Diplomarbeit

Irradiated apoptotic peripheral blood mononuclear cells preserve ventricular function after myocardial infarction

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……..
(Unterschrift)

Die Wissenschaft gibt dem, welcher in ihr arbeitet und sucht, viel Vergnügen, dem, welcher ihre Ergebnisse lernt, sehr wenig.

Friedrich Wilhelm Nietzsche (1844-1900)

DANKSAGUNG

Ich möchte mich bei all jenen bedanken, die mich bei der Durchführung und der Verfassung dieser Arbeit sowohl fachlich als auch persönlich unterstützt haben.

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Kurzfassung

Der akute Herzinfarkt, gefolgt von einem Remodellierungsprozess des Herzens ist eine der Hauptursachen für Herzversagen und Tod in der westlichen Welt.

In vorangegangenen Studien konnte bewiesen werden, dass die Transfusion von apoptotischen Zellen die Transplantation von allogenem Knochenmark erleichtern und eine lethale Graft-versus-Host Reaktion verhindern kann. Dieser positive Effekt dürfte sich auf das immunsuppressive Profil, das apoptotische Zellen besitzen, zurückführen lassen.

Die immunmodulatorische Effizienz von irradiierten, apoptotischen Zellen wurde in dieser Studie mittels Mixed-Lymphocyte-Reaction und Co-Inkubation Assays mit LPS stimulierten Zellen in *in vitro* Experimenten verifiziert. Die Expression und Sekretion von pro-angiogenetisch wirkenden Faktoren (Interleukin-8, Vascular endothelial growth factor, VEGF and Matrix metalloproteinase-9) in Zellkulturen von lebenden und apoptotischen Zellen (peripheral blood mononuclear cells, PBMC) wurde mittels Reverse transcription polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) bestimmt. Anschließend wurden in einem *in vivo* Tiermodell des akuten Herzinfarkts die protektiven Eigenschaften dieser Zellsuspensionen in der Ratte untersucht. Immunhistologische Auswertungen wurden durchgeführt um inflammatorische und pro-angiogenetische Zellen 72 Stunden nach Herzinfarkt im Myokard zu detektieren. Mittels Echokardiographie wurde die Herzfunktion 6 Wochen danach bestimmt. Das Ausmaß des Infarktschadens wurde mit planimetrischen Analysen ausgewertet.

Irradiierte, apoptotische PBMC reduzierten die inflammatorische Reaktionen in *in vitro* Experimenten und wiesen eine starke Aufregulierung und Sekretion der pro-angiogenetischen Faktoren Interleukin-8, VEGF und Matrix metalloproteinase-9 auf. Ratten, die einen akuten Herzinfarkt erlitten und denen Suspensionen von apoptotischen Zellen verabreicht wurden zeigten eine verstärkte Infiltration von Endothelprogenitorzellen in das

geschädigte Myokard innerhalb der ersten 72 Stunden verglichen mit Tieren aus den Kontrollgruppen (Infusion von Zellkulturmedium oder lebenden PBMC). In der Echokardiographie und der planimetrischen Auswertung zeigte sich eine signifikante Verbesserung der Herzfunktion und eine Reduktion des Schadensausmaßes nach Herzinfarkt ($p<0.05$ und $p<0.001$).

Diese Daten zeigen, dass die Infusion von irradiierten, apoptotischen PBMC Suspensionen nach experimentellem Herzinfarkt für eine reduzierte Entzündungsreaktion, ein verstärktes Einwandern von Endothelprogenitorzellen und den Erhalt der Herzfunktion sorgt.

Abstract

Acute myocardial infarction (AMI) followed by cardiac remodeling is a major cause of congestive heart failure and death. Of clinical relevance are reports that demonstrated that infusion of apoptotic cells lead to allogeneic hematopoietic cell engraftment in transplantation models and to a delay of lethal acute graft-versus-host disease by initiating immunesuppressive mechanisms.

Immunemodulatory function of irradiated apoptotic peripheral blood mononuclear cells (PBMC) was evaluated by mixed-lymphocyte reactions and co-culture assays using LPS stimulated cells *in vitro*. Reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assays (ELISA) were utilized to quantify pro-angiogenic Interleukin-8, Vascular endothelial growth factor (VEGF) and Matrix metalloproteinase-9 in cell culture supernatants obtained from viable and apoptotic PBMC. Cell suspensions of viable and irradiated apoptotic PBMC were infused in an experimental rat AMI model. Immunehistological analysis was performed to detect inflammatory and pro-angiogenic cells within 72 hours after myocardial infarction. Cardiac function was analyzed by echocardiography and determination of infarction size was conducted by planimetry after six weeks.

Irradiated apoptotic PBMC attenuated immune reactivity and evidenced secretion of pro-angiogenic Interleukin-8, VEGF and Matrix metalloproteinase-9 *in vitro*. Rats with experimental AMI that were infused with irradiated apoptotic PBMC cell suspensions showed enhanced homing of endothelial progenitor cells within 72 hours as compared to controls (medium alone, viable PBMC). Echocardiography and planimetric analysis showed a significant reduction of infarction size and improvement of post AMI remodeling as evidenced by an attenuated loss of ejection fraction ($p<0.05$ and $p<0.001$).

These data indicate that infusion of irradiated apoptotic PBMC suspensions in experimental AMI circumvented inflammation, caused preferential homing of regenerative EPC and preserved cardiac function.

Introduction

Pathophysiology

Once adequate oxygen supply to the myocardium is interrupted, ischemia of the heart muscle and subsequently the formation of an infracted tissue is initiated. This disruption can be caused by occlusion of a coronary artery due to the advanced stages of atherosclerosis, thromboembolism or vascular spasms[1,2,3,4].

The rupture of an atherosclerotic plaque is the most common cause leading to myocardial infarction. This event starts the blood clotting cascade which leads to a total or subtotal occlusion of the affected artery in the end. Thus oxygenation of the myocardial area which is aligned to the occluded vessel is prevented which subsequently leads to ischemia[5,6,7,8]. Following this process, cardiomyocytes not supplied sufficiently with oxygen and nutrient products undergo cell death, i.e. necrosis and to a lesser extent also apoptosis[9,10].

Because of this cellular necrosis, an inflammatory process is started which begins with infiltration and accumulation of immune cells in the damaged parts of the myocardium. In the early phase this cell populations mainly consists of neutrophils and as this re-organization of the necrotic myocardium continues also macrophages migrate towards the infracted area and begin to phagocytize cellular debris[11,12,13].

Over the next days, myofibroblasts join these above mentioned cell populations and a phase of tissue stabilization is initiated[14,15].

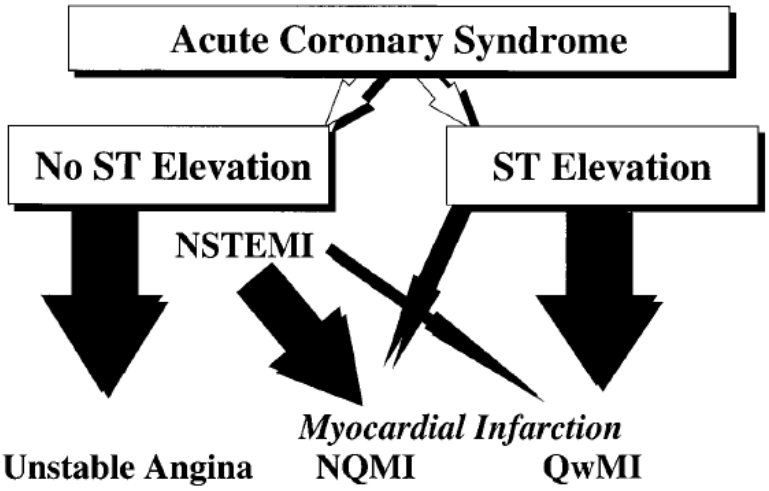
These periods of myocardial restructuring can be divided into acute, healing or healed myocardium. An acute event of infarction is characterized by infiltration and presence of polymorphonuclear leukocytes within the damaged tissue area. The presence of mononuclear cells and fibroblasts and the absence of polymorphonuclear leukocytes characterizes a healing infarction. This process takes place approximately 7 days after the initial event of myocardial ischemia. A healed infarction is manifested as scar tissue and collagen deposition without cellular infiltration. The entire process leading to a healed infarction usually requires approximately five to six weeks[16,17]. Additionally, reperfusion

alters microscopic and macroscopic appearance of the necrotic area with large quantities of extravasated erythrocytes.

Clinical Symptoms

The process of myocardial ischemia can be identified from the patient's anamnesis and by utilizing electrocardiography (ECG). Patients suffering from acute cardiac ischemia usually show symptoms including chest, epigastric, arm, wrist or jaw discomfort or pain. These symptoms can occur during rest or exercise. During the period of intense exercise and subsequent recovery the risk to develop clinical symptoms related to myocardial infarction is about 6-fold higher in people in physically good condition as compared to resting periods. For patients in poor physical condition, this rate is more than 35-fold higher[18]. The discomfort associated with acute myocardial infarction usually lasts at least 20 minutes. The discomfort may develop in the central or left chest and then extends to the arm, jaw, back or shoulder. Acute ischemic symptoms can also be associated with dyspnea, sweating, nausea and vomiting[19,20,21]. Although many patients have symptoms such as those, these complaints may go unrecognized or may be erroneously labeled as another disease entity, such as indigestion or a viral syndrome. Myocardial ischemia or infarction may also occur without symptoms. Symptomless ischemia can predominantly occur in diabetes patients due to a loss of neurosensitivity [22,23]. Also women often present a status of reduced symptoms after acute cardiac ischemia. In this case it may be detected only by the ECG, blood sampling for cardiac enzymes (e.g. Troponin T, Troponin I, CK-MB) or cardiac imaging (MRI or angiography)[24].

This group of symptoms related to reduced oxygen supply to the heart is termed clinically as acute coronary syndrome (ACS). To further distinguish this symptoms as cardiac angina, acute ischemia or infarction, diagnostic techniques have to be applied.



NQMI=non-Q wave myocardial infarction; NSTEMI=non-ST elvation myocardial infarction; Q wave MI=Q wave myocardial infarction; ST=ST segment of ECG tracing.

Figure 1

Figure 1 shows clinical classification of acute coronary syndrome (ACS).

Adapted from Myocardial infarction redefined--a consensus document of The Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of myocardial infarction. *Eur Heart J. 2000 Sep;21(18):1502-13.*

Epidemiology

Although death from myocardial Infarction has declined over the last decades, it still remains a leading cause for both morbidity and mortality in most of the industrialized countries in the western world.

Despite this reduction in early mortality from myocardial infarction by new therapeutic modalities, the risks to die of ischemic heart disease still remain very high[25]. Since clinicians have the option to intervene with PTCA (percutaneous transluminar coronary angioplasty) and pharmaceuticals that may reopen the occluded vessel, acute myocardial syndromes can be therapied more successfully than in the last decades. In clinical trials focusing on post myocardial infarction thrombolytic therapies, a 30 day mortality of 6–10% has been reported. In cases of primary angioplasty mortality has been as low as 2.5% in large scale randomized studies[26].

Chronic symptoms caused by ischemic heart disease tend become more and more predominant in patients having survived the initial ischemic process[27]. These chronic dysfunctions are mainly due to the process of remodeling, initiating the loss of ventricular geometry and cardiac functional parameters. These changes involve myocyte necrosis and apoptosis, scar formation, ventricular dilatation and hypertrophy of non-infarcted myocardium[28,29].

Furthermore, in an estimation by the European Society of Cardiology more than ten million Europeans are currently suffering from heart insufficiency, most cases causes by ischemic heart disease[30,31].

The notable Framingham-Study[27] shows an even worse picture, with a five years survival rate for men of 25% and for women of 38%, whereas in advanced stages the median survival declines to just one year. Rates of incidence are at 0,1% in younger collectives of patients and start to climb to approximately 2,5% in 75 years old patients. Cardiovascular diseases tend to assume an almost epidemic shape in the industrialized world as the population pyramid indicates a rise of life expectancy over the last decades[32,33].

Risk Factors for Myocardial Infarction

As atherosclerosis is in most cases the underlying disease entity for the development of myocardial infarction, these two pathologic conditions share common risk factors [34,35,36,37,38].

- Hypertension[39,40,41]
- Hyperlipoproteinaemia[42,43,44,45,46,47,48]
- Smoking [49,50,51,52,53,54,55,56,57,58]
- Diabetes[59,60,61,62,63,64]
- Abdominal obesity [65,66,67,68,69]
- Sedentary life style [70,71]
- Family history of heart disease[72,73,74]

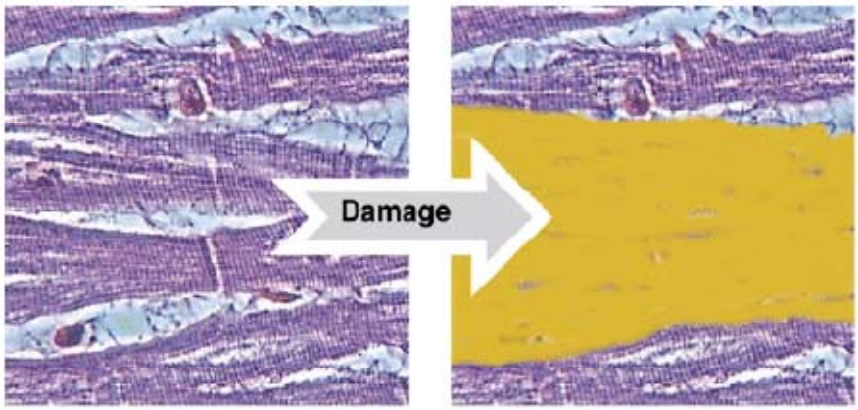
- Age[75,76,77,78]
- Psychosocial stress[79,80]

Studies have demonstrated that women experience their first AMI on average 9 years later than men. The younger age of onset of acute myocardial infarction in men is largely explained by higher levels of risk factors including abnormal lipid levels and smoking before the age of 60 years among men. Women seem to have a reduced risk to develop myocardial infarction before the onset of menopause. Although after the onset of menopause, possible due reduced levels of endogenous estrogens and their vasoprotective effects, women are equally at risk[81].

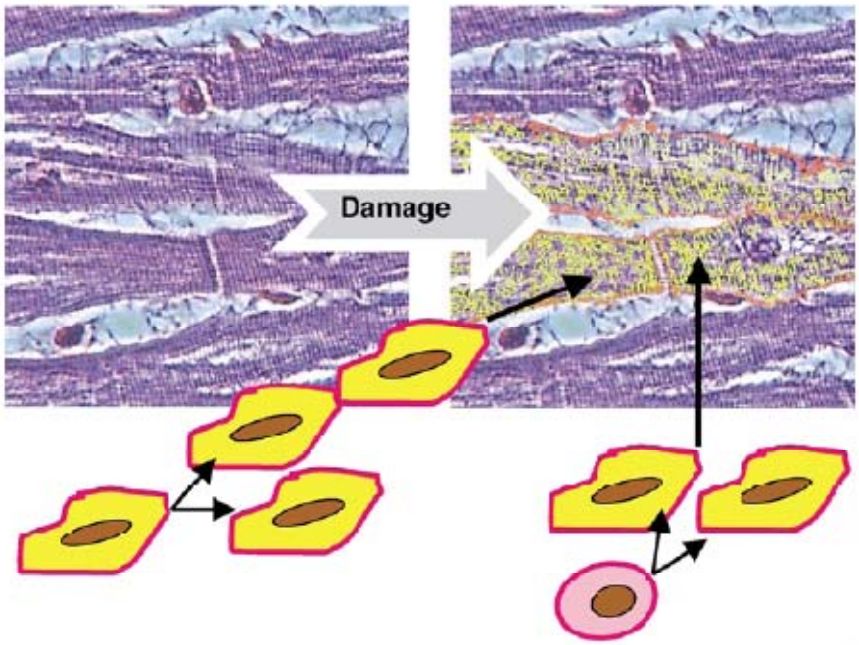
Stem Cell Therapies Trying to Replace Infracted Myocardium

Although myocardial infarction is and was considered as an irreversible injury[82], a plethora of investigations on cardiac repair utilizing different types of stem or progenitor cells was conducted over the last decade[83].

Based on recently published observations, it appears that there is a low level of myocyte proliferation accompanied by attraction of stem or progenitor cells even in the postnatal heart[84,85]. Whether this occurs by replication of pre-existing mature cardiomyocytes or by conversion of stem cells into new cardiomyocytes is now an area of intense research[86] (Figure 2).



(a) Common concept: replacement of cardiomyocytes by scar tissue



(b) New concept: repair by replication of endogenous cardiomyocytes or conversion of stem cells into new cardiomyocytes

Figure 2

Adapted from Schwartz Y, Kornowski R. Autologous stem cells for functional myocardial repair. *Heart Fail Rev. 2003 Jul;8(3):237-45.*

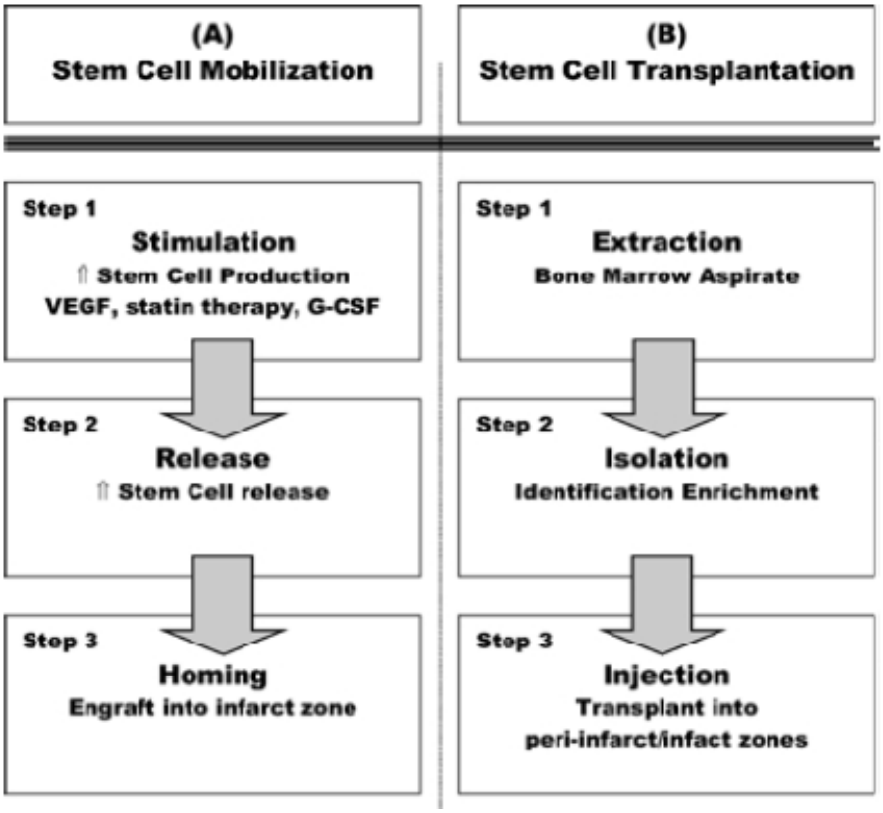


Figure 3

Two different methods of autologous stem cell therapy after myocardial infarction. Stem cell mobilization (A), cell expansion and release is induced in the native bone marrow by Vascular endothelial growth factor (VEGF), statin therapy, Granulocyte colony stimulation factor (G-CSF) or other therapeutic drugs or mediators. The released cells then home to and engraft into the damaged zones of the myocardium. In extrinsic stem cell transplantation (B), autologous stem cells are extracted from the bone marrow and injected into the peri-infarct or infarct zones of the myocardium.

Adapted from Schwartz Y, Kornowski R. Autologous stem cells for functional myocardial repair. *Heart Fail Rev. 2003 Jul;8(3):237-45.*

Regeneration of infarcted myocardium utilizing pluripotent stem cells can be accomplished by two main approaches (see Figure 3). The first technique is stem cell mobilization, which involves stimulating the expansion of specific populations of stem cells within the bone marrow of the patient[87,88,89,90], and then directing released stem cells or progenitor cells to the infarct zone without any extracorporal techniques (such as cell separation or *in vitro* cultivation). This form of transplantation requires developing pharmacological or genetic

means to stimulate specific populations of stem or progenitor cells and direct their homing to the damaged tissue. The second more feasible approach is autologous stem cell transplantation. This technique involves isolating autologous stem cells from bone marrow or other tissue and reinjecting them directly into the myocardium, intracoronary or intraveneously after myocardial infarction[91,92,93]. A few studies suggest that regeneration of damaged myocardium by stem cell mobilization is possible. Beltrami et al. demonstrated the presence of proliferating cardiac myocytes in the hearts of humans who died 5 to 12 days after myocardial infarction[85]. It was suggested that the Ki-67 positive cells identified as proliferating cells in the post-infarct human hearts were probably differentiating or proliferating progenitor cells originating from the bone marrow[85]. As recently demonstrated in a mouse model of myocardial infarction, conducted by Jackson et al., bone marrow cells can target infarcted myocardium and differentiate into cardiomyocytes and vascular structures[87]. Ten weeks before ligation of the left anterior descending artery (LAD) for 60 min, animals underwent bone marrow transplant with a highly enriched population of hematopoietic cells from the bone marrow of a syngeneic transgenic mouse whose cells expressing the reporter gene lacZ. Therefore, any cells in the recipient animal that expressed lacZ had to be derived from the donor bone marrow. Two and four weeks after 60 minutes of LAD ischemia, lacZ-positive endothelial cells and cardiomyocytes were identified in the myocardium[87]. Although the number of lacZ-positive cells was small (0.02% cardiomyocytes, 1 to 2% endothelial cells), these results suggest that by optimized release and targeting of stem cells from the bone marrow after myocardial infarction, it may be possible to enhance the repair and regeneration of myocardial tissue.

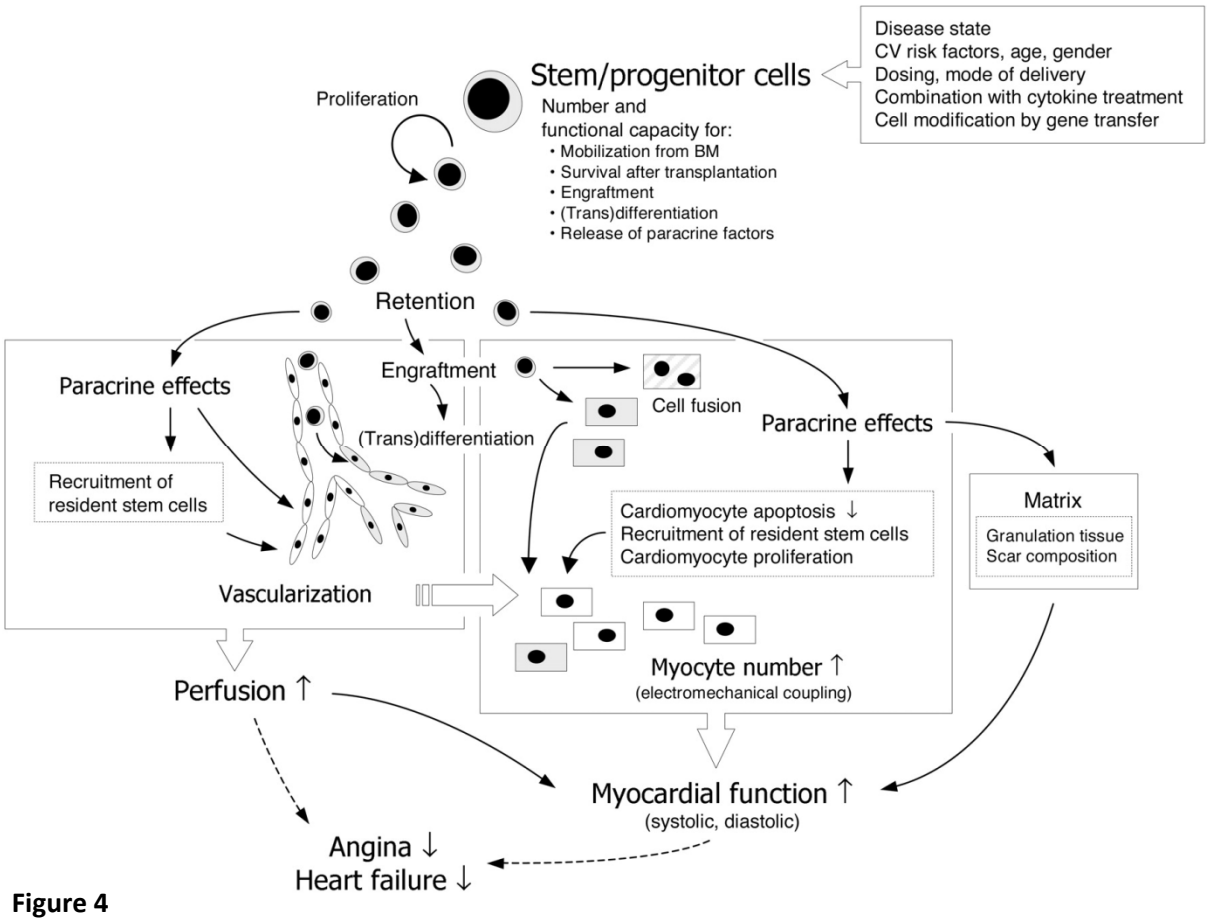


Figure 4

A working hypothesis of therapeutic stem cell transplantation for myocardial regeneration. Stem and progenitor cell transplantation is sought to promote vascularization and myocyte formation. Improved vascularization may facilitate beneficial effects in the myocyte microenvironment. Depending on the stem cell type and local milieu, the relative contribution of cell incorporation or paracrine effects may vary.

Adapeted from Wollert KC, Drexler H. Clinical applications of stem cells for the heart. *Circ Res. 2005 Feb 4;96(2):151-63.*

Over the last decade many different types of stem or progenitor cells have been investigated in animal studies or clinical trials in order to improve cardiac function after myocardial infarction. The passage on the next few pages should give a brief overview of cell types used for this purpose:

Cell Types Used for Myocardial Regeneration

Endothelial Progenitor Cells

Endothelial Progenitor cells (EPCs) have originally been defined by their cell surface expression of the hematopoietic marker proteins CD133 and CD34 and the vascular endothelial growth factor receptor-2 (VEGF receptor, FLK). Furthermore, they possess the capacity to incorporate into sites of neovascularization and to differentiate into endothelial cells in situ[94]. Increasing evidence suggests that EPCs expanded by cell culture also contain a $CD14^{+}/CD34^{-}$-mononuclear cell population with "EPC capacity", which mediates its angiogenic effects by releasing certain paracrine factors[95,96]. Notably, EPC numbers and their angiogenic capacity are impaired in patients with coronary artery disease, which may limit their therapeutic usefulness[97,98].

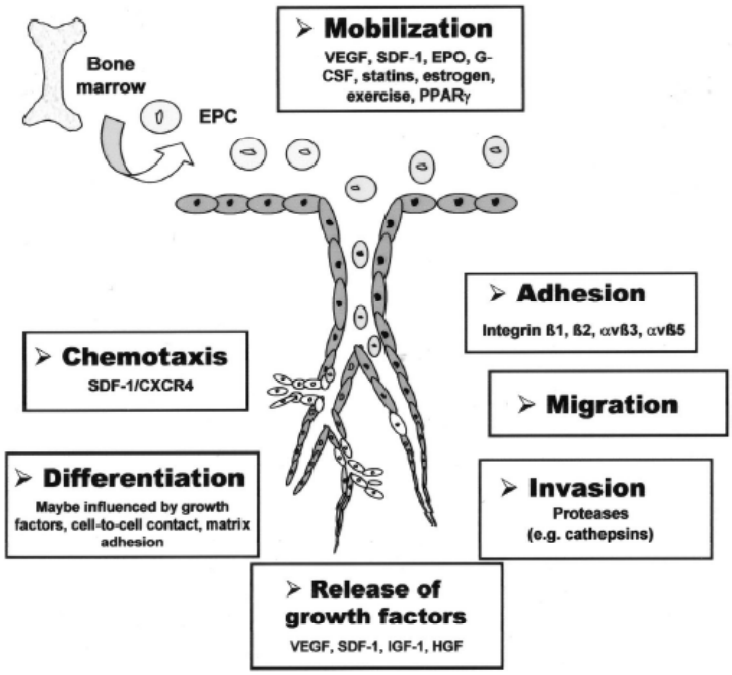


Figure 5

Mechanism of EPC homing and differentiation after an ischemic event. Recruitment and incorporation of EPCs into ischemic tissue requires a coordinated multistep process including mobilization, chemoattraction, adhesion, transmigration and in situ differentiation.

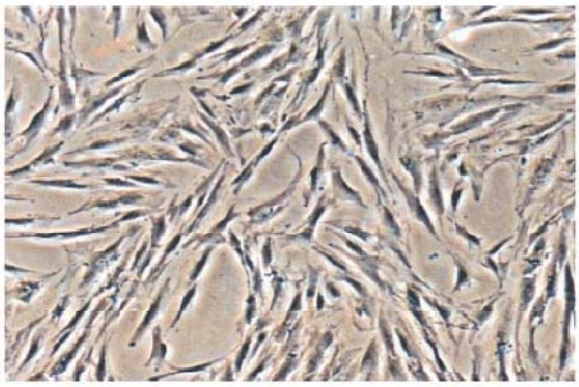
Adapeted from Urbich C, Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res. 2004 Aug 20;95(4):343-53.*

CD133^{+} Cells

The cell surface antigen CD133 is expressed on early hematopoetic stem cells (HSCs) and EPCs. Both cell types collaborate to promote vascularization of ischemic tissues[99]. CD133^{+} cells can integrate into sites of neovascularization and differentiate into mature endothelial cells. Less than 1% of nucleated BMCs are CD133^{+}, and because these cells cannot be expanded *ex vivo*, only limited numbers of CD133^{+} cells can be obtained for therapeutic purposes.

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) represent a rare population of CD34^{-} and CD133^{-} cells present in bone marrow stroma and other mesenchymal tissues[100]. MSCs can differentiate into many different cell types, such as osteocytes, chondrocytes and adipocytes. Differentiation of MSCs to cardiomyocyte-like cells has been observed under specific culture conditions *in vitro* and after injection into healthy or infarcted myocardium in animals. When injected into infarct tissue, MSCs may enhance regional wall motion and also prevent remodeling of the noninfarcted myocardium[101,102,103]. Additionally, cultured MSCs secrete many different angiogenic cytokines, which improve collateral blood flow recovery in a murine hind limb ischemia model[104]. Because MSC clones can be expanded *in vitro*, and as reported, have a low immunogenicity, they might be used in an allogeneic setting in the future, as suggested by Pittenger et al.[100].



Human MSCs in culture condition evidence a spindle shaped phenotype in phase contrast microscopy.

Figure 6

Adapeted from Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res. 2004 Jul 9;95(1):9-20.*

Skeletal Myoblasts

Skeletal myoblasts are progenitor cells that are normally in a quiescent state under the basal membrane of mature muscular fibers. Myoblasts can be isolated from skeletal muscle biopsies and expanded by *in vitro* techniques. These isolated myoblasts can differentiate into myotubes and retain skeletal muscle properties when transplanted into an infarct scar. Although myotubes do not couple with resident cardiomyocytes electromechanically, myoblast transplantation has been shown to augment systolic and diastolic performance in animal models of myocardial infarction[105,106].

Resident Cardiac Stem Cells

In investigations conducted over the last years it was determined that resident cardiac stem cell (RCSC) populations are capable to differentiate into cardiomyocyte or vascular lineages. This suggests that these cells could be used for cardiac tissue repair[107,108]. It was shown that RCSCs can be clonally expanded from human myocardial biopsies[109]. It has been reported that intramyocardial injection of these cells after AMI in mice promotes cardiomyocyte and vascular cell formation and leads to an improvement in systolic function.

Embryonic Stem Cells

Embryonic stem cells (ESC) are totipotent stem cells derived from the inner cell mass of blastocysts. Under specific culture conditions, ESCs can differentiate into multicellular embryoid bodies containing differentiated cells from all three germ layers including cardiomyocytes. Human ESC cell–derived cardiomyocytes display structural and functional properties of early-stage cardiomyocytes. As shown by Kehat et al., these cells can couple electrically with host cardiomyocytes when transplanted into normal myocardium[110,111]. However, unresolved ethical and legal issues, concerns about the tumorigenicity of these cells, and the need to use allogeneic cells for transplantation currently hamper the use of ESCs in clinical studies.

Activated Macrophages

Activated macrophages are a rich source of cytokines, growth, and survival factors and play a key role in wound healing. The processes of post-myocardial infarction repair and dermal wound healing have many common pathways. Furthermore, macrophages are involved in inflammatory injury suppression, myocyte protection, and regeneration[112,113,114]. In a study by Leor et al. it was shown that the infusion of *ex vivo* activated macrophages can improve ventricular function after myocardial infarction by improving endogenous repair mechanisms[114].

Meta-Analysis of Clinical Application of Cellular Therapies for Myocardial Infarction

In a meta-analysis conducted by Singh et al.[115] seven randomized controlled trials were enrolled (Suarez de Lezo et al.[116], Schächinger et al.[117], Lunde et al.[118], Ge et al.[119], Janssens et al.[120], Wollert et al.[121] and Chen et al.[122]), evaluating changes in left ventricular ejection fraction (LVEF) following intracoronary bone marrow cell (BMC) therapy in comparison to control groups. A meta-analysis of 516 patients (BMC group, 256; control group, 260) was performed (see table on the next page). There were no significant differences in patient characteristics between the two groups (data not shown).

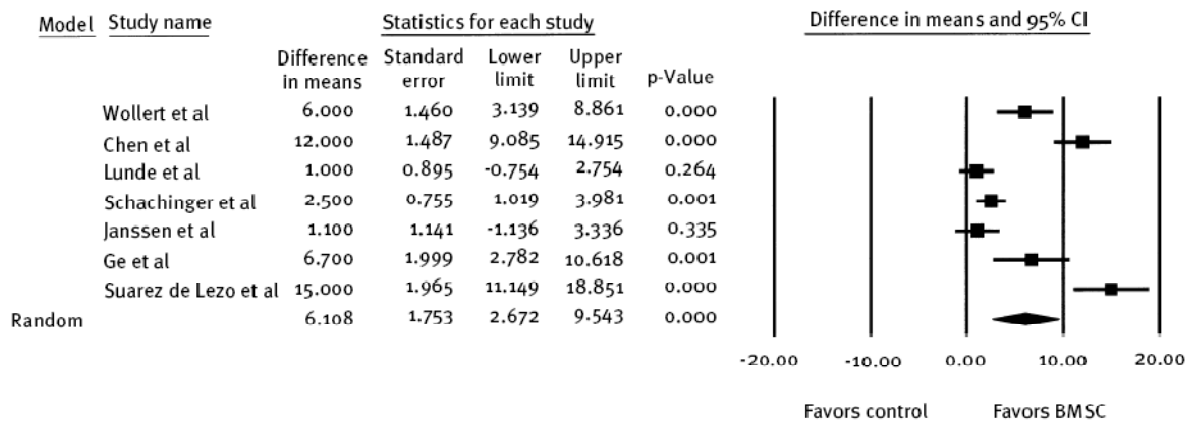
Investigator Group	Setting	Design	No. of Cells Administered in BMT Group	Results
Wollert et al	Acute myocardial infarction followed by PCI	Randomized trial LVEF assessed by MRI	Approximately 2.5×10^9 unfractionated BMC 5 d after MI	At 6 mo: LVEF 6% greater in BMC group than in control group
Chen et al	Acute myocardial infarction followed by PCI	Randomized study LVEF assessed by ECHO	Approximately 8 to 10×10^9 BMC suspension 18 d after MI	LVEF in the BMSC group increased significantly (67%±11% vs 49%±9% and 53%±8%)
Lunde et al	Acute myocardial infarction followed by PCI	Randomized trial LVEF assessed by SPECT, echocardiography, and MRI	Approximately 7×10^7 Ficoll-separated BMC 6 d after MI	No significant difference in LVEF between the 2 groups
Schächinger et al	Acute myocardial infarction followed by PCI	Randomized, double-blind trial LVEF assessed by left ventricular angiography	Approximately 2.4×10^8 Ficoll-separated BMC 3 to 7 d after MI	Greater absolute increase in LVEF in BMC group than in placebo group (5.5% vs 3.0%)
Janssens et al	Acute myocardial infarction followed by PCI	Randomized, double-blind trial LVEF was assessed by MRI	Approximately 3×10^8 Ficoll-separated BMC within 24 hrs of MI	No significant difference in overall LVEF at follow-up between BMC and placebo group
Ge et al	Acute myocardial infarction followed by PCI	Randomized, double-blind trial LVEF assessed by echocardiography	About 4×10^7 BMC within 24 hrs of MI	A 5.8% increase in the LVEF in BMC group
Suárez de lezo et al	Acute myocardial infarction followed by PCI	Randomized trial LVEF assessed by LV angiogram	About 9×10^8 Ficoll-separated BMC 5 to 12 d after MI	A 20% increase in mean ejection fraction in BMC group

Abbreviations: BMSC = bone marrow stem cell; LVEF = left ventricular ejection fraction; MRI = magnetic resonance imaging; PCI = percutaneous coronary intervention; SPECT = single photon emission computed tomography.

Study	Total (n = 516)	BMSC (n = 256)	Control (n = 260)	Follow-up
Wollert et al	60	30	30	In hospital, 6 mo
Chen et al	69	34	35	In hospital, 3, 6 mo
Lunde et al	100	50	50	In hospital, 6 mo
Schächinger et al	187	92	95	In hospital, 4 mo
Janssens et al	60	30	30	In hospital, 4 mo
Ge et al	20	10	10	In hospital, 6 mo
Suárez de Lezo et al	20	10	10	In hospital, 3 mo

Abbreviation: BMSC = bone marrow stem cells.

Table adapeted from Singh S, Arora R, Handa K, Khraisat A, Nagajothi N, Molnar J, Khosla S. Stem cells improve left ventricular function in acute myocardial infarction. *Clin Cardiol. 2009 Apr;32(4):176-80.*



Based on these data, this meta-analysis suggests that intracoronary bone marrow stem cell infusion is effective in improving the left ventricular systolic function in patients after acute myocardial infarction (AMI).

Adapeted from Singh S, Arora R, Handa K, Khraisat A, Nagajothi N, Molnar J, Khosla S. Stem cells improve left ventricular function in acute myocardial infarction. *Clin Cardiol. 2009 Apr;32(4):176-80.*

Protective Mechanisms Induced by Apoptotic Stem Cells

Cell-protective mechanisms induced by transplanted apoptotic cells might also be of importance in many studies which sought to determine a regenerative potential of stem or progenitor cells applied after myocardial ischemia. Anker et al. described the role of cellular necrosis and apoptosis on immunemodulatory mechanisms after myocardial infarction[123]. They assume that myocyte necrosis plays the predominant part in triggering the pro-inflammatory state in the ischemic microenvironment. Apoptosis occurs preferentially in the border zone of the infracted area and was supposed to interfere with local inflammation by inducing a counter-regulatory mechanism. In clinical trials, stem or progenitor cells from the peripheral blood flow or whole bone marrow aspirates were isolated from patients with acute infarction and were either injected into the coronary artery after percutaneous coronary intervention (PCI) adjected to the infarct area or directly into the ischemic heart muscle. The viability of cells before injection was determined to be in the range of 75% to 95%[121,124,125,126]. This data indicate that a proportion of injected cells was already apoptotic or necrotic at the time of administration. A further proportion of healthy injected living cells likely become apoptotic within the heart tissue because of exposure to various pro-apoptotic or cytotoxic factors secreted by damaged cardiomyocytes in the local environment.

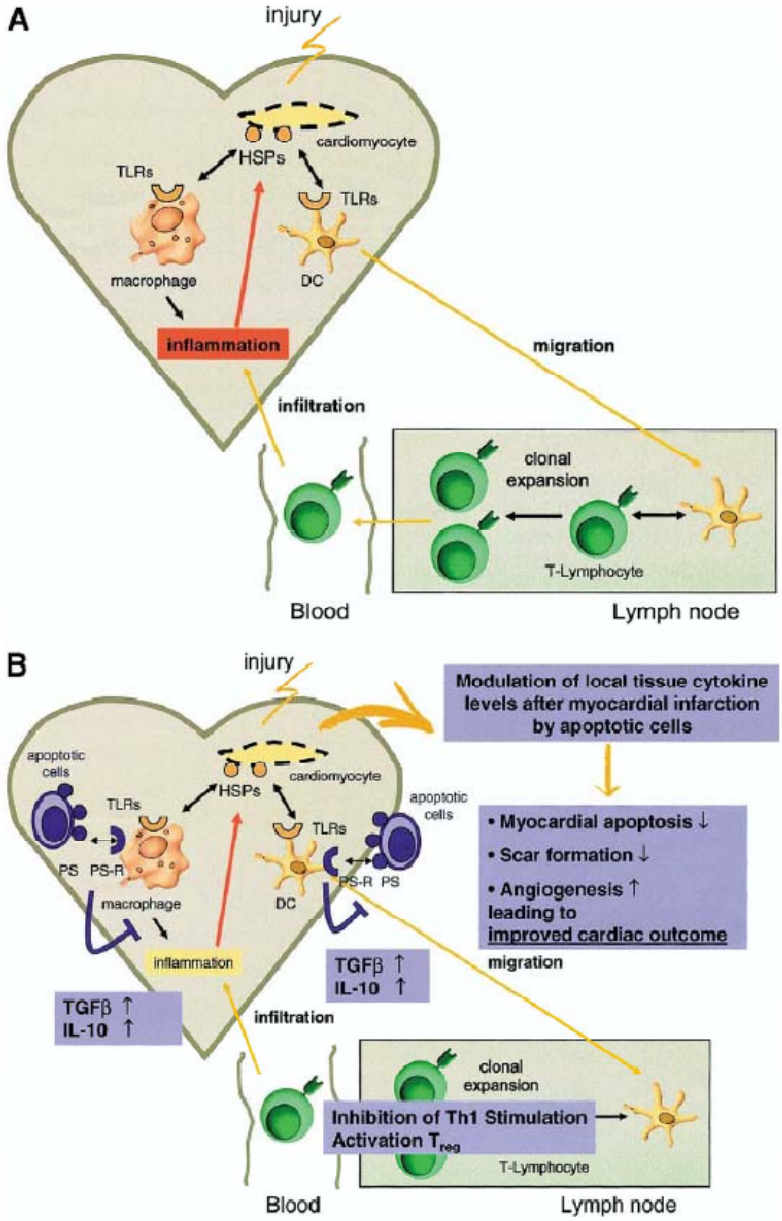


Figure 7

Immune regulation and pathophysiology of myocardial injury. Ischemic injury damages cardiac cells, resulting in up-regulation of heat-shock proteins (HSPs) that triggers macrophages and immature dendritic cells (DC). Apoptotic cells interact with this immune regulation after myocardial injury. Transplanted, *ex vivo* prepared apoptotic cells or cells undergoing apoptosis *in vivo*, inhibit macrophages and DCs via interaction of their surface phosphatidylserine with the respective receptors (PS-R) on the immune cells. This results in less myocardial inflammation, less myocardial apoptosis and scar formation as well as enhanced angiogenesis.

Adapted from Thum T, Bauersachs J, Poole-Wilson PA, Volk HD, Anker SD. The dying stem cell hypothesis: immune modulation as a novel mechanism for progenitor cell therapy in cardiac muscle. J Am Coll Cardiol. 2005 Nov 15;46(10):1799-802.

Phagocytic cells, such as macrophages and other phagocytes clearly discriminate between apoptotic cells and viable cells. Recognition occurs prior to the lysis of the dying cell, implying an early surface change. The most characteristic surface change associated with apoptosis is loss of phospholipid asymmetry and exposure of phosphatidylserine, this change is required for recognition and engulfment.

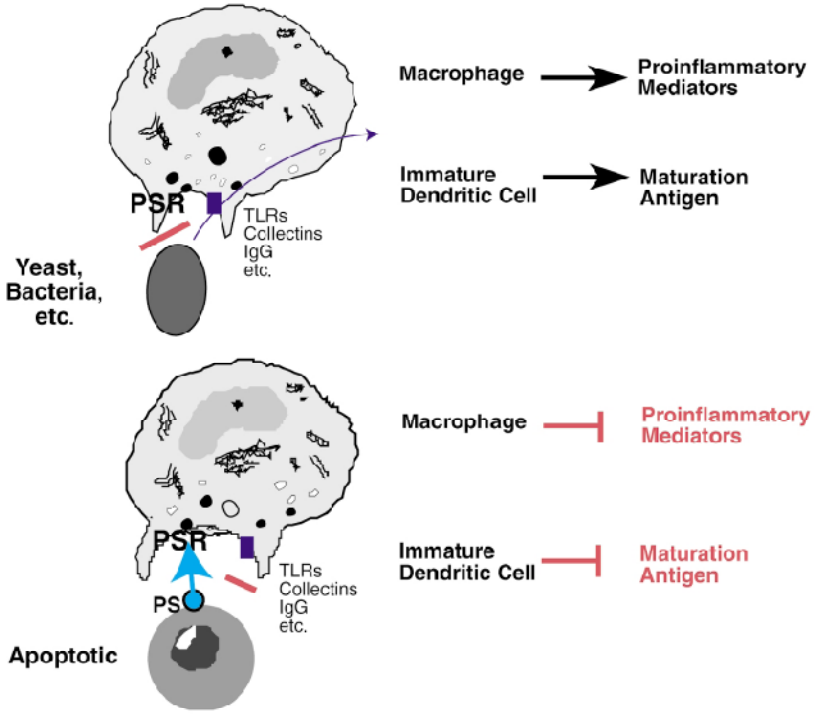


Figure 8

A hypothetical model for the role of the phosphatidylserine receptor in modulating inflammation and immune response[127]. Engulfment of microbial organisms that fail to express phosphatidylserine externally does not engage the phosphatidylserine receptor. Instead, receptors are stimulated by the microbe or its products that transduce pro-inflammatory signals, such as toll-like receptors. In contrast, when apoptotic cells are recognized, they expose phosphatidylserine, thus engaging the phosphatidylserine receptor, which provides an anti-inflammatory signal. TLR, toll-like receptor

Adapted from Fadok VA, Bratton DL, Henson PM. Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. J Clin Invest. 2001 Oct;108(7):957-62.

The interaction of apoptotic cells with phagocytes induces not only potential engulfment but also the delivery of anti-inflammatory and anti-immunogenic signals to the cellular microenvironment. By contrast, necrotic cells are more likely to initiate inflammation and trigger activation of the adaptive immune system[128].

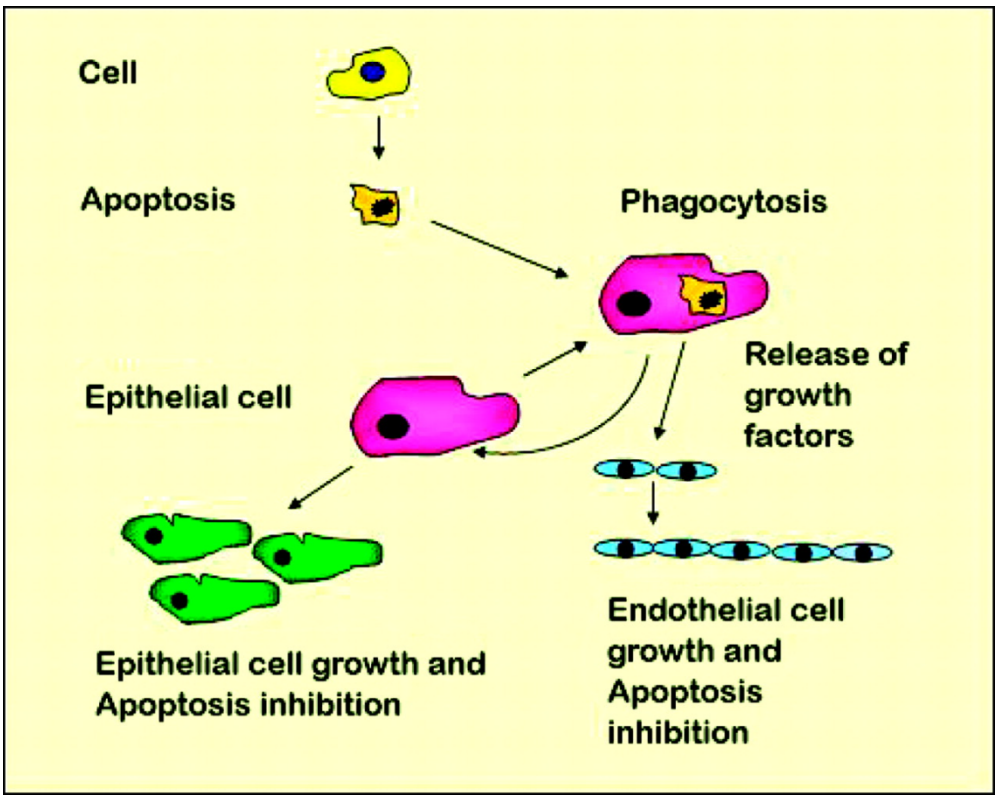


Figure 9

Sequence of events that leads from phagocytosis of apoptotic cells to proliferation and apoptosis resistance of neighboring cells. VEGF secreted by mouse mammary gland epithelial HC-11 cells after engulfment of Jurkat cells stimulates the growth of co-incubated rat lung microvascular endothelial cells and improves epithelial wound closure.

Adapted from Golpon HA, Fadok VA, Taraseviciene-Stewart L, Scerbavicius R, Sauer C, Welte T, Henson PM, Voelkel NF. Life after corpse engulfment: phagocytosis of apoptotic cells leads to VEGF secretion and cell growth. FASEB J. 2004 Nov;18(14):1716-8.

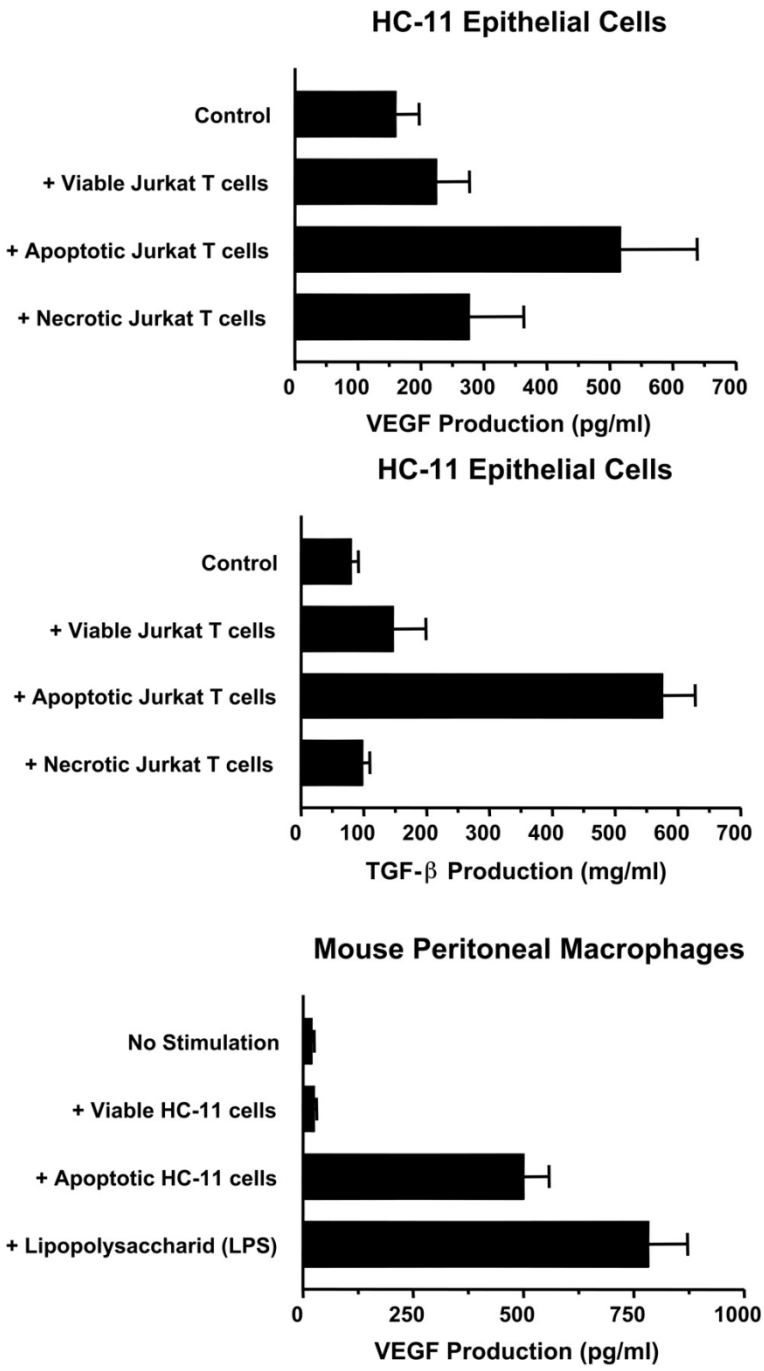


Figure 10

Apoptotic Jurkat T cells induce VEGF and TGF-ß protein expression in HC-11 epithelial cells. VEGF and TGF-ß protein release into the medium were determined by ELISA. HC-11 cells were cultured with viable, apoptotic, or necrotic Jurkat T cells for 24 h in serum-free medium. VEGF secretion was noted in peritoneal macrophages cultured with apoptotic HC-11 cells for 24 h as well as in peritoneal macrophages treated with LPS at 1 ng/mL (*n*=3; ±SEM).

Adapted from Golpon HA, Fadok VA, Taraseviciene-Stewart L, Scerbavicius R, Sauer C, Welte T, Henson PM, Voelkel NF. Life after corpse engulfment: phagocytosis of apoptotic cells leads to VEGF secretion and cell growth. FASEB J. 2004 Nov;18(14):1716-8.

Based on these results, it was presumed that macrophages that had engulfed apoptotic bodies secrete bioactive mediators that lead to endothelial cell proliferation. By utilizing micro-array assays, Fadok et al. demonstrated that VEGF and other growth and angiogenesis-related factors were upregulated in response to the clearance of apoptotic cells. Furthermore, it was shown that transcripts for hepatocyte growth factor (HGF) were increased when phagocytes were exposed to apoptotic but not to necrotic cells (see table below).

Change in expression of growth factor- and angiogenesis-related genes

	Phagocytosis of apoptotic cells (ratio)	**Phagocytosis of necrotic cells (ratio)**
Hepatocyte growth factor	8.14	0.76
TGF-a	1.72	1.30
TGF-b1	1.55	1.41
TGF-b2	3.72	0.12
TGF-b3	0.44	0.95
VEGF-A	2.76	0.54

Paracrine Effects of Cellular Therapies for Myocardial Infarction

Paracrine factors released by transplanted cells after myocardial infarction may influence adjacent cells and exert their actions via several mechanisms. Myocardial protection and neovascularization are the most extensively studied mechanisms in regenerative medicine. Furthermore, the postinfarction inflammatory and fibrogenic processes, cardiac metabolism, cardiac contractility and endogenous cardiac regeneration may also be positively influenced in a paracrine fashion as suggested by Gnecchi et al. (see Figure below)[129]. It is likely that the paracrine mediators are expressed and released in a temporal and spatial manner exerting different effects depending on the microenvironment after an ischemic injury.

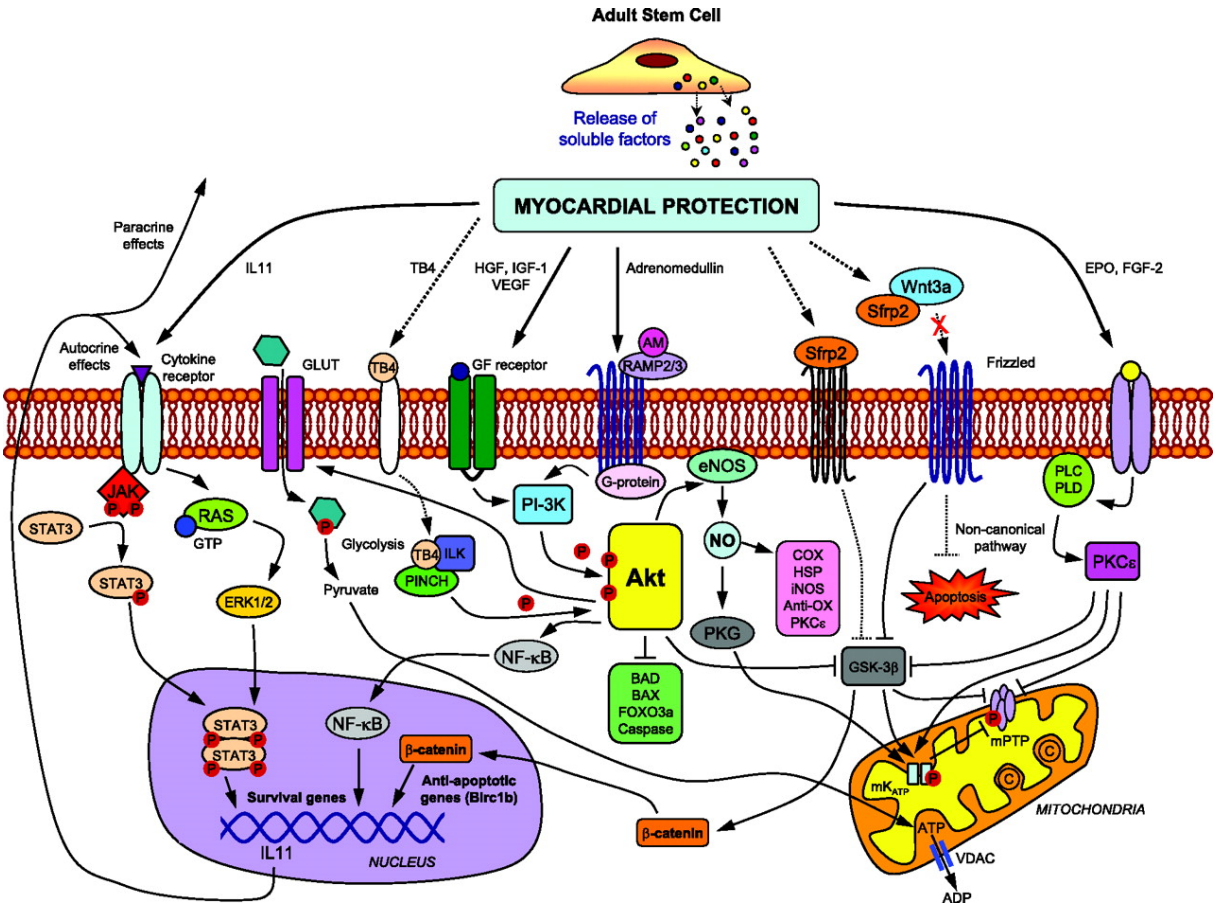


Figure 11

Proposed factors and signaling pathways involved in stem cell–mediated myocardial protection[130]. It has been shown that transfused adult stem cells (ASCs) mediate cardioprotection by producing and releasing soluble mediators with known cytoprotective properties. Most of these factors act through the activation of the prosurvival phosphatidylinositol 3-kinase (PI3K)/Akt pathway.

Adapted from Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res. 2008 Nov 21;103(11):1204-19.

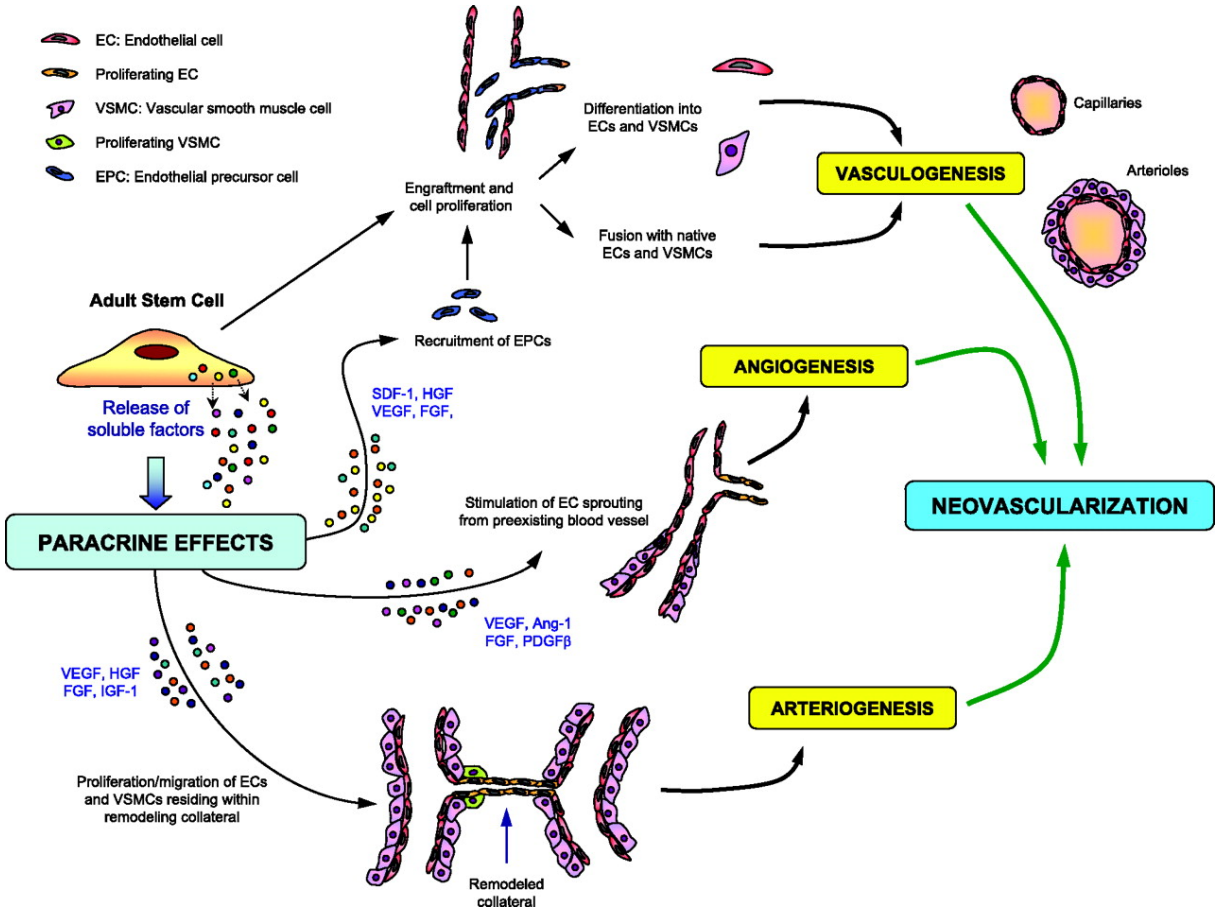


Figure 12

Mechanisms involved in neovascularization. In contrast to the embryonic heart vasculature, the adult heart vessels are quiescent. Only when under stress or in the presence of pathological conditions, such as myocardial infarction, the coronary vascular bed does expand. ASCs are involved in postnatal neovascularization, which encompasses three different mechanisms. The first is referred to as postnatal vasculogenesis, which consists in the assembly of new blood vessels by fusion and differentiation of endothelial progenitor cells originating from the bone marrow. The second mechanism is angiogenesis and consists of the sprouting of new vessels from preexisting vessels. The third mechanism is collateral enlargement and muscularization, namely arteriogenesis. The release of pro-angiogenic and pro-arteriogenic factors by transplanted stem cells positively influences neovascularization in a paracrine fashion leading to a better outcome after myocardial ischemia[131].

Adapted from Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res. 2008 Nov 21;103(11):1204-19.

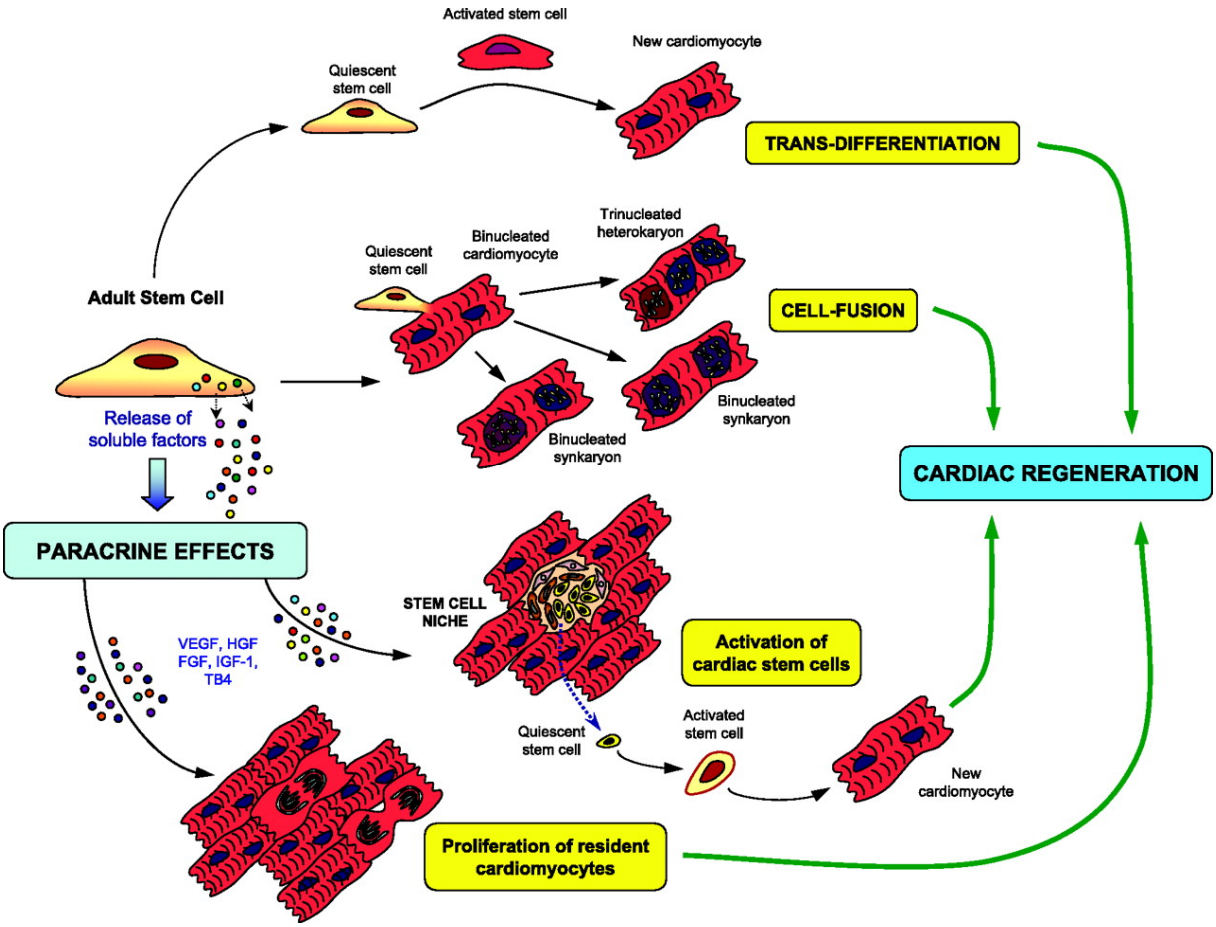


Figure 13

Figure 13 shows possible mechanisms involved in myocardial regeneration. Transplantation of stem cells is sought to induce cardiac regeneration via different mechanisms. Transdifferentiation into newly formed cardiomyocytes has been the first proposed way. Cell fusion of stem cells with native cardiomyocytes represents a second possibility, but the exact biological mechanisms of this event remain unclear. Finally, soluble paracrine factors released by the stem cells may induce activation, migration, and differentiation of circulation bone marrow derived stem or progenitor cells which leads to enhanced proliferation of resident cardiomyocytes.

Adapted from Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res. 2008 Nov 21;103(11):1204-19.

Rationale and Aims of the Study

Since a considerable percentage of transfused stem or progenitor cells used in clinical human trials intended to improve ventricular function after myocardial ischemia is already undergoing apoptosis, we sought to investigate the underlying mechanisms of cellular therapies in regenerative medicine.

In detail the following parameters were of importance:

We first investigated the immunemodulatory potential of apoptotic cells in *in vitro* experiments. As apoptotic cells reduced pro-inflammatory reactions and initiated the up-regulation of proteins and enzymes necessary for progenitor cell liberation from the bone marrow, we applied syngenic apoptotic cells in an *in vivo* rat model of myocardial infarction.

Materials and Methods

In vitro Experiments

Seperation of Peripheral Blood Mononuclear Cells

Venous blood was drawn from healthy young volunteers after informed consent by venepuncture of an antecubital vain using a vacutainer system (Greiner Bio One International, Austria)**.** Blood specimens in EDTA-tubes were processed immediately and PBMC were separated by Ficoll density gradient centrifugation. Therefore whole blood was diluted 1:2 in Hanks balanced salt solution (HBBS, Lonza, Basel, Switzerland) and was shifted carefully in a 50 milliliter tube containing Ficoll-Paque solution (GE Healthcare Bio-Sciences AB, Sweden), then tubes were centrifuged for 15min at 800g at room temperature without brake. This technique generates a layer (buffy coat) of lymphocytes and monocytes in a high degree of purity. Buffy coats of mononuclear cells were obtained, washed in HBSS and resuspended in 1 millilitre of fresh UltraCulture serum-free cell culture medium (Lonza, Basel, Switzerland). Cell concentrations were determined on a Sysmex automated cell counter (Sysmex Inc., USA).

Induction of Apoptosis

To induce apoptosis in human PBMC, cells were exposed to gamma-irradiation (cesium-137), using an irradiation system at the department of Haematology (Medical University Vienna) routinely employed to irradiate blood products such as erythrocyte and platelet concentrates prior to transfusion. Therefore PBMC suspensions in cell culture medium were exposed to two cycles of irradiation (60 Gray).

Verification of Apoptosis

Induction of apoptosis was measured by flow cytometry using Annexin-V-fluorescein/propidium iodide (FITC/PI) co-staining (Becton Dickinson, Franklin Lakes, NJ, USA). Annexin-positivity of PBMC after 24 hours of cultivation was determined to be >70 percent and these cells were termed irradiated apoptotic PBMC (IA-PBMC). Non-irradiated PBMC served as controls and were termed viable-PBMC.

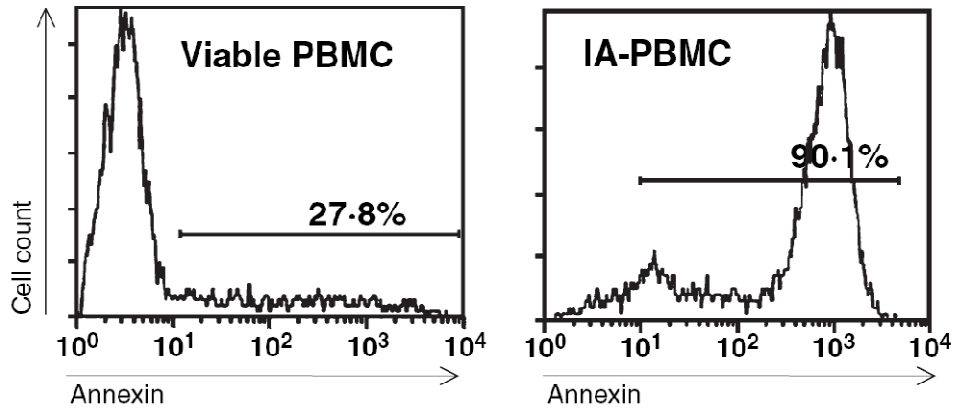


Figure 14

Data from one representative experiment are displayed in Figure 14. Although non-irradiated PBMC evidenced an Annexin-V positivity of 27,8% after 24 hours cultivation maybe due to apoptotic stimuli induced during steps of cell separation or centrifugation, irradiated cells showed levels of Annexin-V staining reaching 90,1%.

Co-incubation of LPS Stimulated PBMC with Irradiated Apoptotic Cells

Human PBMC were obtained as previously described for this experiment. For separation of monocytes from PBMC a magnetic bead system (Monocyte Isolation Kit II, negative selection Miltenyi Biotec, Auburn, CA, USA) was utilized. Therefore cells were incubated with magnetically labeled biotin-conjugated antibodies against CD3, CD7, CD16, CD19, CD56, CD123, and CD235a, thus separating off all non-monocytoid cells, such as T-cells, NK-cells, B-cells, basophils, eosinophils and dendritic cells and separated over a MACS Column. The

obtained cell population of monocytes was of high purity (>95%) as evaluated by flow cytometry.

For co-incubation experiments either purified monocytes or PBMC were stimulated by addition of 1 ng/mL Lipopolysaccharide (serotype 0127-B8, Sigma) to cell culture wells. To determine immunesuppressive of irradiated apoptotic PBMC in co-culture with stimulated autologous PBMC or monocytes, IA-PBMC were added in different concentrations ($1*10^6$, $5*10^5$ and $1{,}25*10^5$).

After an incubation period of 4 hours cell suspensions were harvested, transferred to 1,5 mL tubes and centrifuged for 10 minutes at 400g. Cell culture supernatant were collected and kept frozen at -20° Celsius until further testing.

Cytokine levels of pro-inflammatory mediators Interleukin-1β and Interleukin-6 in supernatants of LPS stimulated cells were evaluated with enzyme-linked immunosorbent assay (ELISA). In detail, measurements were conducted utilizing commercially available ELISA kits (BenderMedSystems, Vienna, Austria) according the manufacturer's instructions. 96-well plates featuring a modified surface with a high affinity for polar groups, found on glycoproteins such as antibodies were coated with an antibody against IL-1beta or IL-6, sealed and incubated over night at 4°C. The next day, plates were washed three times using a washing buffer solution (Phosphate buffered saline (PBS) + 0.05% Tween20) and blocked with Assay buffer (PBS, 0.05% Tween20, 0.5% bovine serum albumin (BSA)) for two hours at room temperature. Standard proteins in decreasing concentrations or samples and a biotin-conjugated detection antibody were added to each well and incubated for an incubation period of two hours. Plates were washed and streptavidin-horseradish peroxidase (HRP) conjugate was added for 20 minutes. After another washing step, 3,3',5,5'-Tetramethylbenzidine (TMB) was used as substrate solution (Sigma, St. Louis, MO, USA) and the enzymatic colour reaction was stopped using 1N sulphuric acid (Merck Chemical, Germany). Plates were read for 0,1 second per well at 450nm on a Wallac Multilabel counter 1420 (PerkinElmer, Waltham, MA, USA). Cytokine concentrations were calculated by comparing optical density (OD) values of samples with OD of known concentrations of the standards utilizing calculation software (Microsoft Excel, Microsoft, USA).

Monocyte-derived Dendritic Cell (DC) Preparation and T-cell Stimulation

PBMCs were isolated from whole blood of healthy donors by standard density gradient centrifugation with Ficoll-Paque, as described above. T cells and monocytes were separated by magnetic sorting using 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 %). Dendritic cells (DC) were generated by culturing purified blood monocytes for 7 days with a combination of granulocyte macrophage colony stimulating factor (GM-CSF) (50 ng/ml) and Interleukin-4 (100 U/ml). Subsequently, DCs were differently stimulated. Maturation was induced either by adding 100 ng/ml LPS from Escherichia coli for 24 hours alone or by adding LPS for 2h and further culturing the dendritic cells with apoptotic cells in a 1:1 ratio for 22 hours. Additionally, DCs were treated with apoptotic cells alone (1:1) for 24h. For the mixed leukocyte reaction (MLR), allogenic, purified T cells ($1x10^5$/well) were incubated in 96-well cell culture plates (Corning Costar, 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 of cultivation. Cells were harvested after 18h and incorporated [methyl-3H]thymidine was detected on a microplate scintillation counter.

RNA Preparation for Polymerase Chain Reaction (PCR)

To evaluate effects of ionizing gamma-irradiation on PBMC relating to induction of cytokines and growth factors associated with angiogenesis and progenitor cell release from the bone marrow and their subsequent homing to sites of ischemic injury, RNA transcription levels of Vascular endothelial growth factor (VEGF), Interleukin-8 (IL-8) and Matrixmetalloproteinase 9 (MMP9) were determined by real time reverse transcription polymerase chain reaction.

For this experiment, IA-PBMCs and viable PBMC were obtained as previously described. Cells were cultured at a density of $1x10^6$ for 24 hours in serum-free UltraCulture medium. RNA was extracted either initially after separation to determine basal transcriptional profiles or

after an incubation time of 24 hours using RNeasy (QiIAGEN, Vienna, Austria) following the manufacturer's instructions. Cells were pelleted by centrifugation for 5 minutes at 300g and lysed using 350 µL of a highly denaturing guanidine-thiocyanate–containing buffer. This procedure inactivates RNases thus ensuring purification of intact RNA. Ethanol is subsequently added to provide appropriate binding conditions, thereafter samples were then applied to a RNeasy Mini spin column and centrifuged in a microcentrifuge for 2 minutes at 8000g. In this step the total RNA binds to the membrane and all contaminants were efficiently washed off. The purified cellular RNA samples were resuspended in 100 µl of distilled water. Hereafter cDNAs were transcribed from RNA by reverse transcriptase using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA) as indicated in the instruction manual.

Quantitative Real Time PCR

mRNA expression of VEGF, Interleukin-8 (IL-8) and MMP9 was quantified by real time reverse transcriptase 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[132]. The relative expression of the target genes was calculated by comparison to the house keeping gene β2M using a formula described by Wellmann et al.[133]. The efficiencies of the primer pairs were determined as described[132].

Effects of PBMC Derived Cell Culture Supernatants on RNA Transcription Profiles in Human Fibroblasts

Human fibroblasts were obtained from Cascade Inc. (Portland, USA) and cultured at a density of $1x10^5$ in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Austria) supplemented with 10% fetal calf serum (FCS, PAA, Austria) and 1% penicillin/streptomycin (Gibco, Invitrogen, Austria) on a 12 well plates (Perkin Elmer, USA). To evaluate influences of either irradiated apoptotic or viable PBMC derived supernatants on fibroblasts, conditioned medium from PBMC cultivation was added to DMEM at a 1:1 ratio. In this setting, fibroblasts were cultured for 4 and 24 hours to determine short and long term influences on transcription of VEGF, IL-8 and MMP9 RNA. RNA samples were extracted at multiple time points (0, 4 and 24 hours) and processed as described above.

ELISA Evaluation of Pro-angiogenic Factors and Homing Promoting Mediators Released by Viable PBMC and IA-PBMC During Cell Culture

Irradiated apoptotic and viable human PBMC were obtained as previously described and cultured for 24 hours at a cell concentration of $2.5*10^6$ in a humidified atmosphere at 37° Celsius. Supernatants were collected the next day, centrifuged and immediately frozen at -20°C until evaluation. The release of pro-angiogentic factors such as Vascular endothelial growth factor (VEGF), Interleukin-8 (IL-8), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Granulocyte Colony Stimulating Factor (G-CSF) and MMP9, an accepted liberating factor of c-kit cells, was analysed utilizing ELISA (DuoSet, R&D Sytems, USA) following the manufacturer's instructions. Plates were read at 450nm on a Wallac Victor 3 Multilabel counter 1420 plate reader and results calculated as described above.

In vivo Experiments

All animal experiments were approved by the committee for animal research, Medical University of Vienna and the ministry for education, science and culture (BMBWK-66.009/0278-BrGT/2005). All experiments were performed in accordance to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH).

Acquisition of Syngeneic IA-PBMC and Viable-PBMC for Acute Myocardial Infarction in *in vivo* Experiments

Nine weeks old male Sprague Dawley rats (weight 250 – 300g) were obtained from ALG Himberg (Abteilung für Labortierkunde und Labortiergenetik Medical University of Vienna, Himberg, Austria). Rats were transferred to the Center for Biomedical Research (Medical University Vienna) and were allowed to acclimatize for two weeks to the laboratory environment before start of experiments.

For the generation of either irradiated apoptotic or viable rat PBMC, animals were anesthetized intraperitoneally with a mixture of xylazin (1 mg/100 g bodyweight) and ketamin (10 mg/100 g bodyweight) and ventilated mechanically. Whole blood was obtained from prior heparinized rats by punctuation of the heart and transferred into a 50 millilitre syringe. Approximately 10-12 millilitre of blood were collected from each animal. PBMC were separated accordingly to the protocol for human cell separation mentioned previously. Apoptosis in rat PBMC was induced by cesium-137 irradiation with 45Gy and cells were cultured for 18 hours. This time point was chosen because rat PBMC evidenced highest Annexin-V positivity after 18 hours compared to non-irradiated cells. Cell density was $8*10^6$ suspended in 300µL.

After cultivation, rat PBMC suspensions, either irradiated or viable were transferred into syringes used for subcutaneous injections. For each animal a volume of 300µL (8 million PBMC) was prepared.

Induction of Myocardial Infarction

Myocardial infarction was induced in adult male Sprague-Dawley rats by ligating the left anterior descending artery (LAD) as previously described[134,135]. In short, animals were anesthetized intraperitoneally with a mixture of xylazin (1 mg/100 g bodyweight) and ketamin (10 mg/100 g bodyweight) and ventilated mechanically. Rats were placed in a dorsal position and a left intercostal thoracotomy was performed. The thorax was opened through the third or fourth intercostal space and a rib-spreading retraction device was placed in position thus simplifying operational proceedings. The pericardium was opened and the heart was then delivered to the surface of the thoracic incision. The left coronary artery was located beneath the cardiac auricle and was subsequently transfixed by passing a 6.0 atraumatic silk suture around the vascular structure. The adjected myocardium immediately began to pale out in comparison to the surrounding viable heart muscle.

Instantly after the onset of ischemia $8x10^6$ apoptotic PBMCs suspended in 300µL 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 animal model experimental design is shown in Figure 15**.**

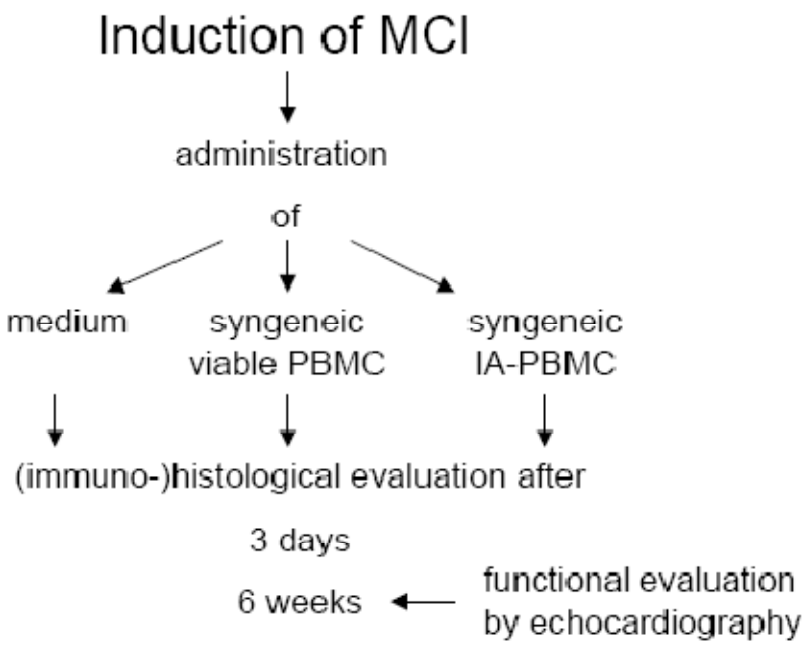
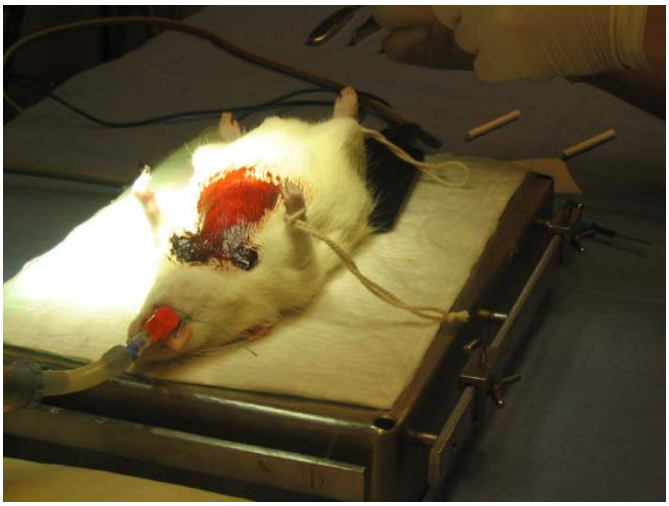
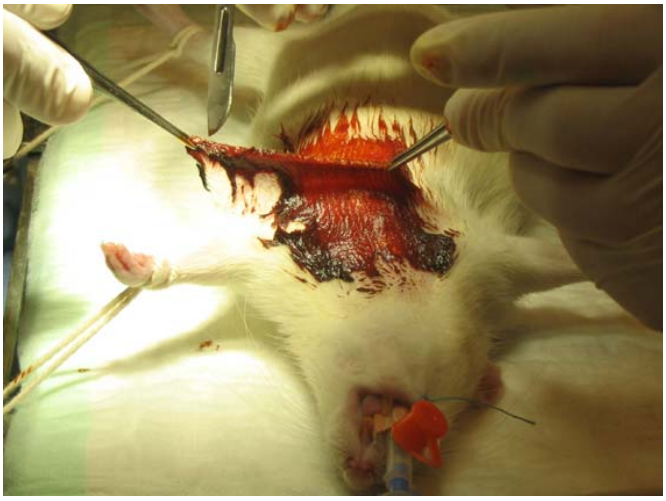
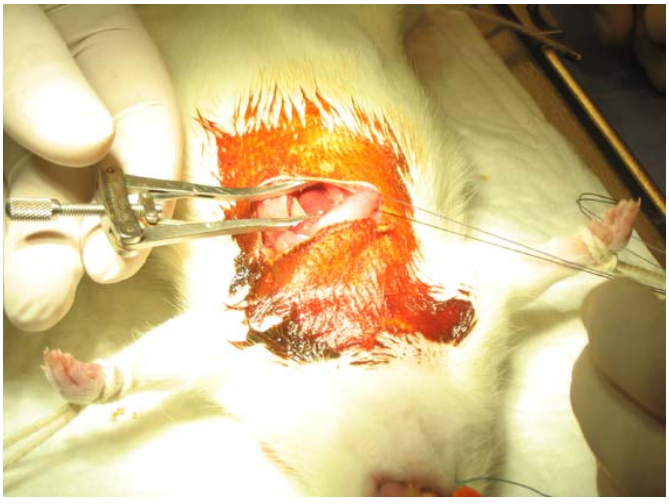
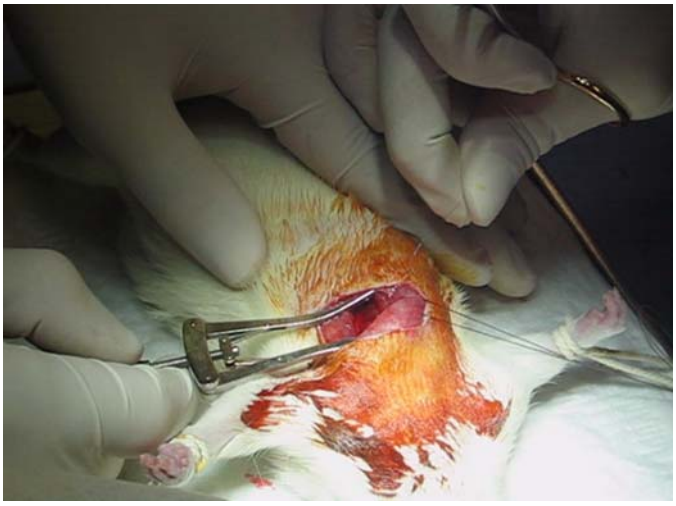


Figure 15

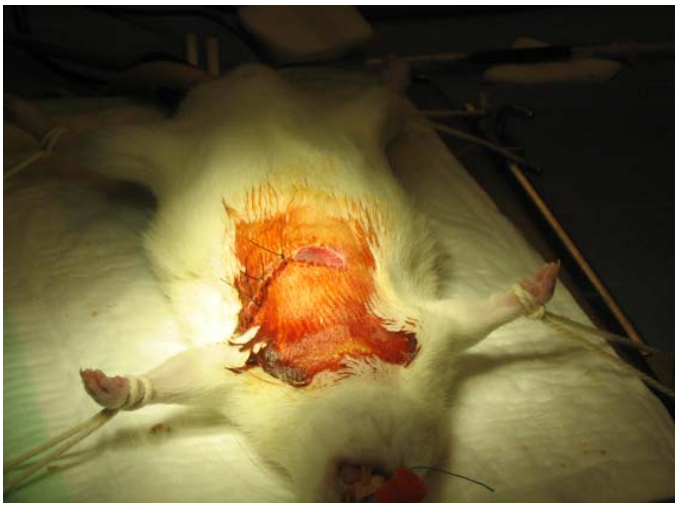

anesthetized and mechanically ventilated rat


dermal incision


intercostal thoracotomy



ligation of the coronary artery



dermal suture

Figure 16

Figure 16 shows LAD ligation in rats and induction of myocardial infarction.

Tracking of Apoptotic Cells

In order to distinguish direct from paracrine effects of injected irradiated apoptotic PBMC an assay utilizing carboxyfluorescein diacetate succinimidyl ester (CFSE) labeled PBMC was established. CFSE, once added to cell suspensions, diffuses without carrier substances through cellular memberanes where intracellular esterases can alter the molecule's structure into a green fluorescent dye. This transformed molecule shows a signifiacantly reduced ability to re-diffuse through cell membranes. The dye will remains captured within

the cell by binding covalently to available primary amines. This method offers a convenient and unbiased way to detect infused cells in solid tissue such as heart, lungs, spleen or liver.

For this purpose, $8x10^6$ syngeneic rat PBMC were labelled with 15µM CFSE dye (Fluka BioChemika, Buchs, Switzerland) at room temperature for 10 minutes. Labelling was stopped by the addition of fetal calf serum (FCS, PAA Laboratories, Linz, Austria). Apoptosis was induced as described above, Annexin-V was determined to reach a level of at least 70% or higher and cells were injected after coronary artery ligation procedure. 72 hours after operation rats were anesthetized intraperitoneally with xylazin and ketamin and sacrified by cardiac explantation. Liver, spleen and heart were fixed in 10% buffered formalin solution and processed following a standard procedure for frozen sections. Tissue samples of heart, liver and spleen specimens were analyzed on a confocal laser scanning microscope (ZEISS LSM 510 laser scanning microscope, Jena, Germany) as described previously by Petersen et al.[136]. Positively stained cells were quantified as labelled cells per high power field and evaluated statistically.

Histology and Immunehistology

All animals were sacrified after intraperitoneal injection of xylazin and ketamin at two different time points either 72 hours or 6 weeks after experimental infarction was induced. Hearts were explanted after median sternotomy and dissection of cardiac vasculature (aorta, cavernous vein, pulmonary arteries). The obtained organs were preserved in 10% buffered formalin and then sliced at the level of the largest extension of infarcted area. For further histological processing, slices were embedded in paraffin for (immune-)histological staining. Tissue specimens for conventional histological evaluation were stained with hematoxylin-eosin (H&E), chromotrop aniline blue (cab) and elastic van Gieson (evg). Immunehistological evaluation was performed according to a protocol for solid organs embedded in paraffin (Department of Clinical Pathology, Medical University Vienna) 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, CA, USA), CD34 (sc-52478, Santa Cruz Biotechnology, CA, USA), c-kit (sc-168, Santa Cruz Biotechnology, CA, USA), S100 beta (sc-58841, Santa Cruz Biotechnology, CA, USA). Slices of stained tissue

samples were transferred to glass slides and were evaluated on a Olympus Vanox AHBT3 microscope (Olympus Vanox AHBT3, Olympus Optical Co. Ltd., Tokyo, Japan) located at the Department of Clinical Pathology at multiple magnification ranging from 20x to 400x and were captured digitally by using a ProgRes CapturePro C12 plus camera (Jenoptik Laser Optik Systeme GmbH, Jena, Germany). Colour levels, brightness and white colour balance were adjusted using Photoshop CS3 software (Adobe, USA).

Determination of Myocardial Infarction Size by Planimetry

In order to determine the extension of the infarcted area, specimens on glass slides were scanned on a desktop scanner at 9600 dots per inch (dpi) and saved as tiff files. Image files were imported into Image J planimetry software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA) for quantitative analysis. 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 collagenous tissue and viable myocardium. Infarct size was expressed as percent of total left ventricular area.

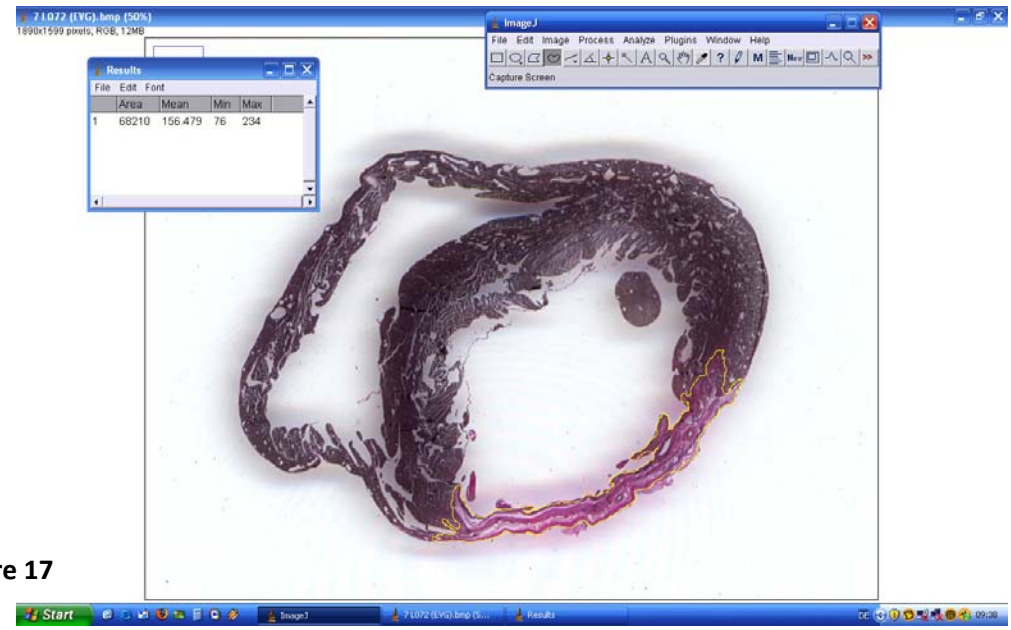


Figure 17

Figure 17 shows planimetric analysis in Image J software.

Cardiac Function Assessment by Echocardiography

Six week after induction of myocardial infarction rats were anaesthetized with 100mg/kg Ketamin and 20 mg/kg Xlyazin prior to echocardiographic evaluation of ventricular function. The sonographic examination was conducted on a Vivid 5 system (General Electric Medical Systems, 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 follow: FS(%)=((LVEDD – LVESD)/ LVEDD)*100%.

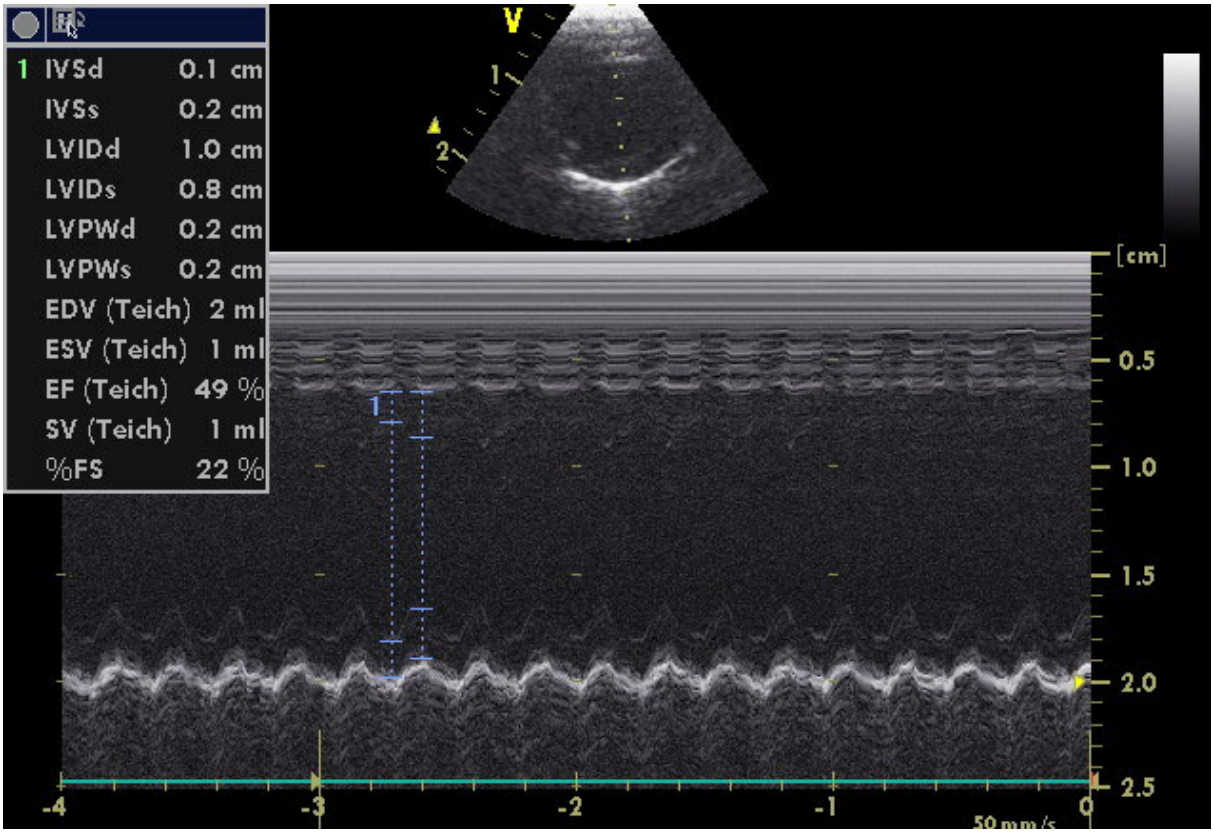


Figure 18

Figure 18 shows echocardiographic determination of cardiac function (m-mode).

Statistical Methods

Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA). All data are given as mean ± standard error of the mean (SEM). 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.

Figures and Illustrations

Figures were generated using GraphPad Prism Pro software (GraphPad Software, USA) and converted into PowerPoint (Microsodt Corp., USA) files for further processing. Illustration of histological samples were edited with Photoshop CS3, for better visualization of infarcted myocardium fibrotic scar was colored in green in specimens obtained 6 week after induction of experimental coronary artery ligation.

Results

Induction of Apoptosis by Cesium Irradiation (IA-PBMC)

To determine the cellular response after exposure to ionizing cesium-137 irradiation, the rate of apoptosis induction in human PBMC was ascertained by flow cytometry utilizing Annexin-V/PI. Irradiation with 60Gy caused positivity for Annexin-V on PBMC in a time dependent manner reaching a peak plateau at 24 hours as compared to viable PBMC. Positivity did not further increase significantly on cell incubated for 48 hours. Viable cells served as controls in this experimental setting. Since Annexin-V binding was highest after 24 hours all further *in vitro* investigations were performed after this culture period for human cells. This time limit was chosen to assure a relatively low positive labelling for PI thus indicating cells were predominantly in the state of apoptosis and not necrosis.

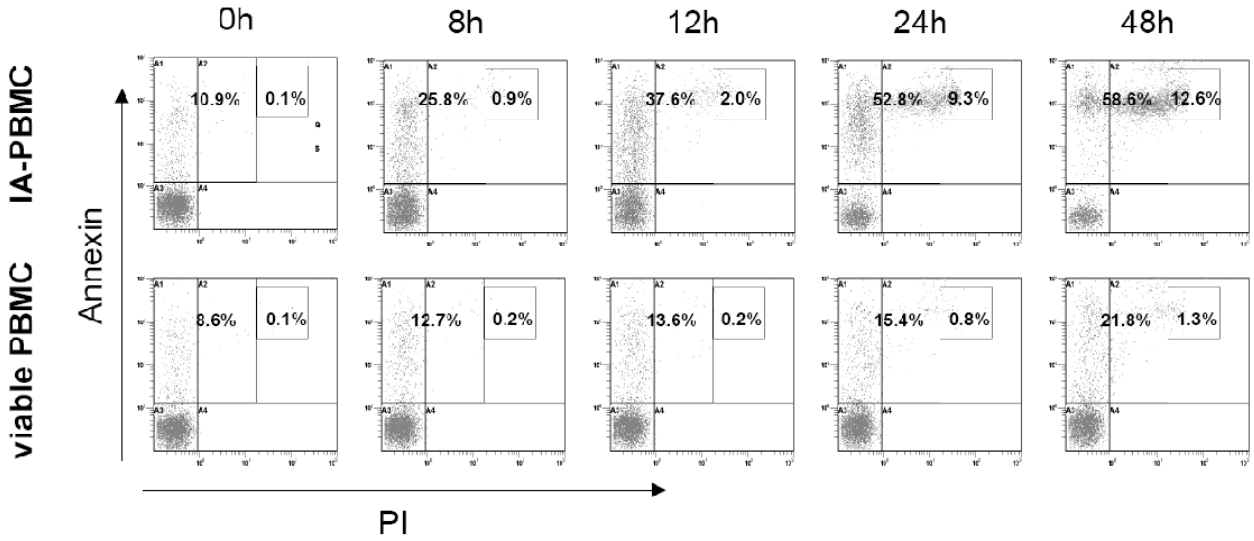


Figure 19

Figure 19 shows staining for Annexin-V/PI by means of flow cytometry.

Irradiated Apoptotic PBMC Evidence Immunesuppressive Features *in vitro* by Reduced Interleukin-1b and Interleukin-6 Secretion

Interleukin-1β as well as Interleukin-6 are recognized as the predominant pro-inflammatory mediators in myocardial infarction *in vivo*. To test the hypothesis whether IA-PBMC have an effect on cellular responses we co-incubated LPS stimulated human monocytes and PBMC with increasing doses of irradiated apoptotic PBMC. A dose dependent decrease in secretion of Interleukin-1β and Interleukin-6 in cultures of both cell types was evident as evaluated by ELISA.

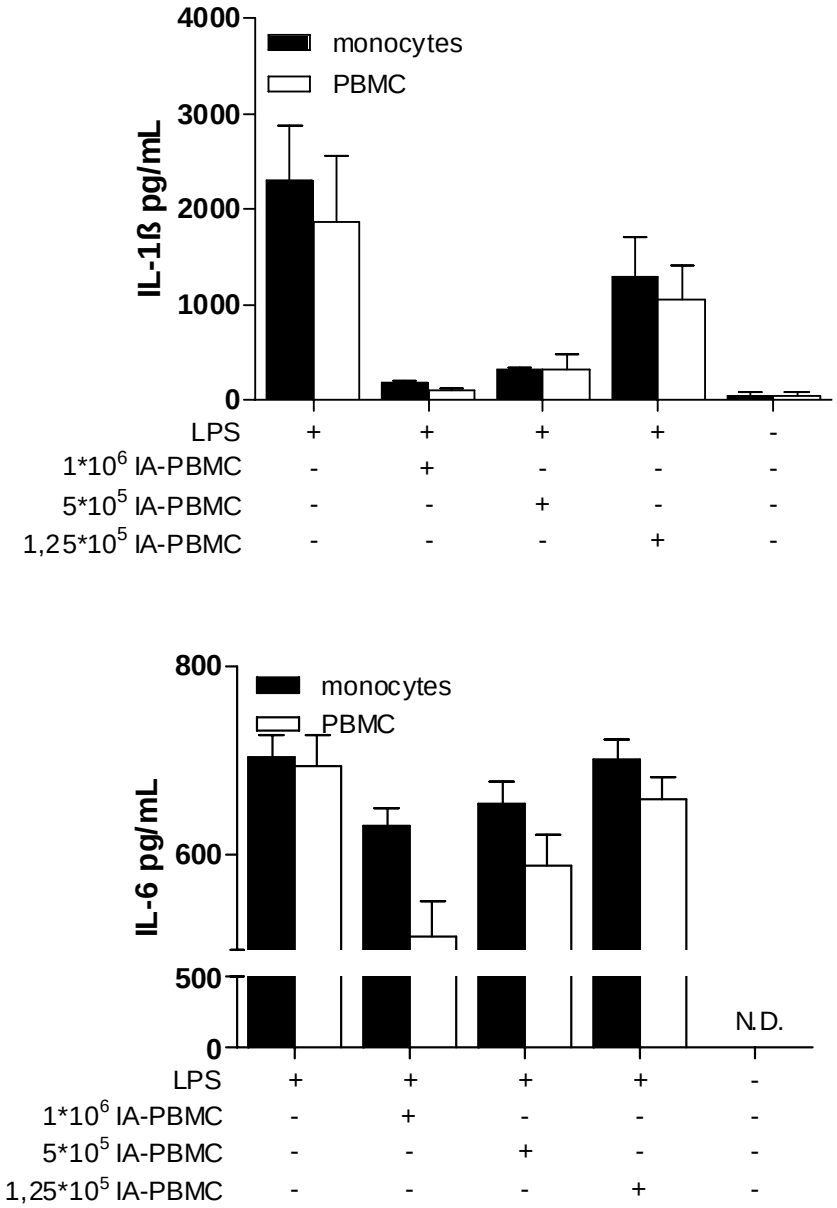


Figure 20

A decrease in pro-inflammatory protein secretion was predominantly found in the case of Interleukin-1beta. Either stimulated PBMC or monocytes co-incubated with $1*10^6$ apoptotic cells evidenced almost secretory levels as found in unstimulated controls. This reduction was less pronounced for Interleukin-6, although LPS triggered PBMC indicated a stronger suppressive response in comparison to monocytes.

Immunesupressive Effects in a Mixed Lymphocyte Reaction

To verify anti-proliferative effects of irradiated apoptotic PBMC in an allogeneic model a mixed lymphocyte reaction (MLR) employed. We utilized allogenic, purified T cells and incubated these effector cells with graded doses of dendritic cells either with or without addition of apoptotic PBMC. The figure below evidences that co-incubation of irradiated cells decreases proliferation rate in a dose-dependent manner as displayed by reduced counts per minute (cpm).

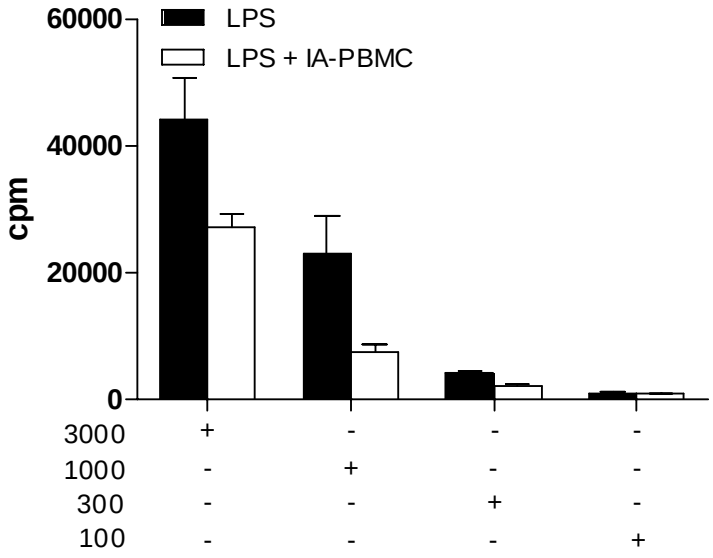


Figure 21

Figure 21 shows results from mixed-lymphocyte reactions investigating immunesuppressive effects of IA-PBMC.

IA-PBMC and Viable PBMC Evidence Increased mRNA Transcription of VEGF, IL-8/CXCL8 and MMP9

Despite this immunesuppressive or immuneregulatory mechanism, we determined whether irradiated apoptotic PBMC can initiate pro-angiogenic responses by up-regulating transcripts of mediators associated with reparative processes in tissue restoration. Therefore real time reverse transcription polymerase chain reaction was used to determine if either VEGF, Interleukin-8 or MMP9, proteins known to be related to mobilization of endothelial progenitor cells are elevated after cesium-137 irradiation. RNA transcription showed little difference in VEGF expression between viable and irradiated PBMC after 24 hours as determined by RT-PCR, however culturing cells overnight seems to trigger an up-regulation as compared to freshly isolated cells. A strong enhancement of IL-8 and MMP9 was evident in irradiated apoptotic PBMC. Peak induction for IL-8 in IA-PBMC was 6 fold versus 2 fold in viable cells, and 30 fold versus 5 fold for MMP9, respectively.

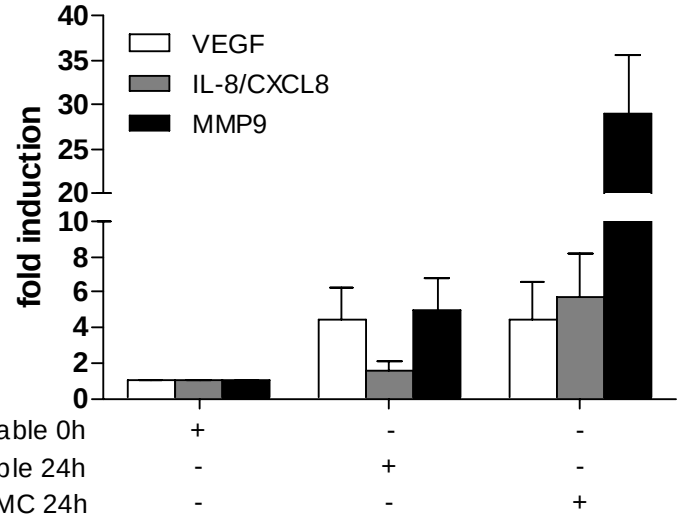


Figure 22

Figure 22 shows the effect of irradiation on transcription profiles for VEGF, IL-8 and MMP9.

Levels of Pro-angiogenic Protein Secretion by Viable and Irradiated PBMC

Supernatants obtained from irradiated apoptotic and viable PBMC were quantified for VEGF, IL-8, GM-CSF, G-CSF and MMP9 utilizing ELISA after 24 hours culture. As seen in Figure 23 VEGF, IL-8 and MMP9 evidenced similar levels in both groups. GM-CSF and G-CSF were not

detectable (data not shown). Of interest was the finding that MMP9 was present in relatively high levels in cell lysates.

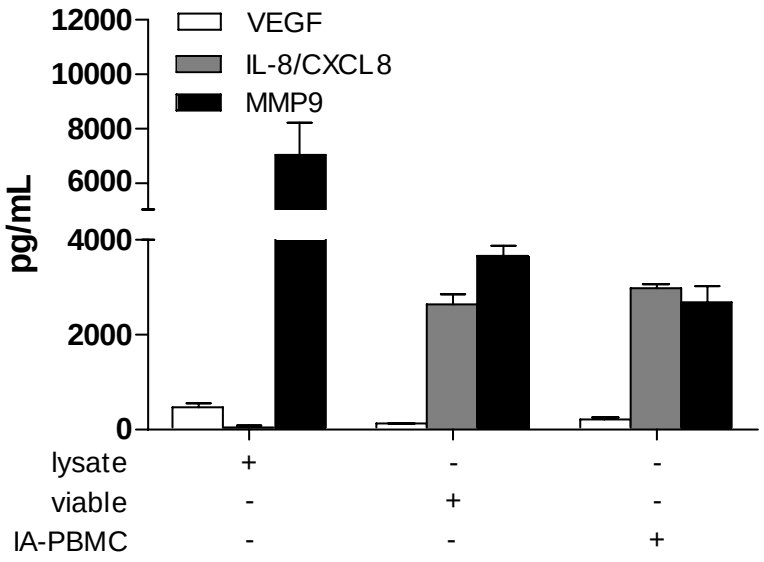


Figure 23

Figure 23 shows supernatant levels of pro-angiogenic mediators. Only minimal differences were detected when viable or apoptotic cells were incubated at a density of $2,5*10^6$ cells per millilitre.

Supernatants of Irradiated and Viable PBMC Cultures Augment Pro-angiogenic mRNA Transcription in Mesenchymal Fibroblasts

PBMC secrete factors attributed to angiogenesis and EPC mobilization during cell culture as previously shown. To further prove this finding we performed a co-culture experiment and suspended human mesenchymal fibroblasts in conditioned medium of either irradiated or viable PBMC.

Since stromal cells in bone marrow are constitutively fibroblasts we sought to investigate whether co-incubation of fibroblasts with supernatants derived from irradiated apoptotic PBMC and viable PBMC had the ability to increase mRNA transcription of factors responsible

for EPC mobilization such as VEGF, IL-8 and MMP9[137]. RT-PCR was conducted at two different time points, at 4 and 24 hours after seeding. Highest levels of induction were detected for IL-8 in cells cultivated in irradiated PBMC supernatants reaching an almost 120-fold induction at 4 hours as compared to controls. This response is also present at 24 hours. A comparable response was found for VEGF, whereas MMP9 up-regulation was predominantly found after 24 hours. This data indicate that supernatants contain paracrine factors that can enhance fibroblasts to augment mRNA products responsible for pro-angiogenic effects in the bone marrow environment.

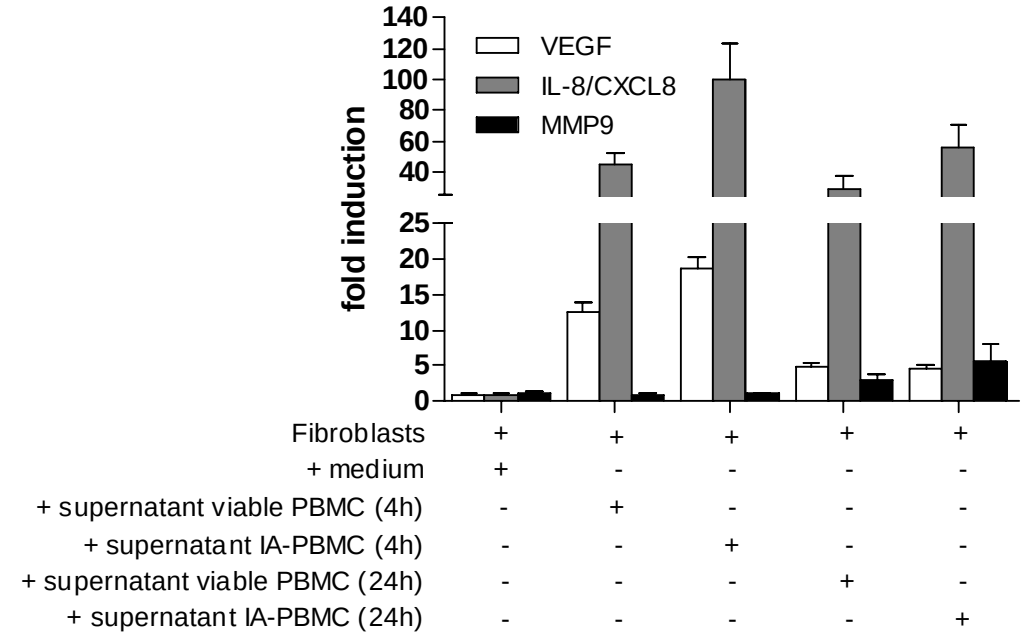


Figure 24

Figure 24 shows results of RT-PCR from fibroblasts incubated in conditioned supernatants.

Adoptive Transfer of CFSE Labelled IA-PBMC in a Rat Myocardial Infarction Model

Because we were able to determine that cultured irradiated apoptotic PBMC can induce both anti-inflammatory and pro-angiogenetic processes in *in vitro* conditions, we sought to investigate if transfused cells evidence a direct cellular response in the infarcted myocardium. Therefore we infused irradiated apoptotic PBMC initially after ligation of left anterior descending coronary artery (LAD). First we sought to determine where these

cultured cells are homing after myocardial ischemia and infarction. CFSE labelled apoptotic cells were injected into the tail vein shortly after LAD artery ligation. Three days after experimental infarction was induced, rats were scarified and explanted hearts, livers and spleens were analysed by confocal laser scanning microscopy for positively labelled cells. A representative histology is seen in Figure 25. The majority of CFSE-labelled PBMC was trapped in the liver (a) and spleen (b) tissue within 72 hours. No cells were observed in the heart (c) suggesting that infused PBMC do not evidence any direct cellular effects in the microenvironment of the infarcted myocardium.

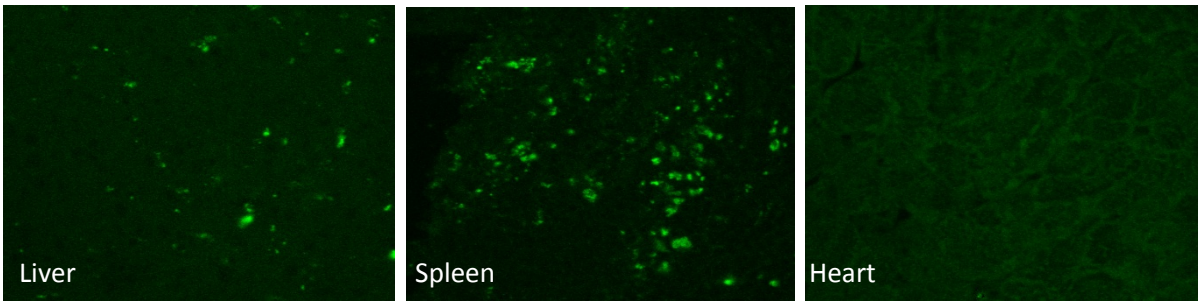


Figure 25

Figure 25 shows CFSE-labelled cells in tissue specimens after myocardial infarction.

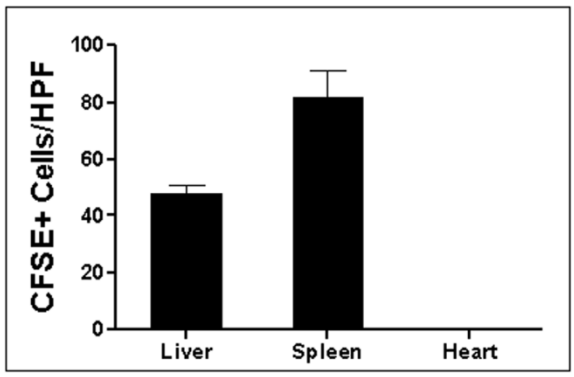


Figure 26

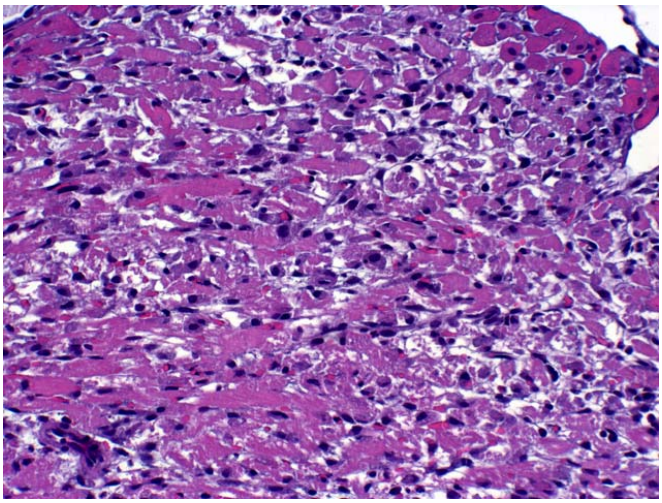
Figure 26 shows Quantification of CFSE-labelled cells in tissue specimens.

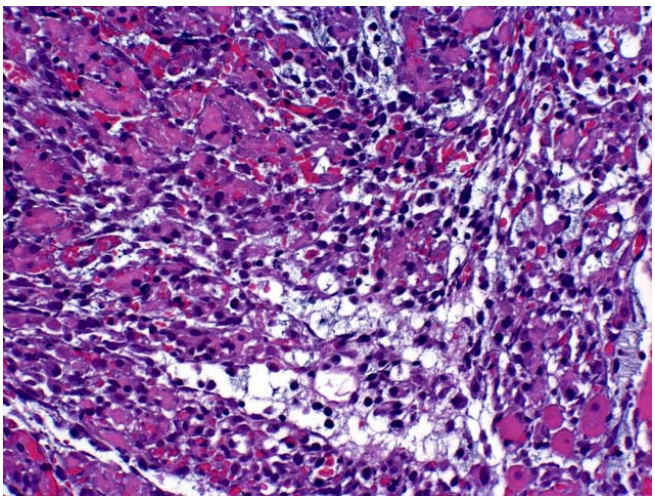
Diverted Early Inflammatory Immune Response in IA-PBMC Treated AMI

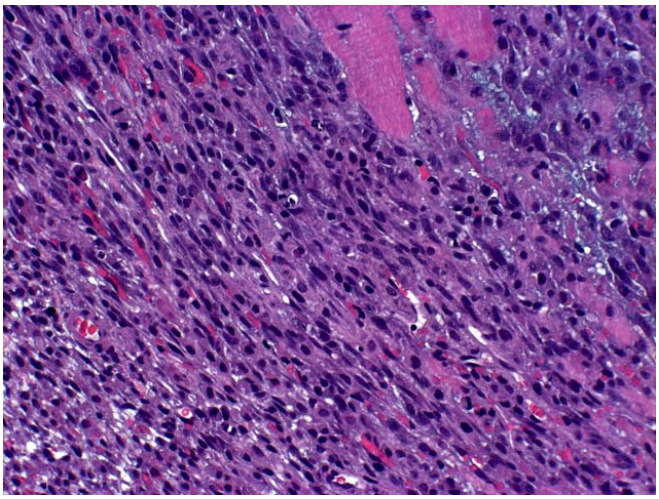
Upon closer investigation in H&E staining, control infarction and viable PBMC infused rats evidenced a mixed cellular infiltrate in the wound areas in accordance to granulation tissue with abundance of neutrophils, macrophages/monocytes, lymphomononuclear cells,

fibroblasts and activated proliferating endothelial cells admixed to dystrophic cardiomyocytes within 72 hours after coronary artery ligation. In contrast, rats treated with irradiated apoptotic PBMC evidenced a much denser monomorphic infiltrate in infarcted areas that consisted of medium sized monocytoid cells with eosinophilic cytoplasm, dense nuclei and a round to spindle shaped morphology. In addition, few lymphomononuclear cells, especially plasma cells, fibroblasts and endothelial could be detected.

H&E


control infarction

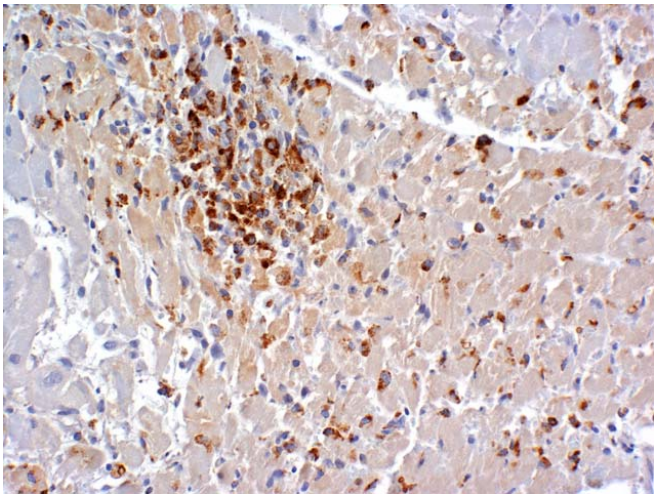

viable PBMC



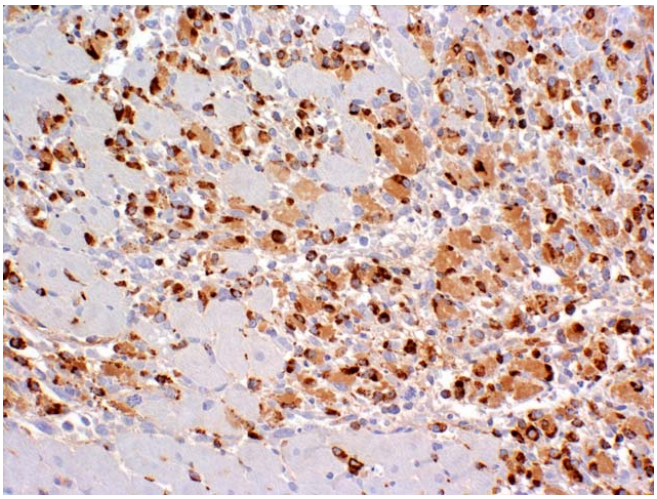
irradiated apoptotic PBMC **Figure 27**

Furthermore, an immunehistological analysis was conducted. Tissue specimens of explanted hearts revealed that the cellular infiltrate in rats injected with irradiated apoptotic PBMC was composed of $CD68^+$ monocytes/macrophages. The other two groups evidenced fewer positively stained cells in the infarcted myocardium. High power field quantifications at a magnification of 400X showed a mean of 60.0±3.6 positively stained cells in the control group, 78.3±3.8 in viable PBMC injected rats and 285.0±23.0 in apoptotic cells transfused animals (±SEM).

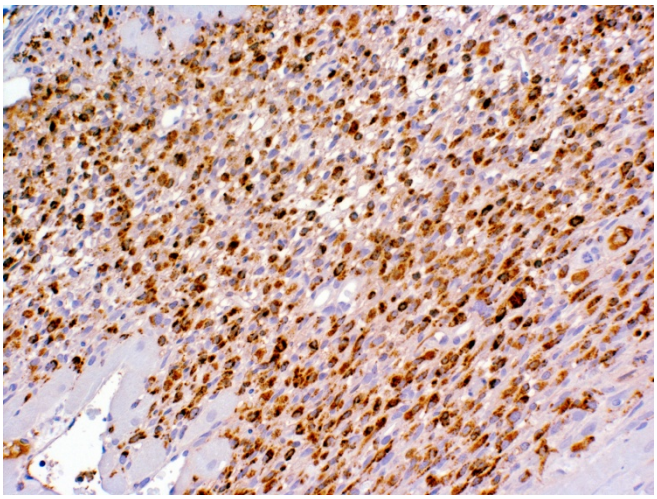
CD68



control infarction



viable PBMC



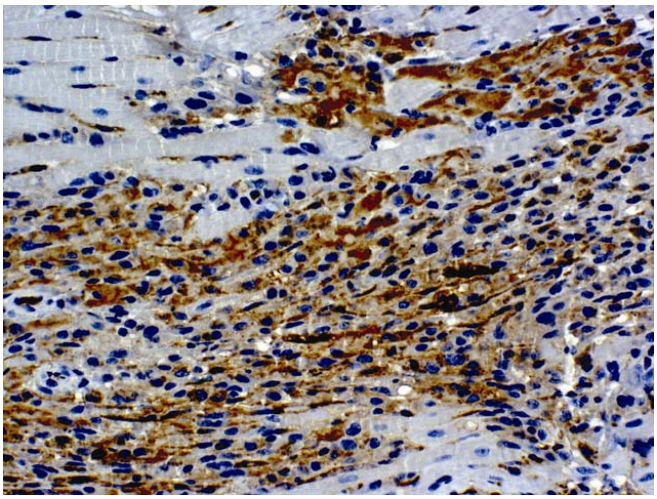
irradiated apoptotic PBMC

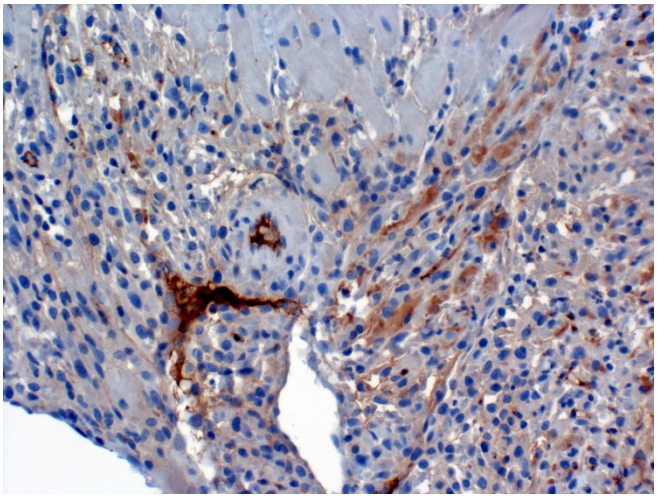
Figure 28

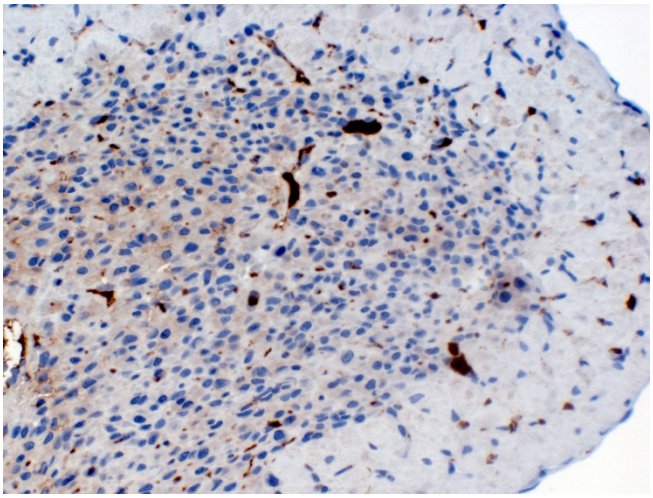
As exposing PBMC to cesium-137 radiation reduced the pro-inflammatory cytokine response after LPS stimulation in *in vitro* conditions and showed immunosuppressive effects in a mixed lymphocyte reaction we sought to investigate whether this mechanism is also of importance *in vivo*. To evaluate this issue an immunohistological staining for S100β positive dendritic cells was performed.

Animals injected with irradiated apoptotic PBMC evidenced slightly reduced amounts of S100β positive cells as compared to untreated controls, although this difference was not as prominent as for $CD68^+$ monocyte/macrophage staining.

S100β


control infarction


viable PBMC


irradiated apoptotic PBMC

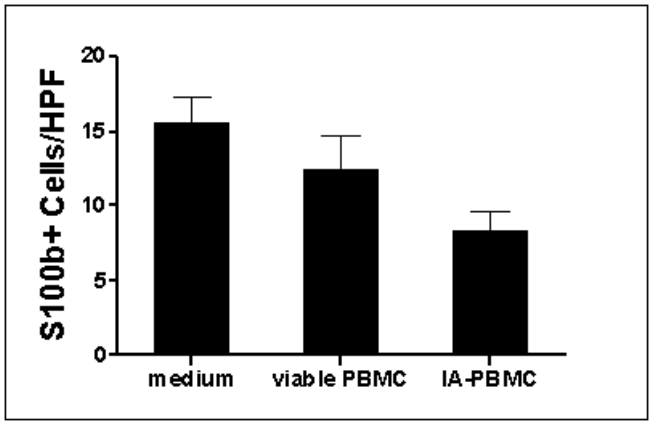
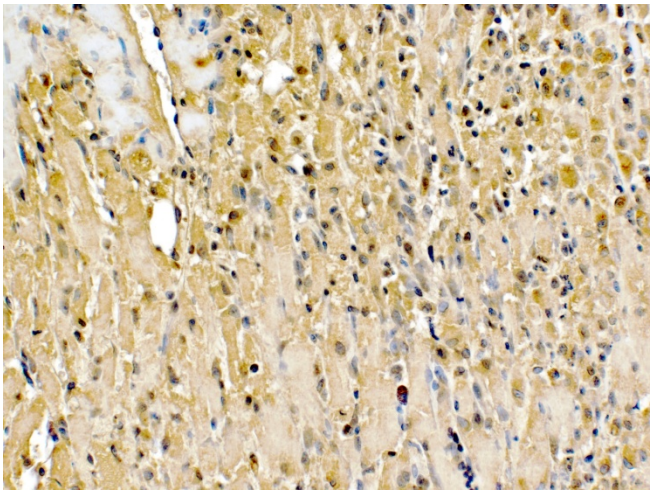


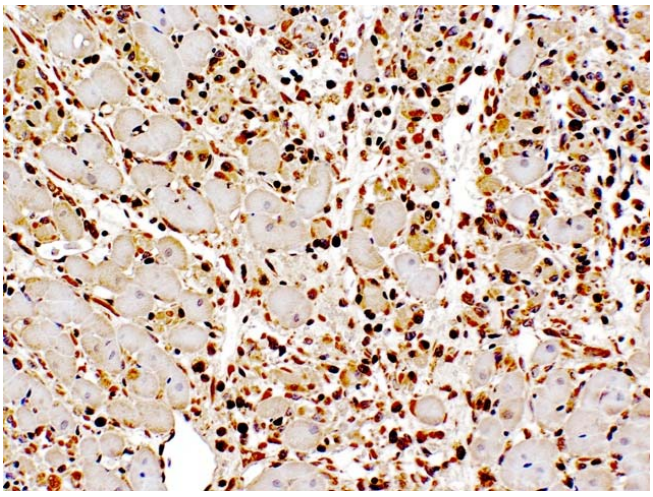
Figure 29

A quantitative analysis revealed that 15.6±1.7 S100β positive cells were present in control animals, 12.4±2.3 in viable PBMC injected and 8.4±1.2 (cells/high power field ± SEM) in apoptotic cells transfused rats.

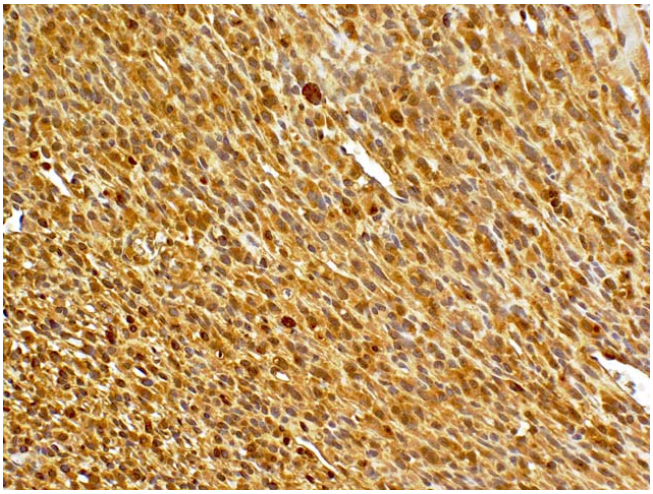
We next sought to determine the pro-angiogenic response after application of irradiated apoptotic PBMC after myocardial infarction. Therefore immunehistological analyses for VEGF were conducted to reveal whether cells infiltrating the myocardium after ischemia express growth factors which can promote angiogenesis.

VEGF


control infarction



viable PBMC



irradiated apoptotic PBMC

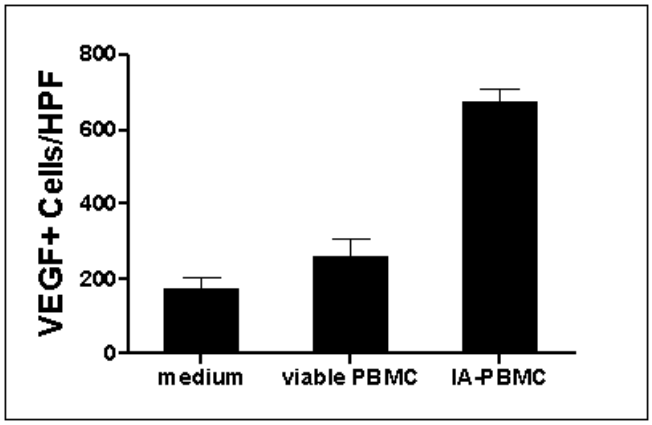
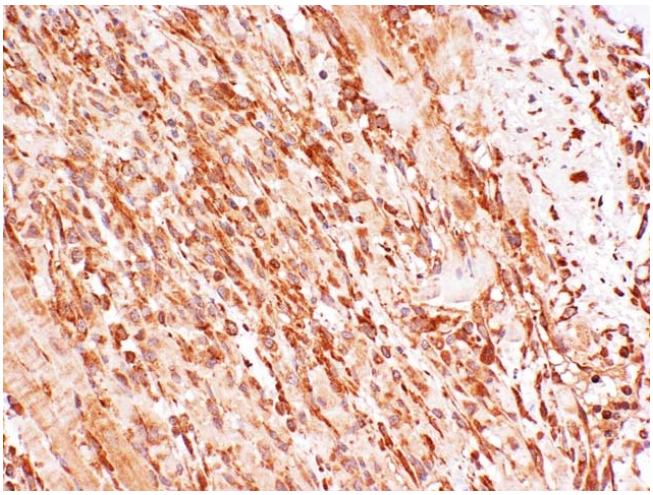


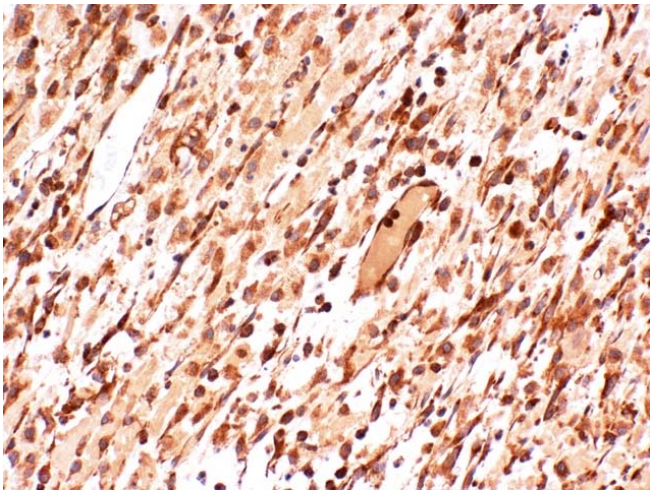
Figure 30

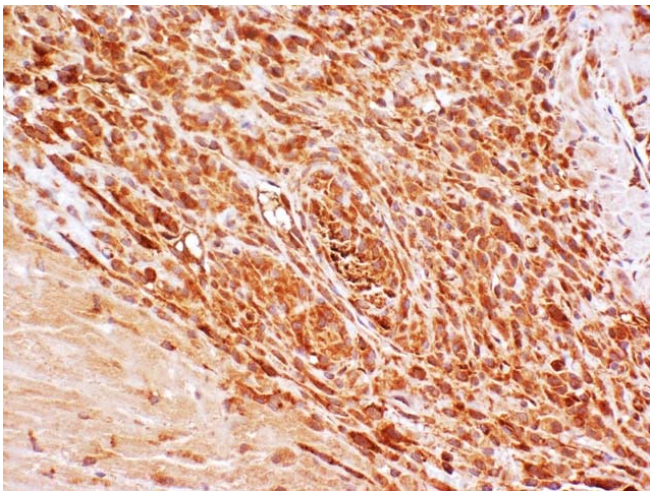
Of interest was the finding that VEGF was up-regulated within the infarcted myocardium in all animals with peak values in rats transfused with irradiated apoptotic PBMC reaching almost three fold higher levels as compared to controls.

To further foster this obtained data the same analysis was implemented for the VEGF receptor FLK-1/KDR. This evaluation showed a comparable pattern of positively labelled cells in the infarcted myocardium as seen for VEGF expression.

FLK-1/KDR


control infarction


viable PBMC



irradiated apoptotic PBMC

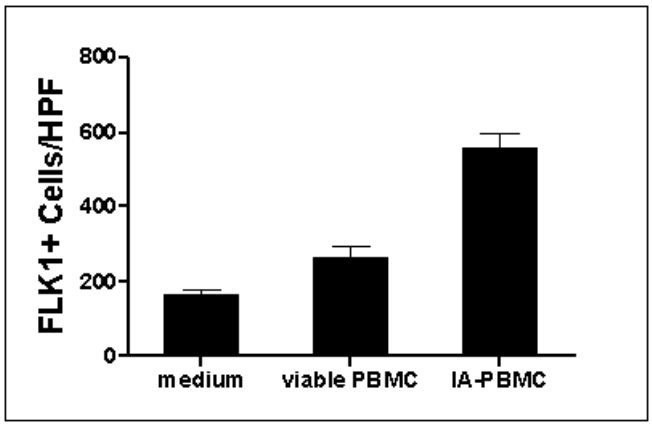
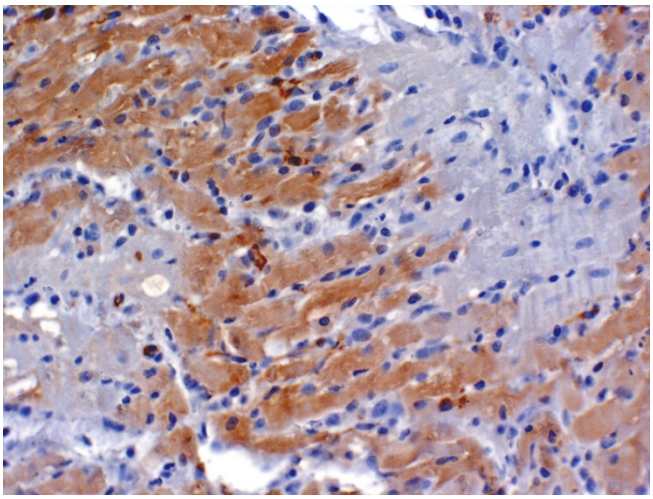


Figure 31

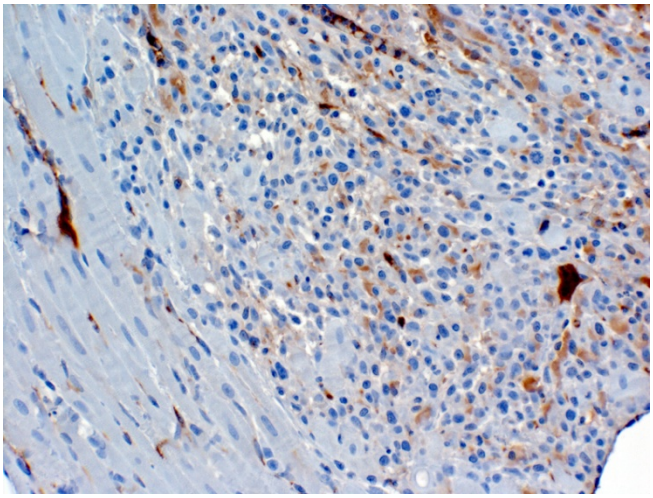
Also specimens analysed for the expression of VEGF receptor evidenced a threefold higher amount of FLK-1/KDR positive cells 72 hours after induction of experimental myocardial infarction.

In the next set of experiments we sought to determine whether the population of infiltrating cells stained positive for cellular markers associated with endothelial progenitor cells and angiogenesis. An immunehistological staining was performed for CD34 and c-kit, both markers usually expressed on the surface of different types of endothelial progenitor cells.

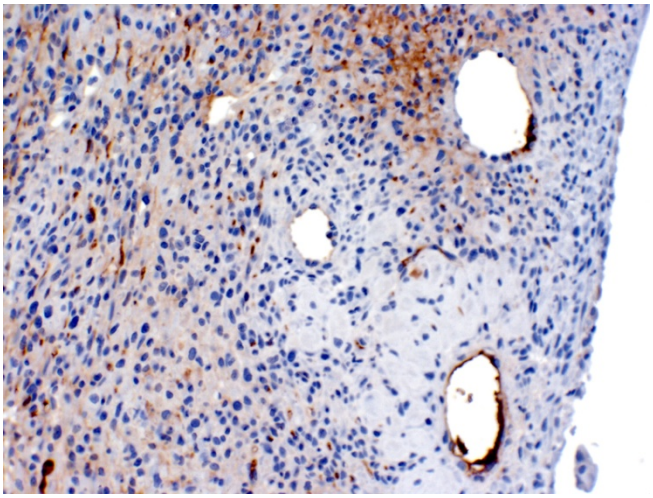
CD34



control infarction



viable PBMC



irradiated apoptotic PBMC

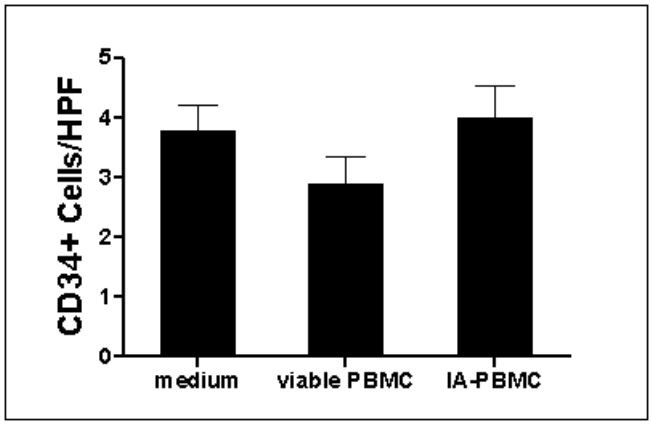
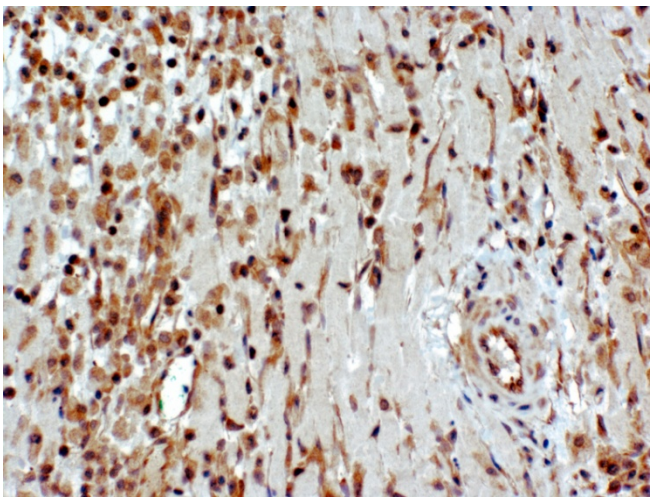
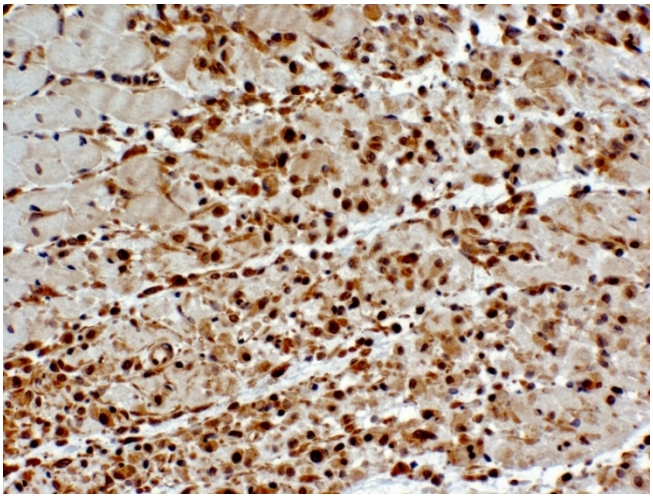
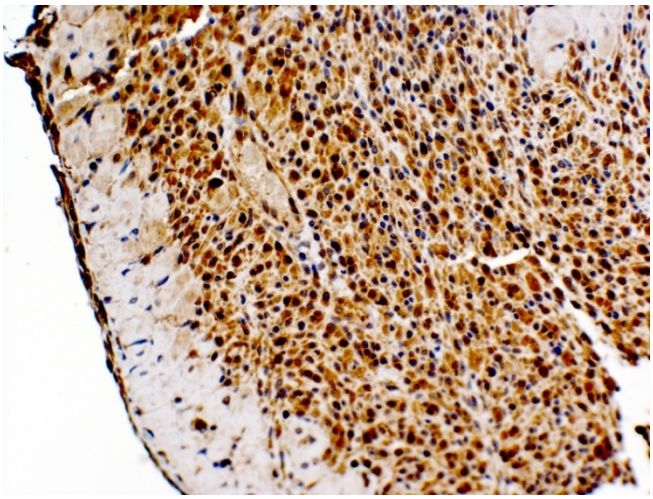


Figure 32

c-kit


control infarction


viable PBMC



irradiated apoptotic PBMC

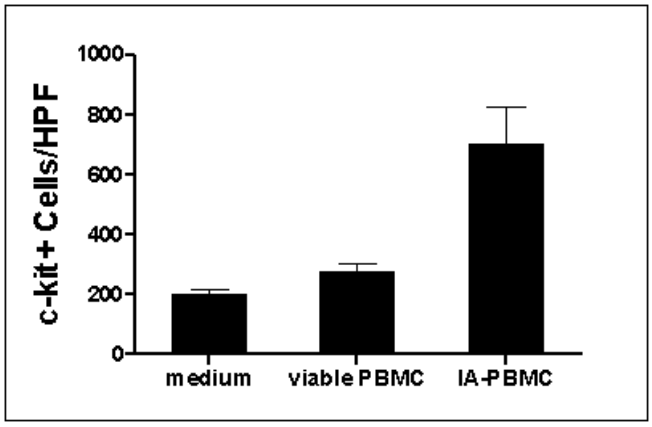
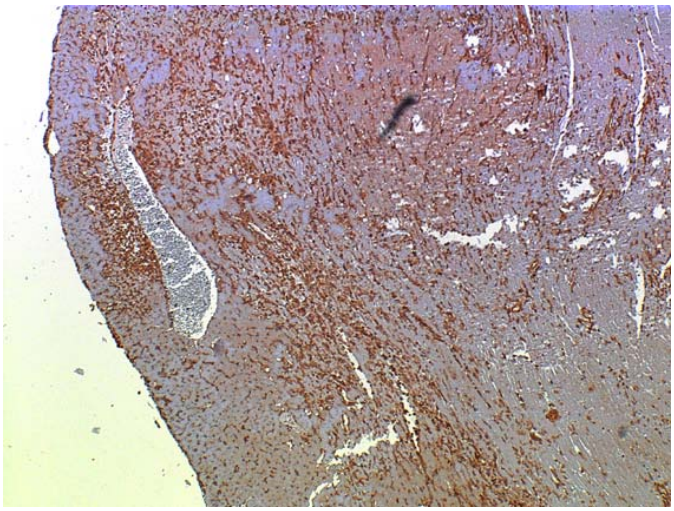


Figure 33

Immunehistological 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 and fewer cells in medium and viable cell receiving animals.

NFκB


control infarction

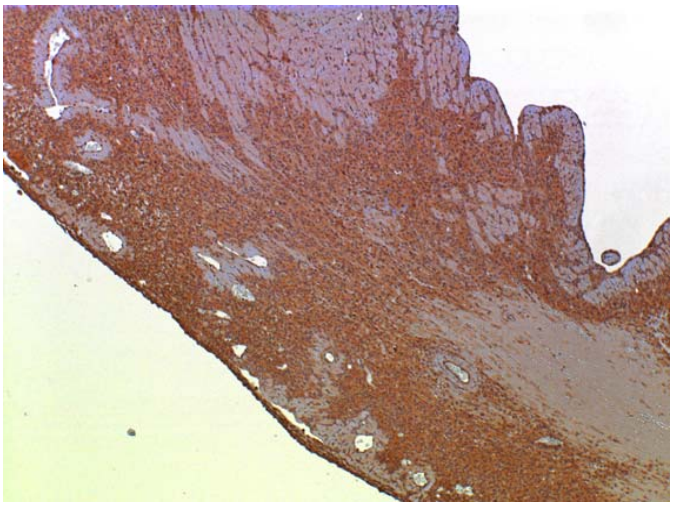
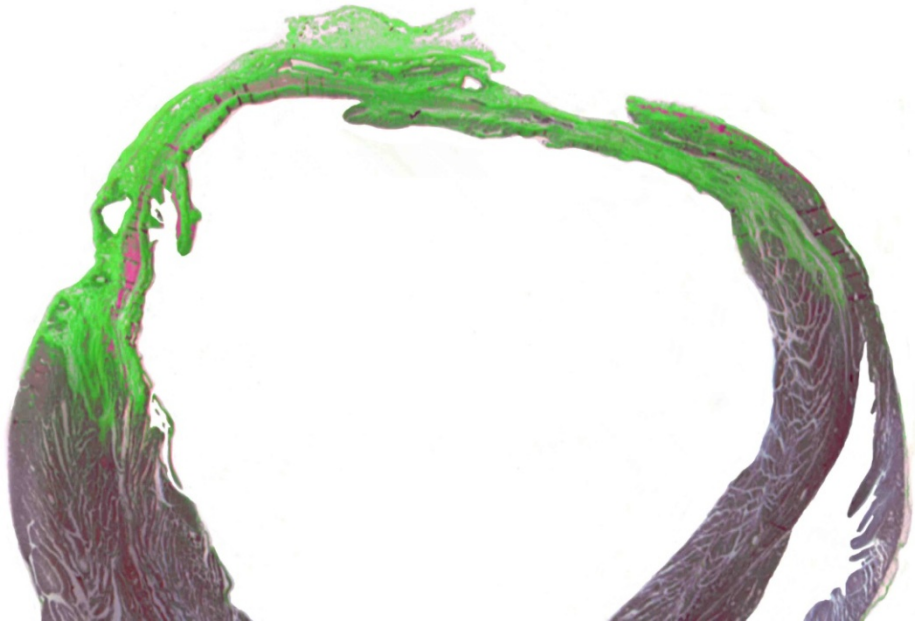

irradiated apoptotic PBMC

Figure 34

Because substantial changes in the infiltration pattern of cells into the infarcted myocardium in rats infused with irradiated apoptotic PBMC were evident after 72 hours, we next sought to evaluate long term responses on ventricular remodelling and cardiac function.

Rats were sacrificed six weeks after experimental myocardial infarction, hearts explanted and processed to histological evaluation. To determine the extent of myocardial scar tissue in relation to viable myocardium a planimetric analysis was conducted. This investigation revealed a mean infarct size of 24,95%±3,58 (SEM) with obvious sign of ventricular dilatation in control animals.

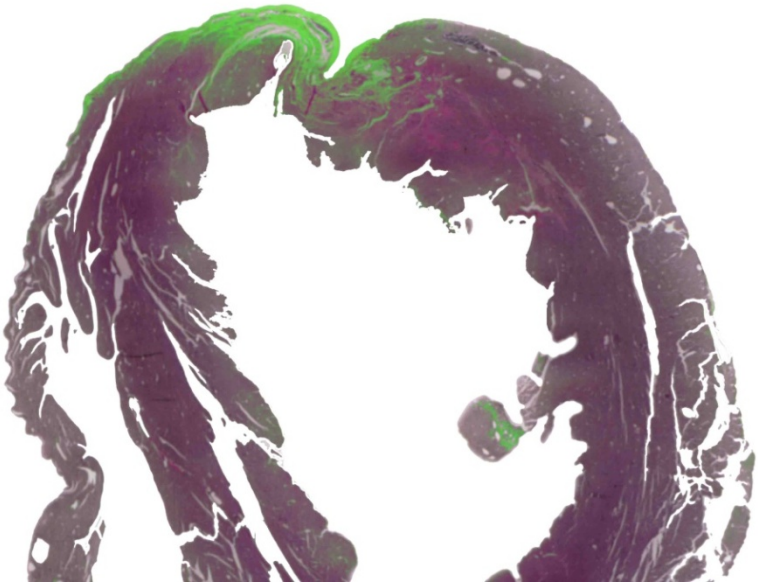


control infarction

Rats transfused with viable PBMC showed fewer signs of dilatation and ventricular remodeling indicated by an infarct area of 14.3% . ± 1.7 (±SEM).

viable PBMC

This improvement of myocardial salvage was further augmented in animals injected with irradiated apoptotic PBMC. The extent of collagenous scar tissue was reduced to 5.81% ±2,02 (±SEM) in this therapy group.



irradiated apoptotic PBMC

Figure 35 shows a statistical analysis of extensions of scarred areas (expressed as percent of the left ventricle) in viable and apoptotic cell injected animals compared to controls.

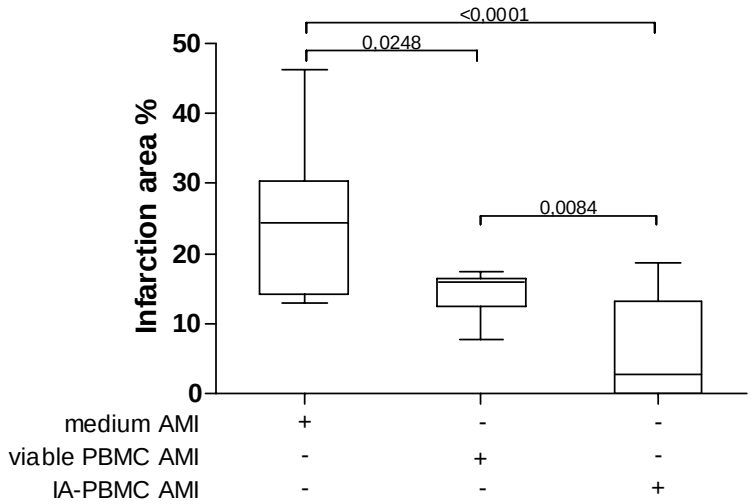


Figure 35

Significant differences were found between viable and apoptotic cells as well as between both treatment groups and controls.

In addition to a massive reduction of left-ventricular remodeling and a macroscopically preserved ventricular geometry echocardiographic analyses were performed six weeks after coronary artery ligation. Assessment of cardiac function was conducted using parameters such as ejection fraction, shortening fraction, end-systolic and end-diastolic diameters and volumes.

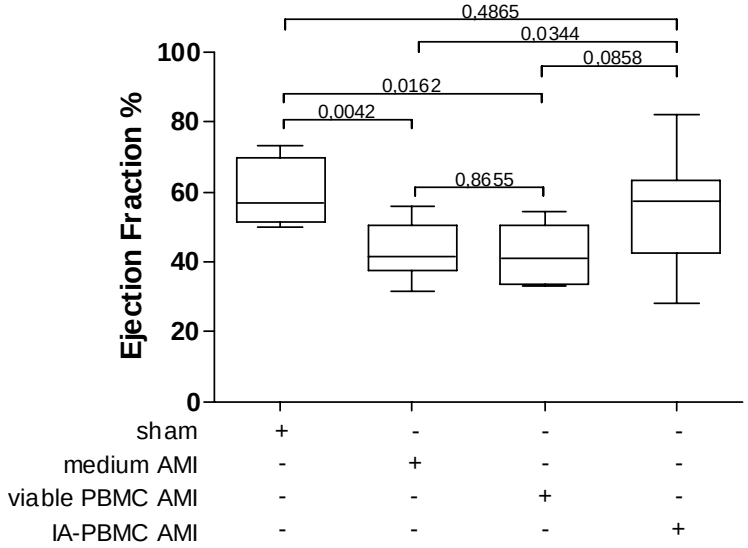


Figure 36

Intravenous application of syngeneic irradiated apoptotic PBMC significantly improved echocardiographic functional parameters as compared with viable PBMC or control animals. Ejection fraction was 60,58%±6,81 (±SEM) in sham operated rats without artery ligation indicating a physiologic cardiac functionality. This value declined to 42,91%±2,14 (±SEM) in control animals six weeks after experimental infarction and to 42,24%±3,28 (±SEM) in animals receiving viable PBMC, whereas rats treated with irradiated apoptotic PBMCs evidenced an EF of 53,46%±4,25 (±SEM).

An analysis performed in order to determine shortening fraction revealed a comparable pattern. Mean values of rats treated with irradiated apoptotic PBMC initially after myocardial infarction were in the same range as sham operated animals although showing a greater variation.

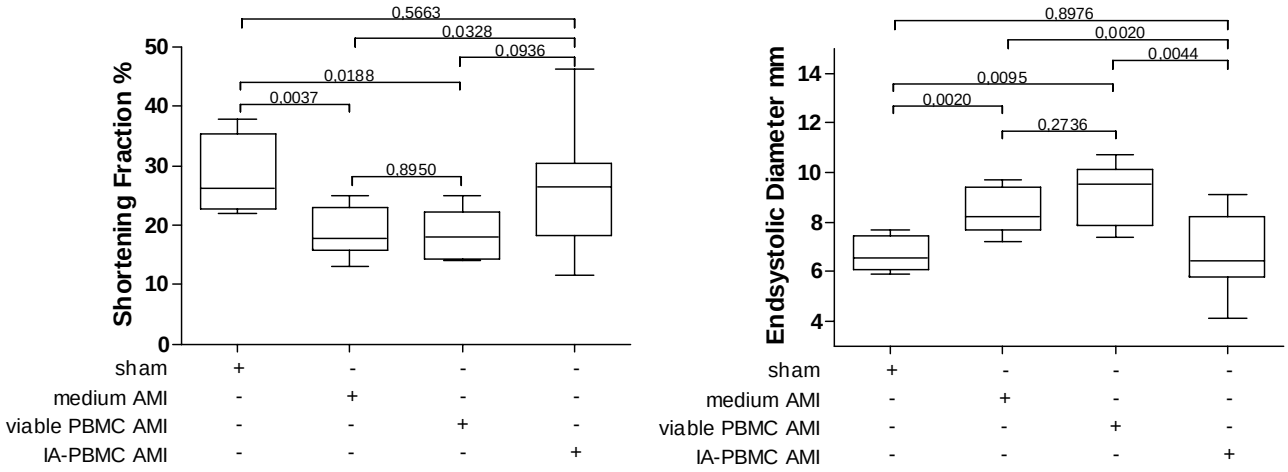


Figure 37

Control animals and viable PBMC treated rats show LVEDD values of 10,43 mm ± 0,21 (±SEM) and 11,03 mm ± 0,40 (±SEM) respectively. Rats transfused with irradiated apoptotic PBMC rats even represent a slightly reduced left-ventricular diastolic diameter of 8,99mm±0,32 compared to 9,47mm±0,64 in sham operated animals. Differences in systolic diameters were less pronounced, but in the same ranking. Left ventricular end-systolic diameter (LVESD) is depicted in Figure 37.

Discussion

In the present study we evaluated *in vitro* and *in vivo* responses to apoptotic cells with special emphasis on the induction of pro-angiogenic and suppression of pro-inflammatory cytokines. Based on this finding we assumed that irradiated apoptotic PBMC induce a state of immunesuppression and secretion of proteins essential for repair mechanisms after myocardial infarction. In order to prove our data obtained in *in vitro* experiments, we infused either viable or apoptotic PBMC initially after acute infarction in a rat model. The obtained results demonstrated that the administration of irradiated cells evoked a massive homing of endothelial progenitor cells into the infarcted myocardium within 72 hours and caused a significant functional recovery within six weeks.

Since inflammation and pro-inflammatory stimuli play an integral part in congestive heart failure after ischemic events[137,138,139], interventions to reduce these processes are necessary to preserve cardiac homeostasis and function. We first sought to investigate the potential of apoptotic cells to interfere with this process of ongoing inflammation. Co-culture of irradiated apoptotic PBMC in immune assays with LPS stimulated mononuclear cells resulted in reduced Interleukin-1β and Interleukin-6 production. This effect was also detectable in an attenuated dendritic cell mixed lymphocyte reaction (MLR).

Both immune parameters were described to have a role in inflammation after myocardial ischemia. In addition we evidenced that viable as well as apoptotic PBMC secrete IL-8, VEGF and MMP9 into the culture medium within a culture period of 24 hours. This effect was even more pronounced in RT-PCR analyses. These proteins and metalloproteinases were described to be responsible for neo-angiogenesis and recruitment of progenitor cells from the bone marrow to the ischemic myocardium. The chemokine IL-8 belongs to the CXC family that consists of small (<10 kDa) polypeptides that bind to and have potent chemotactic activity for endothelial cells[140,141,142]. It has been shown that IL-8 is inevitable for myocardial regeneration and wound healing. Three amino acid residues at the N-terminus (Glu-Leu-Arg, the ELR motif) determine binding of CXC chemokines such as IL-8, Gro-alpha

and other chemokines to CXC receptors 1 and 2 on endothelial cells and are promoting endothelial homing and angiogenesis[143].

In addition MMP9 secretion was identified to be pivotal to mobilize endothelial progenitor cells from the bone marrow into the circulation. Since this matrixproteinase serves as a signal to release soluble kit-ligand (sKitL), a mediator that causes the transition of endothelial and hematopoietic stem cells from the quiescent to proliferative state in the bone marrow, an essential step in the process of tissue restoration. In a further *in vitro* assay we demonstrated that the supernatant (SN) derived from cultured viable and even in a larger extent of irradiated apoptotic PBMC had the ability to enhance mRNA transcription of IL-8 and MMP9 in mesenchymal fibroblasts. These data suggests that cell culture supernatants 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 positive endothelial progenitor cells into the circulation[144].

In order to prove any beneficial effect of this cell suspension *in vivo* we utilized a model of open chest myocardial infarction by coronary artery ligation and infused cultured viable and apoptotic PBMC immediately after onset of ischemia in an experimental rat model. In a 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 of transplanted cells to infarcted myocardium[145,146]. In contrary, it is much more likely that paracrine effects, either by conditioned culture medium alone or by an evoked immune response to this (*ex vivo* cultured) cell suspension exposure is causative for this regenerative effect after myocardial infarction. Since immediate inflammation after acute ischemia determines the road map to ventricular dilatation, we performed histological analysis after 72 hours. Here we proved that rats injected with irradiated apoptotic PBMC evidenced a massive homing of cells positive for markers such as CD68, VEGF, FLK1/KDR and c-kit. This population of infiltrating cells suggest an accelerated clean-up process by heightened monocytes/macrophage levels and also an improved angiogenesis by endothelial progenitor cell homing into the ischemic myocardium within this time period. In addition to these findings, more S100β positive dendritic cells were detected in control animals, indicating a slightly enhanced APC based inflammation in untreated animals.

Our results seen in IA-PBMC treated rats partly foil currently accepted knowledge about the natural course of myocardial infarction. In regards to inflammation: under normal conditions remodeling processes are mediated by cytokines and inflammatory cells in the infarcted myocardium that initiate a wound reparation process that is landmarked by phagocytosis and resorption of the necrotic tissue, hypertrophy of surviving myocytes, angiogenesis and, to a limited extent, progenitor cell proliferation. Any experimental approach so far that intervened into inflammatory response post infarction was shown to be detrimental in AMI models[147]. The administration of glucocorticoids in the treatment of myocardial infarction in order to reduce the post-ischemia inflammatory response showed controversial results. One theory concerning the deleterious effects of steroids in acute myocardial infarction is the phenomenon of delayed or poor healing. This delayed healing appeared largely due to the presence of mummified (dead, but architecturally well preserved) and unphagocytized myocardial cells in the infarct.

When interpreting our histological short term data we argue that transfusion of IA-PBMC cell-medium suspension in AMI results in an advanced transitioning from inflammation to c-kit$^+$ EPC repair phase[148]. In 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[149]. Of note are reports that proved similar repair mechanisms in humans who suffered from myocardial infarction[150]. Recent human data confirm that myocardial infarction reperfusion (MI/R) induces the mobilization of bone marrow progenitor cells. These cells express the c-kit receptor, which has been directly or indirectly implicated in cardiac repair and/or regeneration[108,151]. The requirement for MMP9 in progenitor cell mobilization is also supported by previous work that demonstrated MMP9 to be necessary for the mobilization of c-kit hematopoietic stem cells in response to myeloablative therapy[137]. It was also stated that MMP9, by cleaving membrane KitL to soluble KitL, renders KitL available to c-kit binding on EPCs, as suggested also by recent studies involving haemangiocytes. Fazel et al. also determined the pivotal role c-kit in regenerative responses initiated after myocardial infarction in a mouse knock-out model for c-kit.

The amount of circulating endothelial progenitor cells in wild type and knock-out mice for c-kit were determined and are shown in Figure 38. The release of reparative endothelial progenitor cells after ischemia-reperfusion injury is markedly diminished in knock-out mice for MMP9 and c-kit. In chimeric mice (knock-out mice for c-kit with reconstituted bone marrow from wild-type mice) this response to ischemia was restored.

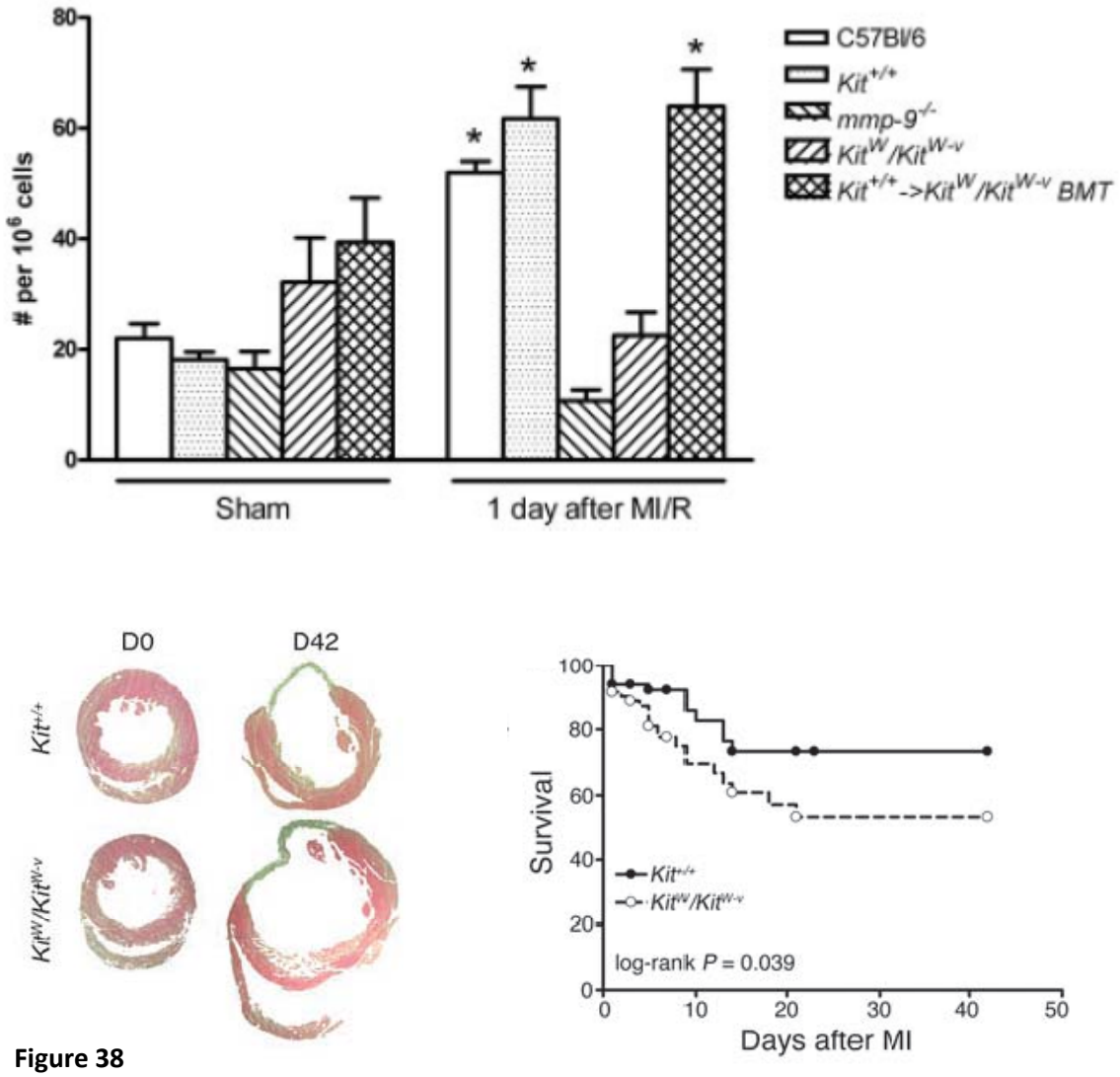


Figure 38

Release of c-kit$^+$ cells after ischemia. H&E stained myocardial section from wild-type and c-kit knock-out mice (KitW/KitW–v) depicting the pronounced ventricular dilatation and larger infarct size in KitW/KitW–v mice. Survival of KitW/KitW–v after myocardial infarction was markedly reduced.

Adapted from Fazel S, Cimini M, Chen L, Li S, Angoulvant D, Fedak P, Verma S, Weisel RD, Keating A, Li RK. Cardioprotective c-kit$^+$ cells are from the bone marrow and regulate the myocardial balance of angiogenic cytokines. J Clin Invest. 2006 Jul;116(7):1865-77.

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 ischemic heart disease. Understanding the molecular mechanisms that include progenitor cell mobilization in response to cardiac ischemia 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[152]. Based on these presented 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.

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Abbreviations

LAD left anterior decending artery

PBMC peripheral blood mononuclear cells

DC dendritic cell

LPS Lipopolysaccharide

PCR polymerase chain reaction

RT-PCR reverse transcriptase polymerase chain reaction

AMI acute myocardial infarction

ECG electrocardiography

CK-MB creatine kinase muscle brain

ACS acute coronary syndrome

NSTEMI non-ST elevation myocardial infarction

QwMI Q wave myocardial infarction

NQMI non-Q wave myocardial infarction

PTCA percutaneous transluminar coronary angioplasty

VEGF Vascular endothelial growth factor

G-CSF Granulocyte colony stimulation factor

EPC Endothelial Progenitor cells

HSC hematopoetic stem cells

MSC mesenchymal stem cells

RCSC resident cardiac stem cell

ESC Embryonic stem cells

LVEF left ventricular ejection fraction

BMC bone marrow cell

PCI percutaneous coronary intervention

HSP heat-shock proteins

PS-R phosphatidylserine receptor

TGF-ß transforming growth factor beta

ELISA Enzyme-linked immunosorbent assay

HGF hepatocyte growth factor

ASC adult stem cells

PBL peripheral blood leukocytes

TUNEL TdT-mediated dUTP nick end-labelling

SN supernatant

FGF fibroblast growth factor

EDTA ethylenediaminetetraacetic acid

HBBS Hanks balanced salt solution

FITC/PI fluorescein/propidium iodide

GM-CSF granulocyte macrophage colony stimulating factor

DMEM Dulbecco's modified Eagle medium

GRO-alpha Growth-related Oncogene alpha

ENA-78 Epithelial cell-derived neutrophil-activating peptide-78

MMP9 matrixmetalloproteinase 9

CFSE carboxyfluorescein diacetate succinimidyl ester

FCS fetal calf serum

H&E hematoxylin-eosin

cab chromotrop aniline blue

evg elastic van Gieson

FS fractional shortening

SEM standard error of the mean

LVEDD left ventricular end-diastolic diameter

MLR mixed lymphocyte reaction

sKitL kit-ligand

MI/R myocardial infarction reperfusion

Appendix

MEDIZINISCHE UNIVERSITÄT WIEN

Applied Immunology
Cardio - Thoracic Research in Surgery

Irradiated cultured apoptotic peripheral blood mononuclear cells regenerate infarcted myocardium

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Introduction

Acute myocardial infarction (AMI) followed by cardiac remodeling is a major cause of congestive heart failure and death. Of clinical relevance are reports demonstrating that infusion of apoptotic cells lead to allogeneic hematopoietic cell engraftment in transplantation models. The mechanism behind this observation responsible for this effect was related to the immunesuppressive nature of apoptotic cells[1,2]. Here we show that culture of irradiated apoptotic peripheral blood mononuclear cells (IA-PBMC) causes decrease of immune function and augments secretion and mRNA transcription of proteins related to angiogenesis and stem cell mobilization (e.g. Interleukin-8) *in vitro*. We therefore infused cultured IA-PBMC in an acute rat AMI model and demonstrated c-kit dependent regeneration of infarcted myocardium[3].

Material and Methods

Immunomodulatory function of irradiated apoptotic peripheral blood mononuclear cells (IA-PBMC) was evaluated by mixed-lymphocyte reaction and co-culture assays using LPS stimulated cells *in vitro*. RT-PCR and ELISA were utilized to quantify pro-angiogenic Interleukin-8 (IL-8/CXCL8), Vascular endothelial growth factor (VEGF)and Matrix metalloproteinase-9 (MMP-9) in cell culture supernatants obtained from viable and apoptotic PBMC. Cell suspensions of viable and irradiated apoptotic PBMC were administered after AMI (rat LAD ligation model). Immunehistological analysis was performed to detect inflammatory and pro-angiogenic cells within 72 hours after AMI. Cardiac function was analyzed by echocardiography and determination of infarction size was conducted by planimetry after six weeks.

Results

Irradiated apoptotic PBMC attenuated immune reactivity and evidenced secretion of pro-angiogenic Interleukin-8 and Matrix metalloproteinase-9 *in vitro*. AMI rats that were infused with irradiated apoptotic PBMC cell suspensions showed enhanced homing of endothelial progenitor cells within 72 hours as compared to controls (medium alone, viable PBMC). Echocardiography and planimetric analysis showed a significant reduction of infarction size and improvement of post AMI remodeling as evidenced by an attenuated loss of ejection fraction ($p<0.05$ and $p<0.001$).

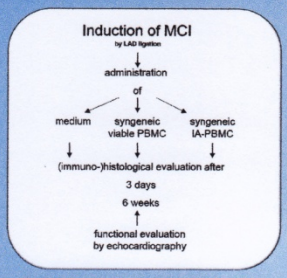

Figure 1 Flow chart of the study design depicts the study protocol and the time points of evaluation of cardiac function and integrity by echocardiography, histology and immunehistology.

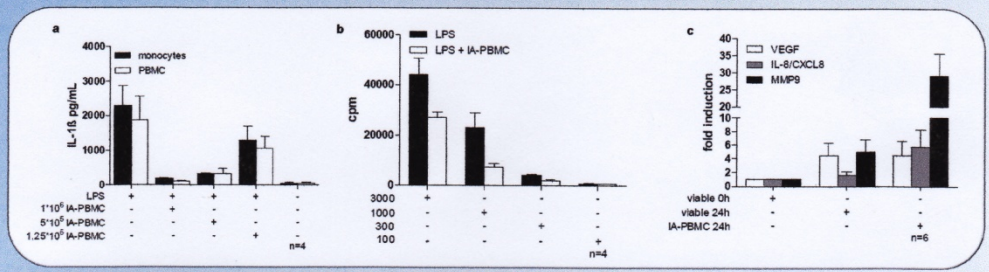

Figure 2 Co-incubation of LPS stimulated PBMC or monocytes with irradiated apoptotic autologous PBMC demonstrates a reduced secretion of the pro-inflammatory cytokine Interleukin-1β in a dose dependent manner (a). Addition of autologous IA-PBMC in mixed lymphocyte reactions after LPS stimulation decreases T-cell proliferation as measured by counts per minute (cpm) (b). RT-PCR RNA expression analysis of Vascular endothelial growth factor (VEGF), Interleukin-8 (IL-8/CXCL8) and Matrixmetalloproteinase-9 (MMP9) transcripts shows an up-regulation of IL-8/CXCL8 and especially MMP9 in irradiated PBMC after a culture period of 24 hours (c).

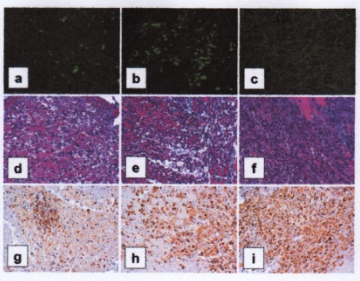

Figure 3 CFSE labeled PBMC administered via the tail vein in rats after myocardial infarction were predominantly found in the spleen (b), to a lesser extent in the liver (a) and no cells in the infarcted heart (c). Histological analysis (HE, immunehistological staining for CD68) shows dense cellular infiltrations in IA-PBMC infused rats (f,i) in comparison to medium or viable PBMC treated animals (d,e and g,h, respectively).

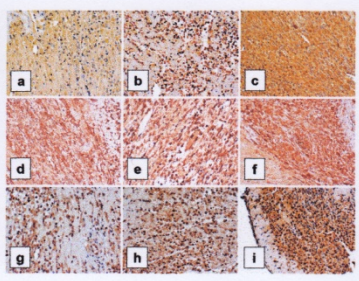

Figure 4 Higher amounts of cells staining positive for VEGF were detected in infarcted myocardial tissue obtained from animals injected with IA-PCMC (c), in comparison with medium (a) or viable cell treatment (b). A similar expression pattern was found for VEGF receptor KDR/FLK1 (d,e,f). Immunehistological 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 (i) and fewer cells in medium (g) and viable cell receiving animals (h).

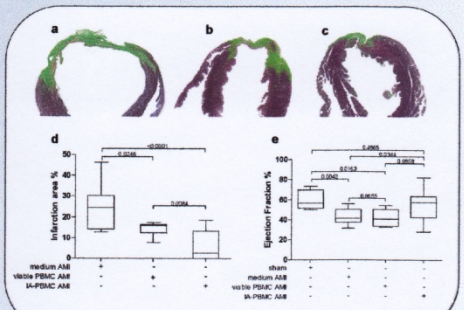

Figure 5 Rat hearts explanted 6 weeks after induction of myocardial infarction (a,b,c). Hearts from medium injected animals (a) appear more dilated and show a greater extension of fibrotic tissue, scar extension was reduced in viable cell injected rats (b) with fewer signs of dilatation. The least amount of scar tissue formation was detected in IA-PBMC injected animals (c). Area of scar extension was 24.95% in medium, 14.3% in viable PBMC and 5.8% in IA-PBMC injected animals (d). Ejection fraction analyzed by echocardiography evidences a better recovery after myocardial infarction in animals injected with IA-PBMC (e).

Discussion

Our findings demonstrate that cultured IA-PBMC suspensions induce immunesuppression and up-regulation of pro-angiogenic markers. These data indicate that infusion of apoptotic PBMC in AMI circumvents inflammation, causes homing of regenerative EPC and preserves cardiac function.

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[2] Bittencourt MC, Perruche S, Contassot E, Fresnay S, Baron MH, Angonin R et al. **Intravenous injection of apoptotic leukocytes enhances bone marrow engraftment across major histocompatibility barriers.** Blood 2001;98:224-30.
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Curriculum Vitae

Michael Lichtenauer

PERSONAL BACKGROUND

Nationality: Austrian
Family Status: Single
Date of Birth: Nov. 16th, 1984
Parents: Heinz and Ingrid Lichtenauer

EDUCATION

2007/02 – Present	Student Research Fellow at the Department of Cardio-Thoracic Surgery, General Hospital Vienna, Medical University of Vienna, Austria
2004/10 – Present	Medical Student at the Medical University of Vienna, Austria
2003/10 – 2004/09	Social Service at the Police Department of Vienna's 19th district
2003/06	Matura (High School Graduation) with Distinction

CLINICAL TRAINING

2009/08	Clinical Clerkship at the Department of Pathology, Landesklinikum Thermenregion Mödling
2009/04	Clinical Clerkship at the Department of Anesthesiology and Intensive Care, Genaral Hospital, Vienna
2009/03	Clinical Clerkship at the Department of Gastroenterology, Hanuschkrankenhaus, Vienna
2009/03	Clinical Clerkship at the Department of Rheumatology, Hanuschkrankenhaus, Vienna
2009/03	Clinical Clerkship at the Department of Hematology, Hanuschkrankenhaus, Vienna

2009/02	Clinical Clerkship at the Department of Cardiology, Hanuschkrankenhaus, Vienna
2008/10	Clinical Clerkship at the Department of Trauma Surgery, Hanuschkrankenhaus, Vienna
2008/10	Clinical Clerkship at the Department of Surgery, Hanuschkrankenhaus, Vienna
2008/08	Clinical Clerkship at the Department of Cardiology, General Hospital, Vienna
2007/08	Clinical Clerkship at the Department of Trauma Surgery, Hanuschkrankenhaus, Vienna
2007/07	Clinical Clerkship at the Department of Cardiology, Hanuschkrankenhaus, Vienna
2006/08	Clinical Clerkship at the Department of Trauma Surgery, LKH Bad Ischl
2006/02	Clinical Clerkship at the Department of Surgery, Evangelisches Krankenhaus, Vienna

CONGRESSES AND MEETINGS

2009/10	Jahrestagung der Österreichischen Gesellschaft für Transplantation, Transfusion und Genetik, Austrotransplant, Seefeld in Tirol
2009/09	40. Jahrestagung der Österreichischen Gesellschaft für Innere Medizin, Wien
2009/09	Hanseatische Klinikkonferenz Pneumologie, Hamburg
2009/09	Kardiovaskuläre Medizin, Hamburg
2009/06	50. Österreichischer Chirurgenkongress, Wien
2009/06	Österreichischer Kardiologenkongress, Salzburg
2009/04	Frühjahrstagung der Österreichischen Gesellschaft für Hämatologie und Onkologie, Salzburg
2009/03	Kardiologie 2009, Innsbruck
2009/02	53rd Annual Meeting of the Society of Thrombosis and Haemostasis Research, Vienna

2008/11	1st EACTS Meeting on Cardiac and Pulmonary Regeneration, Berne, Switzerland
2008/09	39. Jahrestagung der Österreichischen Gesellschaft für Innere Medizin, Graz
2008/05	Österreichischer Kardiologenkongress, Salzburg
2008/05	49. Österreichischer Chirurgenkongress, Innsbruck
2008/02	EAACI-Ga^{2}len Davos Meeting, Pichl/Schladming
2007/10	Jahrestagung der Österreichischen Gesellschaft für Transplantation, Transfusion und Genetik, Austrotransplant, St. Wolfgang

PUBLICATIONS

10 Peer reviewed papers (1 first authorship, 9 co-authorships)
1 Case report

Articles:

Hoetzenecker K, Adlbrecht C, **Lichtenauer M**, Hacker S, Hoetzenecker W, Mangold A, Moser B, Lang G, Lang IM, Muehlbacher F, Klepetko W, Ankersmit HJ.
Levels of sCD40, sCD40L, TNFα and TNF-RI in the Culprit Coronary Artery During Myocardial Infarction.
Labmedicine 2009 (accepted for publication)

Ankersmit HJ, Hoetzenecker K, Dietl W, Soleiman A, Horvat R, Wolfsberger M, Gerner C, Hacker S, Mildner M, Moser B, **Lichtenauer M**, Podesser BK.
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Elevated HSP27, HSP70 and HSP90 alpha in chronic obstructive pulmonary disease: markers for immune activation and tissue destruction.
Clin Lab. 2009;55(1-2):31-40.

Mangold A, Szerafin T, Hoetzenecker K, Hacker S, **Lichtenauer M**, Niederpold T, Nickl S, Dworschak M, Blumer R, Auer J, Ankersmit HJ.
Alpha-Gal specific IgG immune response after implantation of bioprostheses.
Thorac Cardiovasc Surg. 2009 Jun;57(4):191-5.

Lichtenauer M, Nickl S, Hoetzenecker K, Mangold A, Moser B, Zimmermann M, Hacker S, Niederpold T, Mitterbauer A, Ankersmit HJ.
Phosphate buffered saline containing calcium and magnesium elicits increased secretion of Interleukin-1 receptor antagonist.
Labmedicine 2009 May;40(5):290-3

Lambers C, Hacker S, Posch M, Hoetzenecker K, Pollreisz A, **Lichtenauer M**, Klepetko W, Ankersmit HJ.
T cell senescence and contraction of T cell repertoire diversity in patients with chronic obstructive pulmonary disease.
Clin Exp Immunol. 2009 Mar;155(3):466-75.

Szerafin T, Niederpold T, Mangold A, Hoetzenecker K, Hacker S, Roth G, **Lichtenauer M**, Moser B, Dworschak M, Ankersmit HJ.
Secretion of Soluble ST2 – Possible Explanation for Systemic Immunosuppression after Heart Surgery.
Thorac Cardiovasc Surg. 2009 Feb;57(1):25-9.

Hoetzenecker K, Ankersmit HJ, Aigner C, **Lichtenauer M**, Kreuzer S, Hacker S, Hoetzenecker W, Lang G, Klepetko W.
Consequences of a wait-and-see Strategy for Benign Metastasizing Leiomyomatosis of the Lung.
Ann Thorac Surg. 2009 Feb;87(2):613-4.

Soleiman A, Lukschal A, Hacker S, Aumayr K, Hoetzenecker K, **Lichtenauer M**, Untersmeier E, Horvat R, Ankersmit HJ.
An Early Marker of Apoptosis, Caspase-cleaved Cytokeratin-18, is Deposited in Myocardial Lipofuscin.
Eur J Clin Invest. 2008 Oct;38(10):708-12

Hetz H, Hoetzenecker K, Hacker S, Faybik P, Pollreisz A, Moser B, Roth G,Hoetzenecker W, **Lichtenauer M**, Klinger M, Krenn CG, Ankersmit HJ.
Caspase-cleaved cytokeratin 18 and 20 S proteasome in liver degeneration.
J Clin Lab Anal. 2007;21(5):277-81.

Abstracts:

Lichtenauer M, Hoetzenecker K, Dietl W, Soleiman A, Wolfsberger M, Gerner C, Hacker S, Mildner M, Podesser BK, Ankersmit HJ.
Irradiated cultured apoptotic peripheral blood mononuclear cells regenerate infarcted myocardium.
Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Seefeld in Tirol 2009/10
published in Abstractbook

Nickl S, Hoetzenecker K, Mangold A, Moser B, Zimmermann M, Hacker S, Niederpold T, Mitterbauer A, Ankersmit HJ, **Lichtenauer M**.
Heightened extracellular levels of calcium and magnesium induce secretion of chemokines and anti-inflammatory cytokines.
Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Seefeld in Tirol 2009/10
published in Abstractbook

Lichtenauer M, Hoetzenecker K, Dietl W, Soleiman A, Wolfsberger M, Gerner C, Hacker S, Mildner M, Podesser BK, Ankersmit HJ.
Irradiated cultured apoptotic peripheral blood mononuclear cells regenerate infarcted myocardium.
Annual Meeting of the Austrian Society of Internal Medicine, Vienna 2009/09
published in Abstractbook

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Annual Meeting of the Austrian Society of Internal Medicine, Vienna 2009/09
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Hacker S, Lambers C, Hoetzenecker K, Pollreisz A, Aigner C, Mangold A, **Lichtenauer M**, Nickl S, Klepetko W, and Ankersmit HJ.
Elevated HSP27, HSP70 and HSP90α in COPD: Markers for immune activation and tissue destruction.
Annual Meeting of the Austrian Society of Pneumology, Vienna 2008/09
published in Abstractbook

Lichtenauer M, Mangold A, Hoetzenecker K, Hacker S, Pollreisz A, Wliszczak T, Wolner E, Klepetko W, Gollackner B, Szerafin T, Auer J, Ankersmit HJ.
Alpha-Gal Specific Humoral Immune Response after Implantation of Bioprostheses in Cardiac Surgery.
6th EAACI-GA^{2}LEN Davos Meeting, Pichl, Austria. 2008/02
published in Abstractbook

Mangold A, Hoetzenecker K, Hacker S, Pollreisz A, Wliszczak T, **Lichtenauer M**, Wolner E, Klepetko W, Gollackner B, Szerafin T, Auer J, Ankersmit HJ.
Alpha-Gal Specific Humoral Immune Response after Implantation of Bioprostheses in Cardiac Surgery.
Annual Meeting of the Austrian Society of Allergology and Immunology, Alpbach, Austria. 2007/12
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Hacker S, Soleiman A, Hoetzenecker K, Lukschal A, Pollreisz A, Mangold A, Wliszczak T, **Lichtenauer M**, Horvat R, Muehlbacher F, Wolner E, Klepetko W, Ankersmit HJ.
Degenerative Cardiac Pigment Lipofuscin Contains Cytokeratin-18 and Caspase-cleaved Cytokeratin-18.
Annual Meeting of the Austrian Society of Allergology and Immunology, Alpbach, Austria. 2007/12
published in Abstractbook

Oral Presentations:

2009/10 Jahrestagung der Österreichischen Gesellschaft für Transplantation, Transfusion und Genetik, Austrotransplant, Seefeld in Tirol –
Irradiated cultured a poptotic peripheral blood mononuclear cells regenerate infarcted myocardium

Poster Presentations:

2009/09 40. Jahrestagung der Österreichischen Gesellschaft für Innere Medizin –
Irradiated cultured a poptotic peripheral blood mononuclear cells regenerate infarcted myocardium

2008/02 EAACI-Ga^{2}len Davos Meeting –
Alpha-Gal on Bioprostheses in Cardiac Surgery

MEMBERSHIPS

2008/10 Austrian Society of Internal Medicine (ÖGIM)

2008/02 EAACI - European Academy of Allergology and Clinical Immunology

2007/08 Austrian Society of Transplantation, Transfusion and Genetics

AWARDS AND GRANTS

2008/12 Förderungsstipendium/Research Scholarship – Medical University Vienna

2003/06 Matura (High School Graduation) with Distinction

TEACHING ACTIVITY

2007/02 — Teaching Assistent at the Department of Cardio-Thoracic Surgery, General Hospital Vienna, Medical University Vienna, Univ. Doz. Dr. Hendrik Jan Ankersmit

METHODS

Cultivation of human and animal cell lines
ELISA
Histology and Immunehistology
Flow Cytometry

LANGUAGE SKILLS

Native German Speaker
Proficient in English
Knowledge of French and Italian

REFERENCES

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