Original Paper

Exploiting AT2R to Improve CD117 Stem Cell Function *In Vitro* and *In Vivo* – Perspectives for Cardiac Stem Cell Therapy

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Key Words

CD117⁺ stem cells • Transient AT2R stimulation • Cardiac stem cell therapy • HL-1 cell line • Vasculogenesis • Cardioprotection

Abstract

Background/Aims: CD117⁺ stem cell (SC) based therapy is considered an alternative therapeutic option for terminal heart disease. However, controversies exist on the effects of CD117⁺ SC implantation. In particular, the link between CD117⁺ SC function and angiotensin-II-type-2 receptor (AT2R) after MI is continuously discussed. We therefore asked whether 1) AT2R stimulation influences CD117⁺ SC properties *in vitro* and, 2) which effects can be ascribed to AT2R stimulation *in vivo*. **Methods:** We approached AT2R stimulation with Angiotensin II while simultaneously blocking its opponent receptor AT1 with Losartan. CD117 effects were dissected using a 2D-Matrigel assay and HL-1 co-culture *in vitro*. A model of myocardial infarction, in which we implanted EGFP⁺ CD117 SC, was further applied. **Results:** While we found indications for AT2R driven vasculogenesis *in vitro*, co-culture experiments revealed that CD117⁺ SC improve vitality of cardiomyocytes independently of AT2R function. Likewise, untreated CD117⁺ SC had a positive effect on cardiac function and acted cardioprotective *in vivo*. **Conclusions:** Therefore, our data show that transient AT2R stimulation does not significantly add to the beneficial actions of CD117⁺ SC *in vivo*. Yet, exploiting AT2R driven vasculogenesis via an optimized AT2R stimulation protocol may become a promising tool for cardiac SC therapy.

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Introduction

Stem cell therapy is considered an alternative therapeutic option for terminal heart disease. So far, different clinical trials as well as studies using animal models of myocardial infarction showed favorable outcomes following stem cell implantation [1-4]. Interestingly, any stem cell type proved to have at least some beneficial effect on cardiac regeneration following myocardial infarction (MI) [5]. However, widely accepted paracrine effects of these stem cells (SC) in terms of increasing and provoking neovascularization in the injured heart makes them promising candidates for patient-specific autologous cell therapy [6-8].

One of the bone marrow stem cell populations extensively studied are CD117⁺ cells. Besides controversial findings regarding their transdifferentiation potential [9-11], a number of studies point to paracrine signaling as their mechanism of action [12, 13]. Infiltrating as well as the resident CD117⁺ SC increase their expression of VEGF, bFGF and angiopoietin and in this way, establish a pro-angiogenic milieu in the infarct border zone. This stimulation of angiogenesis and potentially myogenesis via paracrine effects in the infarcted region may play a substantially beneficial role for regeneration, as shown for various bone marrow stem cell populations [8, 12, 14-16]. In Mirotsou et al. (2011) as well as Gnecci et al. (2008) factors involved in favorable paracrine signaling of implanted stem cells have been reviewed [6, 14].

Previous studies in our group extensively addressed electrophysiological characteristics of murine CD117⁺ SC prior to implantation [17, 18]. As shown via whole-cell patch-clamp and immunocytochemistry, CD117⁺ SC rather differentiate into an endothelial-like phenotype. We hence assumed it safe to exploit CD117⁺ SC for myocardial regeneration, as their electrophysiological properties make it very unlikely to produce hazardous action potentials or contribute to arrhythmias as found for mesenchymal stem cells or skeletal myoblasts [19, 20].

There is evidence that an interesting link exists between CD117⁺ SC function and the renin-angiotensin system (RAS), which interferes with inflammation and acute cardiac remodeling processes during cardiac injury [21]. Thereby, the angiotensin II type-2 receptor (AT2R) by mediating actions of the active metabolite Angiotensin II (Ang II), opposes effects of the angiotensin II type-1 receptor (AT1R) [22, 23]. It was shown that myocardial hypoxia is associated with an increased activation of RAS, increased Ang II concentration and an increased expression of Ang II receptors [24, 25]. In cardiac regeneration, some studies showed that the AT2R acts cardioprotective, displayed by anti-inflammatory and anti-apoptotic effects and improved myocardial remodeling in animal studies [21, 26, 27].

Yet in contrast, Ichihara et al. describe a negative effect of AT2R expression on cardiac remodeling. Thereby, loss of AT2R signaling was related to suppressed tissue fibrosis and abolished hypertrophic responses [28].

Seeking to resolve these obvious controversies on CD117⁺ SC and the influence of AT2R [21, 28], we indirectly stimulated AT2R signaling in murine CD117⁺ BMSC. After *in vitro* experiments, an *ex vivo* co-culture setup was further used to approximate the situation *in vivo*. Here, the influence of CD117⁺ SC on the survival of the HL-1 cardiac muscle cell line was investigated. Finally, a mouse model of myocardial infarction was used to assess cardiac function, CD117⁺ BMSC retention and cardioprotective effects.

In particular, we addressed the questions whether 1) AT2R stimulation influences CD117⁺ stem cell properties *ex vivo* and, 2) which specific effects can be ascribed to AT2R stimulation *in vivo*. We show here that indirect AT2R stimulation significantly increased CD117⁺ cell elongation and alignment. Untreated CD117⁺ SC further significantly enhanced HL-1 cardiac muscle cell line vitality via cell-to-cell contact and improved cardiac function, capillary density and collagen deposition *in vivo*. However, those beneficial effects could not be additionally enhanced using transient AT2R stimulation. Our results indicate a long-term reduction in eNOS and MMP-2 expression following AT2R stimulated stem cell implantation. It is likely that an altered AT2R stimulation approach for either stem cells or tissue could greatly increase beneficial effects after MI.

Materials and Methods

Isolation and flow cytometry analysis of CD117⁺ SC

CD117⁺ SC were isolated from mice femur and tibia as described before [17]. SC from lung were isolated as previously described by Fang et al. [29]. Fluorescence-activated cell analysis was performed on a FACS LSRII® (BD Biosciences) to evaluate the expression and the purity of the isolated CD117⁺ SC. CD117⁺ SC isolated from C57BL/6-Tg (CAG-EGFP)10sb/J (eGFP⁺ transgenic) mice were also analyzed for their expression of eGFP.

Cells were resuspended in DMEM (PAN Biotech, Aidenbach, Germany) containing 10% fetal bovine serum (FBS, PAN Biotech), 100U/mL penicillin and 100µg/mL streptomycin (1% P/S), and 1.25µg/mL amphotericin B (Sigma-Aldrich). CD117⁺ SC were plated at a field density of 150.000 cells/cm² on 12-well plates.

AT2R stimulation of CD117⁺ cells

CD117⁺ cells were stimulated with 0.1µM Angiotensin II while AT1R was inhibited with 10µM Losartan (2-butyl-4-chloro-1-[p-(o-1H-tetrazol-5-ylphenyl)benzyl]imidazole-5-methanol- mono potassium salt) (both Sigma-Aldrich, Deisenhofen, Germany) for 24h in culture medium (A/L stimulation). For co-culture setting, HL-1 and CD117⁺ SC were stimulated with A/L in the same well. The influence of A/L stimulation of HL-1 cells was assessed prior to co-culture.

Immunocytochemistry

At day 14, cultured cells were fixed with 2% formaldehyde for 10min at room temperature. After permeabilization with 0.1% Triton X-100 for 20min at 37°C and pre-blocking with DAKO Protein Block Serum free (DAKO, Hamburg, Germany), primary antibodies as polyclonal goat anti-VEGFR-2, polyclonal goat anti-CD31 (both Santa Cruz Biotechnology, Heidelberg, Germany) or anti-mouse CD45 (BioLegend, San Diego, USA) were applied overnight at 4°C. After equilibration to room temperature, cells were washed in TBS and incubated with donkey anti-goat Alexa Fluor 488, donkey anti-goat Alexa Fluor 568 or goat anti-mouse Alexa Fluor 488 (all Invitrogen, Carlsbad, USA) for 2h at 37°C. Cells were counterstained for 10min with 4'-6-Diamidino-2- phenylindole (DAPI, Molecular Probes, Karlsruhe, Germany). For qualitative analysis, stained sections were imaged at 400x magnification on LSM 780 confocal microscope (Carl Zeiss, Jena, Germany).

2D-Matrigel Assay

Freshly isolated CD117⁺ SC were cultured onto Matrigel in the presence or absence of A/L or AT2R antagonist PD123319 (5 nM, Tocris Bioscience). 2x10⁵ CD117⁺ SC in 500µl DMEM (ATCC, USA) containing 10% horse serum (Gibco (Life Technologies), Darmstadt, Germany) and 1% P/S were cultured on a Matrigel coated 24-well plate. Blind assessment of the extent of cell alignment was done for the three groups after 10 days. The alteration of cell alignment and sprouting was rated in 5 randomly chosen fields of the 24-well for each of the three independent experiments. Images were obtained at 100x magnification using Axiovert 40 CFL (Carl Zeiss). Scoring was as follows: - No change; + cell sprouting; ++ cell alignment in one direction.

HL-1 cell line culture

The AT-1 mouse atrial cardiomyocyte tumor lineage-derived cell line HL-1 was obtained from Claycomb Lab (LSU Health Sciences Center, New Orleans, USA). Culture protocols for HL-1 were provided by Sigma-Aldrich (SAFC Bioscience, USA). In brief, HL-1 cells were cultured as monolayer in Claycomb Medium supplemented with 10% fetal bovine serum, 1% P/S, 0.1mM norepinephrine and 2 mM L-glutamine (all from Sigma-Aldrich). Well- plates for experimental purpose and tissue culture flasks were pre-coated with 0.02% gelatine and 0.0012.5% fibronectin (both from Sigma-Aldrich). The cells were maintained at 37°C and 5% CO₂.

HL-1-CD117 co-culture setting and hypoxia

To distinguish HL-1 from CD117⁺ SC in direct co-culture setting, cells were labeled with either Vybrant™ Dye Dil (HL-1; prior to culture) (Life Technologies) or anti-mouse CD45 (CD117⁺ SC, prior to

immunocytochemistry). DiI labeling time was 10 min for HL-1 cells. CD45 is not expressed in HL-1 cell line and can thus be used to distinguish cell types during immunocytochemistry.

For co-culture, HL-1 cells were seeded at a density of 70.000 cells per 24-well and 300.000 cells per 6-well in Claycomb Supplemented Medium. The isolated CD117⁺ cells were added to the adherent HL-1 cells in a ratio of 1:2. The co-culturing of both cell types was performed either with direct cell-to-cell contact or without cell-to-cell contact using Millicell® Cell culture inserts (0.4µm PCF, 12mm diameter; Millipore Ireland Ltd.). Cells were co-cultured for 24h before they were subjected to hypoxic conditions for another 24h.

To expose cells to a hypoxic environment, oxygen was replaced in a Modular Incubator Chamber MIC 101, (billups-rothenberg, USA) by stream in of nitrogen in the airtight locked chamber.

MTT-assay

Before MTT-assay CD117⁺ cells were washed off with three subsequent medium changes. After 48h of co-culture (with or without hypoxia) MTT (5mg/ml in PBS) was added to the HL-1 cells and incubated for 4h at 37°C. Then medium was removed completely and dimethylsulfoxide (DMSO) was added until formazan was completely dissolved. 100µl per well were transferred in 96-well plates in triplicates and absorbance was measured at 550nm and 655nm (reference wavelength) using a Microplate Reader (Model 680, Bio-Rad Laboratories GmbH, München, Germany). The results were given as the percentage of vitality in respect to the single culture of HL-1 cells under normoxic conditions (set to 100%).

Quantitative real time PCR analysis

For analysis of AT2R stimulation, RNA from 1x10⁶ CD117⁺ SC in the presence or absence of A/L or PD123319 was isolated following the instructions of TRIzol Reagent (Life Technologies). Samples were analyzed with NanoDrop1000 (Thermo Scientific, Karlsruhe, Germany) to demonstrate consistent quality and to determine RNA concentration. The RNA was reversed to cDNA using RT² first strand kit (SABiosciences, Qiagen, Hilden, Germany). The realtime PCR procedures were performed using RT² SYBR Green/Rox qPCR master mix reagent (SABiosciences). Angiotensin II type 2 receptor (AT2R; PPM04811A) expression levels were normalized to the expression of GAPDH housekeeping gene (PPM02946E). Primer were purchased from SABiosciences (RT² qPCR Primer Assays).

Hearts were removed, embedded in O.C.T.™ Compound (Tissue-Tek®; Zoeterwoude, Niederlande) and snap-frozen in liquid nitrogen. Tissue RNA was isolated following the same protocol of TRIzol Reagent and reversed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems (Life Technologies), Darmstadt, Germany). Primer sets for RT-PCR were purchased from Applied Biosystems: enhanced GFP (Mr04329676_mr); Cxcl12 (Mm00445553_m1); eNOS (Mm00435217_m1); MMP-2 (Mm00439506_m1); CD34 (Mm00519283_m1); BAX (Mm00432051_m1) and Bcl-2 (Mm00477631_m1). Amplification and detection were performed with the StepOnePlus™ Real-Time PCR System (Applied Biosystems) in TaqMan Universal Master Mix (Applied Biosystems) according to the instructions of the manufacturer (Applied Biosystems). DNA extracts were tested in triplicate and negative controls were included in each assay. Cycle thresholds (C_t) for single reactions were determined with StepOne™ Software 2.0 (Applied Biosystems) and the target genes were normalized against GAPDH. Resulting ΔC_t of triplicates was averaged and ΔΔC_t were obtained using Sham group as calibrator sample. The 2^{-ΔΔC_t} method was employed to present the changes in gene expression as x-fold change.

EGFP expression levels are given as ΔC_t (cycle threshold difference between EGFP and GAPDH) as EGFP is not expressed in Sham and MI-C.

Cell sorting and colony forming units-assay

CFU assay was used to analyze the stability of eGFP protein expression in CD117⁺ cells. CD117⁺ cells were sorted with BD FACS Aria (gating: viable eGFP+CD117⁺ cells) and subjected to CFU assay. For one experiment, 5x10⁴ freshly isolated CD117⁺ cells were mixed with 2ml Metho Cult Medium (Mouse Colony-forming unit assay, M3334, StemCell Technologies, Cologne, Germany). CD117⁺ cells were cultured as triplicates for 12 days at 37°C, 5% CO₂. Cells were released from the gel using 1ml Dispase (StemCell Technologies) per dish and analyzed for eGFP expression with BD FACS Aria.

Immunohistochemistry

For immunohistological detection of eGFP+ and VEGF+ stem cells, frozen transverse tissue sections (6µm) of hearts from MI-C and Sham were incubated with goat anti-GFP (conjugated to FITC) (Abcam, Cambridge, UK; ab6662) and rabbit anti-VEGF polyclonal antibodies (Santa Cruz Biotechnology, sc-507). Subsequently, the sections were incubated with donkey anti-rabbit Alexa-Fluor 568 conjugated secondary antibody (Life Technologies). Nuclei were counterstained with DAPI (Molecular Probes). Labelled sections were observed using LSM 780 confocal microscope (Carl Zeiss). Hearts were analyzed 24h, 48h, and 7d after cell injection.

Experimental design of the animal model

The federal animal care committee of LALLF Mecklenburg-Vorpommern (Germany) approved the study protocol (LALLF M-V/TSD/7221.3-1.1-050/12). Male eGFP-transgenic mice (C57BL/6-Tg(CAG-EGFP)10sb/J) and male C57BL6/J mice were purchased from Charles River Laboratories (Sulzfeld, Germany). EGFP-transgenic mice served as CD117+ SC donor. C57BL6/J mice (Charles River Laboratories) were randomly assigned to 4 groups: Sham operation with BD Matrigel™ Matrix (Matrigel) (BD Biosciences USA) injection (Sham, n = 6), myocardial infarction (MI) with Matrigel injection (MI-C; n=6) and two MI groups with implanted CD117+ SC (in Matrigel) with the respective treatment (MI-CD117; MI-CD117+A/L; n = 6). Mice were assessed for functional measurement, histological and qRT-PCR evaluation at three weeks after surgery.

Generation of MI in mouse and intramyocardial stem cell injection

Mice were anesthetized with pentobarbital (50µg/g body weight). After thoracotomy and preparation, the left anterior descending coronary artery (LAD) was permanently ligated. Immediately after LAD-ligation, each mouse received an intramyocardial injection of 2×10^5 CD117+ (± 24 h of AT2R stimulation) SC in Matrigel, or Matrigel alone for MI-C. Along the border of the blanched myocardium $3 \times 5 \mu\text{l}$ injections were given. Sham operated mice underwent identical surgical procedures without LAD-ligation but followed by intramyocardial Matrigel injection without cells.

Left ventricular catheterization

Three weeks after surgery, mice underwent pressure-volume (P/V) loop measurements according to the protocol of CardioDynamics BV (CD Leycom, Zoetermeer, Netherlands) and as established before by our group [30]. Data were collected with the Millar Pressure-Volume System (Ultra-Miniature Pressure-Volume Catheter (model SPR-1030), Millar Pressure Conductance Unit (model MPCU-200) and Millar PowerLab data-acquisition hardware; emka Technologies, Paris, France). Calibration of pressure and volume was performed by equating the minimal (0mmHg) and maximal (100mmHg) conductances. After inserting the catheter into the carotid artery, retrograde access to the left ventricle (LV) was achieved. P/V loops were recorded under normal conditions (baseline) followed by stress conditions mediated by intravenous dobutamine administration (10µg/kg/min, Sigma-Aldrich). Volume signal was corrected by measurement of wall conductance (parallel volume) via hypertonic saline (5%) injection. Data were analyzed with IOX Version 1.8.3.20 software (emka Technologies). After P/V loop measurements, mice were euthanized by a single dose of potassium chloride to arrest hearts in diastole. Each heart was removed, embedded in O.C.T.™ Compound and snap-frozen in liquid nitrogen. For histological and biomolecular investigations the heart tissue has been divided into 4 horizontal levels from top to bottom and, within each, sections of 6µm were cut. The three interlayers between the mentioned levels have been collected separately for RNA isolation.

Determination of functional perfusion and capillary density

Heart sections of 4 horizontal infarct levels (6µm) were stained with Fast Green FCF (Sigma-Aldrich) and Sirius Red (Division Chroma, Münster, Germany). To evaluate fibrosis (n=5 for each group), the Sirius red positive regions of collagen deposition in the remote area (RA) near endocardial border and in the border zone (BZ) of the infarcted area were examined in 5 randomly chosen fields per section (one section per level; 400x) using computerized planimetry (Axio Vision LE Rel. 4.5 software; Carl Zeiss). Collagen density was expressed as the ratio of collagen deposition to myocardial tissue in percentage.

After termination of P/V loop measurements and before euthanasia, mice were perfused with biotinylated lycopersicon esculentum (tomato) lectin (200µg in 250µl 0.9% NaCl; Vector Labs, USA) for

10min using the canula inserted in the jugular vein. Direct contact of lectin with endothelial cells is required for labeling. Therefore, vessels that are not perfused will not be labeled with lectin. Heart sections of 4 horizontal infarct levels were stained with goat anti-biotin (Vector Labs) and in a second step with donkey anti-goat Alexa Fluor 568 (Life Technologies).

Capillary density was assessed by counting the number of capillaries in 5 RA and 5 BZ randomly-chosen fields (400x). Results were expressed as capillaries per high power field (HPF).

Data Analysis

Statistical analyses were performed using GraphPad Prism software version 5.0. Results were expressed as mean±SEM. Two-group comparisons were analyzed by two-tailed Student t-test. Log-rank test was applied to analyze Kaplan-Meier survival curve. P-values <0.05 were considered statistically significant. Data plots were created using GraphPad Prism software version 5.0.

Results

Stimulation for AT2R significantly elevates AT2R mRNA expression

CD117⁺ SC were isolated using MACS and purity was determined using FACS analysis. Only isolations with a purity of at least 80 % were used for further experiments as previously established by us [17]. The efficiency of AT2R stimulation with Angiotensin II (while AT1R was inhibited with Losartan; (A/L stimulation)) was verified by qRT-PCR, showing an 11.38 ± 2.71 -fold ($n = 5$) elevated AT2R mRNA expression compared to unstimulated control cells (Fig. 1). Following AT2R inhibition with its antagonist PD123319, AT2R mRNA was detectable in only 2 out of 5 CD117⁺ SC samples and downregulated AT2R mRNA levels to those observed in control cells (Fig. 1).

CD117⁺ SC increase HL-1 vitality in direct co-culture setting

To approximate the *in vivo* situation, we used HL-1 cells, a cardiac muscle cell line derived from the AT-1 mouse atrial cardiomyocyte tumor lineage, known to have the ability to express cardiac markers and to contract after stimulation in a co-culture setup. The influence of CD117⁺ SC on HL-1 vitality was assessed using the MTT-assay. CD117⁺ SC ($\pm$ AT2R stimulation) were cultured together with HL-1 cells in a ratio of 1:2 under normoxic and hypoxic conditions. Under both conditions, co-culture with untreated CD117⁺ SC significantly increased HL-1 vitality as compared to single HL-1 culture (normoxia: +9.4% $p < 0.01$; hypoxia: +2.6% $p < 0.05$; $n = 4$). The additional treatment with A/L attenuated this effect (Fig. 2a). The role of AT2R in Ang II-mediated apoptosis has been controversially discussed before and seems to differ between the investigated cell types. In this HL-1 co-culture, stimulation of CD117⁺ SC indicates a reversal of anti-apoptotic effects via AT2R. To determine whether direct cell-to-cell contact is necessary for this beneficial effect, cell types were separated by a cell culture insert (filter). No significant differences in HL-1 vitality were detectable when cultured without cell-to-cell contact (Fig. 2b). The influence of Ang

Fig. 1. Quantitative real-time PCR analysis of AT2R mRNA. CD117⁺ SC were treated with A/L or PD123319 for 24h. Only 2 out of 5 experiments showed AT2R expression after PD123319 treatment. The mean mRNA expression levels of untreated control CD117⁺ SC were arbitrarily given a value of 1 (2^0) as indicated by red arrow. Expression levels were normalized to the expression of GAPDH. Data given as mean±SEM; $n = 5$.

Fig. 2. HL-1 vitality in single culture and co-culture with CD117⁺ cells (±A/L). (a) Bar charts illustrate HL-1 vitality determined by MTT –assay after 48h in culture with or without the addition of CD117⁺ SC. Hypoxia treated groups underwent oxygen deprivation for 24h. A significant increase of HL-1 vitality was detectable following CD117 addition, if cells were cultured with cell-to-cell contact (n=4, in duplicates), (b) but not if an inlet filter was used, which prevented HL-1 and CD117⁺ SC cell-to-cell contact (n=2, in duplicates). Data given as mean±SEM; **p<0.01 vs. normoxic HL-1 group; *p<0.05 vs. hypoxic HL-1 group.

Fig. 3. Immunofluorescence analysis of CD117⁺ SC in HL-1 co-culture setting. Representative image of stained 6-day co-cultured HL-1 and CD117⁺ SC showed specific signals for endothelial marker (a) CD31 and (b) VEGFR-2. Pan-hematopoietic marker CD45 was used to distinguish CD117 cells from HL-1 cell line. Cells were stained using anti-CD31 (Alexa Fluor 488), anti-CD45 (Alexa Fluor 568), anti-VEGFR-2 (Alexa Fluor 488) and DAPI (blue) for nuclei counterstaining. HL-1 cells were labeled with Vybrant Dye DiI prior to co-culture. Scale bars: 5µm. Images were acquired with 400x magnification using LSM 780 confocal microscope (Carl Zeiss).

II, Losartan or A/L treatment on HL-1 cell vitality was assessed in advance and yielded no changes in HL-1 vitality (data not shown).

Upon co-culture, CD117⁺ cells were distinguished from HL-1 cells based on CD45 expression analysis, since HL-1 cells do not express CD45. Thereby, after six days in co-culture, CD117⁺ SC expressed endothelial cell surface markers VEGFR-2 and CD31 (Fig. 3a+b). The expression of CD31 on CD117⁺ SC (single culture) was previously described and shown by our group [17].

Fig. 4. Analysis of endothelial properties of CD117⁺ SC. (a) The ratio length to width was analyzed as a measure of cell elongation following a 2-D Matrigel Assay. Five cells in each 5 random fields of view were blindly assessed in three independent experiments. Width and length of chosen cells were measured using ImageJ software. The influence of AT2R treatment and inhibition was compared to control CD117⁺ SC. n=3; *p<0.001 vs. CD117 control; #p<0.05 vs. CD117+PD 123319. Data given as mean±SEM. (b) Images are representative for cell alignment of the three groups in a 2-D Matrigel assay. Cell alignment was blindly assessed in three independent experiments. For each experiment five cells in five pictures (100x) were evaluated. Scale bar: 20µm. Scores were as follows: - No change; + cell sprouting; ++ cell alignment in one direction.

AT2R stimulation significantly increases CD117⁺ cell elongation ratio

A 2-D BD Matrigel™ Matrix (Matrigel) *in vitro* assay was deployed to investigate influences of stimulation on cell alignment and cell morphology. The elongation ratio (length-to-width) of AT2R stimulated cells (3.1 ± 0.4 ; n=3) was significantly increased 1.4-fold compared to control (2.2 ± 0.2 ; *p<0.03; n=3) and 1.7-fold compared to PD123319 (1.8 ± 0.1 ; #p<0.0009; n=3) treated cells (Fig. 4a). We also observed elevated cell alignment and sprouting following A/L addition compared to control and PD123319 treated cells as analyzed with blind assessment (Fig. 4b). Therefore, the AT2R stimulation might be a relevant factor to induce changes in cell morphology as shown here for CD117⁺ SC. This data supports a previous study [17] indicating that AT2R stimulation could be involved in an endothelial-like differentiation of stem cells.

Intramyocardial injection of CD117⁺ SC improves cardiac function and survival rate

The implantation of CD117⁺ SC improved left ventricular functions mostly independent of AT2R stimulation (Fig. 5). The ejection fraction was significantly increased following CD117⁺ SC implantation, while the cardiac output was significantly elevated after AT2R stimulation if compared to the MI-C group (Fig. 5a+b). However, AT2R stimulation did not generally enhance the beneficial effects of CD117⁺ SC implantation. The reversal of an anti-apoptotic effect of CD117⁺ SC following AT2R stimulation as demonstrated with HL-1 cardiac muscle cell line might be responsible for the inconsistent cardiac function. Heart weight/body weight ratio, which is a commonly used index of cardiac hypertrophy, significantly decreased in the groups with implanted cells (Fig. 6a). Mortality was reduced, but did not reach significance as determined with Log-rank test (p=0.209 MI-CD117+A/L compared to MI-C) (Fig. 6b).

SC implantation significantly reduces collagen deposition and capillary density

The implantation of CD117⁺ SC resulted in a significantly increased capillary density (Fig. 7) and a significantly decreased collagen deposition (Fig. 8). AT2R stimulation could not further restore capillary density or collagen deposition.

Fig. 5. Cardiac functions three weeks after MI. (a) Left ventricular functions at both baseline and stress (dobutamine) conditions were assessed by catheterization. Maximal intraventricular developed pressure was increased in both SC implanted groups compared to MI-C. (b) Ejection fraction and (c) cardiac output were significantly increased in MI-CD117 and MI-CD117+A/L group, respectively, under both treatment conditions. (d) Endsystolic pressure was significantly improved in MI-CD117+A/L group compared to MI-C under stress conditions. Data given as mean $\pm$ SEM; n=6; *p<0.05; **p<0.01 vs. MI-C.

Fig. 6. Improved heart to body weight ratio and mortality 3 weeks after MI. (a) Statistical analysis showed the tendency of improved heart to body weight ratio following SC implantation, which did not reach statistical significance (Student's t-test). (b) Therapy related survival rates [%] did not significantly differ among the MI groups as determined with Log-rank test. Data given as mean $\pm$ SEM.

Collagen deposition in the BZ decreased significantly from 25.53 ± 1.43 % in MI-C to 14.81 ± 0.69 % in MI-CD117 ($p < 0.01$, Fig. 4a+b) and 16.92 ± 0.59 % in MI-CD117+A/L ($p < 0.05$, Fig. 8a+b), respectively. Hearts implanted with SC also showed a significant reduction of collagen deposition rate in the remote area (Fig. 8b). Fig. 8a shows representative staining images of collagen deposition (Sirius Red) and myocytes (Fast Green) in the BZ.

Capillary density was determined based on tomato lectin perfusion. Compared to MI-C (22.47 ± 0.9 capillaries per HPF), capillary density in the BZ significantly increased upon SC transplantation (MI-CD117: 35.79 ± 1.82 ; MI-CD117+A/L: 30.55 ± 2.61 capillaries per HPF) (Fig. 7a+b).

Fig. 7. Increased capillary density in remote area of infarct zone. Representative biotinylated tomato lectin perfusion staining in remote area of infarct zone of (a) Sham, (b) MI-C, (c) MI-CD117 and (d) MI-CD117+A/L. Bound lectin was stained with AlexaFluor 568 secondary antibody (A-D). (e) Capillary density in the remote area is significantly increased following SC implantation 3 weeks after MI. Data given as mean±SEM. *p<0.01 vs. MI-C; n=5.

Fig. 8. Collagen deposition in remote area and border zone of infarct. Representative Fast Green FCF (myocytes)/Sirius Red (fibrosis) stainings at the border zone of the infarcted area of (a) Sham, (b) MI-C, (c) MI-CD117 and (d) MI-CD117+A/L. (e) Collagen deposition is significantly decreased in the remote area and border zone of infarct in both SC treated groups three weeks after MI. Data given as mean±SEM. *p<0.05, **p<0.01 vs. MI-C; n=6.

Cell retention analysis is hampered by inconsistent eGFP expression

Despite the proposed ubiquitous expression of eGFP in the C57BL/6-Tg(CAG-EGFP)10sb/J donor mice, only 35.3±2.4 % MACS-isolated viable CD117⁺ BMSC expressed eGFP in contrast to 87.5±6.8 % of lung CD117⁺ SC (n=5). To further assess a possible loss of eGFP expression during culture or differentiation, CD117⁺ SC were purified for eGFP expression with fluorescence activated cell sorting analysis (FACS re-analysis: 85.7 %, data not shown). This purified population was subjected to CFU assay and eGFP expression was determined after 12 d using flow cytometry and fluorescence microscopy. This analysis revealed that eGFP expression was diminished to 49.5±8.1 % (n=2) in viable CD117⁺ cells. We concluded that using EGFP protein expression analysis would result in false negative results and largely underestimate the number of surviving cells. Hence, we did not equal protein expression with cell retention. In contrast, qRT-PCR analysis of eGFP mRNA expression gave more consistent results. CD117⁺ SC freshly isolated from eGFP⁺ transgenic mice or after

Fig. 9. Quantitative real-time PCR analysis of heart and lung tissue 3 weeks after MI. qRT-PCR analysis for eGFP gene in heart and lung tissue. The eGFP expression in heart correlates with the expression found in lung. In MI-CD117+A/L group significantly more eGFP was detected in lung tissue as compared to MI-CD117. EGFP gene levels are given as $\Delta C_t = C_{teGFP} - C_{tGAPDH}$ as eGFP is not expressed in Sham and MI-C. Expression levels were normalized to the expression of GAPDH. Data given as mean $\pm$ SEM; n=6, *p<0.05 vs. MI-CD117.

Fig. 10. Immunofluorescence analysis of eGFP+ implanted cells 24h after intramyocardial injection. Representative cut-out image of eGFP+ cells in the heart 24h after implantation. Few cells were found to express EGFP+, which was not gradable using anti-GFP antibody and not found in later time points. EGFP+ cells were captured to express VEGF (anti-VEGF; Alexa Fluor 568). Nuclei were counterstained with DAPI (blue). Scale bar: 5μm. Images were acquired with 630x magnification using LSM 780 confocal microscope (Carl Zeiss).

12 d in CFU displayed a relative eGFP expression as high as in the positive control (eGFP+ heart tissue). Therefore, investigation of mRNA expression levels more closely resembled the magnitude of recovered cells and was further used in this study.

Three weeks after MI and cell implantation, explanted hearts were co-used for qRT-PCR and immunohistochemistry analysis. Lungs were further analyzed for eGFP expression, since wash-out of intramyocardially implanted cells into the filter organs is an acknowledged process [31, 32]. EGFP mRNA expression was found in the heart sections and correlates with the expression found in lungs (Fig. 9). The eGFP mRNA expression in the organs is plotted as ΔC_t (relative to GAPDH mRNA expression), since it is not possible to compare the groups to Sham or MI-C. MI-CD117 group showed the tendency of a higher eGFP+ cell recovery in the heart, correlating to fewer cells migrating towards the lung in contrast to MI-CD117+A/L group (Fig. 9). A significantly higher cell number (1.2-fold) migrated towards the lung following AT2R stimulation, compared to MI-CD117, offering an explanation for diminished beneficial effects in the heart.

To assess the recovery and localization of (eGFP+) CD117+ SC in the tissue, selected heart slices were observed 24h, 48h and 7d after cell implantation for eGFP expression. Independent

Fig. 11. Quantitative real-time PCR analysis of heart sections 3 weeks after MI. (a) qRT-PCR analysis for AT2R, (b) Bax/Bcl-2, (c) eNOS, (d) MMP-2, (e) CXCL12 and (f) CD34 genes, respectively in heart sections. SC implantation upregulates AT2R as well as eNOS and downregulates Bax/Bcl-2 ratio and MMP-2 expression. The average mRNA expression level in the Sham hearts was arbitrarily given a value of 1 (2^0). Expression levels were normalized to the expression of GAPDH. Data given as mean $\pm$ SEM; n=6, *p<0.05 vs. MI-C; #p<0.05 vs. MI-CD117.

of MI, few eGFP+ cells were found in hearts explanted 24h after cell implantation, but no later. The lack of EGFP positive cells later than 24h is explainable regarding the diminished EGFP protein expression *in vitro* as described above. However, immunohistochemistry analysis of the detected cells showed co-localization of eGFP and VEGFR-2 (Fig. 10) 24h after implantation.

Stem cell implantation induces cardioprotective effects

To clarify cardioprotective and anti-apoptotic effects of the implanted cells, mRNA levels of eNOS and Bax/Bcl-2 ratio were evaluated. An increased eNOS expression in the tissue is associated with an enhanced migratory capacity of HSC and improved neovascularization [33]. As shown in Fig. 11c, eNOS mRNA expression was 2.7-fold elevated in the MI-CD117 group compared to the MI-CD117+A/L group (p<0.05; Fig. 11c). The enhanced eNOS expression following AT2R stimulation, as proposed by other research groups [34], might be an immediate process and thus, not detectable three weeks after MI. Moreover, a reduced Bax/Bcl-2 ratio was reported to alleviate cardiomyocyte apoptosis [35] and is a key factor in cell survival. Our findings revealed Bax/Bcl-2 ratio was significantly reduced by 2.3 and

2.8-fold in MI-CD117 and MI-CD117+A/L, respectively when compared to MI-C (Fig. 11b), pointing to long-term anti-apoptotic effects after SC administration. The cardioprotective effects of stem cell injection were not additionally enhanced using AT2R stimulation.

Stem cell implantation regulates expression of MMP-2 and AT2R, but not Cxcl12

Previous studies revealed that inhibition of matrix metalloproteinase-2 (MMP-2) activity improves the survival rate after MI and increased MMP-2 ratios can be reversed with AT1R blockers [36, 37]. MMP-2 mRNA was considerably down-regulated in MI-CD117+A/L hearts compared to MI-CD117 (Fig. 11d). Different studies showed down-regulation of MMP-2 expression in the context of anti-fibrotic activity following AT2R stimulation [36, 37]. We contribute to this topic, showing a long-term inhibition of MMP-2 as an effect of AT2R stimulation. However, the MMP-2 expression does not accompany remarkably reduced fibrosis if compared with MI-CD117 group. No significant differences in Cxcl12 (Fig. 11e) and CD34 (Fig. 11f) mRNA regulation were detectable upon CD117⁺ SC injection, independent of stimulation. Thus, we speculate that the process of bone marrow stem cell migration is immediate and not perceptible after the remodeling process is completed. The expression of AT2R mRNA in both stem cell treated groups was visibly increased compared to MI-C (Fig. 11a).

Discussion

The current study aimed to assess the potential of bone marrow derived CD117⁺ SC to contribute to cardiac regeneration after MI. We further addressed the existing controversies on the beneficial effects of AT2R stimulation and their underlying mechanisms. A regenerative potential of the CD117⁺ SC population might be attributed to their differentiation into an endothelial-like phenotype, as shown in a 2D-Matrigel assay and with patch-clamp measurements in recent studies published by our group [17]. Using a co-culture setting involving the well characterized HL-1 cell line, we could show proof-of-principle that cell-to-cell contact is a pre-requisite for CD117⁺ SC to improve cardiomyocyte vitality [38, 39]. Therefore, our findings are in line with a recent study demonstrating that cell-to-cell contact induces anti-apoptotic pathways in hematopoietic stem cells (HSC) indicating cardioprotective effects of HSC on cardiomyocytes in a co-culture of HSC with neonatal rat ventricular myocytes [40]. Moreover, enhanced paracrine effects, such as the secretion of VEGF and the expression of VEGFR-2, may be responsible for the quantitative increase in capillary density. In conjunction with our previous study, we demonstrate here that the improved cardiac function is likely a result of cardioprotective actions of CD117⁺ SC and not their electromechanically coupling to native cells. Contrary to previous studies [34], AT2R could not enhance the significant effect of stem cell implantation on cardiac function. Here, we approached AT2R stimulation by simultaneous treatment of CD117⁺ SC with Ang II and the AT1R inhibitor Losartan. Therefore, we could exclude a possible influence of AT1R signaling on the actions of stimulated CD117⁺ SC. Most studies describe significant effects of AT2R signaling either in short-term *in vitro* experiments [41], by using undefined cell populations [34] or in a chronic, systemic AT2R stimulation approach [21]. We were able to reproduce short-term influences of AT2R stimulation in a 2-D Matrigel assay and in a co-culture of HL-1 cardiac muscle and CD117⁺ SC. However, the transient stimulation of the defined CD117⁺ SC population is insufficient to add to proposed long-term beneficial effects for cardiac regeneration.

The detection and analysis of eGFP mRNA levels indicated that the implanted CD117⁺ SC physically contribute to the maintenance of cardiac function and to improved neovascularization. However, the retrieved eGFP⁺ cell number was very low and no injected cells were found 48 h after implantation using immunohistochemistry. Cell retention is limited by 1) the immediate washout following injection and thereafter because of 2) the hostile environment offered in the infarcted area, which lacks supporting cells or tissues,

oxygen or nutritive substrates, but contains an excess of neutrophils and scavenger cells [31, 42, 43]. However, it has to be pointed out that CD117⁺ BMSC display inconsistent eGFP expression in the investigated transgenic mice strain, which further hampers cell retention analysis. Limitations of the use of GFP transgenic mice in bone marrow stem cells were described before [44] and should be taken into account for future studies.

Important unresolved mechanisms of AT2R signaling are its association with fibrosis, apoptosis and its action in angiogenesis [45, 46]. We therefore chose to assess the influence of onetime AT2R stimulation of CD117⁺ SC on certain key factors involved in these mechanisms three weeks after MI.

A reduced Bax/Bcl-2 ratio has been reported to alleviate cardiomyocyte apoptosis. Increased Bcl-2 levels in the acute stage of MI and upregulation of Bax expression by chronic cellular responses of the affected myocytes against (mechanical) stress was described previously [35]. We demonstrated that the Bax/Bcl-2 ratio significantly decreased in both SC implanted groups, when compared to MI-C, pointing to long-term anti-apoptotic effects after SC administration. Despite the negative effect of AT2R stimulation on HL-1 vitality, Bax/Bcl-2 ratio was not significantly regulated by the transient AT2R stimulation of implanted CD117⁺ SC.

Moreover, an upregulation of eNOS expression has improved the migratory capacity of bone marrow-derived stem cells [33]. eNOS was further shown to play a role in ischemic neovascularization [47-49]. In our mouse model of myocardial infarction, eNOS mRNA expression in hearts injected with MI-CD117+A/L was significantly lower than in the MI-CD117 group. With respect to BMSC recruitment from the bone marrow, we could not find significant differences in mRNA levels of CD34 stem cell marker or stem cell homing factor CXCL12 (SDF-1). We speculate that SC recruitment is immediately induced, and not subject to changes in chronic ischemia. Likewise, it is unlikely associated with eNOS regulation at later time points.

MMP-2 is a key factor for micro-environmental adhesion, maturation and differentiation of stem cells [50, 51], but also for the breakdown of extracellular matrix in normal physiological processes and tissue remodeling. Controversies exist with respect to the regulation of MMP-2 after MI. Studies suggest that inhibition of MMP-2 activity improves the survival rate after acute MI by preventing cardiac rupture and delays post-MI remodeling through a reduction in macrophage infiltration [52]. Interestingly, blockade of both Ang II receptors resulted in increased stiffness, fibrosis and increased MMP-2 activity, which was reversed with AT1R blockers [36, 37]. This is in line with our observations, as remarkably decreased MMP-2 mRNA levels were detected in the MI-CD117+A/L group.

However, the stimulation of AT2R on CD117⁺ SC provoked no additional improvement of cardiac functions, fibrosis or capillary density. Species differences, heterogenous SC subpopulations and variation in experimental setups in terms of stimulation might contribute to the different outcomes of previous studies and hamper a reliable comparison. Moreover, it has to be considered that the RAS is activated following MI and that this endogenous activation of AT1R/AT2R might mask specific effects of MI-CD117+A/L stimulated SC after transplantation. Yet, we observed a significant increase of cell alignment and cell elongation after AT2R stimulation *in vitro*, again pointing to a mechanism that might be masked by AT1R related-effects *in vivo*.

Therefore, our work contributes to the clarification of controversies about positive vs. negative AT2R effects after MI. We suggest that the positive effects seen after transient AT2R stimulation are short-term only and cannot significantly add to the beneficial effects of CD117⁺ SC implantation. Yet, an optimized stimulation approach of these cells (i.e. chronic and/or locally restricted to the site of transplantation) may provide a suitable means to exploit AT2R signaling for cardiac stem cell therapy.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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