 ± 6.36 BMCs/10 cryosections). Specific accumulation of SDF1-GPVI in the injured myocardium was proved by immunohistochemistry. SDF1-GPVI accumulation 7 days after transient ischemia...
was substantially enhanced in the infarcted myocardium compared to control treated animals. 28 days after transient ischemia a positive SDF1-GPVI signal was almost absent (Figure 6E). In parallel, detection of CXCR4-positive cells was significantly increased in infarcted myocardium of SDF1-GPVI treated mice compared to control group (61.77 ± 1.88% CXCR4-positive cells versus 35.82 ± 3.45% CXCR4-positive cells, Figure 6F). 28 days after LAD ligation the percentage of CXCR4-positive cells within the infarcted myocardium was not different between SDF1-GPVI and control treated animals (24.04 ± 2.98% CXCR4-positive cells versus 23.59 ± 4.36% CXCR4-positive cells, Figure 6F). Capillary density in the infarct border zone was detected with a PECAM-1 antibody. In the SDF1-GPVI treated group the average capillary density was enhanced at day 7 compared to GPVI control group (991 ± 80 capillaries/mm² versus 476 ± 113 capillaries/mm²) as well as at day 28 compared to FcIgG2 control group (528 ± 19 capillaries/mm² versus 282 ± 41 capillaries/mm², Figure 6G). Reduction of infarct size and enhanced capillary density were functionally relevant because echocardiography 28 days after LAD ligation showed significantly improved cardiac LV-function by approximately 69 % in mice treated with SDF1-GPVI compared to FcIgG2 treated mice (36.1 ± 1.8% fractional area change (FAC) versus 21.3 ± 1.3% FAC, Figure 6H). Echocardiography 7 days after LAD ligation showed no significant difference in regard to FAC between both groups. Next, we analyzed the effect of SDF1-GPVI on LV-function in infarcted mice in the absence of G-CSF. We found that in the absence of G-CSF, SDF1-GPVI preserved myocardial function 28 days after transient ischemia (15.7 ± 1.1% FAC versus 11.7 ± 1.9% FAC) by approximately 34% by trend without reaching statistical significance (p<0.15, Figure 6I).
Discussion

The major findings of the present study are: 1) We successfully generated a bispecific protein with SDF-1- and GPVI-domains that binds with high affinity to the CXCR4 receptor and extracellular matrix proteins such as collagen. 2) SDF1-GPVI triggers chemotaxis of CXCR4-positive cells and preserves cell survival in vitro. 3) SDF1-GPVI enhances endothelial differentiation of progenitor cells in vitro, reveals pro-angiogenic effects and 4) SDF1-GPVI administration reduces infarct size, recruits BMCs, enhances capillary density and preserves cardiac systolic function after MI in vivo.

The findings strongly imply that the bifunctional molecule SDF1-GPVI recruits and modulates function of BMCs at sites of myocardial injury after transient ischemia. Systemic administration of SDF1-GPVI preserves myocardial function and might be an attractive tool to promote myocardial repair/regeneration after ischemic injury.

At sites of vascular or tissue injury, discontinuity of the endothelial barrier and exposure of subendothelial matrix within the (micro-) vasculature leads to platelet adhesion and activation. Platelet adhesion at the injured vessel wall is mediated by adhesion receptors including GPVI that recognizes proteins of the subendothelial matrix such as collagen and fibronectin leading to platelet activation and secretion of granula constituents. We found that platelets secrete SDF-1 upon activation in mice and humans. Platelet-derived SDF-1 recruits CXCR4-positive progenitor cells to the microenvironment of activated platelets and favors migration and endothelial differentiation.

SDF-1 and its receptor CXCR4, regulate trafficking and homing of bone marrow–derived cells to bone marrow or peripheral organs. SDF-1 is the most potent chemokine of stem and progenitor cells, as well as of monocytes, lymphocytes and platelets. SDF-1 gene expression
is regulated by the transcription factor hypoxia-inducible factor-1 (HIF-1) in endothelial cells, resulting in selective expression of SDF-1 in ischemic tissue in direct proportion to reduced oxygen tension \textit{in vivo} \cite{39}. HIF-1-induced SDF-1 expression increases the adhesion, migration and homing of circulating CXCR4-positive progenitor cells to ischemic tissue \cite{39}. Blockade of SDF-1 in ischemic tissue or CXCR4 on circulating cells prevents progenitor cell recruitment to sites of injury \cite{39}. Adenoviral overexpression of SDF-1 in the heart after MI significantly enhanced bone marrow-derived cell recruitment in injured myocardium \cite{40}. SDF-1 has been shown to be cardioprotective after hypoxemia \cite{1} and stimulates recruitment of circulating progenitor cells that promote angiogenesis and support myocyte survival \cite{1}. Administration of a protease-resistant SDF-1 mutant limits MI in mice \cite{41} and promotes angiogenesis in a hind limb ischemia model \cite{42}.

We constructed a bifunctional fusion protein that combines binding activity to the extracellular matrix proteins like collagen and binding to the SDF-1 receptor CXCR4 (SDF1-GPVI). We hypothesized that the "anchor" molecule GPVI binds preferentially to the injured vasculature of the microcirculation and thereby increases the concentration of SDF-1 within the injured myocardium resulting in enhanced accumulation of "repair" cells \cite{23}. Indeed, we found enhanced cell accumulation within the injured myocardium in mice treated with the SDF1-GPVI fusion molecule (\textbf{Figure 6D}). Furthermore, we provide \textit{in vitro} and \textit{in vivo} evidence that our fusion molecule SDF1-GPVI promotes angiogenesis (\textbf{Figure 5} and 6G) and sustains cell survival (\textbf{Figure 3B}). After transient myocardial ischemia (LAD ligation) systemic administration of SDF1-GPVI significantly reduces infarct size and preserves myocardial function in mice (\textbf{Figure 6C} and H).
Following MI, SDF-1 expression in the heart is rapidly upregulated\textsuperscript{18}. However, SDF-1 levels decline between day 4-7 after MI, so CXCR4-positive BMCs are mobilized\textsuperscript{2} at a time, when myocardial SDF-1 expression is not optimal for their recruitment. Furthermore, cardiac myocytes of the infarct border zone as well as cardiac stem cells also start to express CXCR4 after MI at a time of declining myocardial SDF-1 expression\textsuperscript{43}. Therefore, it is believed that sustained expression of SDF-1 is required to decrease cardiac myocyte death and improve cardiac function. Several studies followed this strategy to enhance myocardial SDF-1 levels by invasive means\textsuperscript{44}. These invasive strategies include adenoviral SDF-1 gene delivery\textsuperscript{40}, transplantation of SDF-1 expressing fibroblasts\textsuperscript{2}, intracardial injection of SDF-1\textsuperscript{1} and intramyocardial injection of a modified protease-resistant SDF-1 protein\textsuperscript{41}. They all together preserved cardiac function and reduced heart remodeling, but are limited by the fact that only one single administration of exogenous SDF-1 can be applied to the patient directly after MI. Therefore our aim was to provide a non-invasive pharmacological tool to enrich CXCR4-positive cells by a bispecific molecule targeting a component of the subendothelial matrix (collagen) that is exposed at injured vessels and providing SDF-1 to bind and recruit CXCR4-positive cells to damaged tissue. The molecule allows individual therapeutic application to the patient and might overcome low grafting efficiency of stem cell-based regenerative therapy\textsuperscript{45}.

In addition, we combined our bispecific molecule with G-CSF to increase recruitment of BMCs according to a recent report by Zaruba and colleagues\textsuperscript{46} that demonstrates increased mobilization of progenitor cells from the bone marrow after transient ischemia by pharmaceutical administration of G-CSF. They verified that endogenous SDF-1 concentration after myocardial ischemia and reperfusion is not sufficient to mobilize a number of BMCs that achieve a positive effect on myocardial regeneration\textsuperscript{46}. This therapeutic strategy leads to
systemic mobilization of progenitor cells that might be accompanied by cardiovascular risk as shown in ApoE<sup>-/-</sup> mice with plaque destabilization after widespread EPC mobilization<sup>47</sup>. In contrast, binding of the bifunctional protein to injured and inflamed tissue via the anchor molecule GPVI leads to increased concentration of SDF-1 and consequently to local stimulation of BMC recruitment after tissue damage that may even effectively support endothelial and myocardial healing as shown in our MI model.

In conclusion, we provide evidence that the bispecific protein SDF1-GPVI is highly effective in preserving myocardial function after transient ischemia. Administration of SDF1-GPVI during reperfusion and thereafter might be a promising strategy to treat MI in order to promote myocardial repair and limit heart failure.

**Acknowledgements:** We thank I. Flohrschtütz, L. Laptev, I. Epple, H. Schnell and J. Kwiatkowska for excellent technical assistance. We are indebted to S. McCurdy and Dr. H. Langer for critical reading of the manuscript. We thank Dr. F. Bischof for providing us with the transgenic GFP-mice.

**Funding Sources:** This work was supported in part by the German Ministry of Education and Research (BMBF) #01GU0727, the Deutsche Forschungsgemeinschaft (Transregio-SFB19) and the Klinische Forschergruppe (KFO 274): Platelets – Molecular mechanisms and Translational Implications.

**Conflict of Interest Disclosures:** None

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**Figure Legends:**

**Figure 1.** Generation of the bispecific SDF1-GPVI. (A) Schematic illustration of the construct. Structures of the two constructed proteins GPVI-SDF1 and SDF1-GPVI are illustrated (upper panel). Predicted 3D models of both proteins and a predicted binding model of SDF1-GPVI to CXCR4 receptor (lower panel). SDF1-GPVI shows a theoretical high binding affinity to CXCR4 receptor with a $K_D$ of 105 nM in contrast to the low binding affinity from GPVI-SDF1 to CXCR4 with a $K_D$ of 188 $\mu$M. (B) Purity of the fusion protein SDF1-GPVI (1 $\mu$g) and control GPVI (1 $\mu$g) was verified by silver staining under reducing (left panel) and non reducing conditions (right panel). (C) Purified SDF1-GPVI (600 ng) and the corresponding control proteins were immunoblotted under reducing (left panel) and non reducing (right panel) conditions using SDF-1, GPVI and IgG antibodies.

**Figure 2.** SDF1-GPVI binds to collagen and the SDF-1 receptor CXCR4. (A) The GPVI-mediated collagen binding capacity of the constructed proteins was proved using a collagen
binding ELISA (left panel). All GPVI-domain containing proteins (SDF1-GPVI, GPVI-SDF1 and GPVI) revealed collagen binding in a concentration-dependent manner. In contrast FcIgG2 was not able to bind to collagen (n\geq 3, significance between SDF1-GPVI and FcIgG2 in each concentration category with p<0.001). SDF1-GPVI (10 µg/ml) and control GPVI were preincubated with soluble collagen to compete binding to immobilized collagen (right panel). Soluble collagen almost completely inhibits binding of GPVI-domain containing proteins and immobilized collagen (n=3, significance to SDF1-GPVI with p=0.03 and to GPVI with p=0.03).

(B) Binding of SDF1-GPVI to CXCR4 was proven by a CXCR4-competition assay and measured by flow cytometry. Bar graph and representative FACS images show that only SDF1-GPVI displays a clearly detectable CXCR4 binding capacity in contrast to GPVI-SDF1 and other control proteins (n\geq 3, significance between SDF1-GPVI and FcIgG2 in each concentration category with p<0.001). (C) CXCR4-positive BMCs displayed increased adhesion on SDF1-GPVI under flow conditions compared to other coating surfaces (n=3, general significance p=0.001 (Kruskal-Wallis Test), significance between SDF1-GPVI and GPVI with p<0.001 and between SDF1-GPVI and hSDF-1 with p=0.001). Representative images were taken, fluorescence microscopy analysis of cells stained with DAPI (left panel) and phase contrast analysis of cells (Scale bar: 20 µm, magnification: 400x).

**Figure 3.** SDF1-GPVI triggered chemotaxis and preserved cell survival. (A) The chemotactic capacity of SDF1-GPVI was analysed by cell migration using a Transwell system (upper and lower, left panel). SDF1-GPVI induced increased cell migration in a dose dependent manner whereas GPVI-SDF1 was not able to trigger chemotaxis (p<0.001). hSDF-1 (100 ng/ml) was used as positive control (scale bar: 100 µm, magnification: 100x, n\geq 3, significance between
negative control and SDF1-GPVI (77pM, 155pM) with p<0.001). Cell migration was abolished by addition of an anti-CXCR4 antibody (lower, right panel, n≥3, significance between anti-CXCR4 and IgG2b with p=0.04). (B) BMCs were incubated for 6 hrs under apoptosis-inducing culture conditions (upper panel). In the presence of SDF1-GPVI preserved cell survival was observed by number of live cells (%) demonstrating the survival effect of SDF1-GPVI in contrast to FcIgG2 control protein (n=3, p<0.001). TGFβ1 and hSDF-1 served as positive controls. Detection of live, apoptotic and dead cells was achieved using propidium iodide and annexin V staining by flow cytometry. (lower panel, n=3, general significance p=0.002 (Kruskal-Wallis Test)).

Figure 4. SDF1-GPVI enhanced endothelial differentiation of BMCs in vitro. (A) In vitro endothelialization was performed by colony forming unit (CFU) assay. BMCs cultured on SDF1-GPVI coated surfaces revealed enhanced endothelial colony formation compared to other coating surfaces (right panel). Images of CFU experiments display the state of differentiation on day 3, 5, 7 and 14 (left panel) (Data represent mean ± SEM; n=6; significance between SDF1-GPVI and uncoated in each time category with p<0.001 (one-way ANOVA) and significant increase of number of colonies on SDF-1-GPVI, GPVI and collagen coated surface over time with p<0.006 (repeated measures ANOVA)). (B) SDF1-GPVI-coated surfaces displayed more rapid and more efficient uptake of DiI-Ac-LDL (red fluorescence). Detection of living cells was achieved using Calcein staining (green fluorescence, scale bar: 100 μm). (C) BMCs cultured on SDF1-GPVI coated surfaces show enhanced interendothelial VE-cadherin expression (red fluorescence) (Calcein, green fluorescence, scale bar: 100 μm). (D) Endothelial marker expression of
differentiated BMCs on SDF1-GPVI within 14 days revealed a comparable expression pattern to naïve hCAECs (scale bar: 50 μm).

**Figure 5.** SDF1-GPVI revealed pro-angiogenic effects *in ovo*. (A) Schematic illustration of the experiment. (B) CAM was explanted at day 13 and blood vessel orientation was examined. Blood vessels growing towards the test solution (a), and those growing centrifugally away from the test solution (b), were counted and a/b ratio was determined to evaluate the degree to which the test substance induced pro- or anti-angiogenic effects. Application of PBS or the control protein GPVI resulted in equal angiogenesis without any chemoattractant impact. Addition of SDF1-GPVI and hSDF-1 resulted in a comparably strong doubling of pro-angiogenic effects. When VEGF was applied pro-angiogenic events were further enhanced (n≥3, general significance p=0.006, significance between SDF1-GPVI and PBS with p<0.001).

**Figure 6.** SDF1-GPVI treatment reduced infarct size and preserved cardiac systolic function in a mouse model of myocardial ischemia. (A) In C57BL/6J mice SDF1-GPVI (n=7) or control FcIgG2 (n=6) were injected i.v. after LAD ligation and 48 hrs later (two times administration). All mice were treated with G-CSF (100 μg/kg body weight) for three days. 28 days after reperfusion mice were analysed. (B) Pharmacokinetics of SDF1-GPVI in mouse serum samples. Serum levels of SDF1-GPVI before, 6, 12, 24 and 48 hrs after intravenous injection were analysed by ELISA and Western Blot (n=4). (C) Representative transversal cardiac sections show reduced infarct size in SDF1-GPVI treated mice (left panel, pale area). Quantitative analysis of the infarct size in percent of area at risk (right panel, n≥6, p=0.02). (D) Cardiac sections from mice 24 hrs after MI. Representative images of recruited GFP-positive BMCs.
(green) into the heart (left panel, scale bar: 25 μm, magnification 200x). Quantification of recruited BMCs (right panel, n=3, p=0.081). (E-G) Cardiac sections from mice 7 and 28 days after MI were stained with hematoxylin and eosin (HE) and immunostained for FcIgG2, SDF-1, CXCR4 and PECAM-1. Control group at day 7 was treated with GPVI and on day 28 with FcIgG2 (scale bar: 25 μm, magnification 75x and 200x). Quantitative analysis of CXCR4-positive cells (F, right panel, significance on day 7 with p=0.0002 and no significance on day 28 with p=0.94). Quantitative analysis of capillary density (G, right panel, significance on day 7 with p=0.02 and on day 28 with p=0.006). (H) Representative M-mode echocardiograms (left panel). Fractional area change (FAC) 28 days after MI was substantially improved in SDF1-GPVI treated mice compared to control (right panel, n≥6, p<0.0001). At time point 7 days no significant difference was found in regard to FAC between both groups (p=0.56). (I) In the absence of G-CSF FAC was improved in SDF1-GPVI treated mice compared to control by trend (p=0.15).
**a** Protein 1: GPVI-SDF1

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Protein 2: SDF1-GPVI

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GPVI-SDF1  

SDF1-GPVI  

CXCR4

**b**

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Figure 2

(a) Collagen binding (OD 450nm) as a function of concentration [μg/ml].

(b) CXCR4 binding %Gated (Low Right) as a function of concentration [μg/ml].

(c) Adherent cells stained with DAPI and Phase contrast.
**Figure 3**

(a) Representative images of cell migration (migrated cells) for different conditions:
- **hSDF-1**
- **Negative control**
- **SDF1-GPVI 155 pM**
- **GPVI-SDF1 155 pM**

(b) Cell survival (% Live cells) for different conditions:
- **Freshly Isolated cells**
- **Ø**
- **SDF1-GPVI**
- **FclgG2**
- **TGFβ1**
- **hSDF-1**

Legend:
- **PI**
- **Annexin V**
- **70% 12% 24% 62% 48% 28% 24% 57% 43% 28% 57% 18% 48% 23% 18% 28% 18%**
Figure 4a

- Uncoated collagen I
- Collagen I + GPVI
- Collagen I + SDF1-GPVI

Number of colonies over time:
- 1d
- 3d
- 5d
- 7d
- 10d

Figure 4b

Images showing cell colonies at different time points for each condition.
Ziegler et al. Figure 5

**a**

- **Test substance**
- **Silicone ring**
- **CAM (chorio allantois membrane)**

**b**

- Graph showing ratio a/b for different substances:
  - PBS
  - hSDF-1
  - GPVI
  - SDF1-GPVI
  - VEGF

- Significant differences indicated by ***

Downloaded from http://circ.ahajournals.org/ by guest on January 15, 2018
e SDF1-GPVI ctrl SDF1-GPVI ctrl SDF1-GPVI ctrl

7d

HE HE anti-hlgG anti-hlgG anti-SDF-1 anti-SDF-1

28d

HE HE anti-hlgG anti-hlgG anti-SDF-1 anti-SDF-1

f

SDF1-GPVI ctrl

7d

Day 7

% CXCR4-positive cells

Day 28

SDF1-GPVI ctrl

***
Figure 6b

- **Day 7**
  - SDF1-GPVI
  - ctrl

- **Day 28**
  - SDF1-GPVI
  - ctrl

Graph showing capillaries/mm² for SDF1-GPVI and ctrl groups at Day 7 and Day 28. Significant differences are marked with asterisks: * for Day 7 and ** for Day 28.
Figure 7

LV-systolic function (% FAC)

Day 7

Day 28

SDF1-GPVI + G-CSF

ctrl + G-CSF

SDF1-GPVI

ctrl

n.s.

Day 28

n.s.

Day 28

LV-systolic function (% FAC)
The Bispecific SDF1-GPVI Fusion Protein Preserves Myocardial Function after Transient Ischemia in Mice
Melanie Ziegler, Margitta Elvers, Yvonne Baumer, Christoph Leder, Carmen Ochmann, Tanja Schönberger, Tobias Jürgens, Tobias Geisler, Burkhard Schlosshauer, Oleg Lunov, Stefan Engelhardt, Thomas Simmet and Meinrad Gawaz

_Circulation_. published online January 5, 2012;
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2012/01/05/CIRCULATIONAHA.111.070508

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals
Specific pathogen-free C57BL/6J mice were obtained from Charles River (Sulzfeld, Germany). Animal studies were approved by the district government of Tübingen.

Chemicals and antibodies
Human collagen I was purchased from Becton-Dickinson (BD, Heidelberg, Germany). Acetylated low-density lipoprotein labeled with 1,1’-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) (Biomedical Technologies, USA), calcein (Molecular Probes, Leiden/Netherlands) and vascular endothelial growth factor (recombinant human VEGF165, PeproTech, London, UK) were used. 4’,6-diamidino-2-phenylindole (DAPI, 500 ng/ml in PBS) was obtained from Sigma-Aldrich (Steinheim, Germany), phalloidin from Invitrogen (Carlsbad, CA, USA). The antibodies against vWF and VE-cadherin were from Santa Cruz (CA, USA). Antibodies against VEGFR-2 and PECAM-1 were obtained from Cell Signaling (Frankfurt, Germany), antibodies against claudin 5 and Tie-2 were purchased from abcam (Cambridge, UK).

Human cells
Monocytes were freshly isolated as described previously. CD34+ cells were isolated from remnants of leukapheresis transplants as described previously. All experiments were approved by the local ethic committee of the University of Tuebingen.

Cloning and protein production for SDF1-GPVI
The cDNA sequence coding for SDF1-GPVI includes a C-terminal FcIgG2-tag and a N-terminal IgK leader. This fragment was produced by gene synthesis and cloned into the mammalian expression vector pcDNA5-FRT using HindIII and BamHI restriction sites.
(GENEART, Regensburg, Germany). The Flp-In™ CHO cell line (Invitrogen, Carlsbad, CA, U.S.A.) was stably transfected with either pcDNA5-SDF1-GPVI-FcIgG2-FRT, pcDNA5-GPVI-SDF1-FcIgG1-FRT, pcDNA5-GPVI-FcIgG2-FRT or pcDNA5-FcIgG2-FRT using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s protocol to generate a stable SDF1-GPVI-FcIgG2-Flp-In™-CHO, GPVI-SDF1-FcIgG1-Flp-In™-CHO, GPVI-FcIgG2-Flp-In™-CHO and FcIgG2-Flp-In™-CHO cell line as described previously\(^2,4\). The stable cell lines were cultivated using Hams F12 medium supplemented with 10% FCS, 1% Penicillin/Streptomycin and 250 µg/ml hygromycin B. For protein expression, cells were grown on T-160 cell culture flasks in serum free CHO III (A) medium (Gibco, Paisley, Scotland), supplemented with 4 mM Glutamine, 4.5 g/l D-Glucose and 1% Penicillin/Streptomycin. Cell culture supernatants were harvested 8 days after subculturing and purified using Protein G Agarose Beads (Pierce, Rockford, IL, U.S.A.) according to the manufacturer’s protocol.

**Molecular modeling**

Computational docking and scoring studies of the interaction of SDF1-GPVI with CXCR4 were performed with Molegro Virtual Docker 2008\(^5\). The original parameters of blind docking were used in combination with evaluation scheme based on binding free energy ($\Delta G$)\(^6\). The structure of CXCR4 was obtained from the OPM Data Bank (3OE6).

**Western blot analysis**

Purified supernatant from SDF1-GPVI producing CHO cells was detected via Western blot. Supernatant was prepared with reducing or non reducing sample buffer, separated on SDS-polyacrylamide gel\(^7\) and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). Subsequently, the membrane was blocked using 5% powdered skim milk in PBST (PBS with 0.1% Tween 20) and probed with anti-hSDF1 (R&DSystems, Wiesbaden, Germany) in a dilution of 1:500, anti-human IgG-HRP (Jackson Immuno Research, PA, USA) in a dilution of 1:5000 and anti-GPVI in a dilution of 1:7500. Anti-GPVI was purified by the supernatant of hybridoma 5C4\(^3\). Afterwards the membrane was incubated
with fluorescently labeled secondary antibodies in darkness. The membrane was detected by the Odyssey infrared imaging system (LI-COR, Bad Homburg, Germany).

**Silver staining**

Purified protein was prepared with reducing or non reducing sample buffer and separated on 8% SDS-polyacrylamide gel. Afterwards the gel was fixed with 20% ethanol and 5% acetic acid for 10 min, followed by washing two times in 25% ethanol. Gel was then incubated in 0.02% sodium thiosulfate for 1 min, washed in deionized water and impregnated in 0.2% silver nitrate for 15 min. After washing in deionized water the gel was immersed in developer (6% sodium carbonate, 37.5 µl formaldehyde (37%)). After silver reduction the staining was stopped with 20% ethanol and 5% acetic acid.

**Collagen binding ELISA**

An Immuno HB 96-well plate (Thermo Fisher, Schwerte, Germany) was coated with 10 µg/ml bovine collagen I in coating buffer (15 mM Na$_2$CO$_3$, 35 mM NaHCO$_3$, 3 mM NaN$_3$ pH 9.6) o/n at 4°C. Control wells were coated with 10 µg/ml murine Laminin in PBS for one hour at RT. Wells were blocked with blocking solution (Candor Bioscience GmbH, Weißenberg, Germany) o/n at 4°C. The four proteins SDF1-GPVI, GPVI-SDF1, GPVI and FcIgG2 were prepared with Low Cross Mild buffer (Candor Bioscience GmbH, Weißenberg, Germany) in the following concentrations: 2.5, 5, 10, 20 µg/ml. For inhibition of the functional binding, proteins (10 µg/ml) were preincubated with 200 µg/ml solubilized collagen for 10 min. Wells were washed two times with PBS and were incubated with 100 µl of the proteins for one hour at RT. Afterwards, the plate was washed five times with PBS and incubated with 100 µl anti-human IgG-HRP (Jackson Immuno Research Europe, Newmarket, UK) 1:10 000 in Low Cross Mild buffer for 1h at RT. After washing 5 times with PBST, 0.05% Tween-20, wells were incubated with 100 µl TMB detection reagent (Serva, Heidelberg, Germany) for 10-15 min. Reaction was stopped by addition of 100 µl H$_2$SO$_4$, and the absorbance was measured.
at 450 nm against reference wavelength 570 nm using the ELISA 550 plate reader (Bio-Rad, München, Germany).

**Competition assay**

Every FACS tube was prepared with $2 \times 10^5$ human monocytes and washed with 1 ml ice-cold FACS buffer (PBS with 0.05% acide and 0.5% BSA).

The four proteins SDF1-GPVI, GPVI-SDF1, GPVI and FcIgG2 were prepared in the following concentrations: 200, 100, 60 and 30 µg/ml and were mixed with 10% Intratect (Biotest, Dreieich, Germany). Cells were incubated with these proteins for 10 min at 4°C. Afterwards, cells were labeled with human anti-CXCR4 PE-conjugated antibody (BD Biosciences, CA, USA) or isotype IgG2a control PE mouse antibody (BD Biosciences, CA, USA) and incubated for 30 min at 4°C. After incubation, cells were washed two times with ice-cold FACS buffer, resuspended in 500 µl FACS buffer and gently mixed. Finally, cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences, CA, USA).

**Chemotaxis**

Chemotaxis was performed using 5.0 µm pore size Transwell migration chambers (Costar Corning, NY, USA). SDF1-GPVI in three different concentrations (155 pM, 77 pM and 15 pM), GPVI-SDF1 (155 pM), hSDF-1 (100 ng/ml) or only medium were included in the lower compartments. $1.5 \times 10^5$ CD34$^+$ cells were added to the upper chambers in StemSpan SFEM medium (Stem Cell, Grenoble, France). For inhibition of chemotaxis anti-CXCR4 (10 µg/ml) or control isotype IgG2b (10 µg/ml) antibodies (R&DSystems, Wiesbaden, Germany) were mixed with cells 30 min before the addition of the cells to chambers. The cultures were incubated for 6 hrs at 37°C. Finally, cells in the lower chamber were mugged and counted using flow cytometry.
**Survival assay**

Freshly isolated CD34+ cells were cultured in IMDM medium supplemented with 1% vitamins, 5% FCS, 1% P/S, and 1% NEAA (Gibco, Paisley, Scotland) overnight. Cell survival assay was performed using a 24-well plate. 1 x 10^5 CD34+ cells/well were incubated for 6 hrs at 37°C in serum- and cytokine-free medium (IMDM + GlutaMAX, Gibco, Paisley, Scotland) in the presence of SDF1-GPVI, FcIgG2 (each 177 pM), 500 ng/ml hSDF-1, 50 ng/ml hTGFβ1 or medium alone. The percentages of apoptotic and healthy cells were determined using Annexin V-FITC and propidium iodide (BD Biosciences, CA, USA) co-staining according to the manufacturer’s protocol and flow cytometry.

**Coating procedure of culture plates**

Culture plates and cover slips were coated in four different modes under sterile conditions. Coatings were performed in two steps (1st: collagen, 2nd: protein). Between these two steps wells were washed 3 times with PBS and blocked with 3 % BSA in PBS for 1 h at RT. Subsequently wells were washed with PBS and cells were immediately added to pretreated wells.

**Dynamic adhesion assay**

Adhesion experiments under flow conditions (flow rate 15ml/h and shear rate 2000 s⁻¹ corresponding to the native flow rate of blood in human arterioles) were performed with 1.5x10^5 cells/ml CD34+ cells diluted in IMDM medium. Rectangular coverslips were coated with collagen type I as described and fixed in the flow chamber. Cells were perfused through the flow chamber with a 50 ml syringe and a pulse-free pump for 10 min. After perfusion, all experiments were recorded in real time and evaluated offline.

**Colony forming unit assay (CFU)**

CD34+ cells (5 x 10⁵/cm²) were seeded in precoated 96-well plates and cultivated at 37°C and 5% CO₂ in VascuLife medium (CELLSystems®, Troisorf, Germany). After 24 hrs non-
adherent cells were removed and medium was changed every 3 days. Additionally, endothelial colony-forming units were counted at day 1, 3, 5, 7, 10 and 14 using light microscopy (Axiovert, Carl Zeiss, Oberkochen, Germany)\(^9\).

**Uptake of low-density lipoprotein Dil-Ac-LDL**

CD34\(^+\) cells (5 x 10\(^4\)/cm\(^2\)) were seeded as described in the CFU-assay. The uptake of Dil-Ac-LDL (Biomedical Technologies, USA) of seeded CD34\(^+\)-cells was analyzed at day 1, 3, 5, 7, 10 and 14 as described previously\(^4\).

**Immunocytochemistry**

CD34\(^+\) cells (5 x 10\(^4\)/cm\(^2\)) were seeded and cultivated as described in the CFU-assay. Immunohistochemistry was performed as described previously\(^4\).

**Chorioallantoic membrane assay (CAM assay)**

Fertilized Hisex brown chicken eggs were incubated at 37°C and 60% humidity. After two days of incubation, 2 ml albumen was aspirated from the eggs. A window cut into the egg shell was then encircled with silicone paste and the opening was covered with a Petri dish lid. Afterwards, eggs were immediately returned to the incubator\(^10\). At day nine of incubation, a silicon ring (diameter of 9 mm, thickness of 3 mm) was placed onto the CAM (Figure 1A). 50 µl of test substance (diluted in PBS) was poured into the silicone ring, which was subsequently covered with a cover slip and the eggs were returned to the incubator for another 4 days. At day 13, CAMs were fixed in 4% PFA for 6 hrs at RT and collected. Photographs of the CAM were used to determine the orientation of blood vessels in the CAM. Followed by counting the number of blood vessels orientated towards (Figure 1B, red arrow [a]) and backwards (Figure 1B, green arrow [b]) to the implanted test substance charged silicon ring (Figure 1B). Calculations of the a/b-ratio were evaluated and revealed a pro- or anti-angiogenic effect of applied test substances.
Pharmacokinetics

C57BL/6J mice received SDF1-GPVI (10 µg/g body weight) by intravenous injection. At indicated time intervals (before, 6, 12, 24 and 48 hrs after administration), 100 µL blood was collected in a tube taken from the retro-orbital plexus under isoflurane anaesthesia. SDF1-GPVI levels were determined in the serum using a human IgG ELISA (IgG ELISA Kit, Immunotek, ZMC) according to manufacturer’s instruction and by Western Blot analysis detected with human IgG-HRP Ab (Jackson Immuno Research, PA, USA) as described recently\(^{11-13}.\)

Myocardial ischemia and reperfusion in mice

10 to 12 weeks old C57BL/6J mice were anesthetized by intraperitoneal injection of a solution of midazolame (5 mg/kg body weight; Ratiopharm, Ulm, Germany), medetomidine (0.5 mg/kg body weight; Albrecht GmbH, Aulendorf, Germany) and fentanyl (0.05 mg/kg body weight; CuraMed Pharma, Karlsruhe, Germany). Anaesthesia was maintained with isoflurane (Albrecht GmbH, Aulendorf, Germany). Myocardial ischemia was induced by ligation of the left anterior descending artery (LAD) for 45 min. Successful performance of the occlusion was confirmed by visual inspection of color in the apex. After 28 days of reperfusion the ischemic area (area at risk, AaR) was defined by negative staining with 4% Evan’s Blue (after re-ligation of the LAD at the level marked by the suture left in place) and the infarcted area (infarct size, IS) was detected by triphenyltetrazolium staining (Sigma Aldrich, St Louis, Mo, USA)\(^1.\) The ratio of IS/AaR is an accurate measure to analyze IS within ischemic myocardium and is the primary end point, which determines the effect of the treatment strategy. Areas were digitally quantified by video planimetry.

Administration of G-CSF and SDF1-GPVI

Experimental design is shown in Figure 6A. Mice were randomly divided into the SDF1-GPVI group (n=7) or the FcIgG2 control group (n=6). SDF1-GPVI (10 µg/g body weight) and FcIgG2 (4.2 µg/g body weight) control protein was injected intravenously directly after LAD
ligation and repeated 48 hrs later. Both groups were treated intraperitoneally with G-CSF (100 µg/kg body weight) for three consecutive days. G-CSF treatment was initiated immediately after surgical procedure. Further, mice were treated after LAD ligation with SDF1-GPVI (n=4) group or FcIgG2 control group (n=6) in the absence of G-CSF.

**Immunohistochemistry**

Paraffin-embedded cardiac sections of day 7 and 28 were stained with an avidin-biotin-immunoperoxidase method (LSAB + System HRP, Dako, Heverlee, Belgium) using anti-PECAM-1 mAb (Santa Cruz Biotechnology, CA, USA), anti-CXCR4 mAb (RnDSystems, Wiesbaden, Germany), anti-SDF1 mAb (abcam, Cambridge, UK) or anti-hIgG Ab (Jackson Immuno Research, PA, USA) and isotype control Ab according to standard protocol. Corresponding secondary antibodies (Dako, Heverlee, Belgium) were used. The numbers of PECAM-1⁺ capillaries were quantified from six random areas from the border zone of each heart sample (n=3) and were referred to a certain tissue area (mm²). The percentage of CXCR4-positive cells were counted from six random areas in the infarcted myocardium of each heart sample (n=3) in a blinded manner.

**Echocardiography**

Echocardiography was performed using a VEVO 770 ultrasound machine and a 30 Mhz linear transducer as described recently¹⁴,¹⁵.
References


