“The Ligands of CXCR4 in Vascularization”

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ABBREVIATIONS

Ab	Antibody
acLDL	Acetylated LDL
AMPK	Adenosine monophosphate-activated protein kinase
Apoe	Apolipoprotein E
ATP	Adenosine-5'-triphosphate
bFGF	Basic fibroblast growth factor
BMDM	Bone marrow - derived monocytes / macrophages
BMM	see BMDM
BSA	Bovine serum albumin
CAD	Coronary artery disease
Calcein-AM	Calcein acetoxymethyl ester
CCL2	Chemokine (C-C motif) ligand 2, see MCP-1
CCL3	MIP-1α
CCL4	MIP-1β
CCL5	Chemokine (C-C motif) ligand 5, see RANTES
CCR2	Chemokine (C-C motif) receptor 2, a receptor for MCP-1
CD	Cluster of diffentation – to identify cell surface molecules present on leukocytes
CD31	see PECAM
CD54	see ICAM
CD74	Invariant chain of class II histocompatibility antigens
CD106	see VCAM
CD184	see CXCR4
cDNA	Copy DNA
CVD	Cardiovascular disease
CXCR	Receptor for CXC chemokines
CXCR4	Receptor for CXCL12 and MIF
CXCR2	Receptor for CXC chemokines e.g. MIF and CXCL1
CXCL1	see KC
CXCL12	see SDF-1α
Cy3, Cy7	Cyanine, used as fluorescent dyes
DHE	Dihydroethidium

DAPI	4',6-Diamidino-2-phenylindol
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
dPdt	Derivative of pressure increase and decay
ECs	Endothelial cells
ECFCs	Endothelial colony-forming cells
ECM	Extracellular matrix
ecNOS	Endothelial cell nitric oxide synthase
EDTA	Ethylenediaminetetraacetic acid
EPCs	Endothelial progenitor cells
EOCs	Endothelial outgrowth cells
EO-EPCs	Early outgrowth EPCs
FACS	Fluorescent-activated cell sorting
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FIZZ-1	Found in inflammatory zone, cysteine-rich secreted protein
Flk-1	Fetal liver kinase 1, also known as KDR
GAGs	Glycosaminoglycans
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTP	Guanosine 5'-triphosphate
h	Hours
HBSS	Hanks' balanced salt solution
HH-buffer	Henderson-Hasselbalch buffer
HIF	Hypoxia-inducible transcription factor
ICAM	Inter-cellular adhesion molecule, also known as CD54, expressed on endothelial cells
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal

i.v.	Intravenous
KC	Keratinocyte-derived chemokine, also named CXCL1
KDR	Kinase insert domain receptor, also known as vascular endothelial growth factor receptor 2
LAD	Left anterior descending artery
LCM	L292 cell conditioned medium
LDL	Low density lipoprotein
LFA-1	Lymphocyte function-associated antigen 1, an integrin expressed on all T-cells, B-cells, macrophages and neutrophils mediating the recruitment to the site of infection
LPS	Lipopolysaccharide
LV	Left ventricle
LVDP	Left ventricular developed pressure
Ly6	Lymphocyte antigen 6 complex
mAb	Monoclonal antibody
Mac1	Macrophage-1 antigen, an integrin consisting of CD11b and CD18
Mac2	Macrophage-2 antigen, used for macrophage staining
MCP-1	Monocyte chemoattractant protein-1, also named CCL2, important mediator of monocyte recruitment to inflammatory sites
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MI	Myocardial infarction
MIF	Macrophage migration inhibitory factor
min	Minute
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase (that degrade components of the extracellular matrix)
NO	Nitric oxide, known as nitrogen monoxide
O_2	Molecular oxygen
oxLDL	Oxidized low density lipoprotein
pAb	Polyclonal antibody
PAOD	Peripheral artery occlusive disease
PAD	Peripheral artery disease

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PECAM	Platelet/endothelial cell adhesion molecule 1, also named CD31
PerCP	Peridinin – chlorophyll - protein complex
PFA	Paraformaldehyde
PGE_2	Prostaglandin E_2
PIGF	Placental growth factor, abbr. also PGF
PS	Phosphatidylserine
RANTES	Regulated on activation normal T cell expressed and secreted
rm	Recombinant murine
RNA	Ribonucleotide acid
SDF	Stromal cell-derived factor
SEM	Standard error of the mean
SMA	Smooth muscle actin
SMCs	Smooth muscle cells
TG	Trigyceride
TGF	Tumor growth factor
TNF	Tumor necrosis factor
TSST-1	Toxic shock syndrome toxin - 1
TTC	Triphenyl tetrazolium chloride
VCAM	Vascular cell adhesion molecule, also known as CD106, expressed on endothelial cells
VE-cadherin	Vascular endothelial cadherin – adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
wt	Wild-type
YM1	Also called T-lymphocyte-derived eosinophil chemotactic factor (ECF-L), marker of alternatively activated M2 macrophages

I INTRODUCTION

I.1 Cardiovascular Diseases, Myocardial Infarction, and Peripheral Artery Disease

Cardiovascular diseases (CVDs) comprise a class of disorders of the heart and blood vessels including coronary artery- (CAD), cerebrovascular and peripheral arterial disease (PAD). CVDs represent the number one cause of death and morbidity globally. In 2004, 17.1 million people died from CVDs, which represents 29% of deaths worldwide, with approximately 7.2 million related to CAD and 5.7 million to stroke. It is estimated that 27 million people in Western Europe and North America, 10 million in the United States alone, suffer from PAD (Belch et al., 2003; Niiyama et al., 2009). The incidence of CVD-related mortality is growing rapidly due to an aging population, smoking, hypertension, high body mass index and rising prevalence of diabetes mellitus (Badheka et al., 2011; Dick et al., 2007). By 2030, it is expected that this number will have increased to a total of 23.6 million people (WHO, 2011).

CVD is a manifestation of systemic atherosclerosis and CVD patients are often at high risk of developing ischemic distress (i.e. intermittent claudication and angina) stroke and cardiac death (Hirsch et al., 2006). The main cause of CVD is the narrowing of the blood vessel lumen by clogged fatty deposits (atherosclerotic plaque) and calcification of the vessel wall. These atherosclerotic and hardened arteries have lower conductance, and resulting reduced blood flow that will eventually lead to hypoxic tissue and subsequently, chronic pain and even stroke and limb amputation (Comerota, 2001).

Treatment of CVDs is limited and often necessitates palliative interventions to relief malperfusion of the affected tissues and organs with for instance, medication, coronary artery bypass grafting or balloon angioplasty and stenting in CAD patients (Heil and Schaper, 2007).

I.2 Angiogenesis and Arteriogenesis, Two Forms of Vascularization

The formation of a functional and integrated vascular network is a basic process in the growth and maintenance of tissue. Vascularization encompasses three major forms of blood vessel growth: vasculogenesis, angiogenesis and arteriogenesis.
Vasculogenesis, *de novo* formation of vascular structures from mesenchymal angioblasts, is the earliest morphogenetic process of vascular development and takes place during early embryonic stages (Buschmann and Schaper, 1999; Risau and Flamme, 1995). Postnatal vasculogenesis mediated by endothelial progenitor cells (EPCs) derived from the bone marrow may occur as well (Asahara et al., 1999) (Simons and Ware, 2003), however the association of this process with adult neovascularization remains controversial.

After birth, blood vessel development can be observed in two distinct mechanisms referred to as angiogenesis and arteriogenesis. Angiogenesis is the sprouting and tube formation from pre-existing blood vessels, but also from newly recruited EPCs, resulting in a higher capillary density. Arteriogenesis is the rapid proliferation of collateral arteries from pre-existing arterioles and is the only form of blood vessel growth process that can compensate for flow deficiencies caused by arterial occlusion.

I.2.1 Angiogenesis

Angiogenesis is the growth of blood vessels from pre-existing vasculature. It occurs throughout life and is an important component of different physiological and pathological conditions such as wound healing, fracture repair, and pregnancy (Conway et al., 2001). It is essential that angiogenesis is tightly regulated, combining angiogenic and angiostatic stimuli. However, if not properly controlled, it can lead to tumor growth and metastasis, rheumatic arthritis, and retinopathies (Buschmann and Schaper, 1999). The therapeutic value of angiogenesis has become a factor of great interest. Inhibiting or decreasing angiogenesis possess therapeutic potential in treating cancer and rheumatic arthritis, while stimulation of angiogenesis can be therapeutic in ischemic heart disease, peripheral arterial disease, and wound healing by increasing reperfusion of the tissue.

Mechanisms of Angiogenesis

The cardiovascular system is the first organ system that develops in the embryo (Risau, 1997). The endothelial cell develops during embryonic development, from the pluripotent, mesodermal stem cell-derived hemangioblast. These precursors give rise to angioblasts and hematopoietic stem cells. While hematopoietic stem cells eventually differentiate into all cell types found in the circulating blood, angioblasts have the potential to differentiate into proliferative endothelial cells that will participate in vasculogenesis and thereafter in angiogenesis (Schmidt et al., 2007).

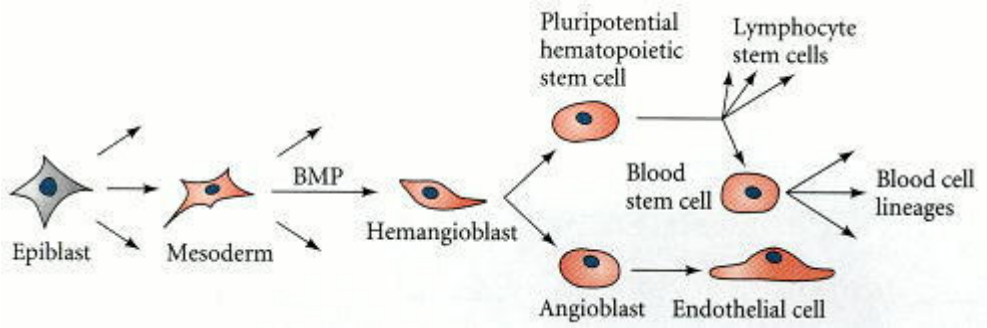

Figure 1: **Origin of the hemangioblast cells.** During embryonic development, hemangioblasts are derived from mesodermal stem cells. They give rise to pluripotential hematopoietic stem cells that generate the blood cells, lymphocytes and angioblasts, which are the precursors of vascular endothelial cells. Figure taken from Gilbert et al. (Gilbert, 2000).

Two types of angiogenesis are known to occur both in utero and in adults: sprouting angiogenesis and intussusceptive angiogenesis (Burri and Tarek, 1990). Sprouting angiogenesis involves the growth of sprouts consisting of endothelial cells towards angiogenic growth factors, such as vascular endothelial growth factor (VEGF), which contributes to new blood vessels (Adair and Montani, 2010).

Intussusceptive angiogenesis, also known as splitting angiogenesis, describes the extension of the vessel wall into the lumen causing a single vessel to split in two vessels (Burri and Tarek, 1990). Intussusceptive angiogenesis plays an important role in vascular development in embryos where growth is fast and resources are limited. But also mechanical stress related to enhanced blood flow can initiate intussusceptive growth in some regions with high blood flow in the adult (Djonov et al., 2002; Kurz et al., 2003). It mainly creates new vessels where vessels already

exist. In addition of building new vessel structures, it is relevant in the formation of vein and artery bifurcations as well as in the pruning of larger microvessels (Djonov et al., 2002).

Angiogenesis depends on a large number of molecules including growth factors and their receptors, cell adhesion molecules, extracellular matrix molecules and proteolytic enzymes. Proliferation and migration of endothelial cells are key steps in angiogenesis. Intriguingly, basic molecules like adenosine, a nucleoside produced in all cells of the body by the dephosphorylation of ATP can trigger this process by its ability to enhance VEGF expression (Adair et al., 2005; Adair and Montani, 2010; Meininger et al., 1988).

Angiogenesis in Hypoxic Conditions

Development of the vasculature occurs where blood vessels have to match nutritive needs of the tissue including the skeletal muscle, the heart, and the brain. Oxygen plays a crucial role in controlling angiogenesis in these tissues. Hypoxic conditions stimulate vessel growth by activation of endothelial cells (i.e. proliferation and migration). The cellular response to hypoxia is mediated via up-regulation of hypoxia inducible transcription factors (HIFs), such as HIF-1α, HIF-1β, and HIF-2α. HIFs up-regulate transcription of numerous genes, thereby affecting endothelial cell growth, smooth muscle cell recruitment, leukocyte attraction, etc. HIF-1 is the best characterized inducer of VEGF expression, the trigger for angiogenesis (Kimura et al., 2000). Most systems in the body are controlled by a negative feedback loop. In the case of angiogenesis, hypoxia induces the release of VEGF, which in turn stimulates blood vessel growth. The newly formed capillaries can increase the supply of oxygen, leading to a reduction of VEGF secretion and in turn stop further development of the vasculature (Adair, 2005; Adair et al., 1990; Adair and Montani, 2010).

The VEGF family of proteins, including VEGF-A, -B, -C, -D and PlGF, are strong signalling proteins involved in vasculogenesis and angiogenesis by binding and thereby activating their receptors (VEGF-R 1-3) (Li et al., 2006). After VEGF is secreted into the extracellular space it can control the onset, extent and duration of angiogenesis via binding to its cognate VEGFR´s. VEGF is responsible for increased

vascular permeability, inflicted through redistribution of intercellular adhesion molecules (Gale and Yancopoulos, 1999), and is followed by extravasation of plasma proteins that serve as scaffold for activated endothelial cells to migrate on (Ferrara et al., 2003). Although permeability supports angiogenesis, excessive vascular leakage can end in circulatory collapse, intracranial hypertension, metastasis or blindness (Carmeliet, 2000; Conway et al., 2001). Therefore permeability is tightly regulated by for instance the natural anti-permeability factor glycoprotein angiopoietin-1 (Carmeliet, 2000).

Angiogenesis also requires degradation of vascular basement membrane and remodeling of extracellular matrix in order to allow endothelial cells to migrate and invade into the surrounding tissue (Rundhaug, 2005). Matrix metalloproteinases (MMPs), a family of zinc endopeptidases participate in this remodeling of the basement membrane by facilitating proteolytically degradation of various components of the extracellular matrix (ECM), such as laminin and fibronection. Via this ECM remodelling, MMPs also regulate the release of ECM-associated growth factors such as VEGF, that in turn animates the communication of endothelial cells to each other allowing them to migrate (Noël et al., 2011). To form a vessel the newly formed endothelial sprout needs to develop a capillary lumen. This is caused by thinning of endothelial cells and fusion of endothelial strands to increase in length and diameter. After forming a new vessel, endothelial cells stop to migrate, become quiescent and survive for years (Carmeliet, 2000).

In addition to endothelial cells, mural cells are linked to the formation of new vessels (Crocker et al., 1970). Smooth muscle cells have been identified as the mural cells of arteries, arterioles and veins, whereas pericytes (multipotent mesenchymal-like cells) have been identified as mural cells of capillaries and venules (Rhodin, 1968). During development and angiogenesis, mural cells play an important role in vascular development (Hughes, 2004). Endothelial cells and pericytes communicate with each other to secure recruitment of pericytes to the external mesenchyme to form small blood vessels (Lu and Sood, 2008). This association between endothelial cells and pericytes stabilizes the newly formed endothelial tubes, promote endothelial survival (Benjamin et al., 1998) and inhibit endothelial proliferation and migration (Hirschi et al., 1998; Sato and Rifkin, 1989). Lack of these pericytes will lead to endothelial

hyperplasia and abnormal vascular remodeling (Hellstrom et al., 2001).

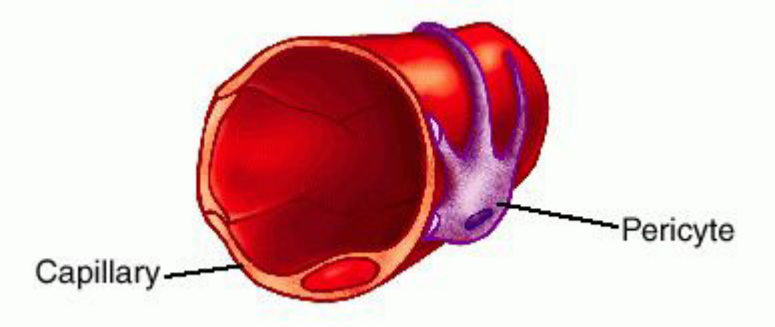

Figure 2: Pericyte wrapped around the capillary. Taken from: (Dorland´s Medical Dictionary for Health Consumers, 2007).

Murine Myocardial Infarction Model, a Model of Angiogenesis

Myocardial infarction is an ischemic heart disease and is caused by interruption of the blood flow to the cardiac tissue. This can be caused by thrombotic occlusion of atherosclerotic coronary arteries. The resulting ischemia induces necrosis of the myocardium along with free radical generation, initiation of the complement cascade, and activation of nuclear factor-κB and Toll-like receptor-mediated signalling pathways (Frangogiannis et al., 2002). These events are accompanied by chemokine and cytokine synthesis, which modulate the inflammatory response. This process ultimately results in replacement of dead cardiomyocytes with collagen-based scar tissue and is important in myocardial healing (Frangogiannis and Entman, 2005; Frangogiannis et al., 2002; Weber, 2007). Leukocytes migrate into the infarcted myocardium followed by activation of proteases and extensive degradation of cardiac ECM. One of the major therapeutic purposes of cardiology is to create strategies to minimize myocardial necrosis and improve cardiac repair and function after infarction. The mouse myocardial infarction model mimics the acute infarcted situation in patients and can be used to study the disease process and test new therapeutic interventions.

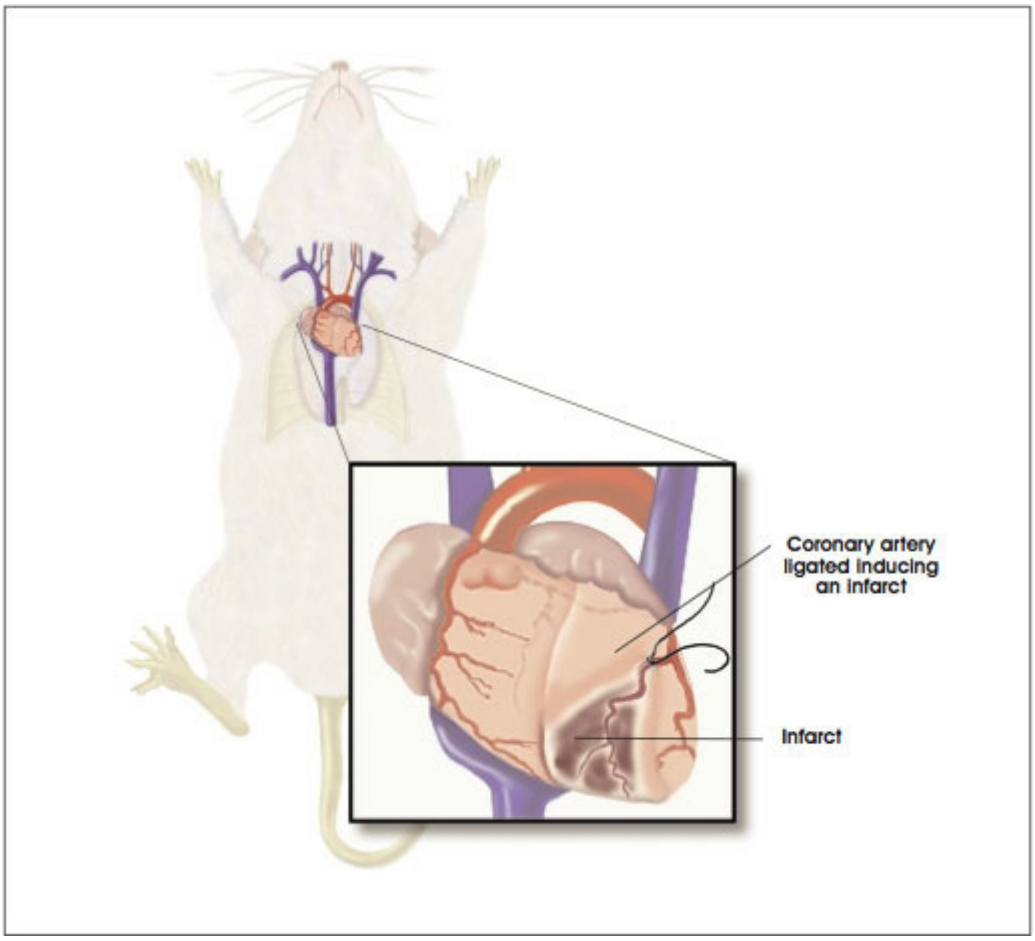

Figure 3: **The mouse model of myocardial infarction**. To induce a myocardial infarction, the left anterior descending coronary is ligated, which deprives the cardiomyocytes of nutrients and oxygen supply. Figure taken from (Winslow and Kibiuk, 2001).

The Role of Endothelial Progenitor Cells

In 1997, Asahara et al. first described the isolation of putative endothelial progenitor cells from the peripheral blood. They isolated bone marrow-derived cells that expressed CD34 and VEGFR-2 (vascular endothelial growth factor receptor-2) and cultured them on fibronectin-coated plates. These cells then differentiate into cells with phenotypic features of endothelial cells ($CD31^+$, E-$selectin^+$, $ecNOS^+$, uptake of oxidized Low Density Lipoprotein (ox-LDL)) and angiogenic potential (Asahara et al., 1997).

Some years later, Peichev et al. defined circulating EPCs as cells with surface markers including CD34, CD133 (originally named AC133), and the vascular endothelial growth factor receptor-2 (VEGFR2, alias Flk-1 or kinase insert domain

receptor KDR in murine and humans, respectively). During culturing, these cells lost their early hematopoietic stem-cell marker CD133, but gained further endothelial markers (i.e. VE-cadherin) (Peichev et al., 2000).

In vitro, two types of endothelial progenitor cells derived from CD34-positive mononuclear cells have been described: early and late outgrowth EPCs (EOCs), also called endothelial colony-forming cells (ECFCs). Both share common characteristics like the expression of CD14, CD31, lectin binding, and ox-LDL uptake, but they have also a distinct morphology, proliferative potential, and vascular tube formation capacity. In contrast to late EPCs, early EPCs do not proliferate but express haematopoietic markers (Ingram, 2004; Rehman et al., 2003). Since early EPCs do not show typical endothelial characteristics *in vitro* and they only increase neovascularization in an indirect way *in vivo*, some researchers have named them angiogenic cells (Rehman et al., 2003; Yoder et al., 2007). The early EPCs are currently being linked to angiogenic cells or macrophages. Raemer et al. described that adherent EPCs derived from blood mononuclear cells demonstrated antigen-presenting capacity and co-stimulated T-cells similar to monocytes, but much more efficiently than human vascular endothelial cells (Raemer et al., 2009). Another study challenges the presence of endothelial cell markers in early outgrowth EPCs. It is considered that during cell culturing, platelet-derived microparticles are taken up by mononuclear cells, providing them with “endothelial characteristics” (von Willebrand factor, CD31, lectin-binding) and angiogenic properties (Prokopi et al., 2009).

Only a small number of circulating $CD34^+$ mononuclear cells in culture gives rise to late EPCs. These late EPCs are thought to be the true endothelial progenitor cells contributing more directly to neovascularization by providing new endothelial cells (ECs) to the vessel lining *in vivo* (Ingram, 2004; Rehman et al., 2003; Yoon, 2005).

Endothelial Progenitor Cells in Angiogenesis and Vascular Repair

Although no clear definition of EPCs exists, they are no longer regarded real endothelial progenitors. Nonetheless, there is no question about their potential to enhance vascular repair and angiogenesis.

EPCs secrete pro-angiogenic factors such as VEGF, granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Rehman et al., 2003). Both, early and late EPCs secrete pro-inflammatory cytokines, whereby the early EPCs release thrombo-inflammatory mediators such as tissue factor (TF) and the late EPCs release chemokines such as MCP-1 (monocyte chemoattractant protein-1). EPCs can also facilitate angiogenesis by secretion of angiogenic factors like cytokines and growth factors as well as matrix-degrading enzymes comparable with monocytes. These “monocytic EPCs” arrive prior to occurring angiogenesis and stimulate the formation of new vessels (Krenning et al., 2009). All EPCs express pro-inflammatory factors and adhesion molecules linking them to atherosclerosis as well (Zhang et al., 2009).

A therapeutic potential of EPCs has been suggested as they can improve vascular repair (Hristov and Weber, 2008). After wire injury in mouse carotid arteries, bone marrow-derived EPCs were detected to adhere to the injured vessel wall and were correlated with increased re-endothelialization and reduced neointima formation (Pearson, 2010). Treatment with statin, a drug applied normally to lower cholesterol levels, boosted the amount of circulating EPCs and adherence of bone marrow-derived cells (Werner, 2002) showing therapeutic potential. Comparable, continual injection of bone marrow-derived progenitor cells from young non-atherosclerotic $Apoe^{-/-}$ (apolipoprotein E) mice in Apoe knockouts fed a high-fat diet (to induce atherosclerosis) prevented atherosclerosis progression in recipients (Rauscher, 2003). On the other hand, George et al. showed that injection of EPCs and bone marrow-derived cells resulted in an increase in atherosclerotic plaque size and increased lesion formation, while the injection of EPCs even correlated with plaque destability (George, 2005). These controversial findings are probably due to the diversity of the cell population used (see previous paragraph), even though they are all called EPCs.

I.2.2 Arteriogenesis

Arteriogenesis is another type of vascular growth and is characterized by rapid proliferation and expansion of pre-existing collaterals. These microvascular vessels are thin-walled and compose an endothelial lining, an internal elastic lamina, and one

or two layers of smooth muscle cells (Longland, 1953). Blood vessels can adapt to changing blood flow conditions. Arteries can enlarge during chronically enhanced blood flow and degenerate when not constantly perfused, or enlarge the thickness of the vessel wall under high blood pressure (Kamiya and Togawa, 1980). These collateral vessels are able to substantially increase their lumen by five- to eight-fold (Scholz et al., 2000) by growing so as to provide increased perfusion to the ischemic regions.

The obstructed region of an artery in which bypassing collaterals will grow, is normally more proximal to the hypoxic zone, and therefore its growth is independent of hypoxia (Heil and Schaper, 2007). In case of stenosis of a major artery, a pressure gradient between the high-pressure pre-occluded area and the low-pressure post-occluded area develops. This pressure difference leads to enhanced blood flow velocity and hence increased shear stress in the interconnecting (collateral) vessels. Mechanical forces caused by this drastically altered blood flow situation are considered driving the induction of collateral growth and remodeling (Unthank et al., 1996).

Increased shear stress leads to an upregulation of distinct processes in collateral arteries and therefore activation of the endothelium, which is a first sign in the development of these vessels (Schaper et al., 1976). The activated endothelial cells upregulate cell adhesion molecules (ICAM, VCAM) and cytokines such as MCP-1 (monocyte chemoattractant protein-1) (Chappell et al., 1998). The enhanced expression of the endothelium-derived chemokine MCP-1 specifically attracts blood monocytes to adhere and to invade the collaterals (Springer, 1994). After the differentiation into macrophages, they secrete a cocktail of factors that are beneficial for collateral artery growth. The secretion of proteases such as the MMPs, which in turn degrade extracellular structures like the elastic lamina, can trigger the proliferation and migration of smooth muscle cells. Even though only a small number of extra endothelial cells is required in the growth of collateral arteries, several layers of smooth muscle cells have to be formed during the development into a larger vessel. The increase of smooth muscle cells leads to a re-arrangement of the cell layers in the vessel wall (Heil and Schaper, 2007).

Stages of Arteriogenesis

The process of arteriogenesis can be divided in four different stages with distinctive morphological and molecular markers, these are: initiation, proliferation, synthesis/migration, and maturation (Scholz et al., 2000).

The initial phase starts minutes after vessel occlusion, at which increased shear stress leads to activation of endothelial cells and, subsequently induces and/or up-regulates intercellular and vascular adhesion molecules. Approximately 12 hours after endothelial activation, monocytes adhere to the endothelium, migrate into the collateral tissue and secrete proteases that digest the elastic lamina (van Royen et al., 2009). *The phase of proliferation* begins 1-3 days after arterial occlusion. During this phase a maximal wave of mitosis of the endothelial cells, smooth muscles cells and fibroblasts is apparent. 3-14 days after occlusion, the most significant growth of the collaterals was found and is considered the *synthetic/growth phase*. While the mitotic activity in smooth muscles cells decreases over time, the synthetic and proteolytic activity rises, leading to a higher production of ECM (i.e. collagen and elastin), which in turn produces a new internal elastic lamina (Buschmann and Schaper, 1999). *The maturation phase* starts about two weeks after the occlusion and is characterized by low proliferation, migration, and proteolytic activity. Most of the “synthetic” smooth muscle cells restore a physiological “contractile” phenotype at this stage.

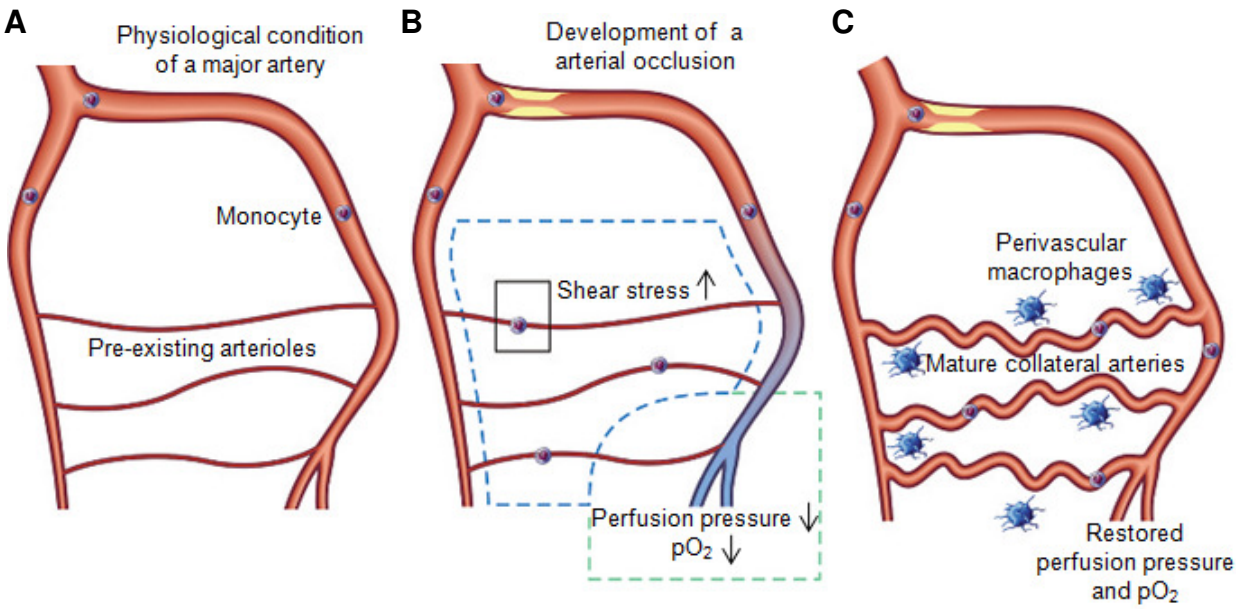

Figure 4: The mechanism of collateral artery growth. (**A**) In physiological conditions, with equal pressure over the pre-existing collateral arteries. (**B**) After arterial occlusion, a drop in pressure leads to a pressure gradient between pre-occlusive and post-occlusive area and lower oxygen saturation more distally (purple-blue color), while the proximal pressure and oxygen saturation stays normal (red color). This pressure gradient increases shear stress in these arterioles, the initial force for arteriogenesis. (**C**) Activated endothelium increases expression of adhesion molecules to attract circulating monocytes, which then transmigrate into the perivascular tissue. Perivascular macrophages secrete growth factors and cytokines that attract more monocytes and stimulate the proliferation of endothelial cells and smooth muscle cells. After adequate development of the collateral arteries the distal perfusion is restored, providing sufficient oxygen to the distal tissue. Figure taken from (Schirmer et al., 2008).

The Role of Monocytes/Macrophages in Arteriogenesis

Monocytes are part of the circulating leukocytes that are derived from hematopoietic stem cells of the bone marrow. Monocytes continually migrate through the vessel wall into the tissue, where they differentiate into macrophages. In response to an inflammatory signal, the number of monocytes increases. Both monocytes and macrophages are phagocytic cells, ingesting and killing bacteria, pathogens, dead cells and cell debris. Since most of the infections with microorganisms occur in the tissue, macrophages exert primarily this protective role. Macrophages orchestrate immune, homeostatic, and inflammatory responses, involving the induction of inflammation, and the secretion of signalling proteins to activate further immune cells

and their recruitment to sites of inflammation (Murphy et al., 2007b). Overall, monocytes and macrophages have an eminent role in angiogenesis, arteriogenesis, and many chemokines/chemokine receptors induce their recruitment.

The adhesion of circulating monocytes to the endothelium of collateral arteries was first observed by Jutta Schaper et al. (Schaper et al., 1976) indicating their important role in collateral vessel formation. A few years later Heil et al. demonstrated the functional link between monocyte concentration in peripheral blood and the improvement of arteriogenesis.

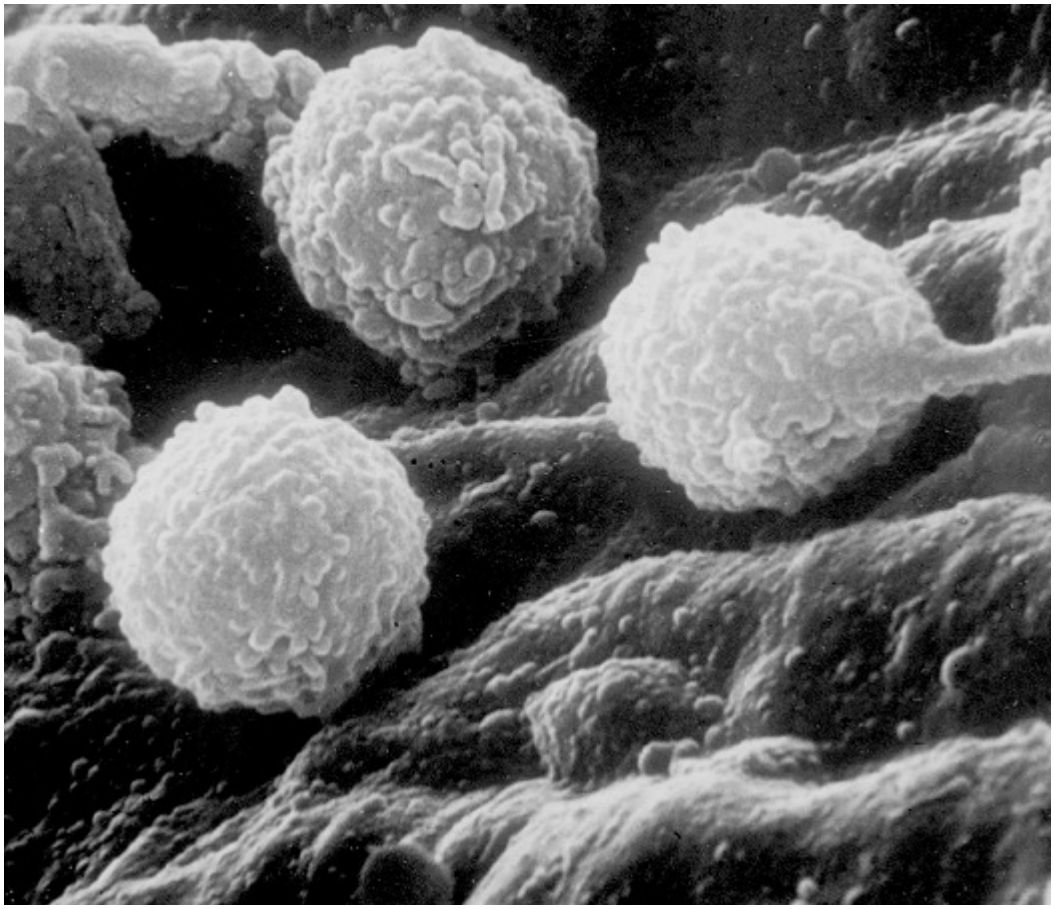

Figure 5: **Monocytes adhere to the inner vascular surface** (Schaper et al., 1976).

In a model of acute femoral artery ligation, it was observed that the pharmacological increase of the content of blood monocytes correlated with collateral conductance and number of collateral arteries and therefore, was regarded to accelerate arteriogenesis. On the other hand, depletion of monocytes almost abolishes the

growth of collateral arteries (Heil et al., 2002). During arteriogenesis, monocytes adhere to the vascular vessel wall at time points before maximal proliferation is observed for the collaterals. These monocytic cells produce large amounts of degrading enzymes, survival factors, growth factors and cytokines such as basic fibroblast growth factor (bFGF), TNF-α and VEGF, and are likely essential for stimulating endothelial and smooth muscle cell adaptation (Arras et al., 1998; Iijima et al., 1993; Sunderkotter et al., 1994). The up-regulation of survival factors such as granulocyte macrophage colony-stimulating factor (GM-CSF) keeps the environment optimal for a stable function of monocytes (Buschmann and Schaper, 1999). These, conversely, produce even more growth factors, such as transforming growth factor beta (TGF-β). The chemokine system contributes not only to angiogenesis in myocardial infarction, but also to the process of arteriogenesis after hindlimb ischemia by directly acting on inflammatory cells and vascular cell types as well. Transcriptional profiling of the adductor muscle in the hindlimb after femoral artery ligation showed an increase in the expression pattern of CC chemokines such as MCP-1 as well as CXC chemokines such as MIP-2 (murine macrophage inflammatory protein-2) (Lee et al., 2004).

MCP-1 is the most extensively studied chemokine in the context of arteriogenesis (Heil and Schaper, 2005). As its name indicates, MCP-1 attracts monocytes to collateral endothelium. Increased fluid shear stress elevates MCP-1 secretion in cultured human endothelial cells, and has been shown to induce monocyte adhesion *in vitro* (Shyy et al., 1994). Local administration of MCP-1 via an osmotic minipump increased the density of collateral arteries after femoral artery ligation in rabbits and enhanced the collateral conductance, reaching values comparable to non-operated rabbits already after 7 days after femoral artery ligation. An increase in monocyte accumulation in the wall of collateral arteries was also observed in these animals revealing that chemoattractants recruit monocytes from the circulation to sites of activated collaterals (Ito et al., 1997b). MCP-1 exerts its function by binding to its receptor CCR2. In ischemia hindlimb studies using mice deficient in CCR2 documented less blood flow recovery compared to wild-type mice and a smaller increase in collateral vessel diameters. Furthermore, the number of invaded macrophages in the perivascular space of the arteries of $CCR2^{-/-}$ was drastically diminished as compared with wild type (wt) - mice (Heil, 2004). On the contrary, Tang

et al. showed normal revascularization of $CCR2^{-/-}$ knockout mice in response to hindlimb ischemia. These CCR2-deficient mice exhibit normal blood flow recovery and arteriogenesis as well as normal monocyte and macrophage recruitment to the thigh (Tang et al., 2004).

Another chemokine receptor, CXCR3 has also been associated with arteriogenesis, contributing to leukocyte recruitment and infiltration into an ischemic area. CXCR3-deficient mice demonstrated a decrease in collateral artery formation and impaired restoration of perfusion after femoral artery occlusion. Interestingly, injection of bone marrow-derived mononuclear cells from wt-mice, but not from $CXCR3^{-/-}$ mice, restored the neovascularisation reaction and collateral artery density in the mutants, suggesting that the expression of CXCR3 in bone marrow-derived cells is necessary for arteriogenesis (Waeckel, 2005).

Besides MCP-1 and CXCR3, intergrins present on monocytes and the intercellular and vascular cell adhesion molecules (ICAM, VCAM) on endothelial cells have been regarded essential in mediating adhesion of monocytes to the endothelium. Integrins are a family of cell surface receptors consisting of two subunits: a variable α-chain and a non-variable β-chain (Giancotti and Ruoslahti, 1999). Integrins signal across the plasma membrane in two directions. This so called inside-out (intracellular to extracellular) and outside-in (extracellular to intracellular) interaction is crucial for maintaining normal cell function (Qin et al., 2004). Integrins mediate sensing and response of cells to its environment, including other cells and ECM, as well as cell functions including movement, growth and survival (Giancotti and Ruoslahti, 1999; Hynes, 1987). Interaction of ICAM-1 on endothelial cells with integrin Mac-1 on monocytes facilitates invasion of monocytes into the vessel wall and accumulation in the perivascular space of growing collateral arteries (Scholz et al., 2000).

Furthermore, growth factors such as VEGF are also involved in monocyte activation by stimulating the expression of leukocyte-specific integrins LFA-1 (lymphocyte function-associated antigen 1) and Mac-1. This, in turn, promotes the adhesion of monocytes to human endothelial cells and transmigration through an endothelial monolayer *in vitro* (Heil et al., 2000).

Hindlimb Ischemia Model, a Model of Arteriogenesis

To treat patients suffering from ischemic vascular diseases, therapeutic strategies for symptomatic relief are limited. There is an urgent need to develop new strategies for treating peripheral artery disease (PAD). To compensate for perfusion deficits, introduction of collateral growth could be a good therapeutic approach. During the last years, numerous treatments have been suggested either with stimulatory substances or different types of cells. Endovascular or surgical interventions can be useful for a handful of people, but long-term indications are often disappointing. Arteriogenesis is so far the most effective form of vascular growth for the survival of the ischemic limbs and organs such as the brain and the heart. The murine hindlimb ischemia model is a complex model of collateral artery formation, which represents PAD, and is useful for testing new therapeutic interventions.

Many studies have used the hindlimb ischemia model in different animals to describe several factors that influence arteriogenesis. The tissue response to ischemia in the hindlimb differs in distinct regions of the leg. After severe ischemic injury, necrosis occurs in the muscle below the knee followed by an acute inflammatory process with angiogenesis and muscle regeneration. On the contrary, arteriogenesis predominates proximal to the ischemic region (Scholz et al., 2002).

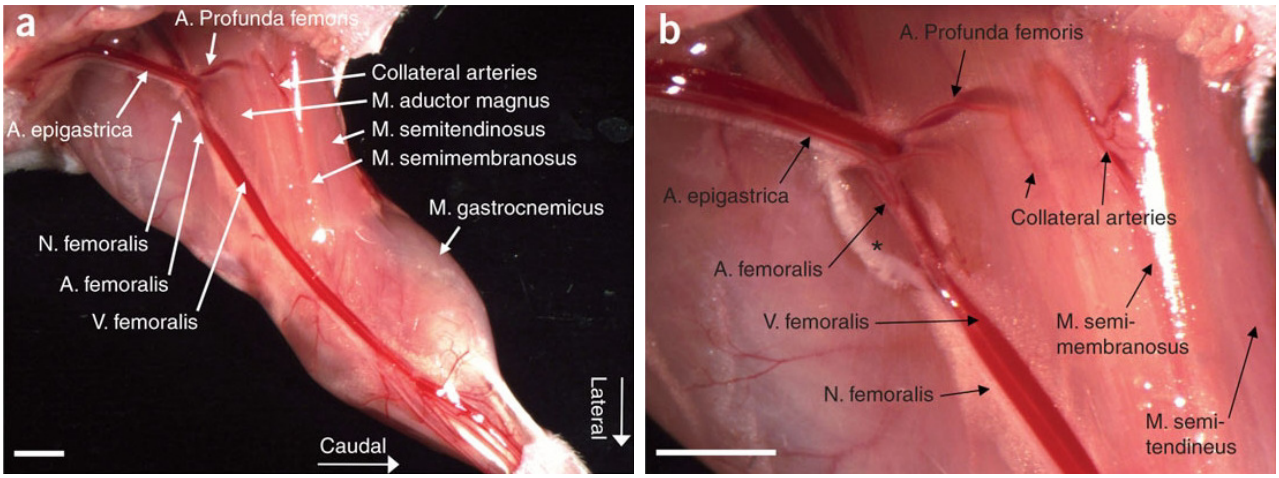

Figure 6: Anatomy of the right mouse hindlimb vasculature. The N. Femoralis has been dissected for a better visualization (b). Scale bars, 2 mm. (Limbourg et al., 2009)

I.2.3 Angiogenesis vs. Arteriogenesis

Both forms of vessel growth, angiogenesis and arteriogenesis, can be monitored after experimental occlusion of a peripheral or coronary artery, but they differ from one another in mechanism of induction, localization, and contribution of growth factors and circulating cells. Angiogenesis is induced by hypoxia and thus occurring in the ischemic region distal to the occlusion site. Importantly, arteriogenesis, independent of the presence of hypoxia, is induced by inflammation and enhanced shear stress due to increased collateral blood flow. A study in rabbits demonstrated that collateral arteries developed in non-ischemic regions without an activation of hypoxia-induced gene transcription and no rise in ischemic markers such as adenosine diphosphate, adenosine monophosphate or lactate, whereas angiogenesis occured in regions of profound ischemia (Ito et al., 1997a).

Although angiogenesis and arteriogenesis are dependent on growth factors in general, the involved growth factors and chemokines are different in each process. Angiogenesis can be largely explained by the action of the growth factor VEGF. Neufeld et al. reviewed that VEGF is one of the most effective inducers and key regulators of angiogenesis, and its expression is potentiated in response to hypoxia (Neufeld et al., 1999). Furthermore, VEGF increases the permeabilization of blood vessels and stimulates vascular endothelial cell proliferation, induces macrophage migration, and inhibits apoptosis. These effects of VEGF are mediated by binding to its high-affinity receptors VEGFR1 and VEGFR2 (murine Flt-1 and Flk-1) expressed on endothelial cells (Neufeld et al., 1999). VEGF is only mitogenic for endothelial cells, but for the growth of collaterals, proliferation of both endothelial cells and smooth muscle cells are required. Neither the increased levels of VEGF nor the expression of the hypoxia-inducible genes were associated with arteriogenesis (Deindl et al., 2001).

In case of arteriogenesis, remodeling and growth of thin-layered pre-existing collateral arteries sustained by growth factors and cytokines produced from circulating cells are essential, whereas in angiogenesis this remodeling process does not take place and it cannot adapt to changes in physiological demands of blood supply (Buschmann and Schaper, 1999). Examples of such growth factors and chemokines reported to be involved in angiogenesis are TGF-α, a-FGF and VEGF

inducing the proliferation of endothelial cells. In arteriogenesis, CCL2 is known as the most potent arteriogenic peptide; TGF-β and GM-CSF were shown to play an important role, also inducing the proliferation of smooth muscle cells (van Royen et al., 2001). Further differences between angiogenesis and arteriogenesis exist in the role of the participating cells. The accumulation of monocytes has a determinant role in arteriogenesis, but not in angiogenesis, which is more dependent on lymphocytes.

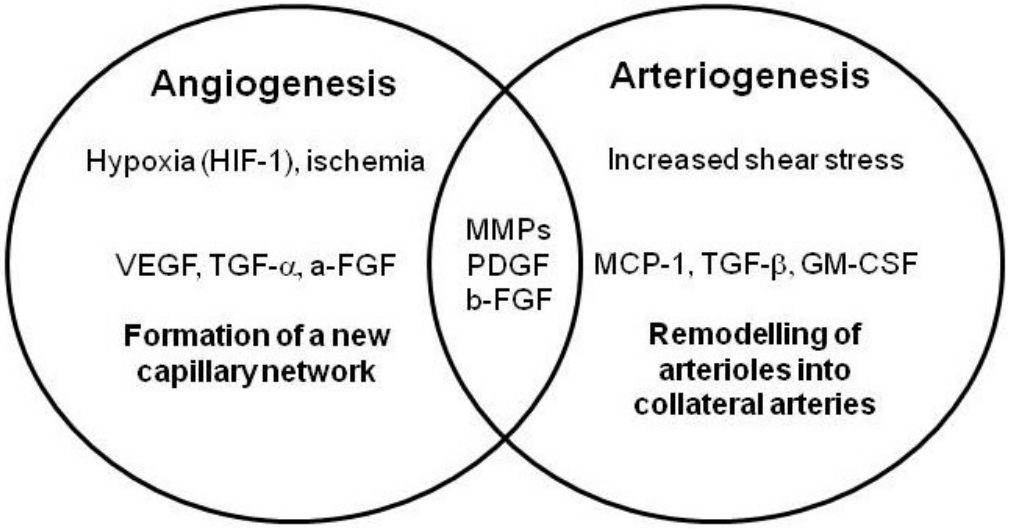

Figure 7: Two forms of vessel growth, angiogenesis and arteriogenesis. This figure summerizes the differences in the underlying inducers, results and the involved factors that are mentioned in the text. Furthermore, it shows the overlap between angiogenesis and arteriogenesis. Figure combined from (Buschmann and Schaper, 1999; van Royen et al., 2001).

I.3 Cytokines and Chemokines

Cytokines are small cell-signalling proteins (about 10 - 25kDa) that are secreted by numerous cells, normally in response to an activating stimulus. They elicit their activity through binding to specific receptors. Cytokines can be divided into at least two main groups dependent on their structure: the hematopoietin group, which includes growth hormones and many interleukins and the TNF group (Murphy et al., 2007a) .

Among the cytokines released by tissues are members of a family of chemoattractant cytokines known as chemokines (chemotactic cytokines). The chemokine super-family exists of approximately 50 structurally related small molecular weight proteins of 8-10 kDa (Reape and Groot, 1999). They are secreted and responsible for leukocyte activation and migration (Charo and Ransohoff, 2006). Chemokines can be classified into four subfamilies according to their structure and function (CC, CXC, CX3C, and C chemokines); where C is a cysteine and X any amino-acid residue (Bazan et al., 1997). The largest subfamily consists of CC chemokines, where the first two of the four cysteine residues are adjacent to each other. CC chemokines attract mononuclear cells to sites of chronic inflammation. Monocyte chemoattractant protein (MCP-1, CCL2) is the most characterized CC chemokine (Charo and Ransohoff, 2006).

A second subfamily, named CXC chemokines has a single amino acid residue interconnected between the first two cysteine residues. Some CXC chemokines, such as interleukin-8 (IL8, CXCL8) contribute to attraction of neutrophils to sites of inflamed tissue, whereas CXCL12/SDF-1 and CXCL1/KC are chemoattractants for T-cells, monocoytes and neutrophils. The CX3C family consist of only one member, fractalkine (CX3CL1), where the N-terminal cysteines are separated by three amino acids. The soluble CX3C chemokine serves as a strong chemoattractant for T cells and monocytes, and the cell-surface-bound protein that is induced on activated primary endothelial cells, promotes strong adhesion of those leukocytes (Bazan et al., 1997). The fourth subgroup is the T-cell attracting C chemokine lymphotactin (XCL1), in which two out of the four cysteines are missing (Moser and Willimann, 2004).

Chemokines exert their biological activity though high-affinity interactions with its complement of chemokine surface receptors; CC chemokines bind to CC chemokine receptors, CXC chemokines to CXC receptors and so forth. These receptors, expressed on different cell types, are seven-transmembrane proteins that signal through coupled G proteins. Upon interaction with their specific chemokine ligands, the receptor activates signal transduction cascades that activate phosphatidyl-inositol-3-kinases and triggers influx of intracellular Ca^{2+}. Besides this ligand-receptor interplay, chemokines interact with low-affinity with proteoglycans consisting of a

protein core, to which glycosaminoglycan (GAG) chains are attached. The GAGs, which are expressed in several forms throughout the body especially on the endothelial cell surface, represent a heterogeneous polysaccharide family (Middleton, 2002). This chemokine-GAG interaction enables the retention of chemokine to the surface of cells, thus creating a high local concentration gradient of chemokines that is needed for cell activation including leukocyte recruitment and migration throughout the tissue (Patel et al., 2001; Proudfoot, 2006).

I.3.1 Receptor / Ligand Interactions in Monocyte Recruitment

Chemokines and their cognate receptors play a pivotal role in directing the movement of mononuclear cells throughout the body (Charo and Ransohoff, 2006). Chemokine receptors are able to regulate inflammation and immunity by recruiting monocytes, neutrophils, and lymphocytes to the site of inflammation and vascular injury (Charo, 2004; Charo and Ransohoff, 2006). The recruitment of activated leukocytes to sites of infection and the migration of leukocytes out of the blood vessel into the tissue is a multi-step process mediated by cell-adhesion molecules. These molecules can be induced on the surface of the endothelium of blood vessels. Three families of adhesion molecules play an important role in leukocyte migration, homing, and cell-cell interactions: selectins, intercellular adhesion molecules and integrins.

The selectins are single-chain transmembrane glycoproteins that can be grouped into E-selectin on endothelial cells, P-selectin on platelets and endothelial cells, and L-selectin on leukocytes. After the endothelium gets activated, selectins are induced allowing monocytes and neutrophils to adhere reversibly to the vessel wall resulting in rolling of circulating leukocytes over the endothelial surface (Frangogiannis et al., 2002).

For transvascular migration, a stronger interaction of rolling leukocytes and endothelium is needed. Inflammatory cytokines are able to induce the expression of intercellular adhesion molecules (ICAMs) on the endothelium. Heterodimeric interactions of ICAM with integrin family members on leukocytes (e.g. LFA-1) facilitate a tighter binding of leukocytes to the vascular endothelium (Murphy et al., 2007a). As a result of this enhanced adhesion, the rolling leukocytes arrest tightly to

the endothelium. Due to the adhesion immunglobulin-related molecules named PECAM (CD31), expressed on both the junction of the endothelial cells and leukocytes, enables leukocytes to squeeze between the endothelial cells (extravasation). With the help of enzymes that break down the basement membrane, the cells penetrate and migrate through the tissue under the influence of a concentration gradient of chemokines.

I.3.2 CXCR4: Structure, Functions and Ligands

CXCR4, a CXC chemokine receptor type 4 also named CD184 or fusin is a G-protein-coupled receptor that was initially described as a leukocyte-derived seven-transmembrane domain receptor (LESTR) (Herzog et al., 1993; Loetscher et al., 1994). A few years later, after its isolation as a co-receptor for HIV-1 (Feng et al., 1996) and the identifying of its ligand stromal-derived-factor-1 (SDF-1, also named CXCL12) (Bleul et al., 1996), an increasing interest to investigate the CXCL12/CXCR4 signalling pathway has developed.

CXCR4 has 21 potential phosphorylation sites (Alkhatib, 2009). The chemokines SDF-1/CXCL12 and macrophage migration inhibitory factor (MIF) are ligands for CXCR4 that activate and signal through CXCR4, and mediate several cellular functions including immune responses, angiogenesis, and leukocyte trafficking (Viola and Luster, 2008). Binding of the ligand to CXCR4 induces a conformational change in the receptor, which allows the binding of the GTP-binding protein (G-protein) to the receptor (Wu et al., 2010). The intracellular G-protein functions as an intracellular signal by inhibiting or activating intracellular enzymes (Alkhatib, 2009).

Stromal Cell-derived Factor-1, CXCL12

Stromal cell-derived factor-1 (SDF-1) belongs to the CXC-chemokine subfamily (i.e. CXCL12) and is a highly effective lymphocyte chemoattractant (Bleul et al., 1996). Six isoforms have been identified so far; SDF-1 alpha, beta, gamma, delta, epsilon and phi (Yu et al., 2006). SDF-1 shows a 99% homology between human and mouse, allowing it to act across species. Unlike other chemokines, which are induced by inflammatory stimuli, SDF-1α is constitutively expressed in normal tissues like the

heart, spleen, and kidney (Takahashi, 2010). Besides its ability to induce chemotaxis, adhesion, and secretion of angiogenic factors and MMPs, SDF-1α regulates recruitment of bone marrow stem/progenitor cells, migration of endothelial cells and mobilization of EPCs and other progenitor cells from the bone marrow (Kucia et al., 2004; Salcedo and Oppenheim, 2003; Schober et al., 2006).

The CXCL12 / CXCR4 Axis in Myocardial Infarction

The CXCL12 / CXCR4 system plays an important role in angiogenesis and myocardial infarction. Occlusion of a coronary artery results in myocardial ischemia, and hypoxia-related induction of HIF-1α. Secondary, HIF-1α increases the expression of CXCL12 and binding to and activation of its receptor CXCR4.

Besides governing haematopoietic cell trafficking, CXCL12 has been shown to promote tissue regeneration by mediating the recruitment of progenitor cells into ischemic areas (Schober et al., 2006). Furthermore, CXCR4 signalling can stimulate proliferation and survival of different cell types, and angiogenesis (Kortesidis et al., 2005; Vlahakis et al., 2002). After vascular injury, CXCL12 expression is up-regulated in response to apoptosis and is involved in CXCR4-dependent recruitment of smooth muscle progenitor cells to injured arteries (Schober et al., 2003; Zernecke et al., 2005). Exogenous CXCL12 applied by myocardial injection, overexpressed in transplanted cardiomyocytes or nanofibre-delivered non-cleavable CXCL12, all induced therapeutic angiogenic/progenitor cell homing (Abbott et al., 2004; Askari et al., 2003), with increasing capillary density and improving cardiac function after myocardial infarction (Hu et al., 2007; Segers et al., 2007). Cardioprotective CXCL12 activates the cell-survival factor protein kinase B (PKB/Akt) via CXCR4 and protects ischemic myocardium, decreasing scar size and mediating neovascularization in mice and rats (Saxena et al., 2008; Schuh et al., 2008). The interaction between CXCL12 and CXCR4 is increasingly exploited and was shown to enhance the efficacy of stem cell therapy after MI (Zhang et al., 2008; Zhang et al., 2007). In addition, over-expression of CXCR4 in mesenchymal stem cells enhanced mobilization and engraftment of mesenchymal stem cells (MSCs) into ischemic area *in vivo* improving cardiac function and left ventricular remodeling after myocardial infarction (Cheng et al., 2008; Zhang et al., 2008).

The intrinsic role of endogenous CXCL12/CXCR4 in MI, however, is far from being conclusively elucidated. For instance, administration of the selective CXCR4 antagonist AMD3100 reduced scar formation and improved cardiac contractility after MI (Proulx et al., 2007). Moreover, CXCL12 can induce both survival and apoptotic signals via CXCR4, which may ultimately determine the fate of afflicted tissues (Vlahakis et al., 2002).

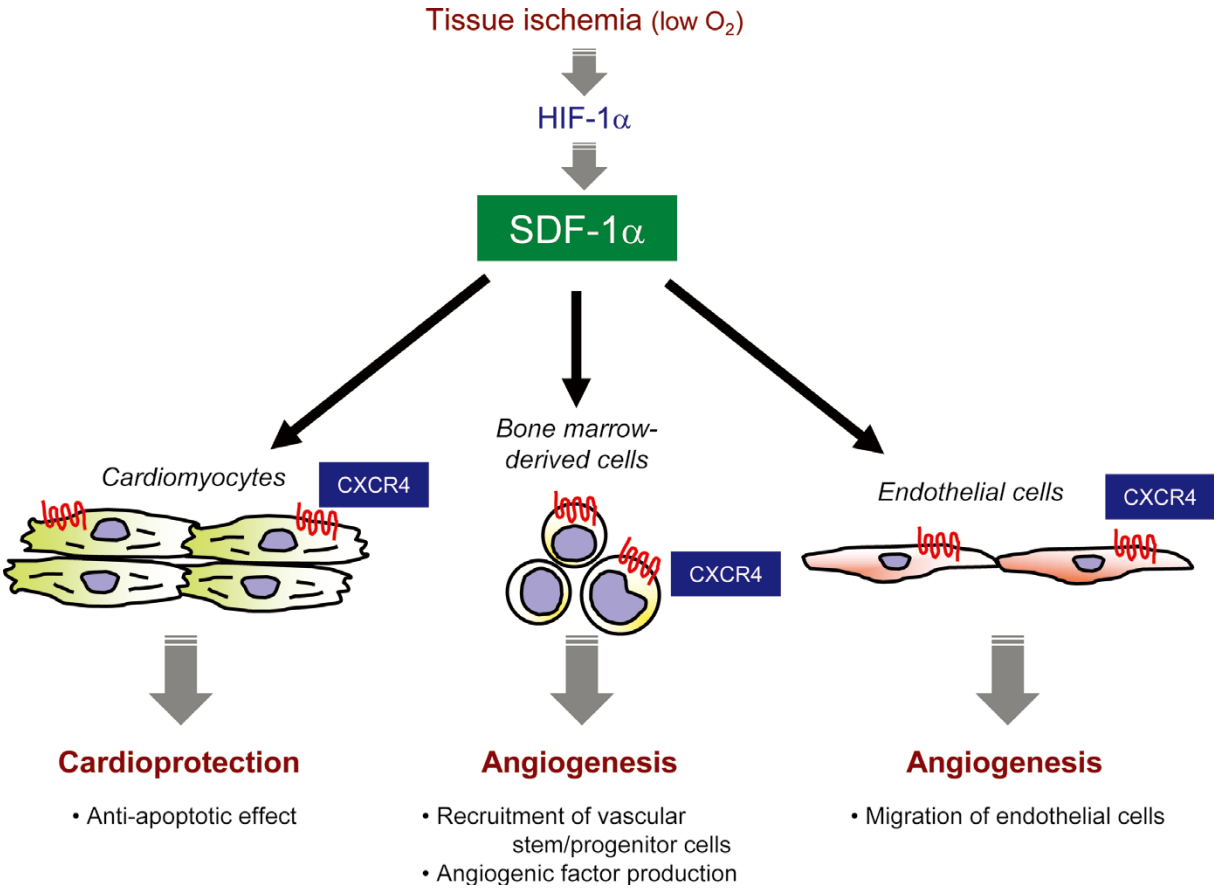

Figure 8: **The role of CXCL12/CXCR4 axis in myocardial infarction.** After an occlusion of a coronary artery, low oxygen up-regulates the expression of HIF-1α that in turn induces the expression of CXCL12. Binding of CXCL12 to its receptor CXCR4 leads to cardioprotection and angiogenesis. Figure taken from: (Takahashi, 2010).

Macrophage Migration Inhibitory Factor

Macrophage migration inhibitory factor (MIF) is the first known member of the cytokine family and was originally described to be secreted by activated T-cells and to inhibit random movement and migration of macrophages (Bloom and Bennett,

1966; David, 1966). Despite its discovery many years ago, the human MIF was cloned first in 1989 and the molecular analysis of biochemical, biological, and biophysical properties have been elucidated in the past 20 years (Bernhagen et al., 1993; Weiser et al., 1989). MIF is a unique protein lacking homology with any other pro-inflammatory cytokine (Donn and Ray, 2004). There is only one MIF gene present in humans and mice genome, located on chromosome 22 (22q11.2) in humans (Budarf et al., 1997) and on chromosome 10 in mice (Bozza et al., 1995), showing a similar gene structure (Bozza et al., 1995). In both species, MIF consists of 115 amino acids and has a molecular weight of 12.5 kDa (Bernhagen et al., 1994). Currently, a second MIF-like molecule, a protein encoded by the homolgous D-dopachrome tautomerase, D-DT gene was discovered (Merk et al., 2011). The genes for MIF and D-DT are located in close proximity to each and share 34% amino acid sequence identity confirming almost identical 3D structures. D-DT´s activation induced a MIF-like signaling cascade triggering the same immunologic pathways as MIF (Merk et al., 2011).

Immune cells such as monocytes/macrophages, blood dendritic cells, T- and B-lymphocytes, neutrophils, nature kill cells, basophils, mast cells and eosinophils express and secrete MIF (Apte et al., 1998; Bacher et al., 1996; Calandra et al., 1994; Chen et al., 1998; Rossi et al., 1998; Takahashi et al., 1999). Different to most cytokines, MIF is quasi-constitutively expressed in mostly all tissues and stored in intracellular pools. Hence, a *de novo* protein synthesis is not needed for early MIF secretion (Calandra and Roger, 2003). Furthermore, several endocrine organs such as the anterior pituitary can produce and release MIF in high levels in response to LPS (lipopolysaccharides) stimulation. This points out that MIF plays a decisive role in the toxic response to endotoxemia and septic shock (Bernhagen et al., 1993; Bucala, 1996; Calandra et al., 2000).

Currently, MIF is recognized to regulate innate and adaptive immunity, acting as a pro-inflammatory cytokine (Bernhagen et al., 1993) with “chemokine-like functions” (Degryse and de Virgilio, 2003). MIF has been linked to various acute and chronic inflammatory diseases such as septic shock (Bernhagen et al., 1993), glomerulonephritis, delayed hypersensitivity (Bernhagen et al., 1996), rheumatoid arthritis (Morand et al., 2006), and atherosclerosis (Zernecke et al., 2008a) by

regulating the recruitment of mononuclear cells to the site of inflammation (Calandra and Roger, 2003).

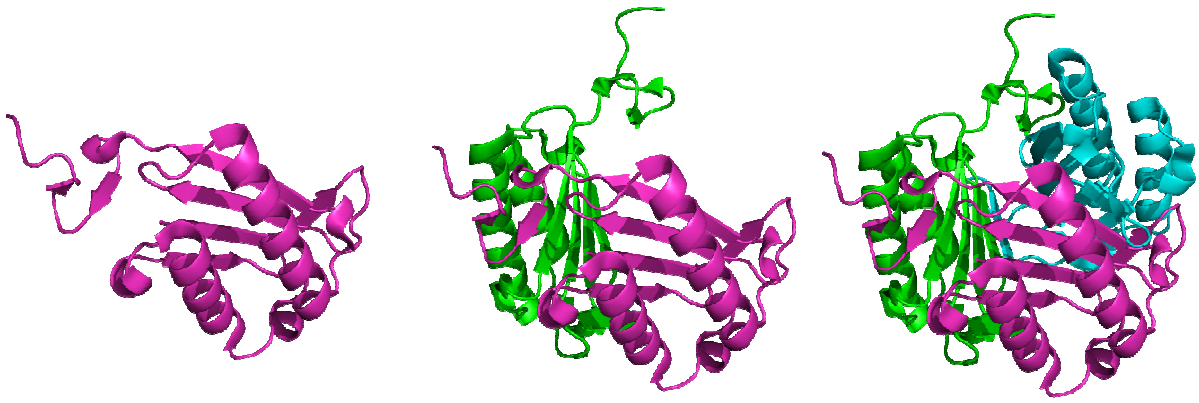

Figure 9: The 3D cystal structure of mono-, di- and trimeric MIF. The images were created with PyMOL. Adapted to Sun et al., 1996.

MIF has been described to bind with high affinity to the extracellular domain of CD74, a type II transmembrane protein. CD74 is also known as a MHC (major histocompatibility complex) class-II-associated invariant chain transporting MHC class II proteins. CD74 is required for MIF-induced activation of extracellular signal-regulated kinase-1/2 (ERK1/2) MAP kinase cascade, cell proliferation and the production of prostaglandin E_2 (PGE_2) (Leng et al., 2003). CD74 functions in immune cell stimulation, in conjunction with CD44, a polymorphic transmembrane protein, which is widely expressed. The recruitment of CD44 into the MIF-CD74 complex might be essential for MIF signalling. Miller et al. demonstrated that MIF is secreted in the ischemic heart and enhances glucose uptake by stimulating the cardioprotective AMP-activated protein kinase (AMPK) pathway through CD74 (Miller et al., 2008).

Bernhagen et al. identified the chemokine receptors CXCR2 and CXCR4 as functional non-cognate receptors for MIF in mediating monocyte/neutrophil and T cell recruitment into atherosclerotic lesions, and enhancing the formation and progression of atherosclerotic plaque (Bernhagen et al., 2007).

CXCR2 is a proteotypic receptor for ELR-motif (ELR^+) chemokines such as CXCL8 (interleukin-8, IL-8) or CXCL1/GRO-α. The CXCL8/CXCR2 axis mediates

pathophysiological processes such as inflammatory leukocyte recruitment, cancer cell migration and angiogenesis (Addison et al., 2000; Charo and Ransohoff, 2006). Recently, flow cytometry, fluorescence microscopy, and pull-down analyses showed, that MIF function in atherogenesis is related to interaction of CXCR2 and CD74, which form a receptor complex regulating leukocyte recruitment and monocyte arrest to the endothelium. Treatment of $Apoe^{-/-}$ mice (apolipoprotein E–deficient mice) on a high fat diet with advanced atherosclerosis with anti-MIF antibodies led to plaque regression and decreased monocyte and T-cell content in the atherosclerotic plaque (Bernhagen et al., 2007; Schober, 2004). Besides CXCR2, MIF is also a functional ligand for CXCR4. Schwartz et al. demonstrated that CD74 is able to form a functional heteromeric complex with CXCR4 as well, and mediate MIF-specific signalling (Schwartz et al., 2009).

Monocytes and macrophages have the ability to migrate within the tissue, to phagocytose and initiate immune responses. In addition, they are a major source of MIF, since they contain already pre-formed pools of MIF and MIF mRNA even in an unstimulated stage. The stimulation of macrophages by a variety of molecules, such as gram-negative bacteria endotoxin LPS, gram-positive bacterial exotoxin (toxic shock syndrome toxin1 TSST-1), and cytokines like TNF-α (tumor necrosis factor) and IFN-γ lead to an rapid secretion of MIF (Bernhagen et al., 1998; Calandra et al., 1994; Calandra et al., 1998).

MIF was identified to be the first protein secreted from monocytes/macrophages upon glucocorticoid stimulation (Bacher et al., 1996; Calandra et al., 1995). Glucocorticoid hormones modulate inflammatory and immune responses and are released within the systemic stress response by the pituitary gland. Low concentrations of glucocorticoids induce rather than inhibit MIF production from macrophages. Once released, MIF acts to override or counter-regulate the glucocorticoid-mediated inhibition of cytokine secretion (Calandra et al., 1995). T-cells also release MIF as an answer to glucocorticoid stimulation (Bacher et al., 1996).

A number of recent studies imply that MIF is involved in processes regulating tumorigenesis, cell proliferation and angiogenesis. Takahashi et al. demonstrated the correlation between MIF expression and enhanced proliferation of murine carcinoma

cells (Takahashi et al., 1998). The administration of neutralizing anti-MIF antibodies to mice dramatically diminished the growth and the vascularization of 38C13 B lymphoma cells (Chesney et al., 1999). MIF stimulates *in vivo* the formation of new blood vessels (angiogenesis) in Matrigel plugs (Amin, 2003; Kanzler et al., 2012) whereas administration of the anti-MIF monoclonal antibody inhibits the neovascularization response (Chesney et al., 1999). Moreover, MIF induces the migration and tube formation of embryonic EPCs in Matrigel *in vitro* and was identified to be released by ecotropic human endometrial cells promoting endothelial cell growth (Yang et al., 2000).

I.3.3 The Role of Chemokines and Cytokines in Myocardial Infarction and Peripheral Artery Disease

Despite the fact that the role of chemokines in the pathogenesis of diseases such as atherosclerosis has been widely explored, the investigation regarding their role in myocardial infarction and PAD still remains incomplete.

After an acute myocardial infarction, the plasma concentration of CC-chemokines such MCP-1/CCL2, macrophage inflammatory protein-1 alpha (MIP-1 alpha) and RANTES (Regulated on Activation Normally T-cell Expressed and Secreted) increases and is correlated with infarct complications including heart failure manifestations and severe left ventricular dysfunction (Parissis et al., 2002). In fact, the best-studied chemokine contributing to myocardial infarction is CCL2, an effective chemoattractant for monocytes, T cells and NK cells.

In a myocardial reperfusion injury model in rats, the administration of an anti-CCL2 antibody resulted in a reduction of the infarction size already 24 h after reperfusion and a decrease of infiltrated macrophages suggesting a pathophysiological function of CCL2 (Ono et al., 1999). Another study confirmed these findings, showing that an anti-CCL2 antibody treatment reduced macrophage recruitment and inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) and furthermore improved contractility and dilation of the ventricle (Hayashidani, 2003). Surprisingly, a single intramyocardial injection of CCL2 into the infarct border zone did not change physiological parameters but induced neo-

angiogenesis and monocyte infiltration (Schwarz et al., 2004). Finally, studies on CCR2- and CCL2-deficient mice revealed its involvement in myocardial remodeling, healing and inflammation after infarction (Dewald et al., 2005; Kaikita et al., 2004). Mice deficient in CCL2 demonstrated decreased and delayed macrophage recruitment in healing myocardial tissue after infarcts and delayed replacement of dead cardiomyocytes with granulation tissue leading to attenuated left ventricular function, whereas mice treated with neutralizing antibody against CCL2 showed no macrophage infiltration (Dewald et al., 2005).

The chemokine ligand 5 (CCL5), also named RANTES has a role in neutrophil and macrophage activation. Treatment with a neutralizing monoclonal antibody (mAb) to mouse CCL5 resulted in a smaller infarct size and reduction of circulating levels of chemokines, associated with less macrophage and neutrophil infiltration within the infarcted heart. Furthermore, post-infarction heart failure was reduced, indicating for cardioprotective effects of blocking CCL5 (Montecucco et al., 2011). The expression of CCR5 binding chemokines macrophage inflammatory protein (MIP)-1α and MIP-1β were markedly induced after myocardial infarction, pointing out that CCR5 signaling prevents uncontrolled inflammation after infarction and protects from adverse remodeling by recruiting mononuclear cells (Dobaczewski et al., 2010).

I.3.4 The Role of Chemokines and Cytokines in Endothelial Progenitor Cells Function

As previously mentioned, the release of chemokines/cytokines in the proximity of angiogenic sites leads to the recruitment of monocytic EPCs, sustaining the process of new vessel formation by stimulation of EPC homing during vascular remodeling. Especially, the CC- and CXC- chemokines and their corresponding receptors play a pivotal role in this process. One example is CCL2-CCR2, which is not only involved in the processes of arteriogenesis and angiogenesis, but also in infiltration of circulating cells due to the receptor expression of CCR2 on endothelial and vascular smooth muscle cells (Buschmann et al., 2003; Goede et al., 1999; Weber et al., 1999). Furthermore, this receptor-ligand interaction also contributed to directing murine $CCR2^+$ EPCs in tumor neovessels respectively to sites of arterial injury by a CCL2

dependent manner, leading to re-endotheliazation associated with the inhibition of neointimal hyperplasia (Fujiyama et al., 2003; Spring et al., 2005).

Another CXC chemokine, namely CXCL12, mediates the adhesion, migration and homing of circulating progenitor cells, which are CXCR4-positive, to match the hypoxic gradients of the ischemic tissue (Ceradini et al., 2004).

Another interaction between the CXC chemokine KC (keratinocyte-derived chemokine, CXCL1) and its corresponding receptor CXCR2 was described in leukocyte recruitment during inflammation (Smith et al., 2004). Moreover, in the case of endothelial dysfunction it sustains inflammatory and atherogenic monocyte recruitment, whereas the KC-CXCR2 axis was also suggested to be required for endothelial recovery after arterial injury (Liehn, 2004). KC is considered to have pro-angiogenic effects on account of its role in promoting EPC arterial homing facilitating tumor growth (Haghnegahdar et al., 2000; Hristov et al., 2007a). A subpopulation of circulating EPCs ($CD14^+KDR^+CXCR2^+$) was reported to be particularly efficient in facilitating endothelial recovery by incorporation at sites of denudation injury, and corresponding to cells with monocytic characteristics (Elsheikh et al., 2005; Hristov et al., 2007a). Thus, EPC homing to sites of denuded areas of arterial injury is dependent on CXCR2 and CXCR4 activation. Furthermore, the early EPC recruitment to the arterial injury site is mediated by CXCL12 and CXCL1 (Hristov et al., 2007b).

Another instance where denudation occurs is after stent implantation. Here, a local administration of VEGF-A to the affected region leads to EPC attraction, achieving stent endothelialization and preventing restenosis (Walter et al., 2004). Homing of EPCs in case of an ischemic muscle, involves the adhesion of endothelial cells, incorporation to capillaries, transendothelial migration into extravascular space and *in situ* differentiation, is accelerated by VEGF leading to neovascularization (Hur et al., 2007; Kawamoto et al., 2004).

II AIM OF THE STUDY

The mechanisms of myocardial protection or regeneration conferred by the CXCL12/CXCR4 axis may involve both recruitment of circulating cells and effects on resident cardiomyocytes. I therefore wanted to study the function of CXCR4 in cardiac remodeling after MI in genetically modified mice to evaluate a potential relevance for unwanted effects of pharmacological compounds.

Progenitor cell-based therapy including the use of EPCs, qualifies as a key to regenerate injured tissue and therefore is considered a great hope in treating ischemic diseases. Stem cell-induced angiogenesis, as a process of neovascularization depends on a cocktail of factors and involves multiple steps. MIF, VEGF, CXCL12 and CXCL1 are likely involved in this process. To better understand the behavior of EPCs in response to different growth factors, I wanted to evaluate the angiogenic potential of these different factors associated with the function of EPCs in angiogenesis.

The pro-inflammatory cytokine MIF which also features chemokine-like functions plays a critical role in inflammatory diseases associated with attracting immune cells to sites of inflammation. MIF is known to promote angiogenesis and most growth factors that have been studied in detail have pleiotropic effects and induce both angiogenesis and arteriogenesis. The aim of this study was to analyze the role of MIF in arteriogenesis in a murine hind limb ischemia model.

III MATERIAL AND METHODS

All solutions were prepared with double distilled water (Heraeus Destamat, Heraeus, Germany) or Millipore water (Milli-Q Plus ultrapure purification, Millipore, MA). Reagents were from Sigma-Aldrich (Deisenhofen, Germany), Roth (Karlsruhe, Germany), or Fluka (Buchs, Switzerland) if not stated otherwise.

III.1 General Equipment

Autoclave	Systec 2540EL (Systec, Wettenberg, Germany)
Balance	Analytical Plus (Ohaus, Pine Brook, NJ, USA)
Centrifuges	Eppendorf 5424 (Eppendorf, Hamburg, Germany), Heraeus Labofuge 400, Heraeus Multifuge 3S-R (Heraeus, Osterode,Germany)
Echocardiograph	Vevo 770 (Visual Sonics, Toronto Canada)
Flow cytometers	FACSCalibur, FACSAria (BD Biosciences, San Jose, CA, USA)
	Beckman Coulter FC500 cell sorter (Beckman Coulter, Inc., Fullerton, CA, USA)
Fluorescence plate reader	SpectraFluor Plus (Tecan, Crailsheim, Germany)
	Infinite M200 (Tecan, Gröding/Salzburg, Austria)
Incubator	Hera Cell 240 (Thermoscientific, Bonn, Germany)
	Innova CO-48 (New Brunswick Scientific CO., Edison, NJ, USA)
Laminar flow hood	Herasafe (Heraeus, Osterode, Germany)
Langendorff apparatus	UP-100 base (Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany)
Laser Doppler	MoorLDI (Moor Instruments, Axminster, UK)
Microscopes	Olympus IX81, Olmypus SZX9 (Olympus Optical, Hamburg, Germany)
	Leica DM2500 (Leica, Wetzlar, Germany)
Microtome	Leica RM 2135 (Leica, Wetzlar, Germany)
PCR thermocyclers	MyCycler (Bio-Rad, Hercules, CA)
pH-meter	InoLab level 1 (WTW, Weilheim, Germany)

RealTime PCR	7900HT Fast Real Time PCR System (Applied Biosystems, Darmstadt, Germany)
Rodent respirator	MiniVent TYPE 845 (Hugo Sachs Elektronik-Havard Apparatus, March-Hugstetten, Germany)
Spectrometer	NanoDrop (Peqlab, Erlangen, Germany)

III.2 General Solutions, Media and Buffers

PBS	37 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4 (PAA laboratories)
PFA	4% paraformaldehyde, 5% sucrose, 0.02 M EDTA, pH 7.4
DMEM / Ham´s F-12	5% FBS, gentamycin (1:1000)
Endothelial Cell Growth Medium M2	MV2 supplement mix, gentamycin (1:1000)
RPMI 1640 medium with L-glutamine	10% FBS, 1% penicillin/streptomycin
FACS staining buffer	PBS, 2% BSA, 2% mouse serum, 2% rabbit serum 2% human serum
Hanks´complete solution	1 x HBSS, 0.1% BSA, 5 mM EDTA
HH-buffer	Hank's buffered salt solution (HBSS), 0.5% (w/v) BSA, 10 mM HEPES, pH 7.4

III.3 Chemokines/Cytokines and Recombinant Proteins

The recombinant murine SDF-1α/CXCL12, KC/CXCL1 and $VEGF_{165}$ were purchased from PeproTech.

Biologically active recombinant mouse MIF and anti-MIF mAb (clone III D.9) was prepared and kindly provided by the Institute of Biochemistry and Molecular Cell Biology, University Hospital (UK) Aachen, Germany.

In short summary: Overexpression of MIF was generated by using E.coli BL21, which was stored as cell pellet at -20°C and thawed on ice, homogenized and resuspended. All steps of cell lysis and protein purification were performed on ice. The lysis was implemented using an EmulsiFlex C-5 homogenizer. The cells were lysed in a single pass at a pressure of 15,000 psi and the cell debris were centrifuged for 30 min at 30,000 x g and 4°C. The supernatant containing the soluble proteins was collected and immediately used for purification.

After protein determination, the first step of MIF purification was performed using an anion exchange chromatography in a FPLC (fast protein liquid chromatography) system with *lowsalt*-buffer. The column was pre-equilibrated and each run, 500 µl of the extract correlating with 1-5 mg total protein was applied. MIF is slightly basic and does not bind to the column. It was therefore eluted with the buffer and appeared in the first pass fraction. The MIF-containing fractions were pooled and the protein concentration was determined. The next purification step was performed by a hydrophobic chromatography using *highsalt*-buffers. The column was equilibrated with 10 ml methanol and 10 ml water before adding 2 mg protein to the column. Through subsequent washing steps low- and non-binding substances were removed. MIF was eluted with 60 % acetonitrile. After determining the protein concentration, the eluate was frozen at -80°C and then lyophilized. The lyophilisates were then stored at -20 °C until further use.

Lowsalt-buffer, pH 7,5	50 mM Tris-HCl + 150 mM NaCl
Highsalt-buffer, pH 7,5	50 mM Tris-HCl + 2 M NaCl

III.4 Antibodies

III.4.1 Primary Antibodies

CXCR2	Rabbit anti-mouse pAb (GeneTax)
CXCR4	Goat anti-mouse pAb (Lifespan Biosciences)
CD74	Goat anti-mouse pAb (Santa Cruz Biotechnology)
MIF	Rabbit anti-mouse pAb (Invitrogen)
Mac2	Rat anti-mouse mAb (clone M3/38, Cedarlane Laboratories)
α-SMA	Mouse anti-human mAb (clone 1A4, Dako)
CD3	Rat anti-mouse mAb (clone KT3, Serotec)
CD31	Goat anti-mouse mAb (clone M-20, Santa Cruz Biotechnology)
F4/80	Rat anti-mouse mAb (clone CI:A3-1, Serotec)

III.4.2 Isotype Controls

Normal goat IgG	Santa Cruz Biotechnology
Normal rat IgG	Santa Cruz Biotechnology
Normal rabbit IgG	Santa Cruz Biotechnology

III.4.3 Secondary Antibodies

Donkey anti-rat IgG	Cy3-conjugated (Jackson ImmunoResearch)
Donkey anti-mouse IgG	FITC-conjugated (Jackson ImmunoResearch)
Donkey anti-rat IgG	FITC-conjugated (Jackson ImmunoResearch)
Donkey anti-goat IgG	FITC-conjugated (Jackson ImmunoResearch)

III.4.4 Directly Conjugated Antibodies

CD31-PE-Cy7	Rat anti-mouse mAb (clone 390, Abcam)
VEGF2R-PE	Rat anti-mouse mAb (clone AVAS 12α1, BD Biosciences)
CXCR4-PE	Rat anti-mouse mAb (clone 2B11/CXCR4, BD Biosciences)
CXCR4-FITC	Rat anti-mouse mAb (clone 2B11/CXCR4, BD Biosciences)

CXCR2-PE	Rat anti-mouse mAb (clone 242216, R&D Systems)
CD74-FITC	Rat anti-mouse mAb (clone In-1, BD Biosciences)
CD11b-FITC	Rat anti-mouse mAb (clone M1/70, eBioscience)
CD11b-PE-Cy7	Rat anti-mouse mAb (clone M1/70, BD Bioscience)
CD45-APC-Cy7	Rat anti-mouse mAb, leukocyte marker (clone 30-F11, BD Biosciences)
CD115-PE	Rat anti-mouse mAb, monocyte marker (clone AFS98, eBiosciences)
Gr-1-PerCP	Rat anti-mouse mAb, mature granulocyte marker (clone RB6-8C5, BD Biosciences)
Ly6C-PE	Rat anti-mouse mAb (clone 1G7.G10, Miltenyi Biotec)
Ly6C-FITC	Rat anti-mouse mAb (clone 1G7.G10, Miltenyi Biotec)

III.4.5 Blocking Antibodies

Anti-$VEGF_{164}$	Goat anti-mouse mAb (R&D Systems)
Anti-CXCL12/SDF-1α	Mouse anti-human/mouse mAb (clone 79014, R&D Systems)
Anti-CXCL1/KC	Rat anti-mouse mAb (clone 124014, R&D Systems)

III.5 Mice

Wildtype C57BL/6J and $CXCR4^{+/-}$ mice were obtained from P. Gierschik, University of Ulm and C57BL/6J (Charles River, Chatillon-sur-Chalaronne, France) $CXCR4^{+/+}$ littermates served as controls.

Mice lacking CXCR4 die perinatally and display profound defects in vascular development, hematopoiesis, and cardiogenesis including severely deficient B-lymphopoiesis, reduced myelopoiesis in fetal liver, and virtual lack of myelopoiesis in the bone marrow (Ma et al., 1998; Tachibana et al., 1998). Therefore, we chose to assess the effects of reduced CXCR4 expression after MI in mice heterozygous for CXCR4, which appear normal, are viable and fertile (Ma et al., 1998) although CXCR4 surface expression on bone-marrow derived mononuclear cells from $CXCR4^{+/-}$ mice is significantly lower compared to wild-type mice (Walter et al., 2005).

All animal experiments were approved either by local authorities and complied with German animal protection law (50.203.2-AC 37, 26/05; -AC 36, 19/05) or by Animal Care and Use committee of the University of Maastricht (2009-077) and were performed according to official rules formulated in the Dutch law on care and use of experimental animals.

III.6 Cell culture and Cell Isolation

Cell culturing was performed under sterile conditions in a laminar flow hood. All cells were cultured at 37°C in a humidified 5% CO_2 incubator. The medium was changed every 2 days. Fetal bovine serum (FBS), a supplement added to culture medium was incubated at 56°C for 30 min to inactivate complement proteins and stored as aliquots at -20°C until use.

III.6.1 Cell Culturing of WEHIs and SVECs

Simian virus 40-immortalized murine endothelial cells (SVECs) were cultured in Dulbecco's modified eagle's medium/Ham´s F-12 supplemented with 5% FBS and gentamycin (1:1000).

The mouse monocytic cell lines, WEHI 274.1 was obtained from the American Type Culture Collection (Rockville, MD, USA) and grown in RPMI 1640 medium with L-glutamine (PAA, E15-840) supplemented with 10% FBS and 1% penicillin/ streptomycin.

III.6.2 Isolation of Angiogenic Early Outgrowth EPCs

Early outgrowth endothelial progenitor cells (EPCs) were derived from spleens of male C57BL/6J wild-type and $CXCR4^{+/-}$ mice. Spleen tissue was minced using a syringe plunger and filtered through a mesh with 40 µm pore size. Mononuclear cells were separated from erythrocytes, dead cells, and tissue debris by density gradient centrifugation (Lympholyte®-Mammal, Cedarlane Lab) at 800 x g for 20 min without brake. After washing the mononuclear cells with PBS, cells were resuspended in microvascular endothelial growth medium MV2 (PromoCell), containing a supplement

mix (C-39226) and gentamycin (1:1000) to avoid contamination with bacteria, and plated in a 6-well plate coated with bovine fibronectin. After four days of culturing, medium was changed and non-adherent cells were removed. At day ten, adherent cells were harvested using accutase, counted and used for further experiments.

III.6.3 Cardiomyocyte Isolation and Hypoxia Experiments

Cardiomyocytes were isolated from wildtype or CXCR4$^{+/-}$ hearts using the Adult M/R Cardiomyocyte Isolation System (Cellutron). After isolation and seeding, cells were cultured in AW medium (without serum, Cellutron). Hypoxia experiments were performed using an Innova CO-48 incubator with O_2 control (1% O_2, 5% CO_2) (New Brunswick Scientific) and hypoxic buffer (HB, 137 mM NaCl; 3.8 mM KCl; 0.49 mM $MgCl_2$; 0.9 mM $CaCl_2$; 4 mM Hepes; supplemented with 10 mM 2-deoxi-glucose; 20 mM sodium lactat; 10 mM sodium ditionit; 12 mM KCl; pH6,2). Cardiomyocytes were incubated with phosphatidylserine (PS) from bovine brain (Sigma) for 3 h before hypoxia. After 1 h of hypoxia, the stress rate was determined by adding dihydroethidium (DHE, Sigma). Stained cells were counted using a fluorescence microscope in five high-power fields and expressed as percentage of total cells.

III.6.4 Isolation of Blood Monocytes

Murine blood was collected from C57BL/6J mice by cardiac puncture using a syringe containing 0.2 ml citrate. Between 1000 and 1500 µl of blood was collected per mouse and diluted with an equal volume of PBS. Cell suspension was loaded on top of Lympholyte®-Mammal and centrifuged at 800 × g for 20 min without the brake. PBMCs were collected from the interface and centrifugated at 800 × g for 10 min. Cells were then resuspended in RPMI 1640 medium with L-glutamine (PAA, E15-840) supplemented with 10% FBS and 1% penicillin/ streptomycin and cultured in 6-well plates.

III.6.5 Isolation of Bone Marrow-Derived Monocytes

In the presence of M-CSF, mononuclear phagocyte progenitor cells derived from femoral and tibia bone marrow. This macrophage growth factor is secreted by L929

cells and is used in the form of L929 cell-conditioned medium (LCM) (Stanley and Heard, 1977). Progenitor cells proliferate and differentiate through monoblast, promonocyte and monocyte stages before maturing into macrophages. At this time point, these cells firmly adhere to the bottom of the culture flask.

For cell isolation, mice were killed by isoflurane and femur and tibia were removed and dissected free from the adherent tissue. Both ends of femur and tibia were cut off and PBS was injected into the bone to flush out the bone marrow tissue in a sterile falcon tube. Cells were dispersed by pipetting and centrifuged at 1200 rpm for 5 min at 4°C. After resuspending in culture medium, the monocytes were plated in 15 cm bacterial plastic plate containing 20 ml culture medium + 15% LCM. After 3 days, 10 ml medium was added and after 6 days, replaced by new medium with 15% LCM. After 8 days, medium was discarded and macrophages were lifted with pre-warmed, filtered 4 mg/ml Lidocaine/10 mM EDTA/PBS solution. After centrifugation, cells were counted and plated in 6-well plates as required.

Macrophages were incubated with rmMIF (5 ng/ml, 10 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml, 400 ng/ml, 500 ng/ml) at 37°C, for 24 h. Macrophages were lysed for mRNA isolation.

III.7 Biomolecular Methods

III.7.1 mRNA Isolation and Reverse Transcription - Polymerase Chain Reaction

mRNA from hearts was isolated with TRIzol Reagent (Gibco) and after reverse transcription (Omniscript Kit, Qiagen), PCR was performed using specific oligonucleotide primers (Table 1).

Table 1: Specific oligonucleotide primers used in this study

Mouse CXCL12	forward primer: 5'-GTCAGCCTGAGCTACCGATG-3'
	reverse primer: 5'-GGGTCAATGCACACTTGTCTGT-3'
Mouse VEGFA	forward primer: 5'-CCTGGCTTTACTGCTGTACCTC-3'
	reverse primer: 5'-GTGCTGTAGGAAGCTCATCTCTC-3'

Mouse VE-cadherin	forward primer:5'-ACTGTGATGATCAGACTGGAGGAC-3' reverse primer: 5'-GTCTGTCTCAATGGTGAAGGTGTC-3'
Mouse vWF	forward primer: 5'-ACTTCTGCAGACCTGCGTAGAC-3' reverse primer: 5'-CCTCAACATATGGGGTGGTAGA-3'
Mouse Bax	forward primer: 5'-TGCAGAGGATGATTGCTGAC-3' reverse primer: 5'-GATCAGCTCGGGCACTTTAG-3'
Mouse Bcl2	forward primer: 5'-AGGAGCAGGTGCCTACAAGA-3' reverse primer: 5'-GCATTTTCCCACCACTGTCT-3'
Mouse ß-actin	forward primer: 5'-AGCCATGTACGTAGCCATCC-3' reverse primer: 5'-CTCT CAGCTGTGGTGGTGAA-3'

III.7.2 Quantification of RNA and Quantitative Real-Time Polymerase Chain Reaction

To determine gene expression of different M1 (IL12, TNF-α, iNOS) and M2 (IL10, YM1, FIZZ-1) macrophage markers, as well as the expression of MIF and its receptors CXCR2, CXCR4, and CD74 after stimulation with different rmMIF concentrations for 24 h, a real time PCR was performed using the Maxima^{TM} SYBR Green qPCR Master Mix and specific primer pairs (Table 2). The SYBR Green dye binds to double-stranded DNA, and after excitation emits light, providing a fluorescent signal, which reflects the amount of double-stranded DNA product generated during the PCR. Thus, as a PCR product accumulates, the fluorescence signal increases and can be directly detected. The advantage of SYBR Green is that it is easy in using, sensitive and not expensive. On the other hand, it binds to any double-stranded DNA in the reaction, which includes non-specific binding and primer-dimers, potentially resulting in an overestimation of the target concentration.

mRNA from macrophages was isolated using ZR RNA MicroPrep™. After measuring the amount of RNA, 1µg total RNA was reverse-transcribed into cDNA and 20 ng cDNA was used per real-time PCR reaction. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene.

Reverse transcription: 2 µl hexamer primer
4 µl reaction buffer
2 µl 5mM dNTPs
1 µl reverse transcriptase
2 µg template RNA
H_2O to a final volume of 20 µl

Real time-PCR: 2 µl cDNA
12.5 µl Maxima^{TM} SYBR Green qPCR Master Mix
2.5 µl forward primer (2 µm)
2.5 µl reverse primer (2 µm)
5.5 µl RNase free water

Real time-PCR program: Initial activation 95°C, 15 min
Denaturation of cDNA 95°C, 30 sec
Annealing of primers 58°C, 30 sec
Elongation of primers 72°C, 30 sec
} x 40
95°C, 2 min
60°C, 2 min

The relative mRNA expression is calculated as follows:

$\Delta C_t = C_t$ (target gene) – C_t (reference gene)

$\Delta\Delta C_t = \Delta C_t$ (sample) – ΔCt (control sample)

Relative mRNA expression: $2^{-(\Delta\Delta Ct)}$

C_t: Cycle number at which the fluorescence intensity exceeds a determined threshold

Table 2: Primers for real-time PCR used in this study (Sigma)

Mouse CXCR2	forward primer: 5'-GCCTTGAGTCACAGAGAGTTG-3'
	reverse primer: 5'-CAAGGCTCAGCAGAGTCAC-3'
Mouse CXCR4	forward primer: 5'-GGCTGTAGAGCGAGTGTTG-3'
	reverse primer: 5'-CAGAAGGGGAGTGTGATGAC-3'
Mouse CD74	forward primer: 5'-CCCATTTCTGACCCATTAGT-3'
	reverse primer: 5'-TGTCCAGCCTAGGTTAAGGGT-3'

Mouse MIF	forward primer: 5'-TTTAGCGGCACGAACGATCC-3'
	reverse primer: 5'-CGTTGGCAGCGTTCATGTC-3'
Mouse YM1	forward primer: 5'-TGGCCCACCAGGAAAGTACA-3'
	reverse primer: 5'-CAGTGGCTCCTTCATTCAGAAA-3'
Mouse IL10	forward primer: 5'-TTTGAATTCCCTGGGTGAGAA-3'
	reverse primer: 5'-CTCCACTGCCTTGCTCTTATTTTC-3'
Mouse TNF-α	forward primer: 5'-CATCTTCTCAAAATTCGAGTGACAA -3'
	reverse primer: 5'-TGGGAGTAGACAAGGTACAACCC-3'
Mouse IL12	forward primer: 5'-GGTGCAAAGAAACATGGACTTG-3'
	reverse primer: 5'-CACATGTCACTGCCCGAGAGT-3'
Mouse FIZZ-1	forward primer:5'-GGAACTTCTTGCCAATCCAG-3'
	reverse primer: 5'-ACACCCAGTAGCAGTCATCCC-3'
Mouse iNOS	forward primer: 5'-TTGCAAGCTGATGGTCAAGATC-3'
	reverse primer: 5'-CAACCCGAGCTCCTGGAA-3'
Mouse GAPDH	forward primer: 5'-ATTGTCAGCAATGCATCCTG-3'
	reverse primer: 5'-ATGGACTGTGGTCATGAGCC-3'

III.8 Protein Assays

III.8.1 Flow Cytometry Analysis

Flow cytometry is the measurement of cells in a flow system, which can be used to analyze cells according to their size, granularity and protein expression or sorting cell populations (FACS = fluorescent activated cell sorting). One cell at a time, suspended in a stream of fluid buffer, passes through a light beam that excites fluorescently labeled cells. Measurements of size (forward scatter) and granularity (sideward scatter) are independent from the fluorescence signal.

Flow cytometry measurements were performed by using the corresponding, fluorescence labelled antibodies in Hank´s balanced salt solution containing 0.1 % bovine serum albumin (BSA) and 0.3 mmol/l ethylenediaminetraacetic acid (EDTA).

Blood and digested hearts were resuspended in FACS staining buffer with specific fluorescence-conjugated antibodies, anti-CD45-APC-Cy7, anti-CD115-PE and anti-

Gr-1-PerCP to determine monocyte subpopulations (Nahrendorf et al., 2007). To analyze endothelial cells and apoptosis, anti-CD31-PE-Cy7, anti-VEGF2R-PE, Annexin-V-FITC (BD Bioscience) staining was performed. Annexin V is a phospholipid-binding protein that has a high affinity for phosphatidylserine, which translocates from the inner to the outer leaflet of the plasma membrane in the early stages of apoptosis. The staining is therefore an important tool to identify apoptosis.
To determine CXCR4 expression on blood cells an anti-CXCR4-PE was used.

Isolated EPCs were characterized by, anti-CD31-PE-Cy7, anti-VEGF-R2-PE, and anti-CD11b-FITC staining. To examine the expression of the MIF receptors on the cell surface, the cells were stained with anti-CXCR2-PE, anti-CXCR4-PE and anti-CD74-FITC.

In vivo shifts in monocyte populations, as a result of administration of rmMIF or anti-MIF mAb during arteriogenesis, were analyzed after whole blood was drawn from the heart of the animals. After erythrocyte lysis, cells were stained with anti-CD11b-PE-Cy7, anti-Ly6C-PE, anti-Ly6C-FITC, and anti-CXCR2-PE in three different antibody mixes per animal. All samples were incubated with antibodies on ice and in the dark for 20-30 min, washed with Hanks´ complete and analyzed immediately by flow cytometry using a FACS Canto II flow cytometer and FLOW JO Software. Non-stained cells served as controls. Cell sorting was performed with the Beckman Coulter FC500 cell sorter (Beckman Coulter, Inc., Fullerton, CA, USA) and analyzed with Kaluza® Analysis Software.

III.8.2 Histochemistry and Immunohistochemistry

Histomorphometry and Determination of Myocardial Infarction Size

Serial sections of the hearts (10-12 per mouse, 400 µm apart, up to the mitral valve) were stained with Gomori's 1-step trichrome stain. The infarcted area was determined in all sections using Diskus software (Hilgers) and expressed as percentage of total left ventricular volume.

To measure the area at risk one day after MI, a staining with tetrazolium (Sigma) was performed. Briefly, hearts were cut into 2 mm thick slices, which were incubated with TTC (2,3,5-triphenyltetrazolium chloride). TCC is a redox indicator, which is used to distinguish between metabolically active and inactive tissue due to the activity of dehydrogenases in living tissues. This enzyme reduces the white compound to red colour compound. After the TTC staining, the hearts were fixed with formalin, and pressed between glass blades to achieve uniform thickness. The pale infarcted area was determined in all sections using Diskus software (Hilgers) and expressed as percentage of total left ventricular area.

Cellular Stainings of Inflammatory Cells in the Heart

Serial sections of the hearts (3 per mouse, 400 µm apart) were stained to analyze the infarcted areas for the content of neutrophils (specific esterase, Sigma), macrophages (F4/80), lymphocytes (CD3), vessels (CD31/PECAM), and myofibroblasts (α-smooth muscle actin). Cells or vessels were counted in 6 different fields per section and expressed as cells or vessels/mm².

To determine apoptosis in the hearts, serial sections (3 per mouse, 400 µm apart) were stained with *In Situ* Cell Death Detection Kit (Roche), and counter-stained with DAPI. Apoptotic cell indexes were expressed as percentage of positive from total cells.

Cellular Stainings of Receptors and MIF in Hindlimb Tissue

Serial sections of the hindlimbs (5 per mouse, 5 µm thick) were stained to analyze the content of macrophages (Mac2), as well as the chemokine MIF and its 3 receptors CXCR2, CXCR4, and CD74. Cells were numbered in 6 different fields per section and absolute number of cells expressing Mac2 and CXCR2 were counted. For CD74 expression, percentage of total cell surface per field, for CXCR4 expression, pixel per field and for MIF expression, percentage of positive tissue per field was analyzed.

III.8.3 AcLDL and Lectin Uptake

EPCs were incubated at 37°C with 10 µg/ml DiI-labelled acLDL (Cell Systems). After two hours, the cells were fixed with paraformaldehyde (1%) for 10 min and subsequently incubated with biotinylated Griffonia Simplicifolia lectin I (100 µg/ml) for 1 hour followed by streptavidin-FITC. Finally, cells were mounted (Vectashield with DAPI, Vector Lab) and analyzed by fluorescence microscopy.

III.9 Functional Assays

III.9.1 Chemotaxis Assay

Chemotaxis assay was performed using a 24-well transwell cell culture chamber with BD Falcon® HTS FluoroBlok™ 3.0 µm colored PET membrane inserts. Serum-starved wild-type and CXCR4$^{+/-}$ EPCs were resuspended in assay medium MV2 containing 0.5% BSA. 20,000 cells in 100 µl medium were applied on top of membrane insert filters. Test factors (rmVEGF (50 ng/ml, 100 ng/ml)), rmSDF-1α (50 ng/ml, 100 ng/ml), rmKC (50 ng/ml), rmMIF (50 ng/ml)) or buffer control were added in 600 µl assay medium and placed in the lower chamber. After incubation at 37°C for 2 h, migrated cells were quantified on the bottom side of the filter after DAPI staining and were counted manually using a fluorescence microscope.

III.9.2 Transmigration Assay

To compare the migratory effect of chemokines and growth factors, a transmigration assay was performed using a 24-well transwell cell culture chamber with Falcon® HTS FluoroBlok™ 3.0 µm colored PET membrane inserts. Brd-U marked SVECs were placed on top of fibronectin-coated filters with 100 µl DMEM/Ham´s F-12 medium. After incubation at 37°C for 24 h, SVECs form an endothelial monolayer on the membrane surface. Inserts were subsequently placed in a new 24-well plate whereby the lower chamber was containing 600 µl assay medium plus the test factors (rmVEGF (50 ng/ml), rmSDF-1α (50 ng/ml), rmKC (50 ng/ml), rmMIF (50 ng/ml), anti-MIF mAb (10 µg/ml). Serum-starved EPCs and WEHIs were resuspended in assay medium MV2 and RPMI containing 0.5% BSA, respectively. 20,000 cells in 100 µl were applied on top of each insert filter. After incubation at

37°C for 2 h, transmigrated cells on the bottom side of the filter were stained with DAPI and were counted using a fluorescence microscope.

III.9.3 Static Cell Adhesion Assay

Static adhesion assay was performed using a 96-well plate. For an endothelial monolayer, 10,000 SVECs were placed in each well at 37°C for 24 h. WEHIs (20,000 or 50,000 cells per well) or isolated blood monocytes (20,000 cells per well) were labelled with calcein-AM (1 µg/ml). Calcein-AM is a cell permeable compound, which can be transported through the cellular membrane into living cells. There it is converted to the strong green fluorescent calcein after hydrolyzing (removing of the acetomethoxy group) by intracellular esterases. Test factors, rmMIF (10 ng/ml, 50 ng/ml, 100 ng/ml) and anti-MIF mAb (10 µg/ml, 20 µg/ml) were added to the assay medium containing calcein-labelled WEHIs or blood monocytes After incubation for 16 h at 37°C, non-adherent cells were removed by washing the cells with PBS. Adherent monocytes were analyzed by multiple fluorescence top reading (excitation 480 nm, emission 520 nm) using TECAN® i-control reader and i-control software.

III.9.4 Flow Cell Adhesion Assay

Chemoattractants recruit monocytes to sites of inflammation, where they adhere to the endothelium and transmigrate through the vessel wall. To study the arrest of monocytes under shear stress, adhesion of WEHIs to an endothelial SVEC monolayer was analyzed under laminar flow conditions. SVEC cells were grown to confluence in 35 mm dishes coated with collagen and stimulated with TNF-α (10 ng/ml, for 2 h at 37°C) to induce adhesion molecule expression. Calcein-labelled WEHIs ($1x10^6$ cells/ml) were then directly incubated with 100 ng/ml rmMIF, 10 µg/ml anti-MIF mAb or PBS for 30 min at 37°C and perfused at 1.5 dyn/cm^2 in HH buffer. After 5 min, the number of adherent cells was quantified in multiple fields by analysis of images recorded with a long integration 3CCD video camera (JVC, Japan).

III.9.5 Matrigel Assay *in vitro*

BD Matrigel™ Basement Membrane Matrix was thawed overnight at 4°C. 300 µl matrigel was placed in each well of a 24-well plate and allowed to polymerize at 37°C for 30 min. EPCs ($1x10^5$ cells/ml/well) were labelled with calcein-AM for 30 min at 37°C and seeded together with murine endothelial cells (SVECs, $3x10^4$ cells/ml/well) in a cell suspension in assay medium on top of the matrigel. The cells were stimulated either with rmVEGF (50 ng/ml), rmSDF-1α (50 ng/ml), rmKC (50 ng/ml), or rmMIF (50 ng/ml). After incubation at 37°C for 24 h, tube formation ability was quantified by counting the total number of tube-like structures in 5 random microscopic fields.

III.9.6 Electron Microscopy

Samples were fixed in 3% glutaraldehyde for 14 h, washed with 0.1 M B2 buffer (pH7.4; 13 mM NaH_2PO_4; 87 mM Na_2HPO_4) overnight, followed by 1 hour in 1% OsO_4 (in 17% sucrose buffer - pH7.4; 88 ml 0.1 M B2 buffer; 12 ml H_2O, 17 g sucrose), rinsed with water and dehydrated with ethanol (30% - 100%) and propylenoxide (100%). Finally, tissues were processed for embedding in Epon, polymerized for 48 h at 60°C, cut into 80-100 nm thick slices and contrasted with uranyl acetate and lead citrate. The samples were analyzed with a PHILIPS EM 400 T at 60 kV and micrographs were taken by an OLYMPUS CCD-Camera MORADA.

III.9.7 Lipid Extraction and HPLC

For lipid extraction, hearts were shock-frozen in liquid nitrogen. After mincing the tissue, chloroform and methanol were added (1:2, 0.5 ml per 150 mg tissue) for 15 min under shaking conditions. Finally, chloroform and water (1:1, 0.2 ml per 150 initial tissue) were added and the samples were centrifuged at 3,000 rpm for 7 min. The lower phase was separated and lyophilized under nitrogen atmosphere. Phospholipid extracts from mouse hearts were separated by HPLC. Briefly, dried lipid films were dissolved in solvent A (hexane–2 isopropanol, 6:8 v/v) and injected in a Spectrasystem 2000 HPLC device with a Betasil Silica-100 column (250×4 mm) from ThermoFischer Scientific (Dreieich, Germany). A linear gradient was applied from 0-100% solvent B (hexane–2 isopropanol–water, 6:8:1.4 v/v/v) over 45 min at 1 ml/min

and lipids were detected at 206 nm. Mobile phases were from Carl Roth GmbH (Karlsruhe, Germany). Peaks were identified using standards for triglyceride, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol (Sigma).

III.9.8 Gas-Chromatography of Fatty Acids

HPLC-isolated lipid fractions were solubilized in hexane and transesterification was done using 2 M potassium hydroxide in methanol. After adding anhydrous sodium sulfate for drying, the solution was vigorous mixed. Next, the filtered solution was neutralized with sodium phosphate monobasic and concentrated in a rotary evaporator. The methylester solution was injected in a Thermo Fischer TR-FAME capillary column (120m x 0.25mm I.D. x 0.25 µm) of a CP-3800 VARIAN gas chromatographer system, equipped with a flame-ionization detector. Peaks were identified according to fatty acid standards (FAME MIX 37, Sigma-Aldrich) and analyzed using automated integration.

III.10 Animal Experiments

III.10.1 Murine Model of Myocardial Infarction

The myocardial infarction surgical procedure in mice imitates the pathobiological and pathophysiological aspects which occur in infarction-related myocardial ischemia (Kolk et al., 2009). $CXCR4^{+/-}$ mice (C57BL/6J) were obtained from P. Gierschik, University of Ulm. $CXCR4^{+/+}$ littermates served as controls. After intubation under general anaesthesia (100 mg/kg ketamine, 10 mg/kg xylazine, i.p.) mice were positive pressure ventilated with oxygen and 0.2% isofluran using a rodent respirator (Liehn et al., 2008). Hearts were exposed by left thoracotomy and MI was produced by permanent ligation of the left anterior descending artery (LAD) with suture ligation. This procedure forms ischemia that can be seen almost immediately after ligation. By closing the LAD, no further blood flow is allowed in that area, whereas the surrounding myocardial tissue is almost not affected. Muscle layer and skin incision were closed with a silk suture.

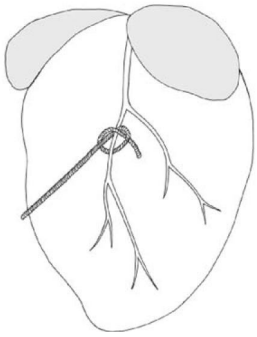

Figure 10: Ligation of the LAD. The heart of the mouse with the pattern of left coronary arteries and the position of the permanent ligation of the left anterior descending artery as performed in the MI procedure (adapted from (Wang et al., 2006)).

III.10.2 Bone Marrow Reconstitution

Bone marrow chimeras were established as described (Lutgens et al., 2010). Wild-type or $CXCR4^{+/-}$ mice received 12 Gray split into two doses. Recipient mice were reconstituted with 2×10^6 $CXCR4^{+/-}$ or wild-type bone-marrow cell injection i.v., respectively. After six weeks, mice were subjected to MI. At the indicated time points, blood samples were taken for leukocyte analysis. Four weeks after the infarction, the hearts were taken for further analysis.

III.10.3 Ultrasound Analysis

Two-dimensional and M-mode (Time-motion mode) echocardiographic measurements (Vevo 770, Visual Sonics, Toronto, Canada) were performed before and after induction of MI. Mice were anesthetized with 1.5% isofluran via a mask and placed in the supine position on a warming pad. The chest hair was removed and the limb leads were attached to the pad. Ejection fraction, fractional shortening, cardiac output, stroke volume, left ventricular end-diastolic and end-systolic dimensions was quantified according to standard protocols (Liehn et al., 2008; Liehn et al., 2010).

III.10.4 Langendorff Perfusion

Langendorff perfusion is an *in vitro* technique to study the cardiac contractile strength, the heart rate, and coronary resistance under known physiological

conditions without the hormonal and neural complications of an *in vivo* animal experiment. After the heart is removed from the animal, the ascending aorta is cannulated and the heart is then perfused in a reverse fashion via the aorta with a nutrient rich oxygenated solution. The backwards pressure causes the aortic valve to close, forcing the solution into the coronary circulation, which usually provides the heart tissue with blood. This feeding solution allows the heart to continue contractions for several hours and heart parameters can be analyzed.

At indicated time points, mice were anesthetized with Ketamin (100 mg/kg) and Xylazin (10 mg/kg) and heart function was analyzed using a Langendorff apparatus and Isoheart software under constant perfusion pressure (100 mmHg) and electrical stimulation to assure constant heart rates (600 bpm). Coronary flow and developed pressure were measured. Finally, hearts were fixed with 10% formalin, paraffin embedded and cut into 5 µm serial slices and used for further analysis.

III.10.5 Vessel Density (High-Resolution Computer Tomography)

For coronary visualization, six mice (three knock-out mice, three wild-type mice) were studied using high-resolution computer tomography. After anesthesia and midline laparotomy, mice were bled to death; the hearts were exposed, retrogradely perfused and fixed with 4% paraformaldehyde. Subsequently, hearts were perfused with Microfil®, a lead-containing contrast agent, which polymerises intraluminally, extracted and scanned in a SkyScan1172 micro-CT (SkyScan, Belgium) by acquiring 681 projections with 1048x2000 pixels at an increment of 0.3°. Reconstruction voxel size was 5.36 µm. All direct branches of the left and right coronary arteries were counted. The cremaster muscle was exposed as described (Soehnlein et al., 2008), and branches of the cremasteric artery were counted. To determine capillary leakage, perfusion of the cremasteric artery with albumin-FITC (Sigma) was performed.

III.10.6 Ischemia Hindlimb Model

Male C57BL/6J mice were investigated in this study. They were blinded and randomly assigned to receive either *(a)* anti-MIF mAb (100 µg, i.p.) the day before

the ligation, *(b)* rmMIF (10 µg, i.p.) for five consecutive days starting one day before ligation, or *(c)* PBS. Considering published data on the effectiveness of the anti-MIF mAb (clone III D.9) shown to neutralize murine MIF bioactivity *in vivo* and *in vitro* (Garner et al., 2003) in different disease models (Calandra et al., 2000; Chagnon et al., 2005; Schober, 2004), I decided not to include an isotype control.

The animals were first anesthetized with 5% isoflurane (flow 1-1.5 l/min) to assure a fast anesthesia. After the mouse is unresponsive to external stimuli, isoflurane was decreased to 2 - 2.5% (flow 0.3 l/min). Using an electric shaver, hair was removed from the limb. The mouse was placed in a supine position on the operating table and connected to a continuous flow of isoflurane. After skin incision was made medial on the right thigh, the vein and nerve were carefully dissected from the femoral artery. Surgical procedure was performed by ligating the right femoral, epigastric and popliteal artery. For analysis of perfusion recovery animals were sacrificed after 14 days (n=6/treatment) after ligation. Animals assigned to histological evaluation and examination of circulating blood monocyte population were sacrificed 48 h and 24 h or 48 h after ligation, respectively (n=3/treatment and time point). Blood for further FACS analysis was drawn from the hearts and collected in EDTA tubes. For histological examination, abdominal aortas were cannulated. After perfusion with PBS, animals were rinsed with 2 ml 0.1% propidium iodide for 2 min followed by fixation with 100 ml PFA (4%). Samples were kept in PFA overnight, and then in 70% ethanol and stored at 4°C.

III.10.7 Pre- and Post Laser Doppler Measurements

Laser Doppler Imaging is used to assess blood perfusion in hindlimbs of mice non-invasively and without the use of tracer dyes under standardized conditions.

A laser beam detects blood cell motion from Doppler broadening of the laser light scattered from the skin. Thereby circulating blood in the microvasculature causes a shift in the Doppler frequency of the scattered laser light that is photo detected and subsequently processed to build up a colour coded map of blood flow. At the same time, a digital camera records the photograph, which corresponds closely to the blood flow (Essex and Byrne, 1991). This allows the comparison of blood flow in

hindlimbs where blood flow has been reduced by occlusion of the femoral artery, relative to the control hindlimb.

Measurements were performed over a number of days, before, immediately after surgery, and on postoperative days 3, 6, 9, and 14 to assess the reperfusion facilitated by the development of collateral vessels. To analyse the perfusion, mean blood flow between the operated and non-operated hindlimbs was compared and the ratio for each animal was calculated (right-to-left (R/L) perfusion ratio).

III.11 Statistical Analysis

Data represent mean ± SEM. Statistical analysis was performed with Prism 4 software (Graph Pad) using unpaired Student-t test or one-way analysis of variance (ANOVA) followed by Newmann-Keuls post hoc test, as appropriate. p-values below 0.05 were considered statistically significant.

IV RESULTS

IV.1 The Role of the CXCL12 / CXCR4 Axis in Experimental Myocardial Infarction

Myocardial necrosis triggers complex remodeling and inflammatory changes. The CXCR4 receptor ligand CXCL12 has been implicated in protection and regeneration after myocardial infarction through recruiting angiogenic outgrowth cells, improving neovascularization and cardiac function. Here I assess the intrinsic functions of CXCR4 in remodeling after MI using CXCR4-heterozygous ($CXCR4^{+/-}$) mice.

IV.1.1 Analysis of MI Size and Inflammatory Cell Content

Four weeks after MI, the infarct size was reduced 42% more in $CXCR4^{+/-}$ than in $CXCR4^{+/+}$ littermates (8.7±1.3% vs. 14.9±1.9% of the ventricular wall; $p<0.05$; Figure 11 A). Area at risk defined by TTC staining showed no difference between the two groups (Figure 11 B). Hence, the reduced infarct size likely reflects an enhanced wound contraction, rather than a difference in the initial extent of cardiomyocyte injury. Moreover, the myofibroblast infiltration (2,600±283 vs. 1,011±165/mm² in controls, $p<0.001$) and collagen content in the infarcted area was significantly higher in $CXCR4^{+/-}$ mice than in wild-type controls ($p<0.05$; Figure 11 C), indicating a more stable and robust scar formation.

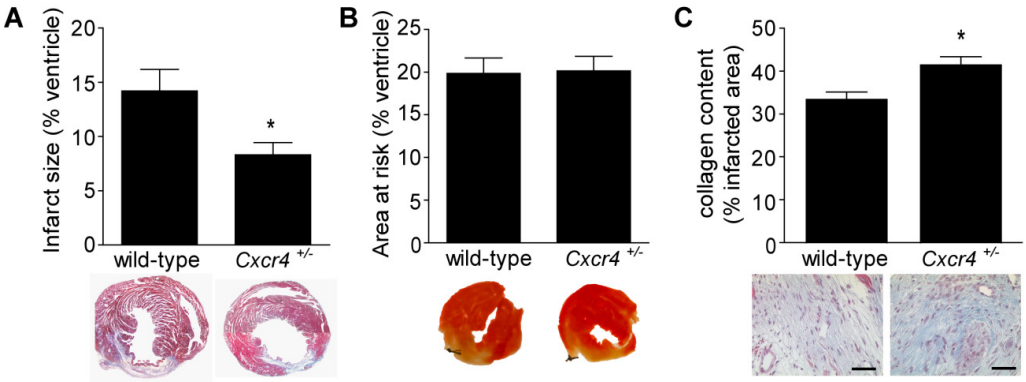

Figure 11: Analysis of myocardial infarction (MI). Compared to wild-type mice, $CXCR4^{+/-}$ mice display a significantly smaller infarct size (**A**) but no differences in the area at risk one day after MI (**B**), and a significant increase in myocardial collagen content (**C**, scale bar 50 µm) 4 weeks after MI, (*$p<0.05$ vs. wild-type).

Next, I analyzed the mobilization and recruitment of inflammatory cells after MI. The MI-induced and transient expansion of neutrophils in the circulation (Figure 12 A) and infiltration of the infarcted area with neutrophils (Figure 12 B) was severely reduced in CXCR4$^{+/-}$ mice 1 day after MI. This shows that the initial inflammatory response differed markedly in CXCR4$^{+/-}$ mice, indicating a prominent role of CXCR4 in post-infarction neutrophil recruitment. Further, while no difference in peripheral blood monocyte levels between conditions was apparent after MI (Figure 12 C), the myocardial infiltration with monocytes/macrophages was diminished 4 days after MI in CXCR4$^{+/-}$ compared to wild-type mice, but subsequently increased resulting in a higher myocardial macrophage content at 14 days after MI (Figure 12 D, E).

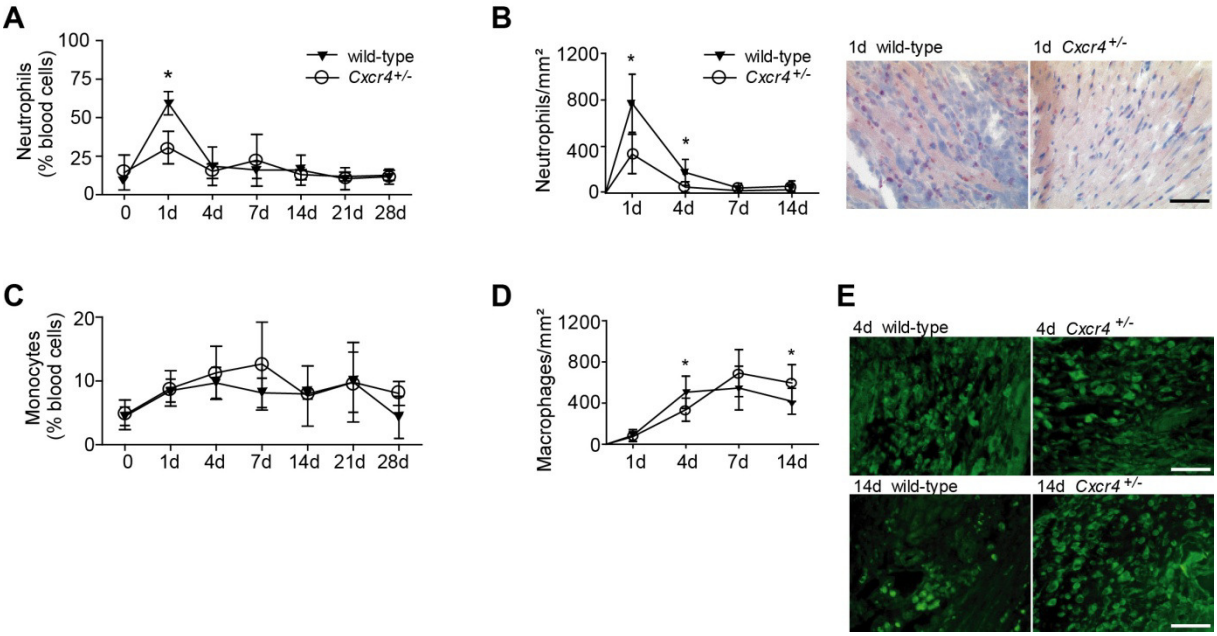

Figure 12: Analysis of inflammatory cells after myocardial infarction (MI). Neutrophil counts in peripheral blood as analyzed by flow cytometry (**A**), as well as neutrophil infiltration in myocardium, as analyzed by immunofluorescence (**B**, scale bar 50 µm) are reduced in CXCR4$^{+/-}$ versus wild-type mice after MI. No differences were observed in total monocyte counts in peripheral blood (**C**), whereas myocardial infiltration with monocytes/macrophages was delayed and enhanced at later stages in CXCR4$^{+/-}$ versus wild-type mice (**D**, **E**, scale bar 50 µm) (*p<0.05 vs. wild-type).

Analysis of monocyte subsets revealed a reduced number of circulating Gr-1^{high} cells and relative expansion of Gr-1^{low} cells and subsequently lower Gr-1^{high}/Gr-1^{low} ratio in peripheral blood of CXCR4$^{+/-}$ after MI (Figure 13 C). These data correspond to diminished infiltration with proinflammatory tissue-degrading Gr-1^{high} monocytes 4 days after MI, whereas Gr-1^{low} monocytes, known to promote wound healing and collagen deposition (Nahrendorf et al., 2007), were increased in the hearts of CXCR4$^{+/-}$ (Figure 13 D). Thus, the inflammatory reaction after MI in CXCR4$^{+/-}$ mice is shifted to an earlier termination of the acute response and onset of a repair process involving Gr-1^{low} monocytes. Notably, whereas CXCR4 expression on Gr-1^{low} monocytes from CXCR4$^{+/-}$ mice (specific mean fluorescence intensity/sMFI 31.5±3.0) was reduced by 43%, as compared to Gr-1^{low} monocytes from wild-type mice (sMFI 61.9±7.4) (Figure 13 A), the low CXRC4 expression on Gr-1^{high} monocytes did not differ between both genotypes (sMFI 6.8±3.5 vs. 5.2±1.5) (Figure 13 B). This suggests a strong adaptation of Gr-1^{low} cells to reduced CXCR4 expression and a possible role of other receptors in their recruitment.

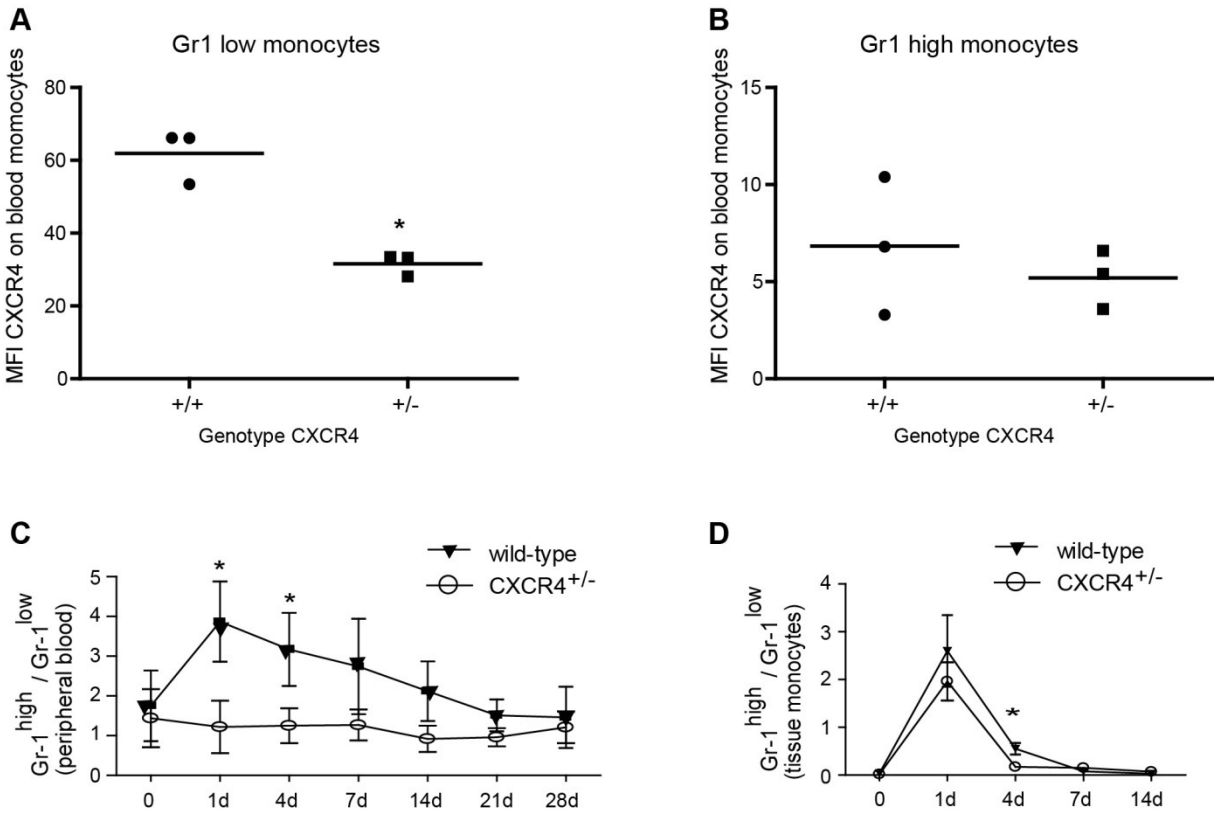

Figure 13: Analysis of Gr-1^{low} / Gr-1^{high} monocytes after myocardial infarction.
Flow cytometry analysis after MI of CXCR4 expression on Gr-1^{low} (**A**) and Gr-1^{high} (**B**) demonstrated a reduced expression of CXCR4 on Gr-1^{low} monocytes from CXCR4$^{+/-}$ compared to wild-type mice (*p<0.05 vs. wild-type), whereas the already low expression of CXCR4 on Gr-1^{high} monocytes did not differ between the groups. The Gr-1^{high} / Gr-1^{low} ratio showed a reduction of Gr-1^{high} cells over time and an expansion of Gr-1^{low} cells in peripheral blood of CXCR4$^{+/-}$ versus wild-type mice, representing a relative shift towards Gr-1^{low} cells (**C**). Analysis of the myocardial infiltration revealed a transient but marked reversal of the Gr-1^{high} / Gr-1^{low} ratio at day 1 after MI in both groups (**D**) and an earlier return to baseline levels with increased at day 4 with increased infiltration of Gr-1^{low} cells in CXCR4$^{+/-}$ versus wild-type mice. (*p<0.05 vs. wild-type).

IV.1.2 Analysis of Cardiac Function after MI

Echocardiography (Table 3) and Langendorff (Table 4) measurements surprisingly failed to reveal changes in ventricular function, in particular no improvement of contractility, after MI in CXCR4$^{+/-}$ versus wild-type mice was found. However, I observed a slightly decreased baseline ejection fraction, as well as a moderate increase in the post-MI ejection fraction in CXCR4$^{+/-}$ mice compared with wild-type mice (Table 3). Moreover, the difference in ejection fraction before and after MI was

significantly decreased in CXCR4$^{+/-}$ mice compared with wild-type (7.6±1.2% vs. 16.8±2.4%, p<0.01), implying a protective or adaptive mechanism in CXCR4$^{+/-}$ hearts.

Table 3: Echocardiography parameters in wild-type and CXCR4$^{+/-}$ mice (LV - left ventricle)

Echocardiography				
Parameter	**Before MI**		**4 weeks after MI**	
	wild-type	CXCR4$^{+/-}$	*wild-type*	CXCR4$^{+/-}$
	n=6-9	*N=7-10*	*n=6*	*n=7*
Heart weight (mg)	128 ± 28	114 ± 35	150 ± 52	153 ± 17
Heart rate (bpm)	476± 66	464 ± 31	442 ± 26	510 ± 37
Respiration rate (bpm)	122 ± 40	116 ± 32	144 ± 26	156 ± 64
Cardiac output (ml/min)	22.1 ± 5.7	18.4 ± 3.8	19.1 ± 3.2	20.3 ± 6.2
Stroke volume (µl)	41.7 ± 9.4	39.3 ± 9.0	40.2 ± 6.8	40.2 ± 10.9
Ejection fraction (%)	74.7 ± 2.0	69.8 ± 9.6	54.8 ± 8.2	63.9 ± 11.0
Fractional shortening (%)	42.8 ± 2.3	39.3 ± 7.5	31.5 ± 4.3	34.7 ± 5.6
LV diameter; d (mm)	3.4 ± 0.8	3.4 ± 0.3	4.2 ± 0.4	4.1 ± 0.6
LV diameter; s (mm)	2.0 ± 0.9	1.9 ± 0.3	2.9 ± 0.3	2.7 ± 0.7

Table 4: Functional parameters of wild-type and CXCR4$^{+/-}$ hearts. (LVDP - left ventricular developed pressure; dPdt - derivative of pressure increase (max) and decay (min); *p<0.05 vs. wild-type.)

Langendorff perfusion				
Parameter	**Before MI**		**4 weeks after MI**	
	wild-type	CXCR4$^{+/-}$	*wild-type*	CXCR4$^{+/-}$
	n=3-4	*n=3-5*	*n=4-5*	*n=5-6*
LVPD (mmHg)	86.7 ± 8.8	78.3 ± 17.0	40.0 ± 10.1	50.0 ± 11.5
increase after Dobutamin (Δ)	40.3 ± 4.0	46.2 ± 5.2	13.8 ± 4.3	10.3 ± 2.6
dPdt max (mmHg/sec)	3189 ± 99	3058 ± 363	1540 ± 97	1590 ± 94
increase after Dobutamin (Δ)	2410 ± 629	1941 ± 187	770 ± 91	704 ± 74
dPdt min (mmHg/sec)	-2695 ± 244	-2011 ± 411	-1267 ± 111	-1280 ± 79
increase after Dobutamin (Δ)	-1756 ± 544	-1407 ± 314	545 ± 28	484 ± 68
Coronary flow (ml)	**3.9 ± 0.2**	**2.0 ± 0.3***	**3.2 ± 0.3**	**1.2 ± 0.3***
Increase after short ischemia (Δ)	**3.4 ± 0.2**	**2.8 ± 0.1***	**1.8 ± 0.4**	**0.2 ± 0.1***

Moreover, coronary perfusion was markedly diminished in CXCR4$^{+/-}$ versus wild-type mice, as determined by coronary flow measurements in isolated perfused hearts. Coronary flow was already reduced under baseline conditions ($p<0.01$; Table 4, Figure 14 A). Ligation of the LAD decreased coronary flow by approximately 50% in both groups after one day, demonstrating an equivalent area of risk in CXCR4$^{+/-}$ and wild-type mice (Figure 14 A, B). Four weeks after MI, the recovery of coronary perfusion was significantly impaired in CXCR4$^{+/-}$ mice, resulting in a reduced flow rate of 1.2±0.3 ml/min compared with 3.2±0.3 ml/min in wild-type mice ($p<0.05$; Table 4, Figure 14 A, B).

To assess whether defective cardiac angiogenesis and neovascularization after MI contributes to these differences in coronary blood flow, myocardial endothelial cells and vessels were quantified. As determined by flow cytometry, the number of myocardial endothelial cells was intrinsically reduced in CXCR4$^{+/-}$ versus wild-type mice (0.60±0.04% vs. 2.04±0.30% CD31$^+$ KDR/VEGF-R2$^+$ cells, $p<0.05$, Figure 14 C). Similarly, neovascularization after MI was impaired in CXCR4$^{+/-}$, as evident by reduced formation of CD31$^+$ blood vessels in infarcted myocardium ($p<0.05$, Figure 14 D). This reduction might contribute to the defective recovery of coronary flow after MI in CXCR4$^{+/-}$ mice. Moreover, the number of primary branches of coronary arteries (56.7±5.2 vs. 30.0±6.9, $p<0.05$, Figure 14 E) as shown by Micro-Angio-CT, as well as epicardial coronary veins, and the number of cremasteric artery branches as quantified by intravital microscopy was diminished in CXCR4$^{+/-}$ versus wild-type mice without evidence for disturbed endothelial permeability, as showed by perfusion of the cremasteric artery with albumin-FITC. This indicates that the endogenous defect in angiogenesis was not restricted to the heart.

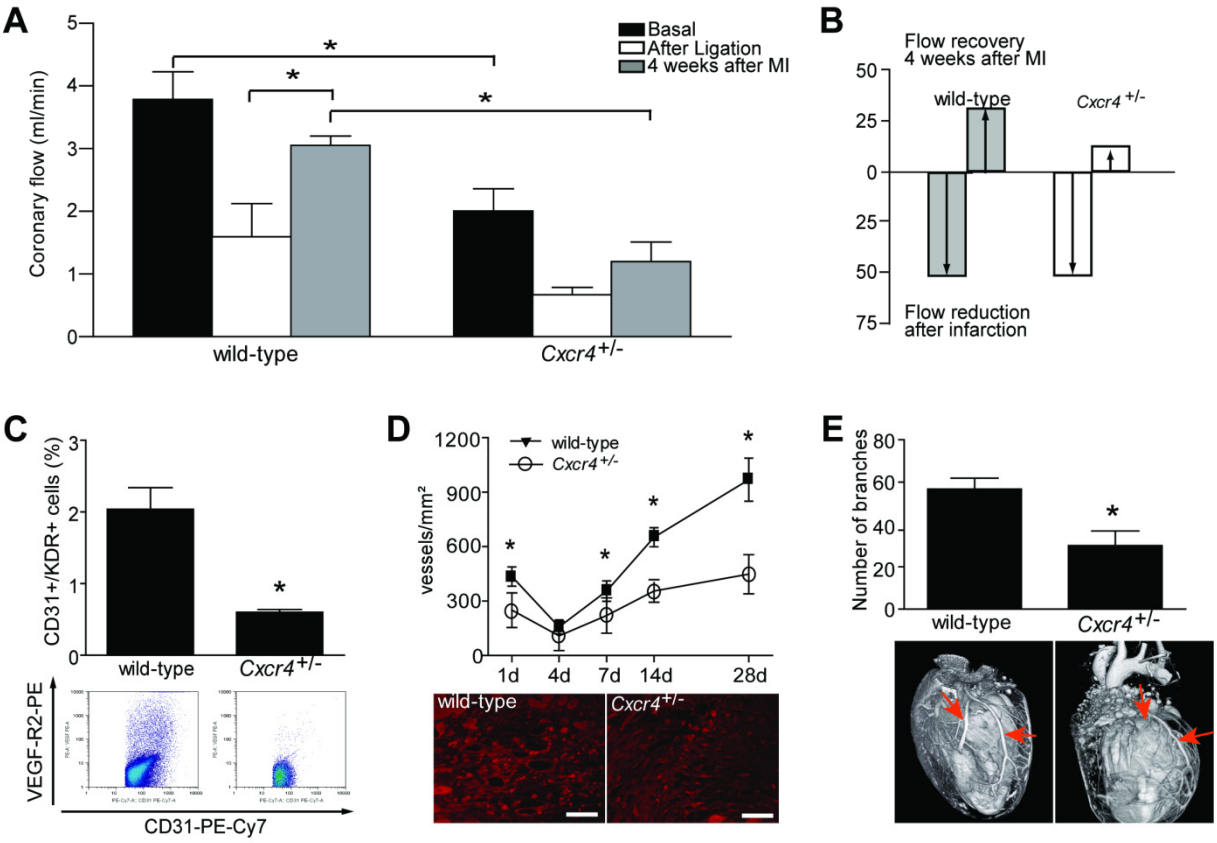

Figure 14: Analysis of coronary flow and angiogenesis. Langendorff perfusion revealed a reduction of coronary flow in CXCR4$^{+/-}$ versus wild-type mice both at basal conditions and 4 weeks after MI (**A**). Despite equivalent flow reduction after MI, recovery of coronary flow after MI, represented as percentage of basal coronary flow, was impaired in CXCR4$^{+/-}$ versus wild-type mice 4 weeks after MI (**B**). Flow cytometry analysis indicated a decreased number of endothelial cells in myocardial tissue, as determined by co-staining for CD31 and KDR (**C**). Immunohistochemical analysis of the MI area at different time points after MI confirmed the impairment in neo-angiogenesis, as assessed by the density of vessels stained CD31 in CXCR4$^{+/-}$ versus wild-type mice (**D**, scale bar 50 µm). The quantification of the branches of the coronary artery (**E**) showed a marked impairment of vessel density in CXCR4$^{+/-}$ versus wild-type mice under basal conditions (red arrows show left anterior descending and circumflex arteries). (* $p<0.05$ vs. wild-type).

To distinguish the influence of the CXCR4 heterozygous background and its role in circulating cells, bone marrow chimera experiments were performed after lethal irradiation. The next day, I reconstituted wild-type mice with CXCR4$^{+/-}$ bone marrow, CXCR4$^{+/-}$ mice with wild-type bone marrow, and wild-type mice with wild-type bone marrow (control). Six weeks later, MI was induced and after another four weeks, the infarction area was significantly reduced in wild-type mice transplanted with CXCR4$^{+/-}$

bone marrow and in CXCR4$^{+/-}$ mice transplanted with wild-type bone marrow compared with control group (14.5±2.2 and 16.2±4.2, vs. 20.8±0.8, $p<0.05$, Figure 15 A). Besides effects attributable to reduced leukocyte infiltration, these data suggest the existence of an additional intrinsic mechanism, which can substantially influence scar formation in our model. Notably, neovascularization after MI was impaired in both groups (wild-types transplanted with CXCR4$^{+/-}$ bone marrow and CXCR4$^{+/-}$ mice transplanted with wild-type bone marrow) compared with controls, as evident by CD31^{+} staining in infarcted myocardium (856±73 vessels/mm² and 527±43 vessles/mm² and 1046±73 vessels/mm² in control, respectively, $p<0.05$ vs. control, Figure 15 B). Whereas heart function, as assessed by echocardiography (ejection fraction) and Langendorff perfusion (left ventricular developed pressure) showed no significant differences between the groups (Figure 15 C). The reduction of neovascularization was more pronounced in CXCR4$^{+/-}$ mice transplanted with wild-type bone marrow and correlated with decreased coronary flow as evident by Langendorff perfusion of isolated hearts (Figure 15 D). This may reflect that the vascularization of the scar is based mostly on vessel formation around pre-existing collaterals, which may explain the markedly reduced neovascularization in CXCR4$^{+/-}$ mice despite reconstitution with wild-type bone marrow.

The myocardial infiltration with neutrophils and monocytes, as well as blood leukocyte subsets (Figure 15 E) after MI did not differ between wild-type and CXCR4$^{+/-}$ mice transplanted with wild-type bone marrow but were significantly reduced in wild-type mice transplanted with CXCR4$^{+/-}$ bone marrow, thus emulating the pattern in CXCR4$^{+/-}$ mice and indicating a shift towards an earlier termination of the acute response and earlier onset of a repair process.

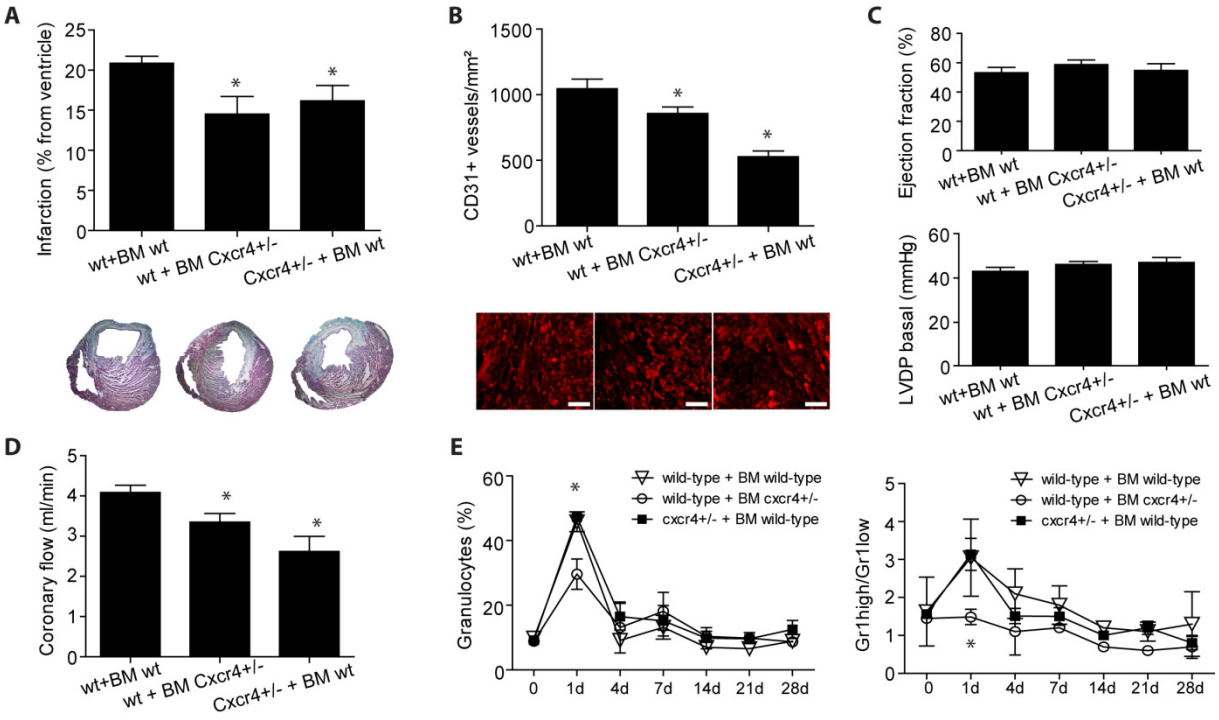

Figure 15: Effect of CXCR4 deficiency in bone marrow cells after myocardial infarction (MI). Four weeks after MI, the infarction area (**A**) and neovascularization, as evident by $CD31^+$ staining in infarcted myocardium (**B**, scale bar 50 µm), was reduced in wild-type mice transplanted with $CXCR4^{+/-}$ bone marrow and in $CXCR4^{+/-}$ mice transplanted with wild-type bone marrow, as compared to wild-type controls transplanted with wild-type bone marrow. Ejection fraction, as assessed by echocardiography, and left ventricular developed pressure, as assessed by Langendorff perfusion, were unaltered (**C**). Coronary flow, as determined by Langendorff perfusion of isolated hearts revealed reduced perfusion in wild-type mice transplanted with $CXCR4^{+/-}$ bone marrow and in $CXCR4^{+/-}$ mice transplanted with wild-type bone marrow compared with control (**D**). As analyzed by flow cytometry, blood leukocyte subsets counts in peripheral blood after MI did not differ between $CXCR4^{+/-}$ mice and wild-type mice reconstituted with wild-type bone marrow, whereas wild-type mice reconstituted with $CXCR4^{+/-}$ bone marrow showed a reduction in blood neutrophils and Gr-1^{high} / Gr-1^{low} ratio (**E**), representing a relative shift towards Gr-1^{low} cells, similarly as observed in $CXCR4^{+/-}$ mice. (*$p<0.05$ vs. wild-type mice reconstituted with wild-type bone marrow).

IV.1.3 The Role of CXCR4 for EPC Trafficking and Function

Since EPCs contribute to post-infarction neo-angiogenesis, I studied the effect of CXCR4 on EPC function. Notably, spleen derived murine $CXCR4^{+/-}$ and wild-type EPCs were analyzed by flow cytometry after culturing for 10 days. Both exhibit endothelial-like properties, namely acLDL uptake, binding of lectin (Figure 16 A), VE-cadherin and von Willebrand factor mRNA expression (Figure 16 B) and surface expression of CD31 and VEGF-R2 (Figure 16 C). Despite this endothelial phenotype, the function of $CXCR4^{+/-}$ EPCs was impaired. In chemotaxis assays, the migration of

EPCs from CXCR4$^{+/-}$ versus wild-type mice towards CXCL12 (but not towards VEGF) was significantly reduced (Figure 16 D). Moreover, endothelial tube formation by CXCR4$^{+/-}$ versus wild-type EPCs *in vitro* (Figure 16 F) and vessel infiltration in subcutaneously implanted Matrigel plugs in CXCR4$^{+/-}$ versus wild-type mice (Figure 16 G) were significantly attenuated.

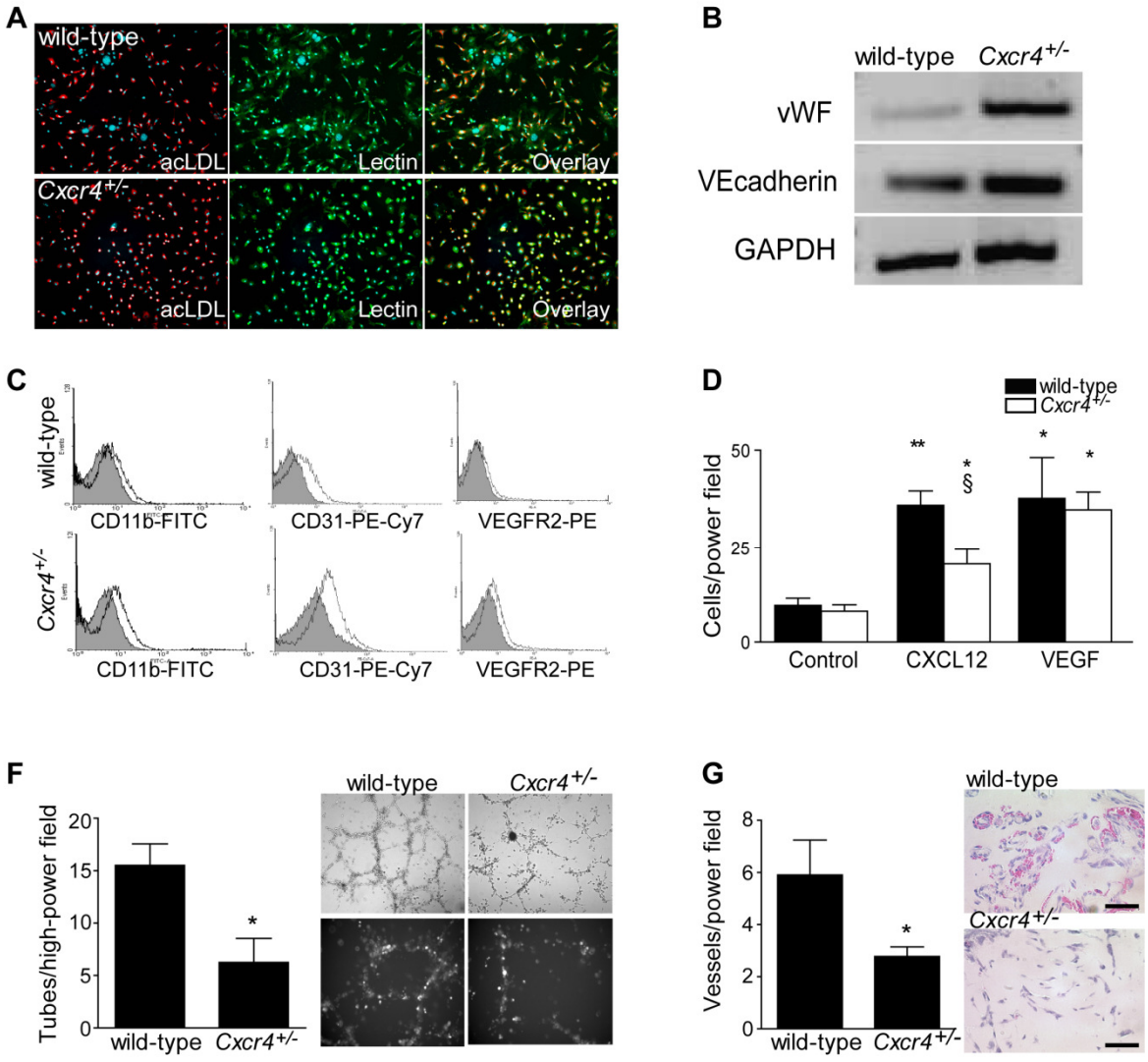

Figure 16: The role of CXCR4^{-} in trafficking and function of angiogenic outgrowth cells. Endothelial progenitor cells (EPCs) isolated from spleens of CXCR4$^{+/-}$ or wild-type mice exhibit endothelial cell-like properties after culture for 10 days, e.g. acLDL up-take, binding of lectin (**A**), vWF and VE-cadherin mRNA expression, as assessed by quantitative RT-PCR (**B**), and surface expression of CD31 and VEGF-R2, as analyzed by flow cytometry (**C**). In chemotaxis assays, CXCR4$^{+/-}$ EPCs showed deficient migration towards CXCL12 but not VEGF as compared to wild-type EPCs (**D**). Tube formation by CXCR4$^{+/-}$ or wild-type EPCs on matrigel *in vitro* (**F**) and neo-angiogenesis in subcutaneously implanted matrigel *in vivo* (**G**, scale bar 50 µm) were both significantly impaired. (*p<0.05 vs. wild-type).

Compared to wild-type mice, CXCL12 mRNA expression was increased in uninjured myocardium of CXCR4$^{+/-}$ mice, possibly reflecting compensatory induction due to reduced vascularization, but its up-regulation after MI was not affected in CXCR4$^{+/-}$ mice (Figure 17 A), indicating that differences in myocardial endothelial cell content and vessel density are rather attributable to defective recruitment of EPCs. No differences were observed in VEGF mRNA expression in CXCR4$^{+/-}$ and wild-type mice, before or after induction of MI.

IV.1.4 Myocardial Apoptosis after Myocardial Infarction

Given the unaltered cardiac function despite the reduced basal myocardial vascularization in CXCR4$^{+/-}$ compared with wild-type mice, I tested whether cardiomyocyte survival and proliferation were affected. The myocardial mRNA expression of Bax (pro-apoptotic marker) and Bcl2 (anti-apoptotic marker) was studied by RT-PCR in uninjured hearts. No difference in Bax and Bcl2 expression was observed between CXCR4$^{+/-}$ and wild-type mice (Figure 17 B). Myocardial apoptosis after induction of MI was slightly decreased 4 days but not 7 or 14 days after MI in CXCR4$^{+/-}$ compared to wild-type mice, as assessed by quantifying TUNEL^{+} cells (Figure 17 C). These data indicate a possible adaptive mechanism of CXCR4$^{+/-}$ cardiomyocytes to the reduced oxygen supply.

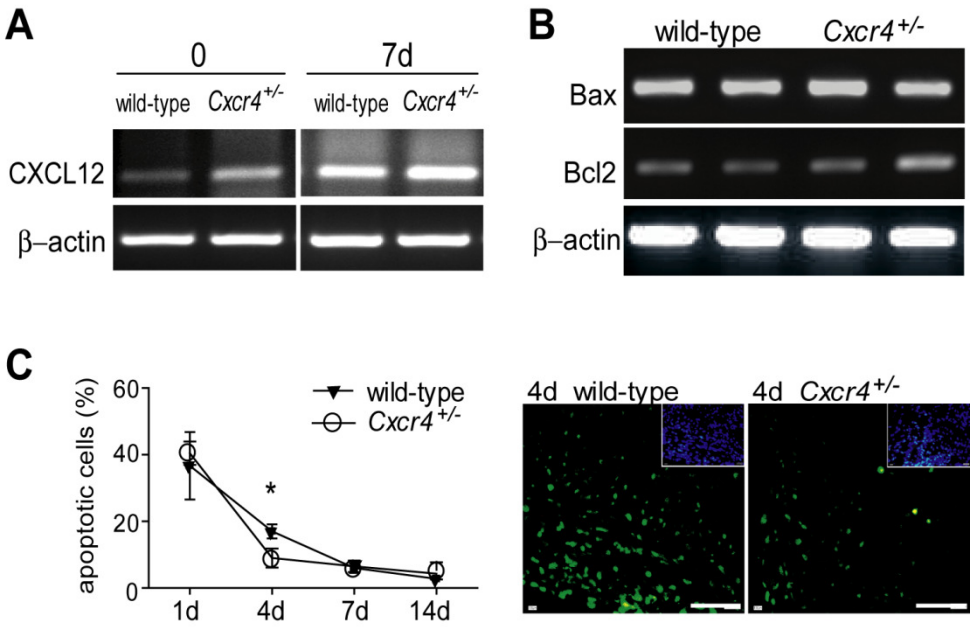

Figure 17: Myocardial apoptosis after myocardial infarction (MI). Expression of CXCL12 mRNA was increased in uninjured myocardium of CXCR4$^{+/-}$ mice but it's up-regulation after MI was not affected in CXCR4$^{+/-}$ mice (**A**), as shown by RT-PCR. Moreover, no differences in myocardial Bax and Bcl2 mRNA expression were observed in CXCR4$^{+/-}$ versus wild-type, as assessed by RT-PCR from uninjured hearts (**B**). Myocardial apoptosis assessed by TUNEL staining at different time points after MI showed reduced apoptosis in CXCR4$^{+/-}$ versus wild-type 4 day after MI, and no differences at later time-points (**C**, scale bar 50 µm, *p<0.05 vs. wild-type).

IV.1.5 Electron Microscopy and Characterization of Lipid Extracts

To detect structural cellular alterations, I performed electron microscopy in CXCR4$^{+/-}$ and wild-type hearts before or 1 day after MI. Wild-type myocardium displayed extensive signs of necrosis with dramatic structural changes and cellular disintegration (Figure 18 A). In infarcted CXCR4$^{+/-}$ myocardium, signs of myofibril disorganization and cardiomyocyte swelling were present but cellular structures were still distinguishable (Figure 18 A). Notably, atypical lipofuscin-like lipid accumulations were found in uninjured CXCR4$^{+/-}$ myocardium with strong osmium tetroxide fixation (Figure 18 A). Such lipid accumulations have been described after dietary fish oil feeding, containing long-chain monounsaturated fatty acids and a degraded form of peroxidized lipids, and have been associated with reduced ischemic damage in rat hearts (Nalbone et al., 1988; Yang et al., 1993).

Subsequent lipid extraction and HPLC analysis indicated a marked accumulation of phosphatidylserine (PS, fractions 1-3) in CXCR4$^{+/-}$ hearts, which was absent in wild-type hearts (Figure 18 B). Using gas chromatography, I analyzed the unsaturated fatty acid index of cardiac lipid extracts (triglyceride, phosphatydilcholine/-ethanolamine, PS). The triglyceride fraction (TG) obtained from CXCR4$^{+/-}$ hearts contained 10% more unsaturated fatty acids than wild-type hearts, and six different unsaturated fatty acids appeared in TG of CXCR4$^{+/-}$ hearts, namely C16:1, C18:3n6, C20:1, C20:3n6, C22:1n9 and C22:2. No differences were noted for saturated/unsaturated fatty acid content in phosphatydilcholine or -ethanolamine. The fatty acid composition of the three PS fractions is detailed in Table 5.

	Saturation (%)		Fatty acids (%)	
PS 1	Saturated	59.8	C14:0	25.7
			C16:0	10.5
			C18:0	14.3
			C22:0	9.3
	Unsaturated	26.4	C18:1n9c	4.2
			C18:2c	5.5
			C18:3n6	3.9
			C20:4n6	11.5
			C22:6n3	11.4
PS 2	Saturated	47.2	C16:0	13.8
			C18:0	23.0
			C22:0	10.4
	Unsaturated	42.5	C18:1n9c	12.0
			C18:2c	7.7
			C22:6n3	22.8
PS 3	Saturated	19.0	C16:0	4.3
			C18:0	8.4
			C22:0	6.3
	Unsaturated	76.9	C14:1	1.8
			C16:1	4.7
			C18:1n11c	11.6
			C18:2c	11.2
			C18:3n6	9.5
			C20:1	11.3
			C20:3n6	9.5
			C22:1n9	3.0
			C22:2	4.6
			C22:6n3	9.2

Table 5: Fatty acid composition of PS fractions from CXCR4$^{+/-}$ hearts.

To evaluate a potential contribution of the PS fractions to cardioprotection in $CXCR4^{+/-}$ mice, isolated cardiomyocytes were pre-incubated with the PS fractions 1, 2 or 3 for 3 hours and the response to hypoxic stress was analyzed. As evident by DHE staining to monitor radical formation, all PS fractions protected cardiomyocytes against hypoxic injury (Figure 18 C). Notably, PS fraction 1 with the highest percentage of saturated fatty acids offered the best protection to hypoxic injury. These data indicate that $CXCR4^{+/-}$ hearts could be intrinsically adapted to hypoxic injury, together with an attenuated inflammatory response explaining the smaller MI size despite impaired neovascularization.

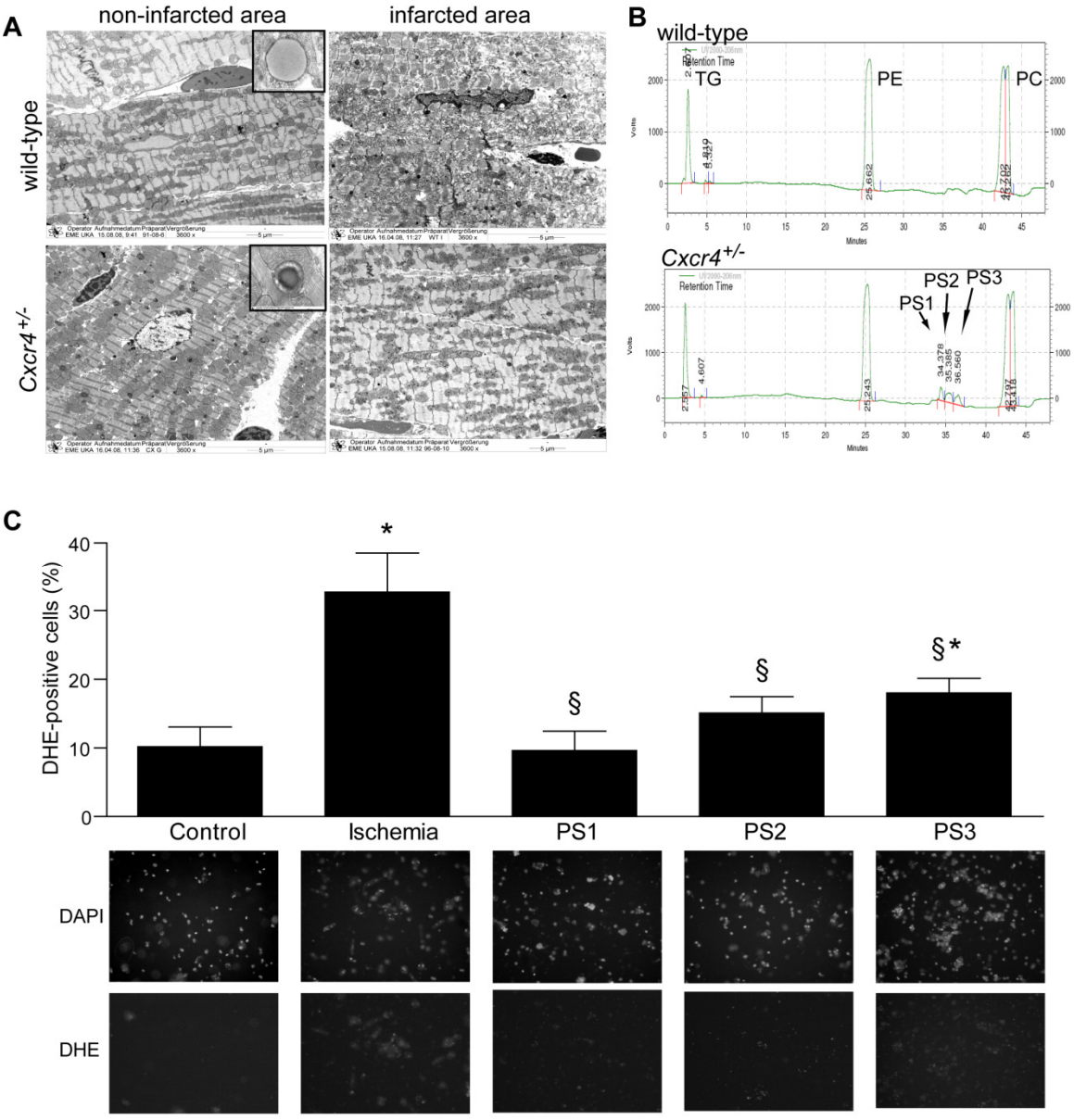

Figure 18: Myocardial electron microscopy and characterization of lipid extracts. Electron microscopy showed atypical lipofuscin-like lipid accumulation with a strong osmium fixation (**A**, left side, one representative lipid vesicle in insert). After MI, CXCR4$^{+/-}$ myocardium exhibits signs of myofibril disorganization and cardiomyocyte swelling but still distinguishable cellular structures, whereas wild-type myocardium shows signs of necrosis with dramatic structural disintegration (**A**, right side). Lipid extraction and HPLC analysis indicates, beside the triglyceride (TG, first peak), phosphatydilethanolamine (PE, second peak) and phosphatydilcholine (PC, third peak), an up-regulation of the phosphatidylserine (PS) in CXCR4$^{+/-}$ hearts, which is not present in wild-type hearts (**B**). *In vitro* hypoxia experiments, pre-incubation of isolated cardiomyocytes with PS fractions protects against hypoxic injury, as shown by DHE staining (**C**, *p<0.01 vs. control, §p<0.05 vs. ischemia, n=4).

IV.2 MIF-induced Angiogenic Potential of EPCs

Stem cell-induced angiogenesis, including the use of endothelial progenitor cells, as a process of neovascularization has obtained great interest for treating cardiovascular ischemic diseases. Angiogenesis is a highly regulated complex process depending on a cocktail of factors and involving multiple steps. MIF, but also VEGF, CXCL12 and CXCL1 are likely involved in this process. To better understand the behavior of EPCs in response to different growth factors, I evaluated the angiogenic potential of these different factors; associated with the function of EPCs in angiogenesis.

IV.2.1 Characterization of Spleen-derived EPCs

Flow cytometry analysis of spleen-isolated EPCs revealed combined expression of endothelial (CD31, VEGFR-2) and myeloid cell markers (CD11b) (IV.1.3). Furthermore, CXCR2 and CXCR4 were present on the cell surface of these cells (Figure 19), whereas CD74, which can bind MIF and potentially functions as a co-receptor for CXCR2 and CXCR4, was not expressed.

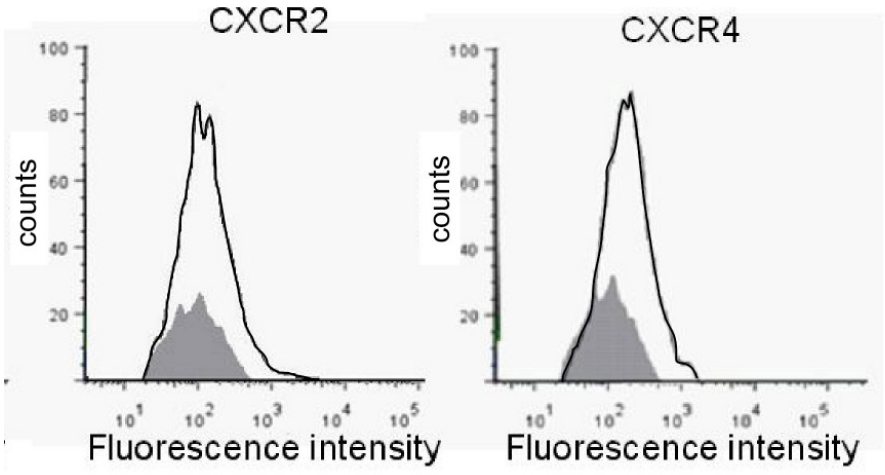

Figure 19: Characterization of spleen-derived EPCs. The expression of CXCR2 and CXCR4 was assessd in spleen-derived EPCs by flow cytometry; representative histograms are shown, unstained cells served as control (grey graph).

IV.2.2 *In vitro* Chemotaxis and Transmigration of EPCs

Chemotaxis assay was performed in a 24-well cell culture chamber. EPCs were added into the membrane inserts. The culture chamber contained assay medium only as control or enriched with different chemokines/cytokines in the lower chamber which served as chemoattractants for these cells. Chemotaxis experiment showed that EPCs migrate through the filter over 2 hstimulation time. The potential of EPCs to migrate was multiplied towards chemokine/cytokine stimulation compared to unstimulated cells (assay medium only). rmVEGF and rmMIF increased fivefold and fourfold the chemoattraction of EPCs compared to control, respectively. The chemotactic effect of rmCXCL12 and rmCXCL1 has tripled compared to control (Figure 20 A, $p<0.05$ vs. control). In contrast to the chemotaxis assay, the transmigration assay was performed using an additional SVECs monolayer on the surface of the filter membrane. The transmigration capacity of EPCs over this endothelial monolayer was examined using a modified Boyden chamber. In contrast to the effect of the stimuli rmVEGF, rmMIF, rmCXCL12 and rmCXCL1 on EPC chemotaxis, transmigration was highest for MIF, inducing transmigration 2.6-fold. This was followed by rmCXCL1 with a significant lower number 1.7-fold to higher than control (Figure 20 B, $p<0.05$ vs. control). Contrary to the chemotaxic assay, rmCXCL12 and rmVEGF presented with transmigration capacity compared to control (Figure 20 B). These data demonstrate that rmMIF is a potent trigger for chemotaxis and transmigration of EPCs.

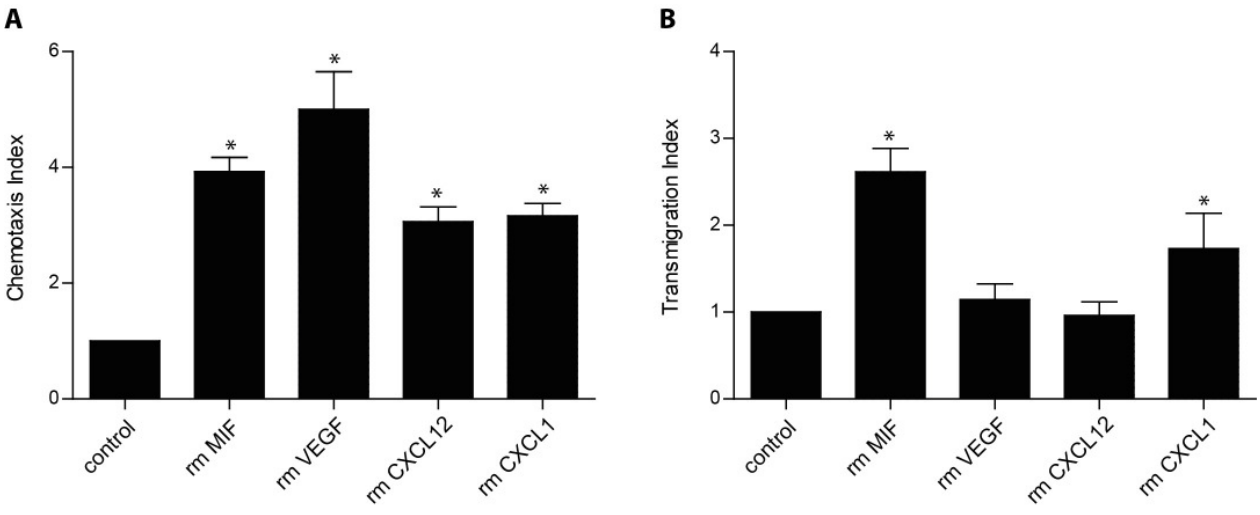

Figure 20: The role of chemokines and growth factor in EPC recruitment. rmMIF, rmVEGF, rmCXCL12 and rmCXCL1 enhance the chemotactic potential of EPCs to migrate through the filter (**A**). EPCs transmigration over an additional monolayer of SVECs was enhanced after stimulation with rmMIF and rmCXCL1, whereas rmVEGF and rmCXCL12 failed to increase these capacity (**B**). For statistical analysis, controls were set at 1 and the experimental groups were normalized to 1 (*$p<0.05$ vs. control, n=3).

IV.2.3 Tube Formation Potential of EPCs *in vitro*

To study the angiogenic potential of rmMIF, rmVEGF, rmCXCL12 and rmCXCL1 on tube formation capacity of EPCs, matrigel experiments were performed. EPCs on the matrigel alone showed no capability to form tube-like structures. Therefore, co-culture of EPCs and SVECs were used and placed together on matrigel basement. After incubation for 24 h, sprouting of SVECs was enhanced. All test factors significantly increased the integration of EPCs and the formation of tube-like structures on the matrigel *in vitro* (Figure 21 A). Thereby rmCXCL12 and rmCXCL1 showed the highest potential to form tubes followed by rmVEGF and rmMIF compared to control (12.5 +/- 0.95 > 12.0 +/- 1.3 > 11.4 +/- 0.67 > 9.0 +/- 0.54 vs. control 4.3 +/- 0.66, respectively, $p<0.05$ vs. control). In figure 21 B, representative pictures show the alterations in tube forming potential of EPCs according to the added test factors.

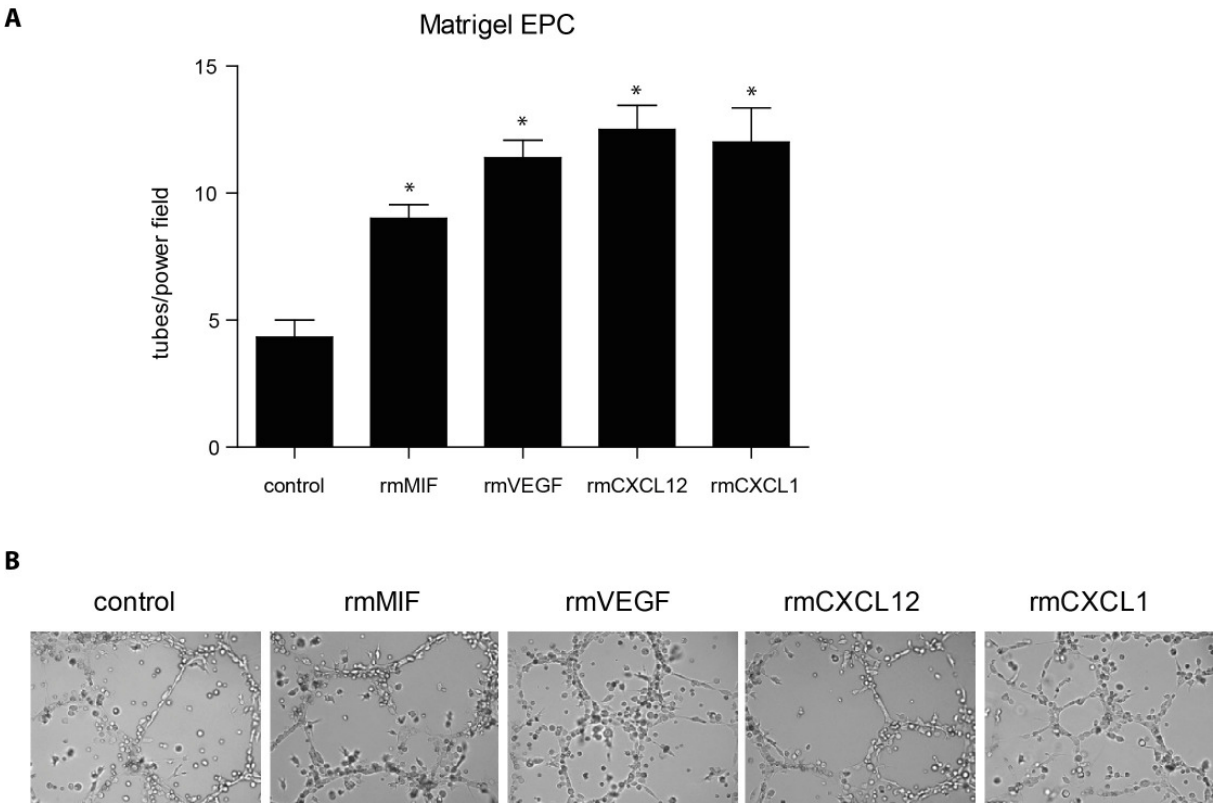

Figure 21: Effect of stimulation on EPC-SVEC tube formation. Number of formed tubes on matrigel by co-culture of EPCs and SVECs after 24 h *in vitro* triggered by rmMIF, rmVEGF, rmCXCL12 and rmCXCL1 (*$p<0.05$ vs. control, n=6) (**A**). Representative pictures show the tube formation by all test factors on matrigel *in vitro* (**B**).

IV.3 The Role of Macrophage Migration Inhibitory Factor in Collateral Formation

The importance of leukocytes in collateral artery growth (arteriogenesis) has been recognized. Proper recruitment of monocytes and their differentiation into macrophages are indispensable for arteriogenesis. Macrophage migration inhibitory factor (MIF) known as an inflammatory cytokine with chemokine-like functions has been associated with macrophage function related to atherogenesis and angiogenesis. Here, I assess the effect of MIF in collateral formation.

IV.3.1 Analysis of Collateral Growth and Inflammatory Cell Content

To determine whether MIF can affect arteriogenesis, paw reperfusion was examined in a murine ischemic hindlimb model as a reflection of collateral remodeling.

Reperfusion of ischemic hindlimb in mice treated with saline (control, CTL), recombinant murine MIF (rmMIF), or anti-MIF murine antibody (anti-MIF mAb) was measured by Laser Doppler imaging at day 3, 6, 9, and 14 after ligation.

All treated mice showed perfusion recovery of the occluded hindlimb although differences in reperfusion were apparent (Figure 22 A). At all time points analyzed rmMIF-treated mice showed depressed perfusion rates in comparison to CTL, whereas the anti-MIF group presented the opposite effect (increased recovery). Significant differences (relative rates) in perfusion rate were apparent between anti-MIF and rmMIF groups at day 6 (0.49+/-0.04 vs. 0.39+/-0.02, $p<0.05$), day 9 (0.55+/-0.03 vs. 0.42 +/-0.03, $p<0.05$) and day 14 post-ligation (0.55+/-0.02 vs. 0.37+/-0.4, $p<0.05$). Furthermore, anti-MIF mAb treatment increased reperfusion compared to control but did not reach significance. Comparing rmMIF with control, reperfusion was diminished after rmMIF treatment. Significant differences were observed at day 14 between groups (0.37+/-0.04 vs. 0.5+/-0.02, $p<0.05$) (Figure 22 A, B).

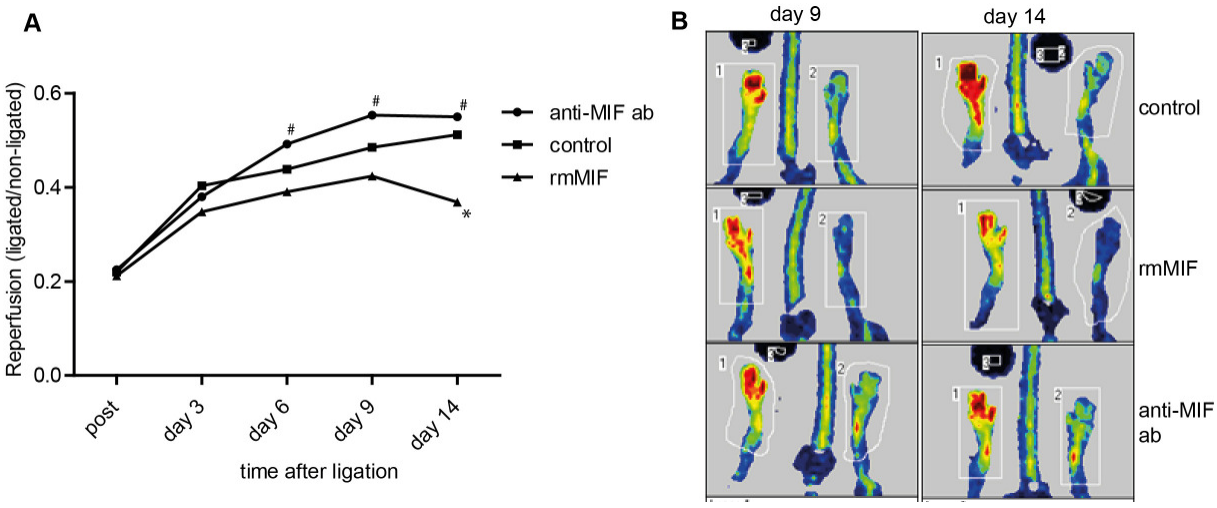

Figure 22: Analysis of Collateral Growth. (**A**) Reperfusion data of the ligated hindlimb presented as ratio of hindlimb perfusion right (ligated) to left (non-ligated). Anti-MIF mAb treatment enhanced reperfusion, whilst rmMIF decreased reperfusion. At day 6, 9 and 14, significant differences were observed between the anti-MIF mAb and rmMIF group (#$p<0.05$ vs. rmMIF) and at day 14 between the rmMIF and control group (*$p<0.05$ vs. control) (**B**) Representative Laser Doppler Images of the experimental groups at day 9 and 14 post ligation. Blue indicating minimal and red maximal perfusion. Mice treated with anti-MIF mAb or CTL showed clear recovery of perfusion for the ligated paw (area 2). Recovery in the rmMIF group was much lower at both day 9 and 14 compared with both anti-MIF and CTL group, as reflected by lower perfusion rate of the ligated paw in the rmMIF group.

Considering published data, macrophages express high levels of CXCR2 (Baggiolini, 1998; Boisvert et al., 1998; Patterson et al., 2002). CXCR2/Mac2 double staining showed that macrophages in the ischemic skeletal muscle express CXCR2. The immunohistochemical stainings of ischemic skeletal muscle tissue at day 2 post-ligation for macrophages (Mac2) and functional MIF receptor CXCR2 illustrated that CXCR2 was at a significantly lower level in the rmMIF group compared to the anti-MIF group (13,69±1,25 cells/field vs. 36,16±1,8 cells/field) (Figure 23 A). Also Mac2 staining revealed a similar tendency in the rmMIF group with a lower amount of macrophages being present in hindlimb tissue as compared with control (16.54±0.69 cells/field vs. 36.25±1.68 cells/field in controls), whereas a significant higher amount was found in the anti-MIF group (44.81±2.49 cells/field) (Figure 23 B). CD74 expression in the ischemic skeletal muscle increased after both treatments (rmMIF 15.80+/-1.1, anti-MIF 33.35+/-2.55, ctr 9.33+/-0.71) but doubled after anti-MIF mAb treatment compared to rmMIF treatment (Figure 23 C). CXCR4 and MIF staining in the skeletal muscle, expressed as pixel per field (CXCR4 staining: rmMIF 1468+/-2.13 > anti-MIF 968.8+/-99.62 > ctr 388.3+/-57.33) and percentage of positive tissue (MIF staining: rmMIF 42.60+/-2.35 > anti-MIF 33.71+/-2.71 > ctr 14.61+/-2.13), respectively, showed an increase after both treatments with a higher amount in rmMIF treated compared to anti-MIF mAb treated mice (Figure 23 D, E).

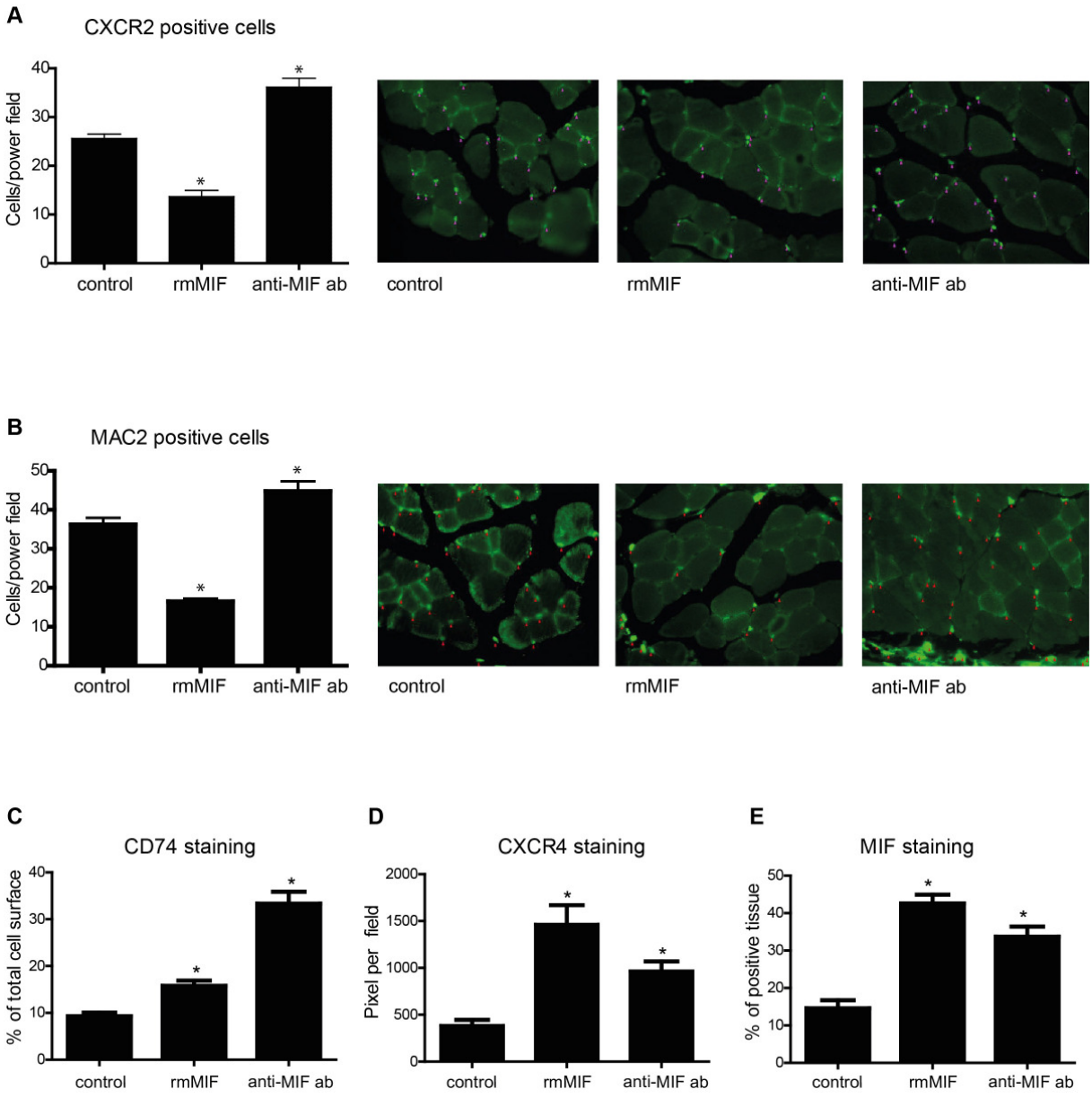

Figure 23: Immunohistochemical stainings of the ischemic skeletal muscle 48 h after ligation for macrophages, MIF and its receptors. rmMIF diminished, whereas anti-MIF mAb increased the number of CXCR2-positive cells (**A**) and macrophages (**B**) in the ischemic skeletal muscle (*p<0.05 vs. control). Violet (**A**) and red (**B**) dots indicating the positive stained cells. (**C**) CD74 expression as percentage of total cell surface increased in both treatments, but doubled after anti-MIF mAb treatment (*p<0.05 vs. control). Staining for CXCR4 (**D**) and MIF (**E**) in skeletal muscle revealed an increase in both groups, with a higher level in the rmMIF group (*p<0.05 vs. control).

IV.3.2 The Role of MIF in Macrophage Polarization

As differences in arteriogenesis for the rmMIF group can relate to altered macrophage polarization, we tested the effect of rmMIF on polarization of mice bone marrow-derived macrophages (BMM). Bone marrow-derived monocytes were isolated and cultured over 8 days while they differentiated into macrophages. These cells were stimulated with different concentrations of rmMIF. Within this context M1 macrophages are considered pro-inflammatory whereas M2 macrophages are pro-angiogenic. In contrast with classical inducers of macrophage polarization INF-γ (M1) and IL-10, but also IL4 and IL13 (M2), rmMIF stimulation had no clear effect on polarization neither to M1 (TNF-α) nor to M2 (IL-19, YM-1) macrophages in this setting (Figure 24), but decreased CXCR2 expression of BMM on mRNA level (Figure 25 A). CXCR4 mRNA content depended on the used rmMIF concentrations. The expression level was increased for 5, 400 and 500 ng/ml rmMIF stimulation, but decreased after stimulation with 10, 50 and 200 ng/ml, reflecting a biphasic effect of rmMIF stimulation on these cells (Figure 25 B). MIF mRNA content was significantly increased after stimulation with 5, 100, 400 and 50 ng/ml, but decreased with 10 ng/ml (Figure 25 C). Only stimulation with 5 and 200 ng/ml rmMIF raised significantly the mRNA expression of CD74 (Figure 25 D).

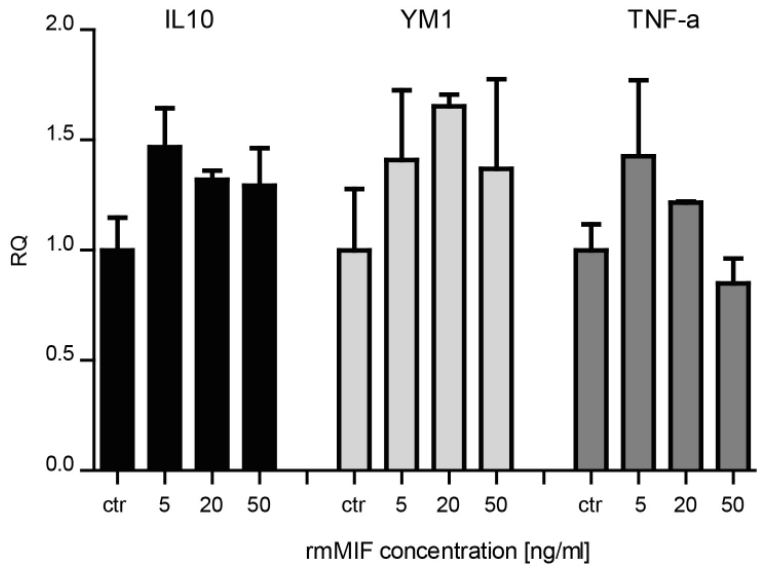

Figure 24: **Expression of M1 (TNF-α) and M2 (IL-10, YM-1) markers after MIF stimulation.** Bone marrow derived macrophages were stimulated with different rmMIF concentrations. After 24 hours, the mRNA was isolated and gene expression was assessed by real-time PCR analysis.

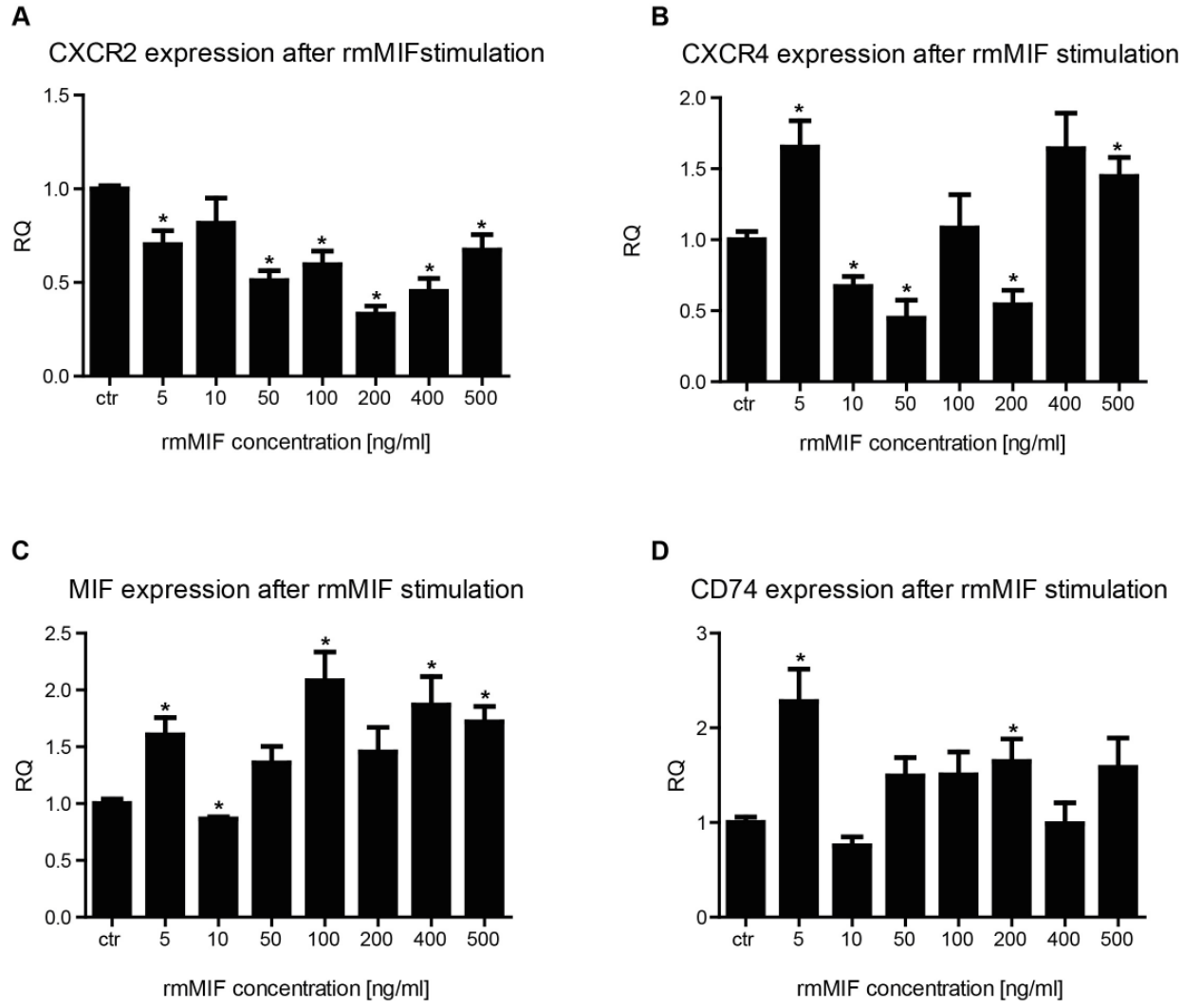

Figure 25: Analysis of mRNA expression of CXCR2, CXCR4, MIF and its receptor CD74 in BMMs after rmMIF stimulation. Cultured bone marrow-derived monocytes that differentiated into macrophages were stimulated with different rmMIF concentrations. RT-qPCR defined expression levels of CXCR2 (**A**), CXCR4 (**B**), MIF (**C**) and CD74 (**D**) (*p<0.05 vs. control, n=3).

IV.3.3 Analysis of Circulating Blood Monocytes

Flow cytometry analysis of circulating blood monocytes at 24 and 48 h post hindlimb ischemia revealed differences in monocyte subpopulations for groups treated with rmMIF and anti-MIF mAb. Blocking MIF resulted in an increase of $CD11b^{+}$ monocytes, whilst rmMIF reduced the level of these circulating cells (Figure 26 A). In all three groups, the number of circulating $Ly6C^{high}$ monocytes were raised after 48 h compared to 24 h after ligation. The level of $Ly6C^{high}$ monocytes did not differ between rmMIF-treated and control mice neither after 24 nor after 48 h. The number

of Ly6C^{high} monocytes was significantly reduced in the anti-MIF group after 24 h, which almost doubled after 48 h reaching content of 71% of all CD11b^{+} monocytes. In contrast, only anti-MIF mAb-treated mice showed an increased of Ly6C^{low} monocyte content after 24 h, followed by an substantial drop to levels comparable in rmMIF and control, which did not show altered Ly6C^{low} monocytes numbers neither after 24 nor after 48 h (Figure 26 C). Similar results were found for circulating CXCR2^{+} monocytes after anti-MIF mAb treatment. Anti-MIF mAb namely enhanced the number of CXCR2^{+} monocytes after 24 h and reduced it after 48 h. All three treatments showed a decrease in the number of circulating CXCR2^{+} monocytes after 48 h when compared with 24 h after ligation (Figure 26 B). Blocking MIF resulted in a higher level of circulating blood monocytes, particularly in an increase of CXCR2^{+} and the pro-arteriogenic Ly6C^{low} monocytes after 24 h.

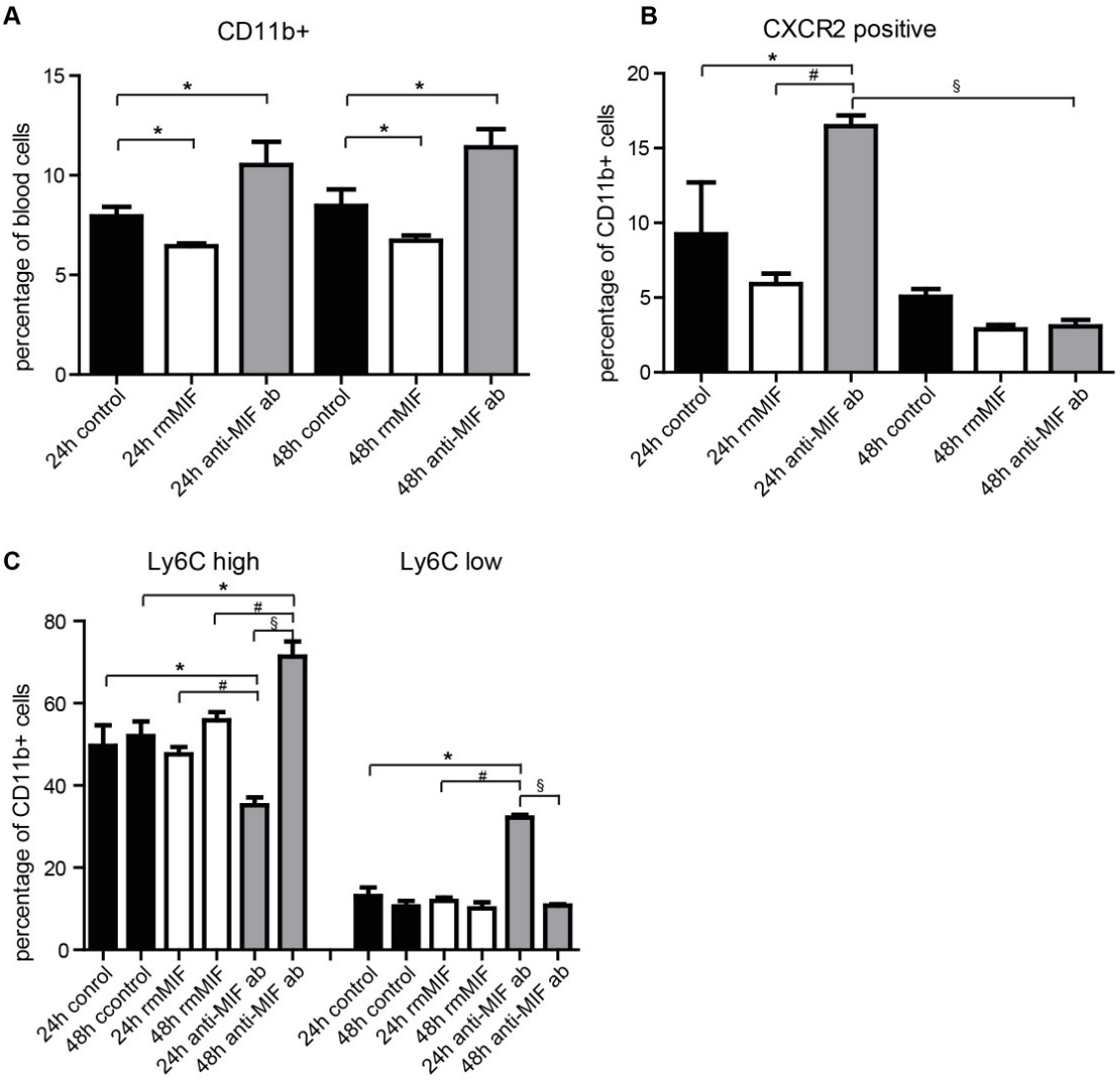

Figure 26: Analysis of circulating blood monocytes after hindlimb ischemia. The number of $CD11b^+$ monocytes in peripheral blood as analyzed by flow cytometry were reduced after rmMIF treatment, whereas blocking endogenous MIF enhanced the number of $CD11^+$ monocytes (*p<0.05 vs. control) (**A**). After 24 h, $CXCR2^+$ monocytes are not significantly reduced after rmMIF, but almost tripled after anti-MIF mAb treatment compared to rmMIF (*p<0.05 vs. control, #p<0.05 vs. 24 h rmMIF) and returned to lower levels after 48 h (§p<0.05 vs. 24 h anti-MIF mAb) (**B**). No differences between the rmMIF and control group were observed in $Ly6C^{high}$ and $Ly6C^{low}$ monocytes, neither 24 h nor 48 h after ligation. $Ly6C^{high}$ monocytes were reduced after 24 h (*p<0.05 vs. control, #p<0.05 vs. 24 h rmMIF) and doubled after 48 h (*p<0.05 vs. control, #p<0.05 vs. 48 h rmMIF, §p<0.05 vs. 24 h anti-MIF mAb). Flow cytometry analysis showed further a strong increase in $Ly6C^{low}$ monocytes after anti-MIF mAb treatment (*p<0.05 vs. control, #p<0.05 vs. 24 h rmMIF) with a drop to the levels of rmMIF and control group (§p<0.05 vs. 24 h anti-MIF mAb) (**C**).

IV.3.4 Adhesion and Transmigration of Monocytes *in vitro*

To analyze the effect of rmMIF on the adhesion of monocytes to an endothelial layer, I performed static adhesion experiments. After 24 h, SVECs form an endothelial monolayer on 96-well plates. Calcein-labelled WEHIs, a mouse monocytic cell line, or isolated blood monocytes (20,000) plus the test factors (rmMIF and anti-MIF mAb) were added on top of the layer. After incubation for 16 h, adherent cells were analyzed. Different conditions were used for the adhesion assay.

First, I tested whether the assay medium has an influence on the adhesion capacity of WEHIS to the endothelial SVEC-layer. Therefore I used minimal medium (RPMI + 0.5% FBS + 1% penicillin/streptomycin) and BSA medium (RPMI + 0.1% BSA + 1% penicillin/streptomycin). Increased fluorescence intensity compared to that of SVECs and PBS-containing wells were used to verify the success of the adhesion. I observed no significant differences between the two assay media, not even within the groups. Only when using BSA medium, a tendency of enhanced adhesion of the WEHIs after anti-MIF mAb incubation was found (Figure 27 A).

Next, I checked whether the number of cells makes a difference in the adhesion after the differential stimuli. Using more cells (50,000 WEHIs) I observed an increased fluorescence signal reflecting a higher level of adherent cells compared to 20,000 cells. Anti-MIF mAb slightly increased the adhesion in both conditions but did not reach statistical significance. rmMIF has no effect (Figure 27 B).

Furthermore, I tested the adhesion of isolated blood monocytes from mice after rmMIF and anti-MIF mAb stimulation. I detected no differences after both stimulations (Figure 27 C). Considering the data on the adhesion of monocytes, I conclude that rmMIF and anti-MIF mAb have no effect after 16 h on the static adhesion capacity of monocytes to an endothelial monolayer.

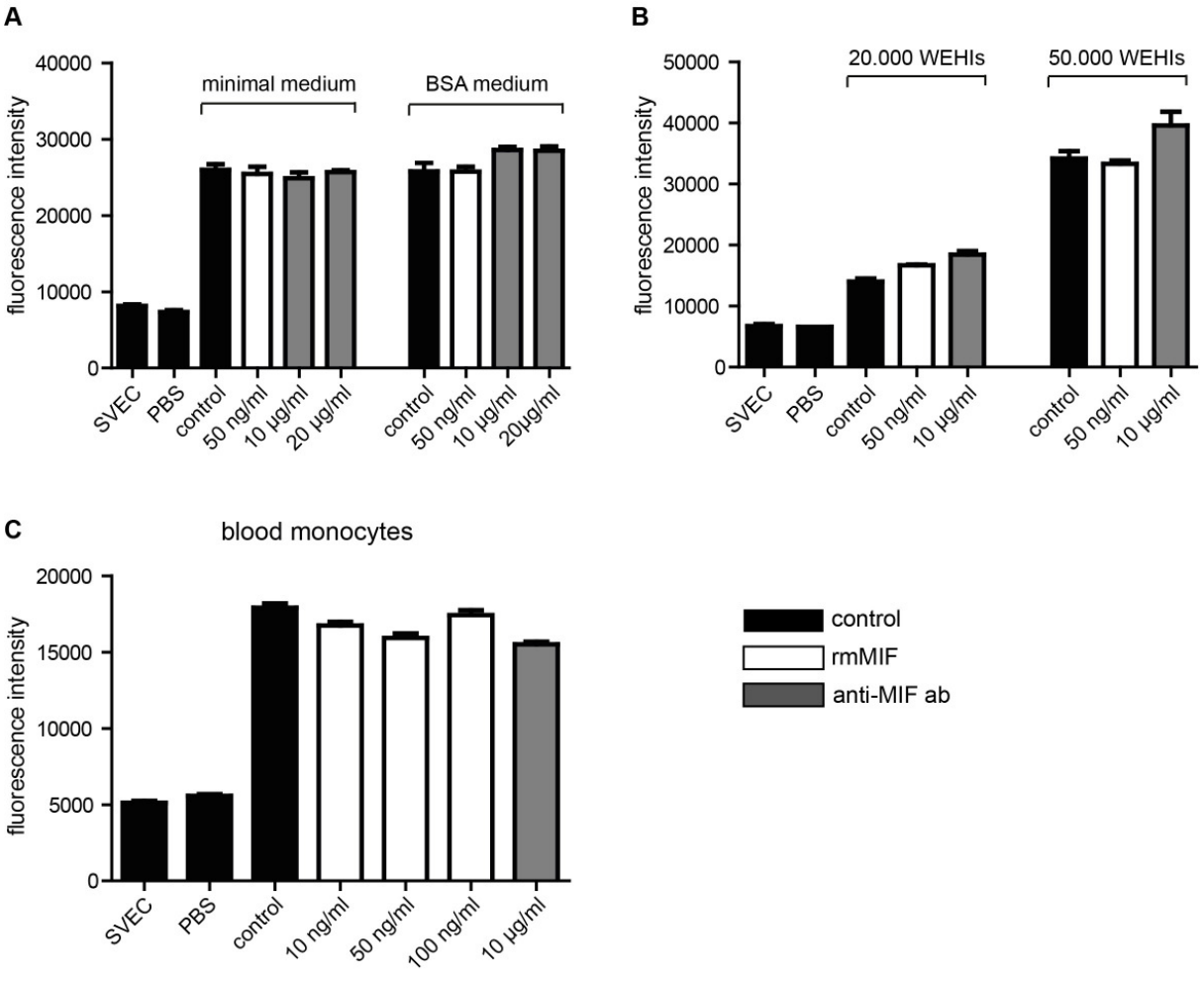

Figure 27: Adhesion of monocytes to an endothelial monolayer. Different assay media were compared with regard to the adhesion of the monocytic cell line (WEHIs). Neither the minimal medium nor the BSA medium affects the adhesion capacity (**A**). More cells (50,000 WEHIs) show more fluorescence signal but no differences after different stimuli were observed (**B**). rmMIF and anti-MIF mAb failed to induce altered adhesion of isolated blood monocytes compared to control (unstimulated) (**C**).

To test whether the adhesion of WEHIs to an endothelial monolayer under flow conditions, representing physiological conditions, differ between rmMIF and anti-MIF mAb incubation, I performed flow cell-adhesion experiments. Both treatments induce and enhance the adhesion of monocytes (rmMIF 63.55+/-5.55, anti-MIF 84.77+/-6.73 vs. ctr 41.00+/-8.04, $p<0.05$) (Figure 28 A).

Moreover, I tested the effect of rmMIF and anti-MIF mAb stimulation on the transmigration capacity of WEHIs through the filter membrane over an additional SVEC-layer. rmMIF decreased the transmigration (rmMIF 10.62+/-0.74 vs. ctr 21.23+/-2.3). Contrary, anti-MIF mAb showed no difference compared to control (anti-MIF 22.80+/-3.0 vs. ctr 21.23+/-2.3), but significantly differs from the rmMIF stimuli (anti-MIF 22.80+/-3.0, $p<0.05$; vs. rmMIF 10.62+/-0.74, $p<0.05$) (Figure 28 B).

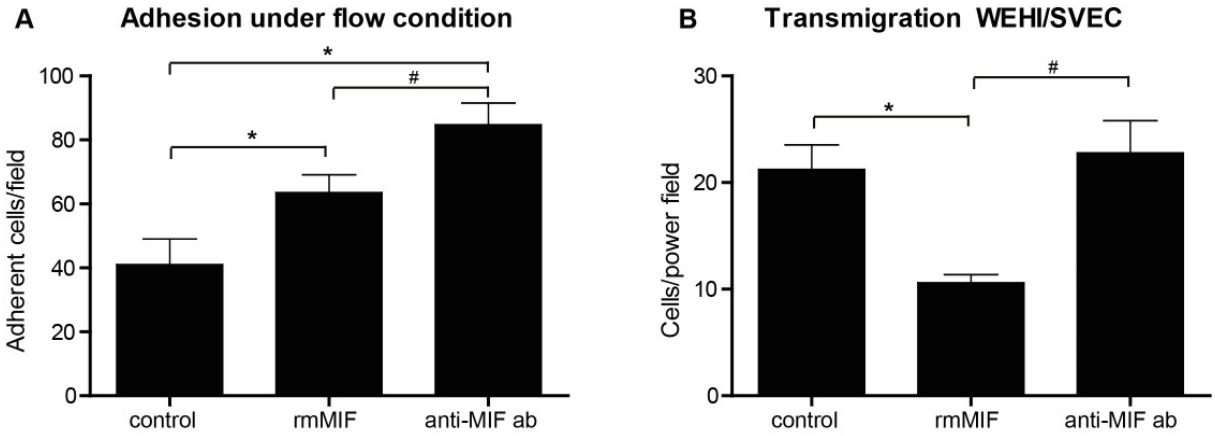

Figure 28: Role of MIF in monocyte recruitment. Both treatments, rmMIF and anti-MIF mAb triggered the adhesion of WEHIs to an endothelial monolayer under flow conditions (*$p<0.05$ vs. control) with a significant higher level after anti-MIF mAb treatment (#$p<0.05$ vs. rmMIF). SVEC monolayers were perfused with rmMIF or anti-MIF stimulated WEHIs. Firmly adherent monocytes were counted (**A**). rmMIF lowers the transmigration capacity of WEHIs through an endothelial monolayer (*$p<0.05$ vs. control), whereas after anti-MIF mAb treatment no differences were observed compared to control. However, compared to rmMIF, anti-MIF mAb treatment doubled the transmigration capacity (#$p<0.05$) (**B**).

V DISCUSSION

V.1 The Double-edged Role of the CXCL12 / CXCR4 Axis in Experimental Myocardial Infarction

Myocardial infarction caused by the thrombotic occlusion of an atherosclerotic coronary artery represents a leading cause of death in western countries. Hence, new therapeutic strategies are urgently required to treat myocardial infarction and ischemic cardiomyopathy. The CXCR4 receptor ligand CXCL12 has been implicated in protection and regeneration after MI through recruiting angiogenic cells, improving neovascularization and cardiac function. Here, I evaluated the intrinsic functions of CXCR4 in remodeling after MI.

These data demonstrate double-edged effects of CXCR4 on myocardial remodeling after MI and point to a variety of possible mechanisms with major clinical implications. Compared with wild-type, $CXCR4^{+/-}$ mice revealed smaller and more stable MI scars due to an altered post-inflammatory pattern characterized by an attenuation of the acute inflammatory recruitment of neutrophils, a shift towards a more regenerative monocyte response and better adaptation of cardiomyocytes to hypoxic stress. This was paralleled by impaired EPC function, myocardial neovascularization and coronary flow recovery, overall amounting in a lack of improvement of ventricular function. Given the major efforts to exploit the CXCL12/CXCR4 axis therapeutically to promote angiogenesis and cellular regeneration, our data provide important insights as to the endogenous function of CXCR4 after MI.

First, I found an altered inflammatory pattern in $CXCR4^{+/-}$ mice after MI characterized by diminished myocardial infiltration with neutrophils and tissue-degrading $Gr1^{high}$ monocytes and earlier infiltration with $Gr1^{low}$ monocytes, which have been associated with deposition of collagen (Nahrendorf et al., 2007). Accordingly, we found that the relative collagen content in the smaller MI scars was increased. Neutrophils release reactive oxygene species, proteases and express mediators capable of amplifying cell recruitment. Hence, a reduced neutrophil infiltration itself represents a self-sufficient mechanism to impair myocardial injury and reduce infarction size (Eash et al., 2009; Tarzami et al., 2003). Recently, CXCR4 has been identified as a central regulator of neutrophil homeostasis directing their release from the bone marrow

under stress conditions (Eash et al., 2009). Although a complete disruption or deficiency of CXCR4 caused an expansion of less mature neutrophils in the circulation in the chronic context of atherogenesis (Zernecke et al., 2008b), the group of *Karshovska* found that an acute mobilization of neutrophils induced by severe vascular injury was blocked by the potent CXCR4 antagonist AMD3645 (Karshovska et al., 2008). Similarly, I observed that MI caused an acute expansion of circulating neutrophils and their myocardial recruitment, which was attenuated in $CXCR4^{+/-}$ hearts. This is in line with a recent study, which failed to detect neutrophil mobilization after various forms of stimulation or *Listeria monocytogenes* infection, when CXCR4 signaling was abrogated (Eash et al., 2009). It seems that there are different mechanisms of neutrophil release in normal physiological state and after acute stress, which both involved CXCR4, but in a different manner. Thus, these data confirm a role of CXCR4 in injury- or stress-induced neutrophil mobilization allowing their subsequent recruitment.

Besides neutrophils, monocytes play an important and finely tuned role in cardiac repair (Nahrendorf et al., 2007). I found that after MI overall monocyte/macrophage infiltration into the myocardium was delayed but enhanced at later stages in $CXCR4^{+/-}$ mice. This corresponds to a reduced infiltration with Gr-1^{high} inflammatory monocytes, which enter the infarcted myocardium during the initial phase, in a process that may be governed by neutrophil secretory products (Liehn et al., 2008). Preventing Gr-1^{high} monocytosis results in a delayed or inefficient removal of apoptotic cells and necrotic tissue but does not impede the healing (Nahrendorf et al., 2007). Conversely, Gr-1^{low} monocytes, which promote healing via myofibroblast accumulation and collagen deposition, were more prevalent and recruited earlier after MI in $CXCR4^{+/-}$ mice. This shift to a more regenerative response may contribute to smaller and stable scar formation. Interestingly, I found that Gr-1^{high} monocytes from CXCR4+/- mice did not display reduced CXCR4 expression. Whereas one may hypothesize that the regenerative Gr-1^{low} cell subset employs additional receptors to compensate for lower CXCR4 levels in recruitment, these data generally imply an important role of other receptors, namely CCR2, in the recruitment of Gr-1^{high} monocytes.

Despite the reduced MI size, the ventricular function was not significantly improved in CXCR4$^{+/-}$mice 4 weeks after MI. This could be due to the reduced basal coronary flow and to the impaired coronary flow recovery in CXCR4$^{+/-}$ hearts 4 weeks after MI. As an underlying mechanism, we studied the function of EPCs as important contributors to neovascularization after MI. The SDF-1/CXCR4 interaction is crucially involved in the mobilization and recruitment of stem and progenitor cells to the heart after MI (Abbott et al., 2004; Lapidot and Petit, 2002). Ones EPCs arrive at the ischemic infarction site, they are able to differentiate and gain endothelial markers. Despite appropriate expression of typical endothelial differentiation markers, such as uptake of acLDL and Lectin, VE-cadherin, vWF, CD31 or VEGFR2, splenic EPCs from CXCR4$^{+/-}$ mice showed deficient chemotaxis towards CXCL12 (but not VEGF) and reduced tube formation *in vitro*. EPCs failed to establish contact with other cells and subsequently failed to invade and integrate into the new vessel structures. Accordingly, myocardial vessel density, endothelial cell content and arterial branching *in vivo* was impaired in CXCR4$^{+/-}$ mice, indicating a defect in angiogenesis and neovascularization after MI. This is in line with a previous study showing that EPCs from CXCR4$^{+/-}$ mice were also significantly impaired to restore blood flow in ischemic nude mice compared with wild-type EPCs in the hindlimb ischemia model (Walter et al., 2005). Showing *in vitro* that these effects are mostly due to a dysfunction of EPC, I cannot exclude that a decrease in the surrounding vascular density also plays a supportive role in our *in vivo* models through reducing the number of circulating cells, which are available at the site of injury. Conversely, a lack of functional improvement in CXCR4$^{+/-}$ mice cannot be explained by a modulation of cardiomyocyte contractility by CXCR4, because CXCL12 has been shown to exert negative inotropic effects (weaken the contraction of the heart muscel) (Pyo et al., 2006) so that one would rather expect improved ventricular function upon inhibition of deficiency of CXCR4.

Moreover, I performed bone marrow chimera experiments to distinguish the influence of the CXCR4 heterozygous background and its effect on circulating cells in normally developed wild-type mice. Despite a reconstitution with wild-type bone marrow, a reduction of infarction area and neovascularisation persisted in CXCR4$^{+/-}$ mice. Moreover, a significant albeit less marked reduction was observed in wild-type mice reconstituted with CXCR4$^{+/-}$ bone marrow. This indicates that reduced CXCR4 levels on the circulating cells, namely progenitor cells and leukocytes, may also contribute

to the effects observed after MI in CXCR4 heterozygous mice, independently of their abnormal cardiovascular development.

Notably, the reduced basal and neovascularisation of $CXCR4^{+/-}$ hearts without any sign of physiological dysfunction raises several questions. Diminished blood supply should lead to a series of histopathological and structural changes of the myocardium with an increase in ventricular mass and volume, and progressive decline in left ventricular performance. None of these parameters, however, differ in $CXCR4^{+/-}$ mice compared with wild-type mice. Hence, I assumed that a protective or adaptive mechanism in $CXCR4^{+/-}$ myocardium would ensure cardiac function even under reduced oxygen conditions. Indeed, no sign of increased apoptosis was observed. After MI, $CXCR4^{+/-}$ myocardium is spared and hypoxic injury seems to be less extensive compared with wild-type mice. Using electron microscopy, I observed lipofuscin-like lipid accumulations, which resembled those found in rat hearts after dietary fish-oil feeding (Nalbone et al., 1988) and may include long-chain monounsaturated fatty acids and degraded forms of peroxidized lipids. Although the result of a clinical trial using polyunsaturated fatty acids showed only a minimal effect on mortality (Svensson et al., 2007; Tavazzi et al., 2008), another study demonstrated a diet enriched with unsaturated n-3 fatty acids can reduce ischemic damage to the heart (Hock et al., 1990). Higher levels of unsaturated fatty acids triglyceride fraction $CXCR4^{+/-}$ hearts may thus represent a possible lead to protection but this clearly requires further investigation into underlying mechanisms.

Another notable difference in the lipid extracts of $CXCR4^{+/-}$ myocardium, I detected high levels of phosphatidylserine (PS), known as a marker of cell death releasing an important destruction signal for macrophages when exposed on the cell surface (Hunter et al., 2005). Moreover, PS supports other cell functions, including mitochondrial membrane integrity for energy production and activation of protein kinase C, which plays an important role in tolerance to hypoxia during late preconditioning (Baxter and Ferdinandy, 2001). This has been involved in the inhibition of specific immune responses, which may extend to local inflammation (Hoffmann et al., 2005). Within this context, specially the PKCε isoform seems to be for major importance in cardioprotection from ischemia (Budas and Mochly-Rosen, 2007). For activation, PKCε requires diacylglycerol (DAG) and PS. Although the

protective role of DAG in MI was shown (Baxter et al., 1997), the effect of PS on the hypoxic myocardium has not been investigated yet. On the other hand, PS are involved in reducing the specific immune response (Hoffmann et al., 2005) and might possess ergogenic properties during physical exertion (Kingsley et al., 2005). Since the oral administration of PS, which are already commercially available, can speed up recovery, prevent muscle soreness, and improve well-being (Kingsley, 2006; Kingsley et al., 2006; Kingsley et al., 2005).

In this study, the permanently decreased coronary flow in $CXCR4^{+/-}$ mice may induce a chronic ischemia in the heart and thus force cardiomyocytes to adapt even from early stages of embryonic development on. An increase in cardiac PS seems to be a possible cause mediating this adaptive mechanism, since pre-incubation of cardiomyocytes with PS isolated from CXCR4+/- hearts protected cardiomyocytes against hypoxic injury. Although the exact mechanism remains to be established, this suggests that PS may contribute to the adaptation of $CXCR4^{+/-}$ hearts to reduced oxygen supply.

Extensive attempts have been made to directly affect the CXCL12/ CXCR4 axis, e.g. by direct injection, nanofiber-mediated delivery of CXCL12 or overexpression of CXCL12/ CXCR4 in cells transplanted into the myocardium (Abbott et al., 2004; Askari et al., 2003; Hu et al., 2007; Saxena et al., 2008; Schuh et al., 2008; Segers et al., 2007; Zhang et al., 2008), aiming to reduce MI size and to improve ventricular function after MI. However, endogenous functions of CXCR4 in ischemic cardiomyopathy and concomitant adaptive processes had not been studied. The double-edged effects of CXCR4 are illustrated by an alteration of the inflammatory response and protection against hypoxic stress, as well as impaired EPC function, neovascularization and coronary flow recovery, which overall result in a limitation of MI scar size without improved ventricular function. Pharmacological antagonism of CXCR4 with AMD3100 has been reported to reduce infarct size and to improve ventricular function after MI in rats (Proulx et al., 2007). Whereas the decrease in MI size is consistent with the findings in this study, an improved contractility has been explained by a suppression of the hypertrophic response in the non-infarct area. This differs from $CXCR4^{+/-}$ mice, which have intrinsically reduced coronary flow and can be considered as a model for congenitally impaired vascularization and adaptation to

hypoxia. Thus, cell-specific, context-dependent and long-term effects of CXCR4 interference or CXCL12 application need to be carefully taken into account when devising therapeutic strategies for MI and ischemic cardiomyopathy.

V.2 The Angiogenic Potential of MIF and EPCs

Cell therapy, especially stem and progenitor cell based therapy including the use of EPCs, qualifies as a key to regenerate injured tissue (Lyngbaek et al., 2007; Schuh et al., 2008), and therefore is considered one of the greatest hopes in treating ischemic diseases (Jain and Duda, 2003). EPCs derived from human blood and blood stem cells are of major focus but also cells derived from the bone marrow (BMCs) are used for the treatment of acute myocardial infarction.

Stem or progenitor cell therapy provides an opportunity to regenerate functional myocardial tissue. During clinical trials BMCs therapy resulted in improvement of cardiac performance parameters with reduction of left ventricular dilatation (Kuswardhani and Soejitno, 2011). This suggests beneficial effects of BMCs therapy for acute myocardial infarction with an increase in cardiac output but also a prophylactic effect for late complications like chronic heart failure (Kuswardhani and Soejitno, 2011). In the more than 2-years follow-up study “REPAIR-AMI”, a significant lower incidence of major adverse cardiovascular events (i.e. death, revascularization, reinfarction) was observed for patients with acute myocardial infarction treated with autologous BMCs (Assmus et al., 2010).

The peripheral blood of adult humans contains endothelial progenitor cells (EPC). *In vitro*, these cells can differentiate into mature endothelial cells, while in the ischemic tissue models these cells contributed to neovasculogenesis (Murohara, 2001). Experimental myocardial infarction studies have shown that EPCs injected in the ischemic infarct zone migrate to the ischemic region and improve the hemodynamic function of the heart (Kawamoto et al., 2001). Unfortunately, the underlying mechanism of this therapeutic benefit is not clearly understood. Animal studies revealed the cardiomyogenic potential of hematopoietic stem cells and their ability to improve neovascularization, reduce fibrosis and support left ventricular function after myocardial infarction (Jackson et al., 2001; Kawamoto et al., 2001). Also in clinical

studies the potential of EPCs to improve vasculogenesis, angiogenesis and organ functions in ischemic diseases was shown (Hill et al., 2003; Schmidt-Lucke et al., 2005).

Stem cell induced angiogensis, as a process of neovascularization has obtained great interest for treating cardiovascular ischemic diseases. Angiogenesis is a highly regulated complex morphogenetic process depending on a cocktail of factors and involving multiple steps. Chemokines and cytokines are involved in this process, improving chemotaxis, transmigration and integration of EPCs and tube formation *in vitro* (Hristov, 2003; Urbich, 2004). Proliferation and migration of endothelial cells together with growth factors and chemokines are key regulators in angiogenesis. To better understand the behavior of EPCs in response to different growth factors, I evaluated the angiogenic potential of different factors; rmMIF, rmVEGF, rmCXCL12 and rmCXCL1 associated with the function of EPC in angiogenesis.

Endothelial cells belong to one of the few cell types that express receptors that specifically bind the acetylated form of LDL (acLDL). Therefore, uptake of AcLDL is regarded to plead for an endothelial phenotype. These data indicate that EPCs, isolated from mice spleens exhibit endothelial-like properties, namely acLDL uptake, binding of lectin, and expression of mononuclear- and endothelial specific markers, as well as CXCR2 and CXCR4, receptors for e.g. CXCL1, MIF and CXCL12. Both chemokine receptors, CXCR2 and CXCR4 are needed for angiogenesis *in vivo* (Addison et al., 2000; Ma et al., 1998; Tachibana et al., 1998). Mice deficient for CXCR4 die perinatally and display profound defects in the development of arteries of the gastrointestinal tract, as well as defects in vascular development and in the hematopoietic system (Ma et al., 1998; Tachibana et al., 1998). CXCR2 knockout mice showed impaired angiogenesis of the cornea in response to CXCR2-ligand-induced angiogenic activity (Addison et al., 2000).

Mobilization of EPCs and neovascularization mediated by EPCs is strongly regulated. The number of circulating EPCs can be increased by mobilizing them from the bone marrow by factors such as G-CSF, statins, erythropoietin, VEGF, SDF-1 (CXCL12) or NO (Hoenig et al., 2006). Stimulating mobilization can be worthwhile before collecting blood for EPC-culture. However, the quality and efficiency of these mobilized EPCs is

under debate (Sarkar et al., 2007). Another way to increase the yield of EPCs upon cultivation of PBMCs is the addition of factors that stimulate endothelial differentiation. Candidates are VEGF, GM-CSF and statins (Dimmeler et al., 2001).

Recruitment and integration of EPCs requires a well coordinated multistep process including adhesion, chemoattraction, migration and eventually the differentiation to endothelial cells (Hristov, 2003; Urbich, 2004). Here, I showed that EPCs are able to integrate into a vascular network of SVECs on matrigel *in vitro*. A number of growth factors and chemokines promote angiogenesis after birth. The best characterized of these pro-angiogenic factors is VEGF, which triggers angiogensis both *in vitro* and *in vivo* by promoting endothelial proliferation, survival and migration (Ferrara et al., 2003). Here, I demonstrated that rmVEGF, but also rmMIF, rmCXCL12 and rmCXCL1 enhanced the chemotactic capacity of EPCs. Chemoattraction and transmigration are two distinct processes involved in the development of blood vessels. EPCs showed a different transmigration behavior as compared to their chemotactic potential. rmMIF and rmCXCL1 enhanced the number of transmigrated cells through an endothelial monolayer, whereas rmVEGF and rmCXCL12 failed to induce this effect.

When cultured on Matrigel, endothelial cells spontaneously form tubes. Here we show that SVECs were able to form tubes in Matrigel *in vitro* after 24 h. Culturing EPCs on Matrigel and determining their ability to form tubes showed that EPCs alone are not able to form tube-like structures, distinguishing them from a true endothelial phenotype. Nonetheless, the angiogenic potential of EPCs could be proven in co-culture with SVECs showing a high responsiveness of EPCs to all growth factors tested in the initiation of tube formation and integration of EPCs into capillary-line tubules displaying pro-angiogenic properties. It is considered that incorporated EPCs into newly formed capillaries enhance neovascularization and promote cardiac regeneration by releasing pro-angiogenic growth factors such as SDF-1 and VEGF, which increase the migration of mature endothelial cells and tissue resident cardiac progenitor cells (Urbich et al., 2005). In addition, the increased expression of cytokines may improve blood vessel formation after cell therapy (Urbich et al., 2005).

Recently, much literature has arisen concerning EPCs, both as a useful stem cell and as a biomarker for cardiovascular diseases (Fadini et al., 2008; Schmidt-Lucke et al., 2005). However, poor standardized specification of EPC characteristics have resulted in the use of a diversity of cell population for therapeutics and research. This diversity might be directly related to the rather controversial data on EPC isolation, culturing, functionality and thus therapeutic efficacy. Despite the fact that my EPCs, cultured over 10 days, showed endothelial characteristics I call them in accordance with Hue et al. angiogenic early outgrowth cells (Hur et al., 2004). EPCs cultured up to 22 days (maximum of 3 passages) upon which they are phenotyped for endothelial characteristics are called the late EPCs (Hur et al., 2004) and are by Ingram and coworkers regarded to be the true EPCs or endothelial colony-forming cells (ECFCs) (Fadini et al., 2008; Ingram, 2004; Yoder et al., 2007). These cells are described to be able to have 20-30 population doublings in vitro and thus are able to provide a relatively high yield of cells. Long-lasting discussion about EPCs lead to the understanding that these cells are present in blood and spleen and should be considered a special pro-angionenic subset of monocytes, overlapping in expression pattern with endothelial cells (ECs) such as CD34, CD31 and VEGF receptor 2 (Fadini et al., 2008; Yoder et al., 2007). On the other hand, these cells show similarities in endothelial function namely chemotaxis towards VEGF, the capacity to form tube-like structures in matrigel and enhancing neovascularization *in vivo.* However, these cells are not able to fully differentiate into endothelial cells, and are thus no real endothelial progenitor cells (Fadini et al., 2008; Purhonen et al., 2008). Persistent discussions about real EPCs will continue and is of course just a question of definition. In fact, the pro-angiogenic function of EPCs remains. However, pro-angiogenic therapies need to cover multiple steps of angiogenesis including stem/progenitor cells and cocktails of involved factors: chemokines, cytokines and growth factors. MIF, but also VEGF, CXCL12 and CXCL1 are potential factors to increase angiogenesis and might be even more able to promote neovascularization in combination with EPCs. Therefore, considering combined treatment of cell-based therapies and these factors, combinations of these factors, or optimization of the therapeutic angiogenic factor delivery mode gives us the opportunity to manipulate and control the pleiotropic nature of angiogenesis.

V.3 The Role of Macrophage Migration Inhibitory Factor, a CXCR Ligand in Arteriogenesis

Arteriogenesis is critically regulated by leukocyte functions and alterations of the mechanisms of monocyte/macrophage dependent collateral remodeling could affect this process. Cytokines like MCP-1, FGF-2, TGF-beta, VEGF, and GM-CSF are known to stimulate angiogenesis on one hand and arteriogenesis on the other (Buschmann et al., 2003). I showed that the cytokine MIF with chemokine like-functions known to promote angiogenesis (Amin, 2003; Chesney et al., 1999; Simons et al., 2011), negatively influenced arteriogenesis, whereas blocking MIF had a positive effect on collateral remodeling.

Accordingly, exogenous MIF reduced the total number of tissue macrophages and CXCR2 positive cells present in the ischemic skeletal muscle tissue. The link between recruitment of monocytes and, subsequently polarization into tissue resident macrophages in the perivascular space of nascent collaterals and remodeling of the artery towards a functional conduit has been shown for numerous studies (Arras et al., 1998; Heil et al., 2002; Schaper et al., 1976; Scholz et al., 2000). Heil et al. showed the functional link between circulating monocyte concentration in the peripheral blood and the enhancement of collateral growth (Heil et al., 2002). Increasing numbers of $CD11b^{+}$ monocytes enhanced collateral artery formation and blood flow in the impaired regions after acute hindlimb ischemia (Capoccia, 2006; Heil et al., 2002). This was accompanied by an increased macrophage accumulation in the tissue as demonstrated by immunohistology staining (Heil et al., 2002). Here, I demonstrate that blocking MIF raised the circulating $CD11b^{+}$ monocyte content. Additionally, $Ly6C^{high}$ monocytes were strongly decreased 24 h after ligation, whereas the pro-arteriogenic $Ly6C^{low}$ monocytes were up-regulated 24 h after ligation with an enhanced ability to infiltrate the ischemic muscle observed in a drop of these cells after 48 h post-occluded. MIF suppressed CXCR2 expression on macrophages *in vitro*, and hinders monocytes to transmigrate into the tissue.

MIF plays an essential role in the formation of new blood vessel through angiogenesis. In mice, the administration of neutralizing anti-MIF antibodies delayed the growth of B lymphoma cells and dampened tumor growth, which was accompanied by a strong reduction in tumor angiogenesis. Tumor endothelial cells

express MIF *in vivo*, and *in vitro*, microvascular endothelial cells secrete MIF needed for proliferation (Chesney et al., 1999). Similarly, rhMIF induces tube formation in Matrigel of human dermal microvascular endothelial cells *in vitro* and angiogenesis in Matrigel plugs and in the corneae bioassay *in vivo*, further support the pro-angiogenic function of MIF (Amin, 2003).

Although angiogenesis and arteriogenesis can be observed after an experimental occlusion of a peripheral artery, the relationship between these two modes of neovascularization remains undefined. Hypoxia is the principal trigger for angiogenesis whereas arteriogenesis is mainly initiated by local changes in shear stress as a result of altered pressure gradients along the microvessels that later grow out to collaterals (Carmeliet, 2000). On the other hand, most growth factors (e.g. VEGF, PDGF, FGF etc.) that have been studied in detail have pleiotropic effects and induce angiogenesis and arteriogenesis (Molin and Post, 2007). Therefore, the inhibitory effect of MIF on arteriogenesis is surprising and should be subject of further research.

MIF is involved in the inflammatory pathogenesis of atherosclerosis and its consequences, e.g. myocardial infarction, ischemia-reperfusion injury, remodeling after arterial injury or unstable plaque formation (Zernecke et al., 2008a). In a model of wire-induced injury in hypercholesterolemic $Apoe^{-/-}$ mice, blocking MIF resulted in a marked reduction of neointimal macrophages and inhibition of transformation of macrophages into foam cells contributing to the progression into an unstable plaque phenotype (Schober, 2004). The direct participation of MIF in atherosclerotic plaque progression was supported in mice studies using $MIF^{-/-}Ldlr^{-/-}$ and $Ldlr^{-/-}$ mice on an atherogenic diet (Pan et al., 2004). In MIF-deficient $Ldlr^{-/-}$ mice, abdominal aorta lipid deposition and intimal thickening from aortic arch throughout the abdominal aorta was reduced compared with $Ldlr^{-/-}$ mice (Pan et al., 2004). Inhibition of MIF leads to reduced atherogenesis and the combination of pro-arteriogenesis and anti-atherogenesis might be of particular therapeutic value.

Monocytes/macrophages likely stimulate arteriogenesis by stimulating migration and proliferation of endothelial cells and smooth muscle cells through the production and secretion of a cocktail of survival factors, growth factors and cytokines.

Monocytes/macrophages are a heterogenous cell population polarized by chemokines and cytokines and differ in receptor expression, cytokine profile, and chemotactic properties (Gordon and Taylor, 2005). Diverse are the forms of macrophage activation and polarization, which can be induced by different microenvironmental signals. Macrophage activation could be either pro-inflammatory or anti-inflammatory, contributing to tissue destruction or regeneration and wound healing (Martinez, 2011). Polarized macrophages have been classified into two groups: M1 and M2 macrophages. Classically activated M1 macrophages are responsive to inflammatory cytokines such as IFNgamma and microbial products such as LPS. Alternatively activated M2 macrophages covers three subsets: M2a (induced by IL-4/IL-13), M2b (induced by immune complexes in combination with Il-1beta or LPS), and M2c (induced by IL-10 and glucocorticoid hormones) (Benoit et al., 2008; Martinez et al., 2008). M1 macrophages are part of the Th1 response and play a role in tumor resistance and tissue distruction by secreting pro-inflammatory mediators and releasing of reactive oxygen and nitrogen intermediates (Benoit et al., 2008; Mantovani et al., 2009). In contrast, M2 macrophages are diverse but in general involved in Th2 response. Furthermore, these anti-inflammatory cells play a role in tumor promotion, angiogenesis and tissue remodeling based on the down-regulation of the pro-inflammatory mediators (Mantovani et al., 2009). In a murine hindlimb ischemia model, enhanced arteriogenesis was observed due to an increase of tissue-resident M2-like macrophages and their enhanced secretion of arteriogenic factors, facilitating smooth muscle cell growth and recruitment (Takeda et al., 2011). In this study set up, neither the polarization to M1 nor M2 macrophages was observed after rmMIF stimulation indicating that rmMIF does not affect the phenotype of tissue resident macrophages. On the other hand, rmMIF reduced the CXCR2 and concentration-dependent CXCR4 expression on these cells on mRNA level, which might be relevant for further receptor- ligand interactions. Possible interplay of MIF and macrophage polarization needs to be further expanded and performed in different experimental set ups.

However, blocking endogenous MIF affects the circulating blood monocytes including $Ly6C^{low}$ and $Ly6C^{high}$. Diversity of monocytes is demonstrated by the differential expression of surface molecules identifying monocyte subpopulation in human and mice. In mice, monocyte subpopulations are distinguished on the basis of the

chemokine receptors CCR2 and CX3CR1 and Ly6C (Gr1) antigen expression (Geissmann et al., 2003). The CX3CR1^{low}/CCR2^{high}/Gr1^{high} monocytes are recruited at sites of inflammation and the CX3CR1^{high}/CCR2^{low}/Gr1^{low} subset characterized by CX3CR1-dependent recruitment to noninflamed tissues (Geissmann et al., 2003). The pro-angiogenic activity for Ly6C^{low} monocytes, based on their anti-inflammatory profile, tissue repair activities and promoting healing has been proposed, whereas Ly6C^{high} monocytes produce high levels of inflammatory cytokines and digest damages tissue (Arnold et al., 2007; Gordon and Taylor, 2005; Nahrendorf et al., 2007; Sunderkotter et al., 2004). In a model of murine hindlimb ischaemia, both blood Ly6C^{high} and Ly6C^{low} monocyte circulating levels were enhanced after femoral artery ligation (Cochain et al., 2010). More specifically, intravenous administration of Ly6C^{low} monocytes are reported to promote arteriogenesis (Cochain et al., 2010) and circulate in higher numbers upon blocking MIF, suggesting that endogenous MIF keeps these monocytes in check. A similar response is shown for CXCR2 positive cells and in fact these cells may in part or even completely overlap the Ly6C^{low} population of monocytes although I currently have no triple labeling data to further examine that hypothesis. In arteriogenesis, the relation between monocyte subsets (Ly6C^{low}, Ly6C^{high}) and macrophage polarization (M1, M2 macrophages) remains unclear. In a mouse myocardial infarction model, GR1^{low} and Gr1^{high} cells have been shown to differentiate into M2 and M1 cells, respectively (Nahrendorf et al., 2007). However it is still obscure whether this reflects pre-commitment or temporal recruitment and exposition to certain environmental signals (Mantovani et al., 2009).

The maximal number of tissue macrophages were observed 2-3 days after occlusion contributing to enhanced collateral formation (Scholz et al., 2000). rmMIF significantly reduced tissue macrophages in the ischemic skeletal muscle two days after surgery. The anti-MIF mAb-induced drop in Ly6C^{high} and rise in Ly6C^{low} population after 24 h, with a change in the exactly opposite direction after 48 h after ligation could be the result of altered recruitment from the bone marrow or altered migration into the vascular wall. Indeed, in our hands, blocking MIF enhances monocyte adhesion under physiological flow but not under static conditions. However, monocyte migration is not affected by anti-MIF mAb, but reduced after rmMIF treatment. I therefore hypothesize that blocking endogenous MIF increases the recruitment of

monocytes and specific subsets such as pro-arteriogenic Ly6C^{low} monocytes as well as the immigration into the tissue.

Immunohistochemical stainings for CD74, CXCR4 and MIF and the adhesion of monocytes to an endothelial monolayer under flow condition showed positive effects after rmMIF and anti-MIF mAb treatment. With regard to the used concentration of rmMIF and anti-MIF mAb, which were optimized in preliminary experiments and generally in accordance with literature, testing other concentrations for further experiments needs to be taken into consideration. Also, it will have to explore why both neutralization of MIF and the addition of exogenous rmMIF led to enhanced effects under tested conditions.

In conclusion, this study shows that blocking MIF enhanced arteriogenesis likely by affecting circulating monocyte subpopulations and subsequent infiltration into the tissue surrounding nascent collaterals. Whether rmMIF blocks recruitment of the pro-arteriogenis Ly6C^{low} monocytes translating into a reduction of Ly6C^{low} monocyte immigration into collaterals and stimulates recruitment of Ly6C^{high} from the hematopoietc compartment needs to be further investigated.

The divergent effect of MIF blockade on atherogenesis and arteriogenesis offers opportunities for selective therapeutic intervention in peripheral artery disease.

VI SUMMARY

The formation of a functional and integrated vascular network is a basic process in the growth and maintenance of tissues and can be established by two forms of blood vessel growth in adults: angiogenesis and arteriogenesis. In this study, the ligands of the chemokine receptor CXCR4 and its role in angiogenesis (represented by the experimental myocardial infarction) and arteriogenesis (represented by the murine hind limb ischemia model) was investigated.

The first approach identified the CXCL12 / CXCR4 axis in protection and regeneration after myocardial infarction associated with complex remodeling and inflammatory changes. Experimental MI was induced by ligation of the left descending coronary artery in $CXCR4^{+/-}$ and wild-type mice. After four weeks, infarct size was reduced in $CXCR4^{+/-}$ compared to wild-type mice. This was accompanied by altered inflammatory cell recruitment, namely diminished neutrophil content, delayed monocyte infiltration and predominance of $Gr1^{low}$ over classical $Gr1^{high}$ monocytes. Basal coronary flow and its recovery after MI were significantly impaired in $CXCR4^{+/-}$ mice. This was paralleled by reduced angiogenesis, myocardial vessel density and endothelial cell count. Despite defective angiogenesis, $CXCR4^{+/-}$ hearts showed no difference in CXCL12, VEGF or apoptosis-related gene expression. Lipofuscin-like accumulation in $CXCR4^{+/-}$ hearts and high levels of phosphatidylserine, which protect cardiomyocytes from hypoxic stress *in vitro* were detected. These data showed the crucial role of CXCR4 in endogenous remodeling processes after MI, contributing to inflammatory/progenitor cell recruitment and neovascularization, whereas its deficiency limits infarct size and causes adaptation to hypoxic stress. This should be carefully taken into account, when devising therapeutic strategies involving CXCL12 / CXCR4 axis.

Chapter 2 provides new insights into the behavior of EPCs in response to angiogenic factors: MIF, VEGF, CXCL12 and CXCL1. Spleen derived EPCs exhibit endothelial-like properties, namely acLDL uptake, binding of lectin, and expression of mononuclear- and endothelial specific markers, as well as CXCR2 and CXCR4. rmMIF, rmVEGF, rmCXCL12 and rmCXCL1 enhanced the chemotactic capacity of EPCs, whereas only rmMIF and rmCXCL1 stimulation increased the number of

transmigrated EPCs through an additional endothelial monolayer. Culturing EPCs on Matrigel showed that EPCs alone are not able to form tube-like structures, but integrate into capillary-line tubules in co-culture with endothelial cells with a high responsiveness to all test factors. Therefore, considering combined treatment of cell-based therapy including EPCs and angiogenic factors might provide new therapeutic approaches to regenerate injured tissues.

The third part of this work intended to analyze the role of MIF in arteriogenesis in a murine hind limb ischemia model. The pro-inflammatory cytokine with chemokine-like functions plays a critical role in inflammatory diseases associated with attracting immune cells to sites of inflammation. In mice with femoral artery ligation, paw perfusion was diminished after rmMIF stimulation, whereas blocking MIF enhanced paw perfusion and therefore collateral formation. Accordingly, exogenous MIF reduced the number of tissue macrophages and CXCR2 positive cells in the ischemic skeletal muscle whereas blocking endogenous MIF resulted in an increase of macrophages. Monocytes likely stimulate arteriogenesis by stimulating migration and proliferation of endothelial cells and smooth muscle cells. Within this context M1 macrophages are considered pro-inflammatory whereas M2 macrophages are pro-angiogenic. Neither the polarization to M1 nor M2 macrophages was observed after rmMIF stimulation indicating that MIF does not affect the phenotype of tissue resident macrophages. However, blocking endogenous MIF affects the circulating blood monocytes including $Ly6C^{low}$ and $Ly6C^{high}$. I hypothesize that blocking MIF increases the recruitment of monocytes and specific subsets such as $Ly6C^{low}$ monocytes from the bone marrow and the migration into the vessel wall. The divergent effect of MIF blockade on atherogenesis and arteriogenesis offers opportunities for selective therapeutic intervention in peripheral artery disease.

VII ZUSAMMENFASSUNG

Die Bildung eines funktionalen und integrierten Gefäßnetzes ist ein grundlegender Prozess während des Wachstums und der Erhaltung von Geweben und kann durch zwei Formen des Wachstums von Blutgefäßen im Adulten gezeigt werden: Angiogenese und Arteriogenese. In dieser Dissertation wurden die Liganden des Chemokinrezeptors CXCR4 und seine Rolle in der Angiogenese (im Mausmodell des experimentellen Myokardinfarkts) und Arteriogenese (im Hinterbein Ischämie Mausmodell) untersucht.

Im ersten Forschungsansatz wurde die CXCL12 / CXCR4 Achse im Zusammenhang mit Schutz und Regeneration nach Myokardinfarkt, der mit komplexen Umbau- und entzündlichen Veränderungen einhergeht, identifiziert. Der Myokardinfarkt wurde durch die Ligation der linken Koronararterie in $CXCR4^{+/-}$ und Wildtyp-Mäusen induziert. Nach vier Wochen war die Infarktgröße in $CXCR4^{+/-}$ im Vergleich zu Wildtyp-Mäusen reduziert. Dies ging einher mit veränderter entzündlicher Zellrekrutierung, sowie mit einer verminderten Anzahl an Neutrophilen, verzögerter Monozyteninfiltration und das Überwiegen von $Gr1^{low}$ über klassische $Gr1^{high}$ Monozyten. Der basale Koronarfluss und die Wiederherstellung nach dem Infarkt waren in $CXCR4^{+/-}$ Mäusen signifikant beeinträchtigt. Dies wurde begleitet durch reduzierte Angiogenese, myokardialer Gefäßdichte und Anzahl an endothelialen Zellen. Trotz defekter Angiogenese zeigten $CXCR4^{+/-}$ Herzen keinen Unterschied in der Expression von CXCL12, VEGF oder Apoptose-verwandten Genen. Lipofuszinartige Ansammlungen in $CXCR4^{+/-}$ Herzen und hohe Anzahl von Phosphatidylserinen, die die Kardiomyozyten *in vitro* vor hypoxischem Stress schützen, konnten nachgewiesen werden. Diese Daten zeigen die wichtige Rolle von CXCR4 in endogenen Umbauprozessen nach Myokardinfarkt, die bei der entzündungsabhängigen Progenitorzellrekrutierung und Gefäßneubildung beitragen, während CXCR4 Mangel die Infarktgröße begrenzt und die Anpassung an hypoxische Bedingungen bewirkt. Diese Punkte sollten sorgfältig bei der Entwicklung neuer Therapeutika berücksichtigt werden, die die CXCL12 / CXCR4 Achse involvieren.

Das 2. Kapitel liefert neue Einblicke in das Verhalten von EPCs in Reaktion auf angiogene Faktoren: MIF, VEGF, CXCL12 und CXCL1. EPCs, die aus der Milz isoliert wurden, weisen endothelzell-ähnliche Eigenschaften auf, wie die Aufnahme von acLDL, die Bindung von Lektin und die Expression von mononukleären- und endothelzell-spezifischen Markern sowie die Expression von CXCR2 und CXCR4. rmMIF, rmVEGF, rmCXCL12 und rmCXCL1 verbessern die chemotaktische Kapazität von EPCs, während nur rmMIF und rmCXCL1 in der Lage sind weiterhin auch die Zahl der transmigrierten EPCs durch einen zusätzlichen endothelialen Monolayer zu erhöhen. Die Kultivierung von EPCs auf Matrigel zeigte, dass EPCs allein nicht in der Lage sind, tube-artige Strukturen zu bilden, sondern sich nur in Co-Kultur mit Endothelzellen in diese kapillar-ähnlichen Strukturen integrieren, mit erhöhter Intensität nach Stimulierung mit den Testfaktoren. Dadurch könnte die kombinierte Behandlung von zellbasierenden Therapien einschließlich der EPCs und der angiogenen Faktoren neue therapeutische Ansätze bieten, um verletztes Gewebe zu regeneriere.

Der dritte Teil dieser Arbeit sollte die Rolle von MIF während des Arteriogeneseprozesses im murinen Hinterbein Ischämie-Modell untersuchen. Das pro-inflammatorische Zytokin mit chemokinartigen Funktionen spielt eine entscheidende Rolle bei entzündlichen Erkrankungen, wobei MIF Immunzellen zu den Entzündungsherden lockt. In Mäusen, in denen die Femoralarterie ligiert wurde, wurde eine verringerte Durchblutung der Pfote nach rmMIF Stimulierung gezeigt, während das Blockieren von MIF die Durchblutung und somit die Kollateralisierung verbesserte. Dementsprechend reduziert auch exogenes MIF die Anzahl an Gewebsmakrophagen und CXCR2 positiven Zellen im ischämischen Skelettmuskel, während das Blockieren von endogenem MIF zu einem Anstieg an Makrophagen führte. Monozyten sind in der Lage, Arteriogenese zu stimulieren, indem sie die Migration und Proliferation von Endothelzellen und glatten Muskelzellen stimulieren. In diesem Zusammenhang gelten M1 Makrophagen als pro-inflammatorische, während M2 Makrophagen als pro-angiogenetische Zellen angesehen werden. Weder die Polarisation zu M1 noch zu M2 Makrophagen wurde nach MIF Stimulation beobachtet, das darauf hinweist, dass MIF den Phänotyp von Gewebsmakrophagen nicht beeinflusst. Allerdings beeinflusst das Blockieren von endogenem MIF die zirkulierenden Monozyten im Blut, einschließlich $Ly6C^{low}$ und $Ly6C^{high}$ Monocyten. Ich

vermute, dass das Blockieren von MIF zu einer erhöhten Rekrutierung von Monozyten und bestimmten Subpopulationen wie $Ly6^{low}$ Monozyten aus dem Knochenmark und zu einer erhöhten Migration in die Gefäßwand führt. Die unterschiedliche Wirkung von rmMIF und dem Blockieren von MIF bei Arteriosklerose und Arteriogenese bietet Möglichkeiten für gezielte therapeutische Interventionen in der Behandlung der arteriellen Verschlusskrankheit.

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IX APPENDIX

IX.1 List of Figures

IX.2 List of Tables

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XI CURRICULUM VITAE

PERSONAL INFORMATION

Name:	Nancy Tuchscheerer
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EDUCATION

October 2008 – May 2012	PhD Student (International Euregio Cardiovascular International Research Training Group (EuCAR) 'Arterial Remodeling') Institute for Molecular and Cardiovascular Research, Institute of Biochemistry and Molecular Biology, University Hospital Aachen, Germany Department of Physiology, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University Medical Center, The Netherlands
November 2007 – October 2008	Diploma thesis German Institute of Human Nutrition (DIfE), Clinical Nutrition (Diabetes) “Influence of methionine on the activation of the insulin signaling pathway in HepG2 cells”
October 2003 – October 2008	Nutrition Sciences with major in molecular nutrition research (Grade: “sehr gut”) Friedrich- Schiller-University Jena, Germany Degree: Dipl. Troph.
June 2003	„Abitur“ (Grade 1,8) Johann-Wolfgang-Goethe-Gymnasium in Auerbach, Germany
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PUBLICATIONS

Liehn, E.A.*, **N. Tuchscheerer***, I. Kanzler, M. Drechsler, L. Fraemohs, A. Schuh, R.R. Koenen, S. Zander, O. Soehnlein, M. Hristov, G. Grigorescu, A.O. Urs, M. Leabu, I. Bucur, M.W. Merx, A. Zernecke, J. Ehling, F. Gremse, T. Lammers, F. Kiessling, J. Bernhagen, A. Schober, and C. Weber. 2011. Double-Edged Role of the CXCL12/CXCR4 Axis in Experimental Myocardial Infarction. *J Am Coll Cardiol*. 58:2415-2423

Isabella Kanzler*, **Nancy Tuchscheerer***, Sakine Simsekyilmaz, Simone Konschalla, Andreas Kroh, Guy Steffens, Andreas Schober, Christian Weber MD, Jürgen Bernhagen, Elisa A. Liehn MD. 2011. Macrophage migration inhibitory factor plays a pivotal role in EPCs-induce angiogenesis. *Circ. Res.* **submitted.**

N. Tuchscheerer*, I. Kanzler*, Mark Vries, A. Wagenaar, E. Liehn, D.G.M. Molin, J. Bernhagen, M.J. Post. 2011. Macrophage Migration Inhibitory Factor, a CXCR Ligand with Potential Function in Arteriogenesis. *Circ. Res.* **submitted**.

CONFERENCES / POSTER PRESENTATIONS

European Society of Cardiology Congress 2009, August 29th – September 2nd 2009, Barcelona, Spain:
"Double-edged role of the SDF-1/CXCR4 axis in experimental myocardial infarction". Liehn EA, **Tuchscheerer N**, Kanzler I, Drechseler M, Zandler S, Schuh A, Merx M, Koenen RR, Schober A, Weber C

European Society of Cardiology Congress, August, 2010, Stockholm, Sweden:
"Macrophage migration inhibitory factor, a CXCR ligand with potential function in collateral artery growth". **Tuchscheerer N**, Molin DGM, Schulten H, Kanzler I, Liehn EA, Steffens G, Weber C, Bernhagen J, Post MJ

Basic Science Poster Reception, August 2010, ESC Congress Stockholm, Sweden:
"Macrophage migration inhibitory factor, a CXCR ligand with potential function in collateral artery growth". **Tuchscheerer N**, Molin DGM, Schulten H, Kanzler I, Liehn EA, Steffens G, Weber C, Bernhagen J, Post MJ

9th World Congress of Microcirulation, September 26th – 28th 2010, Paris, France:
"Macrophage Migration Inhibitory Factor, a CXCR ligand with potential function in arteriogenesis". **Tuchscheerer N**, Molin DGM, Schulten H, Kanzler I, Liehn EA, Steffens G, Weber C, Bernhagen J, Post MJ