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ORIGINAL ARTICLE

Irradiated cultured apoptotic peripheral blood mononuclear cells regenerate infarcted myocardium

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ABSTRACT

Background Acute myocardial infarction (AMI) is followed by post AMI cardiac remodelling, often leading to congestive heart failure. Homing of c-kit+ endothelial progenitor cells (EPC) has been thought to be the optimal source for regenerating infarcted myocardium.

Methods Immune function of viable peripheral blood mononuclear cells (PBMC) was evaluated after co-culture with irradiated apoptotic PBMC (IA-PBMC) *in vitro*. Viable PBMC, IA-PBMC and culture supernatants (SN) thereof were obtained after 24 h. Reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay were utilized to quantify interleukin-8 (IL-8), vascular endothelial growth factor, matrix metalloproteinase-9 (MMP9) in PBMC, SN and SN exposed fibroblasts. Cell suspensions of viable- and IA-PBMC were infused in an experimental rat AMI model. Immunohistological analysis was performed to detect inflammatory and pro-angiogenic cells within 72 h post-infarction. Functional data and determination of infarction size were quantified by echocardiography and Elastica van Gieson staining.

Results The IA-PBMC attenuated immune reactivity and resulted in secretion of pro-angiogenic IL-8 and MMP9 *in vitro*. Fibroblasts exposed to viable and IA-PBMC derived SN caused RNA increment of IL-8 and MMP9. AMI rats that were infused with IA-PBMC cell suspension evidenced enhanced homing of endothelial progenitor cells within 72 h as compared to control (medium alone, viable-PBMC). Echocardiography showed a significant reduction in infarction size and improvement in post AMI remodelling as evidenced by an attenuated loss of ejection fraction.

Conclusion These data indicate that infusion of IA-PBMC cell suspension in experimental AMI circumvented inflammation, caused preferential homing of regenerative EPC and replaced infarcted myocardium.

Keywords Acute myocardial infarction, c-kit, endothelial progenitor cells, immunosuppression, PBMC.

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Introduction

Acute myocardial infarction (AMI) often leads to congestive heart failure [1]. Despite current pharmacological and mechanical revascularization, no effective therapy is defined experimentally to replace infarcted myocardium. Integral component of the remodelling process after AMI is the inflammatory response and the development of neo-angiogenesis after AMI [2–4]. These processes are mediated by cytokines and inflammatory cells in the infarcted myocardium that phagocytose apoptotic and necrotic tissue and initiate homing of interstitial dendritic cells (IDC) and macrophages [5–7]. Clinical trials aimed at attenuating AMI induced inflammatory response were abducted as systemic immunosuppression (steroids) led to increased infarct size and delayed myocardial healing [8]. From these data, it was concluded that inflammatory response after AMI is responsible for tissue stabilization and scar formation. A new field in regenerative cardiovascular medicine emerged when investigators observed that distant stem cells sense sites of damage and promote structural and functional repair [9–12]. By utilizing this approach, Orlic *et al*. injected c-kit positive endothelial progenitor cells (EPC) into the boarder zone of experimental AMI and increased neo-angiogenesis and regeneration of myocardial and vascular structures [13]. This

work ignited a plethora of publications that demonstrated a regenerative potential of 'cell-based therapy'; however, it still remains elusive whether this therapeutic effect is caused by the transplanted cells themselves, recruitment of resident cardiac stem cells or by activation of as yet unidentified paracrine and immunological mechanisms [14]. Ischaemia in infarcted myocardium causes apoptotic processes and initiates alterations in cell surface lipids on dying cells. The best-characterized modification is the loss of phospholipid asymmetry and exposure of phosphatidylserine (PS). These PS are recognized by macrophages and dendritic cells (DC) (antigen presenting cells, APC) via ligands such as thrombospondin, CD14 and CD36 [15]. Under physiological conditions, these receptors serve to engulf apoptotic and necrotic debris and initiate a silent '*clean up*' process. This process of phagocytosis by APC leads to a phenotypic anti-inflammatory response as determined by augmented interleukin-10 and transforming growth factor-ß (TGF-ß) production and impaired APC function [16]. Of clinical relevance are reports that demonstrated that infusion of apoptotic cells leads to allogeneic haematopoietic cell (HC) engraftment in a transplantation model [17] and to a delay in lethal acute graft-versus-host disease (GVHD) [18]. Moreover, in solid organ transplantation models, infusion of donor apoptotic cells increased heart graft survival [19]. Contrary to inflammation and relevant to progenitor cell recruitment from bone marrow (BM), it was shown that opsonization of apoptotic cells elicits enhanced vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8/CXCL8) production of APC [20–22]. In addition to the latter cytokines, matrix metalloproteinase-9 (MMP9) was identified as vital for EPC recruitment and liberation from the bone marrow [23].

We conclude that the current 'status quo' in AMI treatment is directed towards early reperfusion and reopening the acute occluded coronary artery and that myocardial inflammation post infarction is perceived beneficiary despite the fact that this condition increases myocardial damage and counteracts endogenous repair mechanisms. We therefore speculate that a 'novel therapeutic programme' in AMI treatment should include modification of inflammation after myocardial ischaemia and supply systemic paracrine factors that augment recruitment and consequently homing of EPCs staining positive for the progenitor cell marker c-kit from the bone marrow to the infarcted myocardium. As physiological clearance of apoptotic leucocytes evidences such biological qualities, [16–22] we hypothesized whether irradiation induced apoptosis of peripheral blood mononuclear cells (IA-PBMC) advances such attributes. Here, we show that culture of IA-PBMC causes decrease in immune function and augmented secretion and mRNA transcription of proteins related to angiogenesis and stem cell mobilization (VEGF, IL-8/CXCL8 and MMP9) [23] *in vitro*. We therefore infused cultured IA-PBMC in an acute rat AMI model and demonstrated c-kit dependent regeneration of infarcted myocardium.

Methods

Induction of apoptosis of PBMC and generation of supernatants

For the *in vitro* experiments, blood samples were drawn from healthy young volunteers. Apoptosis of PBMC was induced by Cs-137 caesium irradiation with 60 Gy (human PBMC) or with 45 Gy for *in vivo* (rat PBMC) experiments [24]. Cells were resuspended in serum-free Ultra Culture Medium (Cambrex Corp., North Brunswick, NJ, USA) containing 0·2% gentamycin sulphate (Sigma Chemical Co, St Louis, MO, USA), 0·5% β-mercapto-ethanol (Sigma), 1% L-glutamine (Sigma) and cultured in a humidified atmosphere for 24 h for *in vitro* experiments (concentration of cells, 1×10^6 mL). Induction of apoptosis was measured by AnnexinV-fluorescein/propidium iodide (FITC/PI) co-staining (Becton Dickinson, Franklin Lakes, NJ, USA) on a flow cytometer. Annexin-positivity of PBMCs was determined as > 70% and these cells are consequently termed IA-PBMC. Non-irradiated PBMC served as controls and are termed viable-PBMC. From both experimental settings, supernatants were collected and served as experimental entities as described below (SN-viable- PBMC, SN-IA-PBMC).

LPS-stimulation experiments

Human PBMCs and monocytes (purity > 95%) were separated using a magnetic bead system (negative selection Miltenyi Biotec, Auburn, CA, USA). PBMCs and monocytes were co-incubated for 4 h with different concentrations of apoptotic autologous PBMCs (annexin postivity > 70%, data not shown) and lipopolysaccharide (1 ng mL^{-1} LPS; Sigma Chemical Co). Supernatants were secured and kept frozen at −80 °C until further tests. IL-6 and IL-1β release was determined using commercially available ELISA kits (BenderMedSystems, Vienna, Austria).

Monocyte-derived dendritic cell preparation and T-cell stimulation

PBMCs were isolated from heparinized whole blood of healthy donors by standard density gradient centrifugation with Ficoll-Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). T cells and monocytes were separated by magnetic sorting using the MACS technique (Miltenyi Biotec). Purified T cells were obtained through negative depletion of CD11b, CD14, CD16, CD19, CD33 and MHC class II-positive cells with the respective monoclonal antibody. Monocytes were enriched by using the biotinylated CD14 mAb VIM13 (purity 95%). DCs were generated by culturing purified blood monocytes for 7 days with a

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combination of GM-CSF (50 ng mL^{-1}) and IL-4 (100 U mL^{-1}). Subsequently, DCs were differently stimulated. Maturation was induced either by adding 100 ng mL^{-1} LPS from Escherichia coli (serotype 0127-B8, Sigma Chemie) for 24 h alone or by adding LPS for 2 h and further culturing the DC with apoptotic cells in a 1 : 1 ratio for 22 h. Additionally, DCs were treated with apoptotic cells alone (1 : 1) for 24 h. For the mixed leucocyte reaction (MLR), allogenic, purified T cells (1×10^5 per well) were incubated in 96-well cell culture plates (Corning Costar, Lowell, MA, USA) with graded numbers of differently stimulated DCs for 6 days. The assay was performed in triplicate. Proliferation of T cells was monitored by measuring [methyl-3H]thymidine (ICN Pharmaceuticals, Weiden, Germany) incorporation, added after 5 days. Cells were harvested after 18 h and incorporated [methyl-3H]thymidine was detected on a microplate scintillation counter.

Cell culture, RNA isolation and cDNA preparation of viable PBMC, IA-PBMC and SN exposed fibroblasts

IA-PBMCs, viable-PBMC (1×10^6 cells, both conditions cultured for 24 h in Ultra Culture Medium) and fibroblasts exposed to SN-viable-PBMC/SN-IA-PBMC were investigated [1×10^5 fibroblasts obtained from Cascade Inc. (Portland, OR, USA)] were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% foetal bovine serum (FBS; PAA, Linz, Austria), 25 mM L-glutamine (Gibco-BRL) and 1% penicillin/streptomycin (Gibco) and seeded in 12-well plates; fibroblasts were co-incubated with SN-viable-PBMC, SN-IA-PBMC- for 4 and 24 h respectively). After RNA extraction of PBMC and fibroblasts (using RNeasy; Qiagen, Vienna, Austria) following the manufacturer's instruction, cDNAs were transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) as indicated in the instruction manual.

Quantitative real time PCR

mRNA expression was quantified by real time PCR with LightCycler Fast Start DNA Master SYBR Green I (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocol. The primers for VEGF (forward: 5′-CCCTGATGAGATCGAGTACATCTT-3′, reverse: 5′-ACCGCCTCGGCTTGTCAC–3′), IL-8 (forward: 5′-CTCTTGGCAGCCTTCCTGATT-3′, reverse: 5′-TATGCACTGACATCTAAGTTCTTTAGCA-3′), MMP9 (forward: 5′-GGGAAGATGCTGGTGTTCA-3′, reverse: 5′-CCTGGCAGAAATAGGCTTC-3′) and β-2-microglobulin (β2M, forward: 5′-GATGAGTATGCCTGCCGTGTG-3′, reverse: 5′-CAATCCAAATGCGGCATCT-3′) were designed as described previously [25]. The relative expression of the target genes was calculated by comparison with the house keeping gene β2M using a formula described by Wellmann *et al.* [26]. The efficiencies of the primer pairs were determined as described [25].

Release of pro-angiogenetic factors and MMP9 by viable PBMC and IA-PBMC after culture

IA-PBMC (5×10^5) and viable PBMC were incubated in a humidified atmosphere for 24 h. Supernatants were collected after 24 h and immediately frozen at −80 °C until evaluation. Lysates of respective cells served as controls. Release of pro-angiogentic factors (VEGF, IL-8/CXCL8, GM-CSF, G-CSF) and MMP9, an accepted liberating factor of c-kit cells, was analysed utilizing ELISA (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. Plates were read at 450 nm using a Wallac Multilabel counter 1420 (PerkinElmer, Boston, MA, USA).

Acquisition of syngeneic IA-PBMC and viable-PBMC for AMI *in vivo* experiment

Syngeneic rat PBMC for *in vivo* experiments were separated by density gradient centrifugation from whole-blood obtained from prior heparinized rats by puncturing of the heart. Apoptosis was induced by Cs-137 caesium irradiation with 45 Gy for *in vivo* experiments and cultured for 18 h as described above. (annexin V staining > 80% IA-PBMC, annexin V staining < 30% viable PBMC).

Induction of myocardial infarction

Animal experiments were approved by the committee for animal research, Medical University of Vienna (BMBWK-66·009/0278-BrGT/2005). All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH). Myocardial infarction was induced in adult male Sprague–Dawley rats by ligating the left anterior descending artery (LAD) as previously described [27]. In short, animals were anaesthetized intraperitoneally with a mixture of xylazine (1 mg per 100 g bodyweight) and ketamine (10 mg per 100 g bodyweight) and ventilated mechanically. A left lateral thoracotomy was performed and a ligature using 6-0 prolene was placed around the LAD beneath the left atrium. Immediately after the onset of ischaemia, 8×10^6 apoptotic PBMCs suspended in 0·3 mL cell culture medium were infused through the tail vein. Infusion of cell culture medium alone, viable PBMC and sham operation respectively served as negative controls in this experimental setting. The rat experimental design is shown in Fig. 1a,b.

Tracking of apoptotic cells

8×10^6 syngeneic rat PBMC were labelled with 15 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Fluka BioChemika, Buchs, Switzerland) at room temperature for 10 min. Labelling was stopped by the addition of foetal calf serum

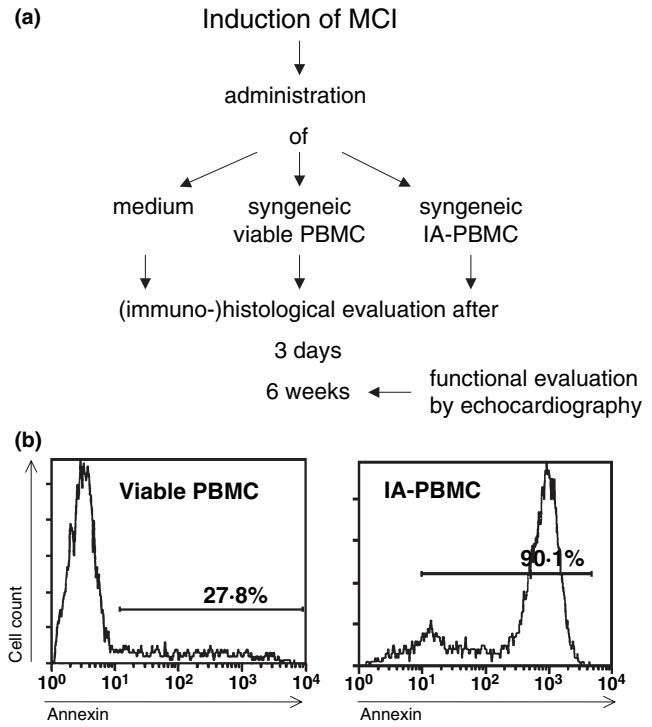

Figure 1 (a) Flowchart of the study design, (a) The study protocol and the time points of evaluation of cardiac function by echocardiography, histology and immunohistology. (b) The percentage of irradiated and non-irradiated rat PBMC positively stained for Annexin V after a culture period of 18 h. $n = 4$.

(FCS). Apoptosis was induced (annexin V > 70%) and cells were injected after ligation procedure. Seventy-two hours after operation, rats were sacrificed and the liver, spleen and heart were processed following a standard procedure for frozen sections ($n = 4$). Samples were analysed using confocal laser scanning microscopy (ZEISS LSM 510 laser scanning microscope, Jena, Germany) as described previously [28].

Histology and immunohistochemistry *in vivo*

All animals were killed either 72 h or 6 weeks after experimental infarction. Hearts were explanted and then sliced at the level of the largest extension of infarcted area ($n = 8$–10). Slices were fixed in 10% neutral buffered formalin and embedded in paraffin for (immuno-)histological staining. The tissue samples were stained with haematoxylin–eosin (HE) and elastic van Gieson (evg). Immunohistological evaluation was performed using the following antibodies directed to CD68 (MCA 341R; AbD Serotec, Kidlington, UK), VEGF (05-443; Upstate/Milipore, Charlottesville, VA, USA), Flk-1 (sc-6251; Santa Cruz Biotechnology, Delaware, CA, USA), CD34 (sc-52478; Santa Cruz Biotechnology), c-kit (sc-168; Santa Cruz Biotechnology), S100 beta (sc-58841; Santa Cruz Biotechnology). Tissue samples were evaluated using Olympus Vanox AHBT3 microscope (Olympus Vanox AHBT3; Olympus Optical Co. Ltd., Tokyo, Japan) at 200× magnification and captured digitally by using a ProgRes CapturePro C12 plus camera (Jenoptik Laser Optik Systeme GmbH, Jena, Germany).

Determination of myocardial infarction size by planimetry

To determine the size of the infarcted area, we used Image J planimetry software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA). The extent of infarcted myocardial tissue (% of left ventricle) was calculated by dividing the area of the circumference of the infarcted area by the total endocardial and epicardial circumferenced areas of the left ventricle. Planimetric evaluation was carried out on tissue samples stained with evg for better comparison of necrotic areas. Infarct size was expressed as percentage of total left ventricular area.

Cardiac function assessment by echocardiography

Six week after induction of myocardial infarction, rats were anaesthetized with 100 mg kg^{-1} Ketamine and 20 mg kg^{-1} Xlyazine. The sonographic examination was conducted on a Vivid 5 system (General Electric Medical Systems, Waukesha, WI, USA). Analyses were performed by an experienced observer blinded to treatment groups to which the animals were allocated. M-mode tracings were recorded from a parasternal short-axis view and functional systolic and diastolic parameters were obtained. Ventricular diameters and volumes were evaluated in systole and diastole. Fractional shortening was calculated as follows: FS(%) = ((LVEDD − LVESD)/LVEDD)×100%.

Statistical methods

Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA). All data are given as mean ± standard of the mean. Normal distribution was verified using the Kolmogorov–Smirnov test. Paired two-sided *t*-tests for dependent, unpaired *t*-tests for independent variables were utilized calculating significances. Bonferroni-Holm correction was used to adjust *P*-values for multiple testing. *P*-values < 0·05 were considered statistically significant.

Results

Induction of apoptosis with caesium irradiation (IA-PBMC)

To evaluate the immunomodulatory potential of apoptotic cells, we first determined the cellular response to induction of apoptosis by cesium irradiation of human peripheral blood mononuclear cells (PBMC) by flow cytometry utilizing Annexin-V/PI

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staining on a flow cytometer. Irradiation caused positivity for Annexin on PBMC in a time-dependant manner and peaked within 24 h as compared to viable PBMC. Viable cells served as controls (Fig. 2a). As Annexin-V binding was the highest after 24 h, all further *in vitro* investigations were performed after this culture period (IA-PBMC). Viable PBMC served as control in RT-PCR and supernatant experiments.

IA-PBMC evidence immunosuppressive features *in vitro*

Interleukin-1β and interleukin-6 is recognized as the predominant pro-inflammatory mediator in myocardial infarction *in vivo*. To test the hypothesis whether IA-PBMC have an effect on cellular response, we co-incubated human monocytes and PBMC with IA-PBMC and stimulated target cells with LPS. We found a dose-dependant decrease in secretion of IL-1β and IL-6 in cultures of both cell types as evaluated by ELISA (Fig. 2b,c). To verify anti-proliferative effects of IA-PBMC in an allogeneic model, we utilized a mixed lymphocyte reaction (MLR). We utilized allogenic, purified T cells and incubated these effector cells with graded doses of DC with/without addition of IA-PBMC. Figure 2d evidences that co-incubation of IA-PBMC decreases proliferation rate in a dose-dependent manner.

IA-PBMC and viable PBMC evidence increased mRNA transcription of VEGF, IL-8/CXL8 and MMP9

To substantiate whether irradiation leads to enhanced mRNA transcription of proteins known to be related to mobilization of EPC, we analysed PBMC after separation and after apoptosis induction (24 h). RNA transcription showed little difference in VEGF expression as determined by RT-PCR, however a strong increase of IL-8/CXCL8 and MMP9. Peak induction for IL-8/CXCL8 in IA-PBMC was sixfold vs. twofold in viable cells and 30-fold vs. fivefold for MMP9 transcripts respectively (Fig. 2e).

IA-PBMC and viable PBMC secrete paracrine factors that cause endothelial progenitor cells liberation

SN derived from IA-PBMC and viable PBMC were quantified for VEGF, IL-8/CXCL8, GM-CSF, G-CSF and MMP9 utilizing ELISA after 24 h culture. As seen in Fig. 2f, VEGF, IL-8/CXCL8 and MMP9 evidenced an increment. GM-CSF and G-CSF were not detectable (data not shown). Of interest was the finding that MMP9 evidenced peak values in cell lysates.

SN derived from IA-PBMC and viable PBMC augment pro-angiogenic mRNA transcription in mesenchymal fibroblasts

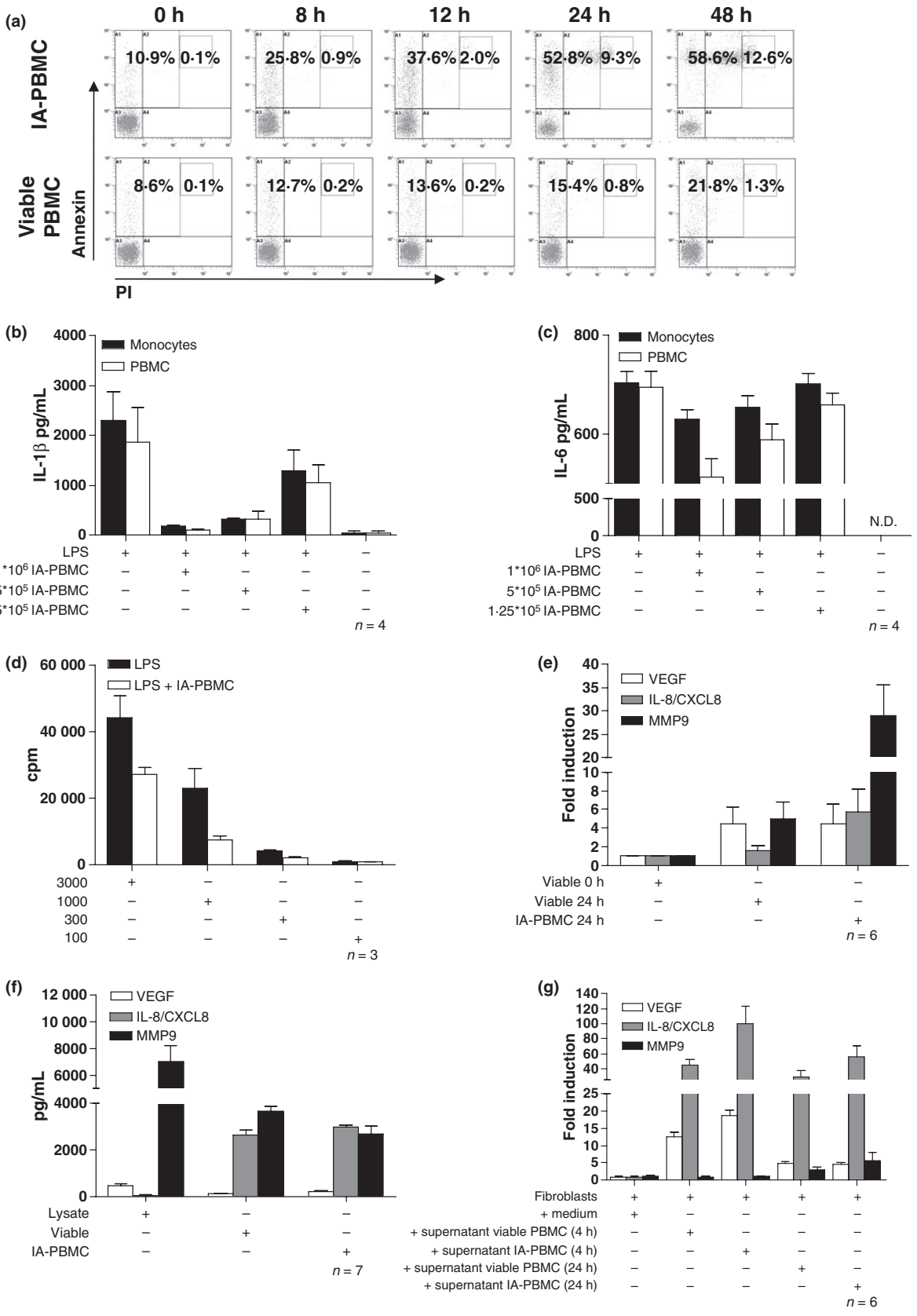
As stromal cells in bone marrow are constitutively fibroblasts, we sought to investigate whether co-incubation of fibroblasts with SN derived from IA-PBMC and viable PBMC had the ability to increase VEGF, IL-8/CXCL8 and MMP9 [23] mRNA transcription, factors responsible for EPC mobilization. RT-PCR was conducted at 4 and 24 h. Highest levels of induction were detected for IL-8/CXCL8 in cells cultivated in IA-PBMC supernatants reaching an almost 120-fold induction at 4 h as compared to controls. This response is also present at 24 h. A comparable response was found for VEGF, whereas MMP9 upregulation was predominantly found after 24 h. This finding indicates that supernatants contain paracrine factors that enhance fibroblasts to augment mRNA products responsible for pro-angiogenetic effects in the bone marrow (Fig. 2g).

Adoptive transfer of CFSE labelled IA-PBMC in a rat myocardial infarction model

As we were able to prove that cultured IA-PBMC are both anti-inflammatory and pro-angiogenetic *in vitro* we infused IA-PBMC and viable PBMC in an acute rat AMI model. First, we sought to determine where these cultured cells are homing after infarction. CFSE labelled IA-PBMC were injected into the tail vein shortly after LAD artery ligation. A representative histology is seen in Fig. 3a–c. A majority of CFSE-labelled IA-PBMC was trapped in the spleen and liver tissue within 72 h. No cells were observed in the heart.

Diverted early inflammatory immune response in IA-PBMC-treated AMI

Upon closer investigation in HE staining, control infarction and viable leucocytes (viable PBMC)-treated AMI rats evidenced a mixed cellular infiltrate in the wound areas in accordance with granulation tissue with abundance of neutrophils, macrophages/monocytes, lymphomononuclear cells, fibroblasts and activated proliferating endothelial cells admixed to dystrophic cardiomyocytes. (Figure 3d,e) within 72 h after AMI. In contrast, rats treated with IA-PBMC evidenced a dense monomorphic infiltrate in wound areas that consisted of medium-sized monocytoid cells with eosinophilic cytoplasm, dense nuclei and a round to spindle shaped morphology (Fig. 3f). In addition, few lymphomononuclear cells, especially plasma cells, fibroblasts and endothelial cells could be detected. Immunohistochemical analysis revealed that the cellular infiltrate in IA-PBMC AMI rats was composed of abundant $CD68^+$ monocytes/macrophages (Fig. 3i) that were much weaker in the other two groups [MCI, viable PBMC and IA-PBMC high-power-field (HPF) cell counts of 60·0 ± 3·6, 78·3 ± 3·8 and 285·0 ± 23·0 (±SEM) respectively] (Fig. 3g,h). Content of vimentin-positive mesenchymal cells was similar in all groups (data not shown), while S100+ DC [29] were preferentially found in control infarction [AMI, viable PBMC and IA-PBMC HPF cell counts of 15·6 ± 1·7, 12·4 ± 2·3 and 8·4 ± 1·2 (±SEM) respectively] as compared to treated groups (Fig. 3j,k,h) (representative histology, $n = 5$).



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Early homing of VEGF+, Flk1+ and c-kit+ cells in IA-PBMC-treated AMI

As IA-PBMC evidenced a dense monomorphic infiltrate in wound areas that consisted of medium-sized monocytoid cells with eosinophilic cytoplasm and dense nuclei, we explored multiple surface markers related to neo-angiogenesis and regenerative potency. This 'peculiar' cell population identified in the HE staining in IA-PBMC-treated AMI group stained highly positive for VEGF, vascular endothelial growth factor receptor (Flk-1) and c-kit (CD 117) (Fig. 4c,f,i). Expression of both markers was reduced in control AMI and viable PBMC-treated AMI group (Fig. 4a,b,d,e,j,k) (Representative histology, $n = 5$).

Attenuated infarct size in IA-PBMC-treated AMI

In a planimetric analysis performed on EVG stained tissue samples from hearts explanted 6 weeks after myocardial infarction was induced, rats receiving medium show a collagenous scar extending to over 24·95 ± 3·58% (±SEM) of the left ventricle with signs of dilatation. In IA-PBMC-treated rats, these signs were almost abrogated with infarct sizes of 5·81 ± 2·02% (±SEM) as compared to 14·3 ± 1·7% (±SEM) treated with viable PBMC (Fig. 5a–c).

LV function improves in IA-PBMC-treated AMI

Intravenous application of syngeneic cultured IA-PBMC significantly improved echocardiographic parameters as compared with viable PBMC or culture medium-treated animals. Ejection fraction (EF) was 60·58 ± 6·81% (±SEM) in sham operated rats and declined to 42·91 ± 2·14% (±SEM) in AMI animals treated with medium, and to 42·24 ± 3·28% (±SEM) in animals receiving viable PBMC, whereas rats treated with IA-PBMCs evidenced an EF of 53·46 ± 4·25% (±SEM) (Fig. 5e).

Analysis of end-systolic and end-diastolic diameters (LVESD, LVEDD), end-systolic and end-diastolic volumes (LVESV, LVEDV, data not shown) showed a comparable pattern to the previously observed values. Medium receiving animals and viable PBMC-treated rats show LVEDD values of 10·43 ± 0·21 mm (±SEM) and 11·03 ± 0·40 mm (±SEM) respectively, IA-PBMC rats even represent a slightly reduced left-ventricular diastolic diameter of 8·99 ± 0·32 mm compared to 9·47 ± 0·64 mm in sham-operated animals. Differences in systolic diameters were less pronounced, but in the same ranking. LVESD is depicted in (Fig. 5f).

Discussion

Our findings demonstrate that irradiated apoptotic PBMC (IA-PBMC) induce immunosuppression *in vitro* and are associated with secretion of pro-angiogenetic proteins. We therefore infused cultured viable-PBMC and IA-PBMC in an acute rat AMI model and demonstrated that this treatment evokes massive homing of FLK1+/c-kit+ positive EPC into infarcted myocardium within 72 h and caused a significant functional recovery within 6 weeks.

Co-culture of IA-PBMC in immune assays resulted in reduced IL-1β and IL-6 production and attenuated allogeneic dendritic mixed lymphocyte reaction (MLR). Both immune parameters were described to have a role in inflammation after myocardial ischaemia. In addition, we evidenced that viable- and IA-PBMC secrete IL-8/CXCL8 and MMP9 into the culture medium within 24 h. These proteins were described to be responsible for neo-angiogenesis and recruitment of EPC from the bone marrow to the ischaemic myocardium. The IL-8/CXCL8 chemokine belongs to the CXC family that consists of small (< 10 kDa) heparin-binding polypeptides that bind to and have potent chemotactic activity for endothelial cells [29–31]. Three amino acid residues at the N-terminus (Glu-Leu-Arg, the ELR motif) determine binding of CXC chemokines such as IL-8 and Gro-alpha to CXC receptors 1 and 2 on endothelial cells and are promoting endothelial chemotaxisis and angiogenesis [32–34]. In addition, MMP9 secretion was identified to be pivotal in EPC mobilization as this matrixproteinase serves as signal to release soluble kit-ligand (sKitL), a chemokine that causes the transition of endothelial and haematopoietic stem cells (EPC) from the quiescent to proliferative niche in the bone

Figure 2 (a) FACS analysis shows that irradiation leads to induction of apoptosis in human PBMC with a time dependent increase of Annexin V expression over 48 h, $n = 4$. Necrosis was defined by high PI positive staining. (b) Co-incubation of LPS stimulated PBMC or monocytes with irradiated apoptotic autologous PCMC demonstrates a reduced secretion of the pro-inflammatory cytokine interleukin-1β in a dose-dependant manner, $n = 4$. (c) To a lesser extent, this finding also correlates with the interleukin-6 secretion profile of LPS stimulated PBMC and monocytes in the presence of IA-PBMC, $n = 4$. (d) Addition of autologous IA-PBMC in a mixed lymphocyte reactions with LPS stimulation decreases T-cell proliferation as measured by counts per minute (cpm), $n = 3$. (e) RT-PCR RNA expression analysis of VEGF, IL-8/CXCL8 and MMP9 transcripts shows an upregulation of IL-8/CXCL8 and especially MMP9 in irradiated PBMC after a culture period of 24 h, $n = 6$. (f) ELISA analysis of VEGF, IL-8/CXCL8 and MMP9 demonstrates that MMP9 is predominantly found in cell lysates, whereas differences in VEGF and IL-8/CXCL8 protein secretion remain approximately at the same level in both viable cells and IA-PBMC, $n = 7$. (g) Human fibroblasts incubated with supernatants obtained from cell cultures of viable or IA-PBMC exhibit a strong upregulation of VEGF, IL-8/CXCL8 and MMP9 transcripts in RT-PCR analysis, peak values were found in fibroblasts incubated in IA-PBMC supernatants. $n = 6$.

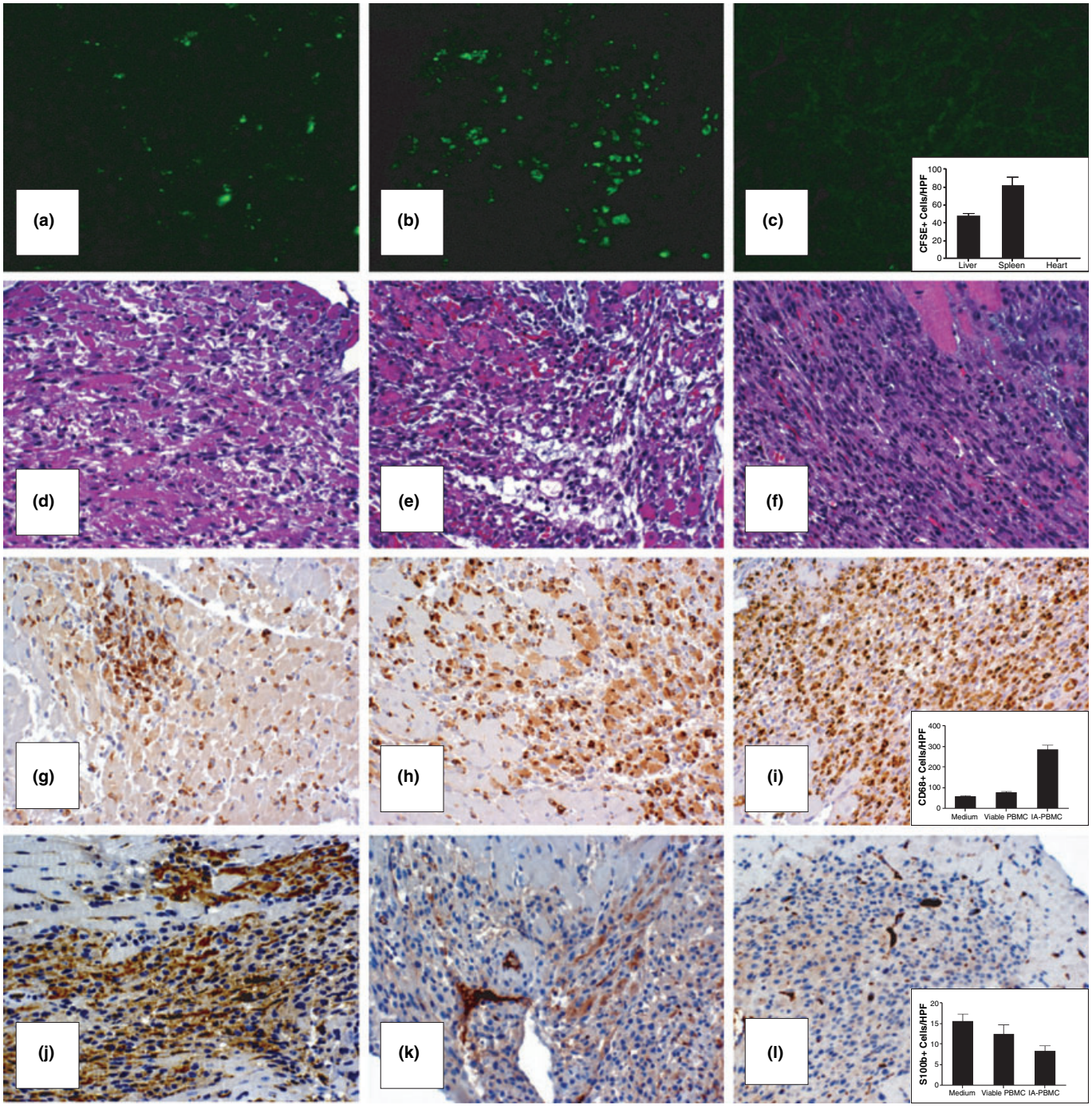

Figure 3 (a, b, c) CFSE labelled syngeneic PBMC administrated via the tail vein in rats after artificial myocardial infarction were predominantly found in the spleen (b), to a lesser extent in the liver (a) and no cells in the infarcted heart (c). (d–f) HE-stained infarct zones of rats injected with either medium (d) or viable PBMC (e) show a comparable pattern of ischaemic myocardium infiltrated by immune cells, tissues obtained from rats receiving IA-PBMC indicate dense infiltrations. (g–i) rats treated with viable cells (h) reveal slightly more $CD68^+$ stained cells in the infarcted myocardium than in medium-treated rats (g), but a threefold higher amount of $CD68^+$ was detected in IA-PBMC injected animals. (j–l) higher levels of S100β+ cells were found in rats receiving medium alone compared with the injection of viable PBMC or IA-PBMC, $n = 5$.

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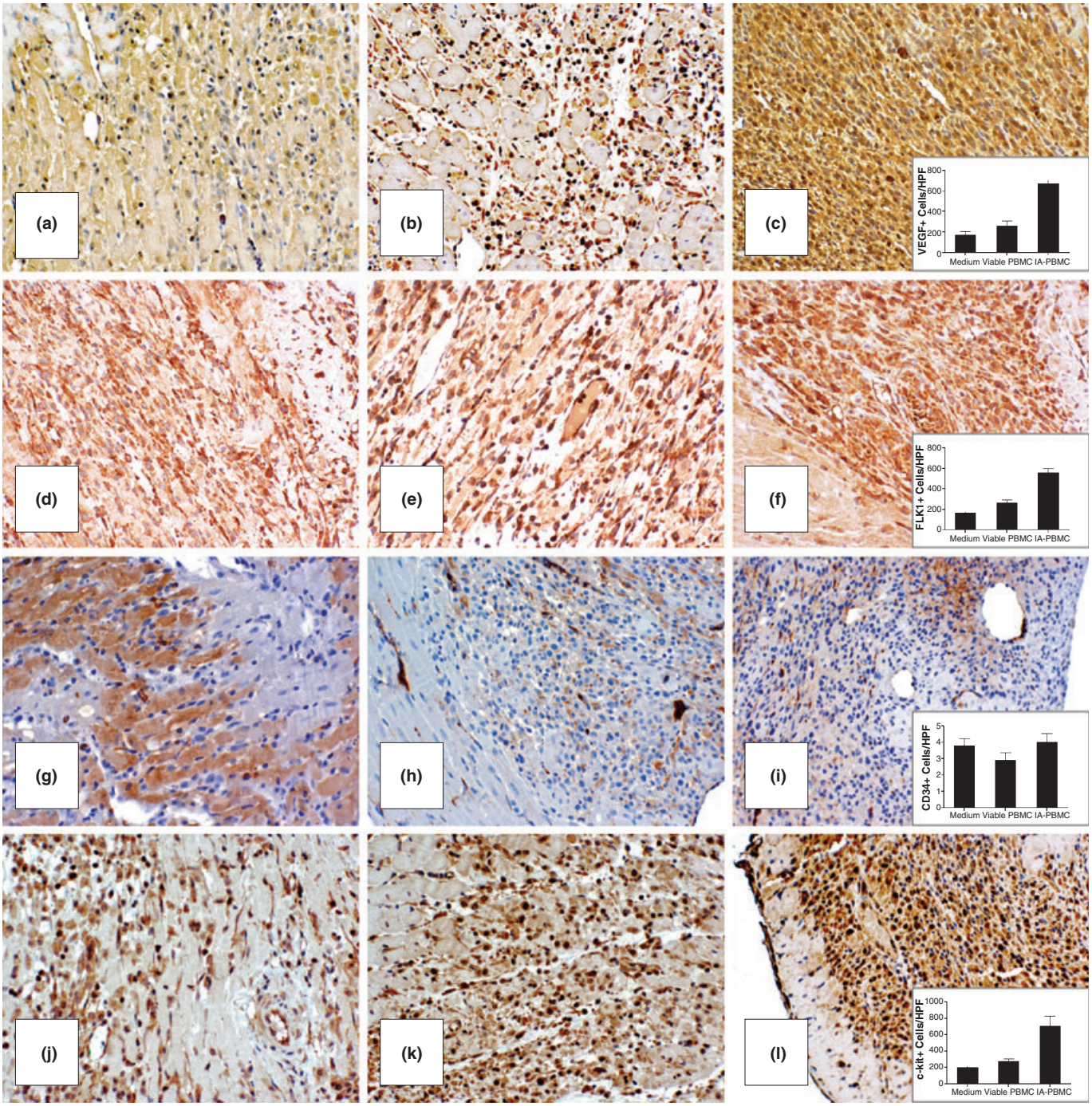

Figure 4 (a, b, c) Almost fourfold higher amounts of cells staining positive for VEGF were detected in infarcted myocardial tissue obtained from animals injected with IA-PCMC (c), in comparison with medium (a) or viable cell treatment (b). (d–f) A similar expression pattern was found for VEGF receptor KDR/FLK1 with peak values in the IA-PBMC group (f) compared to medium (d) and viable cells (e). (g–i) No differences were detected for CD34 in all three groups. (j–l) Immunohistological analysis for the marker c-kit in infarcted hearts shows a high quantity of positively stained cells and dense localization in rats injected with IA-PBMC (l) and fewer cells in medium (j) and viable cell receiving animals (l), $n = 5$.

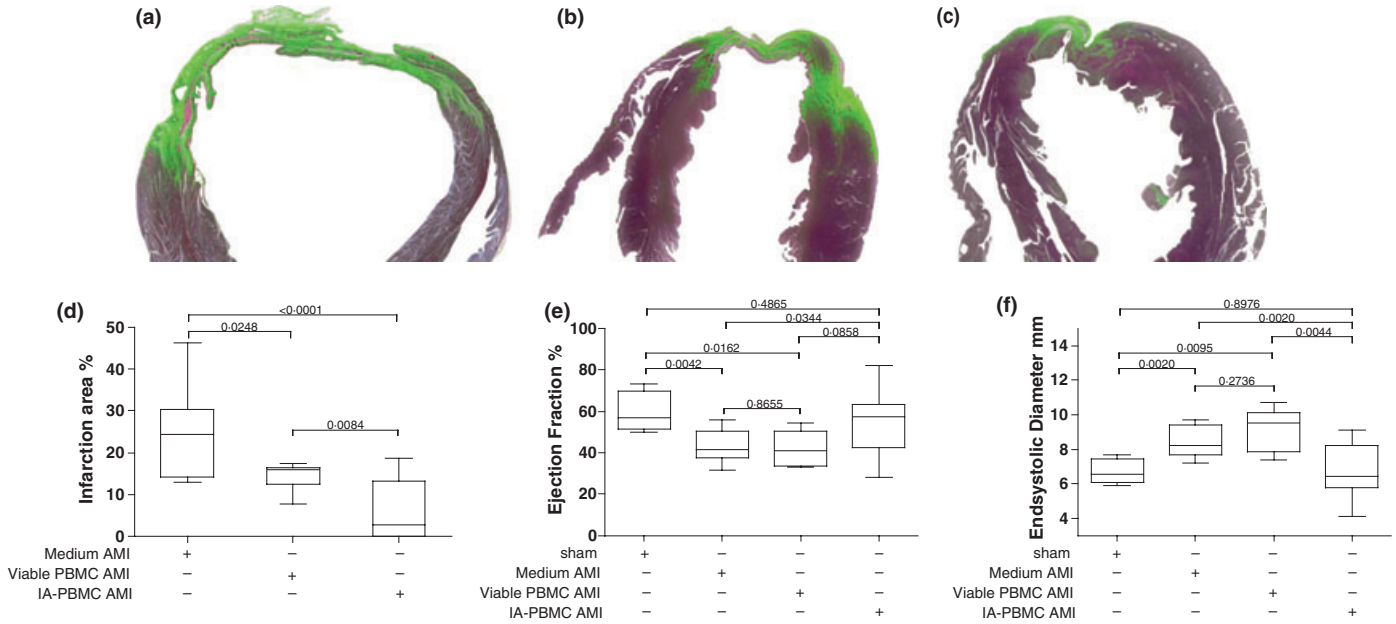

Figure 5 (a, b, c) Histological analysis of ischaemic rat hearts explanted 6 weeks after induction of myocardial infarction (Elastica van Gieson staining), hearts from medium injected animals (a) appear more dilated and show a greater extension of fibrotic tissue (infarcted myocardium was coloured in green for better visualization), scar extension was reduced in viable cell injected rats (b) with fewer signs of dilatations, the least amount of scar tissue formation was detected in IA-PBMC injected animals (c). (d) Statistical analysis of data obtained from planimetric analysis of specimen collected 6 weeks after LAD-ligation shows a mean scar extension of 24·95 ± 3·6% in medium, of 14·3 ± 1·3% in viable PBMC and 5·8 ± 2·0% in IA-PBMC injected animals (mean + SEM). (e, f) Assessment of cardiac function parameters ejection faction and endsystolic diameter by echocardiography evidences a better recovery after myocardial infarction in animals injected with IA-PBMC, n = 8–12.

marrow [23]. In a further *in vitro* assay, we demonstrated that the supernatant (SN) derived from cultured viable- and IA-PBMC had the ability to enhance mRNA transcription of IL-8/CXCL8 and MMP9 in mesenchymal fibroblasts. These data suggest that SN derived from viable and irradiated PBMC contain paracrine factors that confer a biological situation in the bone marrow, which results in elution of c-kit+ EPC into circulation [35–37].

To prove any beneficial effect of this culture-cell suspension *in vivo*, we utilized a model of open chest myocardial injury and infused cultured viable- and IA-PBMC shortly after LAD ligation in a rat animal model. In the first attempt, we proved that CSFE labelled IA-PBMC were trapped in majority in the spleen and the liver. These data indicate, and as suggested by others, that 'cell based therapy' does not induce homing to infarcted myocardium [38,39]. In contrast, it is much more likely that paracrine effects, either by 'modified' culture medium alone or by an evoked 'immune-mediated cytokine storm' due to cell-culture suspension exposure is causative for our regenerative effect in AMI. As immediate inflammation after acute ischaemia determines the road map to ventricular dilatation, we performed histological analysis after 72 h after AMI. We proved that IA-PBMC-treated rats evidenced massive homing of $CD68^+$ and VEGFa/FLK1/c-kit+ positive EPC cell populations within this time period. In contrast, more S100β positive DC were found in control AMI, indicating enhanced APC-based inflammation in control AMI.

Our results seen in IA-PBMC-treated rats partly foil the currently accepted knowledge about the natural course of myocardial infarction. In regards to inflammation: Under normal conditions remodelling processes are mediated by cytokines and inflammatory cells in the infarcted myocardium. This inflammatory response initiates wound reparation, phagocytosis and resorbtion of necrotic tissue. This ''physiologic'' process leads to survival of myocytes, neoangiogenesis and homing of regenerative progenitor cells (EPC). Any experimental approach so far that intervened in inflammatory response post infarction was shown to be detrimental in AMI models [8,40]. When interpreting our histological short-term data, we argue that IA-PBMC cell-medium suspension in AMI results in an advanced transitioning from inflammation to c-kit+ EPC repair phase [41]. Previous work has confirmed

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that bone marrow of circulating progenitor cell therapy after AMI improves cardiac function, regardless of whether transdifferentiation of the cells to cardiomyocytes occurs or not. With regard to c-kit+ EPC, Fazel *et al.* defined the significant role of bone marrow derived cells as indispensable for cardiac repair. Pharmacological inhibition with imatinib mesylate and non-mobilization of c-kit+ EPC resulted in an attenuated myofibroblast response after AMI with precipitous decline in cardiac function [42,43]. Of note are reports that proved similar repair mechanisms in humans who suffered from myocardial infarction [44].

The recent paradigm shift in cardiac biology towards the heart as an organ capable of self-renewal and repair has created new opportunities for treatment of ischaemic heart disease [45]. Understanding the molecular mechanisms that include progenitor cell mobilization in response to cardiac ischaemia provided a necessary road map to develop targets for specific progenitor cell mobilization without induction of a general pro-inflammatory state. Such a population that has been implicated in myocardial regenerative efforts is the c-kit+ EPC [45–48]. On the basis of our present data, we feel justified to claim that IA-PBMC cell-medium suspension circumvented inflammation and caused preferential homing of regenerative c-kit+ EPC with long-term rehabilitation of infarcted myocardium.

Clinical outlook

As infusing 'syngeneic' cultured IA-PBMC in a rat AMI model evidenced regenerative potency, we feel comfortable to suggest that our experimental treatment modality will instigate further experimental investigations, which will lead ultimately to clinical trials in patients with AMI.

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Disclosure

Intellectual property is claimed by the Medical University of Vienna.

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