Evaluation of Different Approaches to Enhancing Arteriogenesis Using Isolated Monocytes and GM-CSF Treatment in an Ischemic Mouse Hind Limb Model

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For my parents and my husband Paul

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1. Introduction

Cardiovascular diseases are the major cause of death and morbidity in adults in most developed and many developing countries. In Germany, according to the statistics in 2004, 23.7 % of all deaths were caused by cardiovascular diseases, in particular by chronic ischemic heart disease, acute myocardial infarction and heart failure [1]. In the same year, 32,241 patients died of stroke which represents the 5th most frequent cause of death. In Germany, 30 % of the sixty year and older population suffers from peripheral arterial occlusive disease. The main causes of death of these patients are myocardium infarction, heart failure and sudden cardiac death [2]. Up to date, treatment involves percutaneous revascularisation with balloon angioplasty and stenting or coronary artery bypass grafting. The long term success of both of these strategies is limited by the development of restenoses. In addition, many patients are still not suitable for conventional treatments. The only hope for those patients could be therapeutic arteriogenesis [3], a non-invasive treatment using growth factors, gene or cell therapy in order to create endogenous “bypassing” vessels.

1.1. Forms of vascular growth

Three different forms of vessel growth are described, of which after birth, only two can be observed: angiogenesis and arteriogenesis.

Angiogenesis is the de novo formation of capillary networks by sprouting of endothelial cells from pre-existing postcapillary networks.

Arteriogenesis refers to the growth of pre-existing collateral arterioles leading to the formation of large conductance arteries that can substitute for an occluded artery.

In the embryo, basic blood vessel growth processes through *vasculogenesis*, which is the in situ differentiation of angioblasts into endothelial cells that form the primary vascular network.

Vasculogenesis is initiated by haemangioblasts (common precursors for both endothelial and haematopoietic cells) in the yolk sac that create cellular aggregates, known as blood islands [4]. Cells of the inner layer of these aggregates differentiate into haematopoietic precursors and cells of the outer layer give rise to precursors of endothelial cells. The earliest markers common to endothelial and haematopoietic precursors have been identified as CD31, CD34 and vascular endothelial growth factor receptor type-2 (VEGFR-2) [5]. Angioblasts

(endothelial cell precursors) can migrate extensively until they differentiate. Some growth factors like vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), basic fibroblast growth factor (bFGF) and insulin-like growth factor-1 (IGF-1) stimulate their differentiation and mobilization [6,7,8].

In summary, vasculogenesis leads to the primary, immature vasculature. The further development of the embryonic vascular system is initiated by angiogenic growth processes.

1.2. Mechanisms of angiogenesis

The term *angiogenesis* was introduced in 1935 by Hertig for the description of placental vessel growth [9]. In 1971, Folkman used this term for neovascularization accompanying tumor growth [10].

Angiogenesis describes the formation of new capillary networks from pre-existing postcapillary microvessels. This process involves the enlargement of venules and can proceed via three mechanisms: by extending of sprouts from sides and ends of pre-existing vessels (sprouting angiogenesis), by longitudinal division of the pre-existing vessel by pillars of peri-endothelial cells (intussusception) or by transendothelial cell bridges formation which separate the pre-existing vessel into capillaries [11]. Newly developed capillaries reach a diameter of 5 to 8 µm.

Angiogenesis is induced in response to tissue hypoxia and initiated by the activation of endothelial cells. Several hypoxia inducible transcription factors (HIFs), such as HIF-1β, HIF-1α and HIF-2α, are upregulated. They bind to specific DNA sequences, hypoxia-response promoter elements, which regulate the transcription of several genes that are involved in the cellular response to hypoxia. For example, HIFs bind to the promoter of the vascular endothelial growth factor gene, which induces expression of VEGF, the strongest known angiogenic factor [12].

The activation of endothelial cells by hypoxia is followed by a nitric oxide (NO) mediated vasodilation of the parent vessel. VEGF, upregulated by NO [13], is responsible for increased vascular permeability followed by extravasation of plasma proteins. The latter serve as a scaffold for migrating endothelial cells. The glycoprotein angiopoietin-1 is a natural inhibitor of vascular permeability and protects the vasculature against plasma leakage which could lead to the circulatory collaps or intracranial hypertension [14].

Basement membrane degradation and extracellular matrix invasion are the next steps in angiogenesis and they are dependent on proteases and protease inhibitors. Many enzymes are involved in this process including matrix metalloproteases (MMPs), chymases, heparanases and serine proteases. The two plasminogen activators u-PA and t-PA convert the plasma protein plasminogen to plasmin, which subsequently activates several MMPs and degrades structural proteins such as laminin, fibronectin etc [15].

After dissolving of the physical barriers, proliferating endothelial cells can migrate to distant sites. This migration is mediated by chemotactic factors released from various cells such as fibroblasts, monocytes, mast cells etc. Angiopoietin-2 is involved in endothelial cell migration by detaching inter-endothelial cell contacts and loosening the peri-endothelial matrix [16]. Growth factors involved in the proliferation of endothelial cells are VEGF and its receptors, placental growth factor (PlGF), fibroblast growth factor (FGF) and platelet–derived growth factor (PDGF). There are several inhibitors of endothelial cell proliferation such as angiostatin, endostatin, antithrombin III and interferon β [14]. NO terminates the proliferative actions of growth factors and promotes the formation of vascular tubes [17].

The next step in angiogenesis is the formation of a capillary lumen. This is caused by thinning of endothelial cells and fusion of pre-existing vessels. Activators of this process are VEGF, angiotensin-1 and $\alpha_v\beta_3$ integrin. An endogenous inhibitor of lumen formation is thrombospondin-1 [14].

Endothelial cells of a newly formed vessel stop to migrate and their survival is controlled by apoptosis. Remodeling and „pruning” of capillary–like vessels results into a structured network of branching capillaries.

In adult organisms, angiogenesis contributes to various physiological and pathological processes such as wound healing, fracture repair, reproduction, tumour growth, retinopathy etc. Understanding the mechanisms of angiogenesis was thought to help forming the base for therapeutic applications in treatment of ischemic cardiovascular diseases or cancer. However, some clinical trials (e.g. VIVA) did not show expected results in treatment of ischemic heart diseases with angiogenic growth factors (e.g. VEGF). The possible reason is that because of their limited ability to conduct blood, capillaries cannot substitute for an occluded artery. In contrast, the stimulation of arterial growth could be a promising therapeutic strategy.

1.3. Mechanisms of arteriogenesis

1.3.1. General principles of arteriogenesis

After an acute occlusion or severe stenosis of a major artery, a steep pressure gradient between the high-pressure pre-occlusive area and the low-pressure post-occlusive region develops. Blood flow is now redirected along this pressure gradient, thereby recruiting the pre-existing collateral network. The increased blood flow velocity within the collateral network leads to the generation of fluid shear stress along the collateral endothelium. A sustained increase in fluid shear stress is the main stimulus for the activation of endothelial cells. The activated endothelium produces a number of cell adhesion molecules (e.g. ICAM-1) and cytokines (e.g. MCP-1), of which the latter attract circulating blood cells [18]. It was suggested that the attraction of monocytes to the vascular wall is followed by their tethering, rolling and transmigration through the endothelium [19]. The presence of monocytes and macrophages was proposed to be important for the development of collateral arteries, since they are producers of numerous cytokines (e.g. MCP-1), growth factors (e.g. TNF-α, b-FGF) and matrix metalloproteases (MMPs). MCP-1 acts as a chemoattractant for monocytes, TNF-α is an inflammatory mediator, b-FGF is a mitogen for both endothelial and smooth muscle cells and MMPs degrade the extracellular matrix and create space for the growing artery.

The presence of pre-existing collateral arteries was first described in 1669 by the English anatomist Lower, who had observed them in the heart. Later studies showed that collateral vessels have the ability to grow by proliferation of endothelial and smooth muscle cells which results in an increase of the collateral diameter up to 20 times [20]. As a result of a twenty-fold increase in collateral diameter and increased number of collaterals, blood flow restoration can reach 30 to 40 percent of the origin value. Pre-existing collateral arterioles usually have an internal diameter between 30 and 50 µm and create an arcade-like network that interconnects territories of arterial subbranches [21]. Pre-existing collaterals are thin-walled conduits composed of the endothelial layer, lamina elastica interna and one or two layers of smooth muscle cells. The presence and development of these interconnecting vessels varies among species and within individuals [22]. Pre-existing collaterals have the ability to increase the lumen by growth and not only by vasodilation.

1.3.2. Biomechanical forces in arteriogenesis

In 1893, the German anatomist Thoma published the so called “Law of Histiomechanics” which describes the relationship between the size of an artery and the velocity of blood flow within it [23]. According to this law, large vessels with a low flow tend to reduce their lumen, whereas small vessels with a high flow tend to widen it (like collateral vessels). As mentioned before, after a relevant stenosis of a major artery occurs, blood flow is redistributed via interconnecting vessels that connect a high-pressure with a low-pressure region. The pressure gradient between two territories causes an increased fluid shear stress (FSS) within the interconnecting arteries. FSS is directly proportional to the blood flow velocity and inversely proportional to the cube of the vessel radius. It ranges from 20 to 30 dynes/cm [24]. The important role of FSS for arteriogenesis was demonstrated in an experiment using a rabbit ischemia hind limb model by performing an arterio-venous shunt distally to the femoral artery occlusion. Artificially increased FSS within collateral arteries of the shunted hind limb led to a 4-fold increase of collateral flow compared with the contra-lateral ligated but not shunted limb [25].

1.3.3. Remodeling of collateral vessels

Remodeling of a pre-existing arteriole into a large artery (arteriogenesis) has some common features with atherosclerotic remodeling which also includes monocyte invasion and neointima formation. However, remodeling of collateral arteries is unique in its extent. The remodeling process starts with the loosening of intercellular connections between smooth muscle cells, which results in a dilatation of the pre-existing arteriole. This is followed by an increase in the vascular permeability of the arteriole, mediated through VEGF, and leads to a plasma leakage. Extravasated plasmatic proteins are probably involved in the formation of new extracellular matrix. Subsequently, the arteriole is invaded by monocytes and lymphocytes. Endothelial proliferation is followed by a disruption of the internal elastic membrane and circular smooth muscle cells of the pre-existing artery undergo apoptosis [26]. The extracellular matrix is digested by matrix metalloproteases to create space for a growing artery. Proliferating smooth muscle cells (SMC) are transformed from the contractile to the synthetic phenotype and create a new extracellular matrix and a new internal elastic membrane [22]. Remodeled collateral arteries have similar histological characteristics like

normal conductive arteries except from increased collagen content between smooth muscle cell layers in the media and an increase in vessel length which causes a typical tortuous pattern.

During later stages of arteriogenesis, the regression of smaller collaterals and the maturation of larger collaterals to optimized tube-like blood vessels can be observed simultaneously. This effect has been called pruning [24]. Pruning may be based on the fact that due to higher resistance in small collaterals blood flow is limited which leads to lower shear stress within these small vessels, while larger vessels with lower resistance conduct increased blood flow. This in turn means higher shear stress which is the driving force for the maturation process.

1.3.4. Time course of arteriogenesis

Histological changes during arteriogenesis can be divided into four stages [18,27]. Despite minor overlap of the phases, this scheme can be used for a description of time course of the collateral development.

The initial phase starts after the vessel occlusion. Transcription factors are upregulated within 30 minutes and mediate expression of adhesion molecules, cytokines and growth factors. Endothelial cells are activated by shear stress and express both intercellular and vascular adhesion molecules (ICAM and VCAM). Blood monocytes are attracted to the endothelium by monocyte chemoattractant protein-1 (MCP-1) and bind to endothelial cells via their integrin receptors LFA-1 and Mac-1. The activated endothelium is characterized by swelling and oedema.

The proliferation phase begins 24 hours after arterial occlusion and peaks at day three to seven. During this phase, an increased mitotic and proliferative activity of endothelial cells, smooth muscle cells and fibroblasts can be observed. Blood monocytes migrate through the vessel wall and accumulate in the adventitia and perivascular space of collateral arteries. Monocytes and macrophages secrete TNF-α and FGF-2 to induce an inflammatory surrounding and matrix-metalloproteases to digest the extracellular matrix.

During the synthetic phase, endothelial and smooth muscle cells assume a synthetic phenotype. The lamina elastica interna is digested by elastases allowing smooth muscle cells to migrate. Accumulated monocytes and macrophages start to degrade and adhesion molecules on endothelial cells are no longer expressed.

The maturation phase starts about two weeks after arterial occlusion. Smooth muscle cells restore a contractile phenotype. Proliferation activity is decreased to a lower level of activity. The vessel numbers and structure are already determined.

1.4. Differences between angiogenesis and arteriogenesis

After the experimental occlusion of a coronary or a peripheral artery, both angiogenesis and arteriogenesis can in principle be observed. Both processes differ in induction, mechanisms, localization and involvement of growth factors and circulating cells. While angiogenesis is induced by hypoxia and therefore occurs in the ischemic region distant to the occlusion site, the stimulus for collateral growth is an increased shear stress caused by increased collateral blood flow. Sustained increase of shear stress activates the collateral vessel endothelium. In the ischemic tissue, upregulation of VEGF can be detected, whereas in the tissue surrounding the occlusion VEGF expression has not been found [28].

Thin layered pre-existing collateral arteries have to undergo remodeling and growth with the assistance of growth factors and cytokines produced from circulating blood cells, unlike angiogenesis, where remodeling does not occur. Growth factors and cytokines reported to be involved in arteriogenesis are TGF-β, MCP-1 and GM-CSF, whereas VEGF, TGF-α and a-FGF were shown to participate in angiogenesis [29]. The accumulation of monocytes was proposed to be specific for arteriogenesis, but not for angiogenesis. Furthermore, only true collateral arteries have the ability to compensate for blood flow deficiencies after arterial occlusions, because as stated in Hagen Poiseuille's law, the ability to conduct blood refers to the fourth potency of the vessel radius. Consequently, capillaries are not recruited to blood conductance over larger distances.

1.5. Recruitment of peripheral blood cells

After onset of ischemia, two different types of cells were previously reported to be recruited from the peripheral circulation: monocytes and endothelial progenitor cells [27]. The invasion of peripheral blood cells is mediated by chemoattraction and the main role of these cells is to produce cytokines and growth factors and to create an inflammatory environment. Platelets also adhere to the collateral endothelium and produce interleukin-4, which increases the expression of adhesion molecules [22]. Basophils, which transform into mast cells after their

migration into a tissue from blood circulation, produce heparin and other substances which are probably involved in collateral artery growth. T-cells have been also observed to be accumulated in the adventitia of a growing artery and they participate in destroying of neighboring cells to create space for a growing artery [24].

1.5.1. Monocytes and macrophages

1.5.1.1. Physiological functions

Monocytes and macrophages were classified by Aschoff as cells of the reticulo-endothelial system. In 1972, Van Furth proposed the mononuclear-phagocyte system classification.

Monocytes are generated in the bone marrow and pass through several developmental stages: stem cells, committed stem cells, monoblasts, promonocytes, monocytes in bone marrow, monocytes in peripheral blood and macrophages in tissue. The process of haematopoiesis is controlled by growth factors. Differentiation from pluripotential stem cells to myeloid progenitor cells is mediated by IL-3. Further differentiation to monocyte/macrophage lineage is mediated by M-CSF and GM-CSF. Moreover, TNF-α has also been implicated in growth regulation of macrophage precursors.

Blood monocytes represent 4-8 % of all blood leukocytes. Circulating monocytes possess migratory, chemotactic, pinocytic and phagocytic activities. After 2 or 3 days in the blood circulation, monocytes migrate through the vessel wall (diapedesis) and commit differentiation to become tissue macrophages [30]. In response to invasive stimuli and inflammation, monocyte numbers increase dramatically.

Macrophages can be divided into resident and inflammatory cells. Resident tissue macrophages are present in large numbers in tunica submucosa, pulmonary alveoli, juxtaglomerular spaces in kidneys, liver, spleen, lymph nodes, bone marrow, serous fluids (pleural and peritoneal), skin etc. but are very rare in skeletal muscles and around blood vessels (own unpublished observations). Resident tissue macrophages are relatively quiescent cells with low oxygen consumption and little or no cytokine production. Their main function is phagocytosis and chemotaxis. The population is maintained by local proliferation of progenitor cells and not via monocyte influx, and the life span of resident macrophages is between 6 and 16 days. Macrophages isolated from different anatomical sites possess diverse

phenotypes and capabilities. Inflammatory macrophages are present in exsudates. They can be characterized by specific markers like peroxidase activity and since they are derived exclusively from monocytes, they share similar properties.

Macrophages in their activated state can secrete a large number of substances (shown in table 1) and their biologic activity range from induction of cell growth to cell death. They can be primed for activation by IFN-γ, M-CSF, GM-CSF, TNF-α etc. In the activated state, they exhibit enhanced MHC class II expression, antigen presentation, oxygen consumption and reduce their proliferative capacity. Macrophages can be deactivated by prostaglandin E_2 (PGE_2) and the so called macrophage deactivating factor (MDF) which was isolated from a tumor cell supernatant [31].

Polypeptide hormones	IL-1, IL-6, IL-8, TNF-α, IFN-α, IFN-γ, PDGF, FGF, TGF-β, GM-CSF, G-CSF, erythropoietin
Complement components	C1, C2, C3, C4, C5, factors B, D, P
Coagulation factors	V, VII, IX, X, plasminogen activator prothrombin, plasmin inhibitors
Bioactive lipids	PGE_2, PGF_2, PGI_2, tromboxans LTB_4, LTC_4, LTD_4, LTE_4
Enzymes	elastase, collagenase, hyaluronidase, arginase acid proteases, peptidases, lysozyme, lipases, ribonucleases, phosphatases
Protease inhibitors	α_2-macroglobulin, plasminogen activator inhibitors, collagenase inhibitor
Proteins of extracellular matrix	fibronectin, thrombospondin, chondroitin sulfate proteoglycans
Reactive oxygen intermediates	superoxide, hydrogen peroxide, hydoxyl radical, hypothalite
Reactive nitrogen intermediates	nitric oxide, nitrites, nitrates
Others	thymidine, uracil, uric acid, neopterin

Table 1. Secretory products of macrophages. Modified from Nathan et al. 1987.

Macrophages are involved in immune, homeostatic and inflammatory responses. They provide an immediate defense against foreign elements prior to leukocyte immigration. Macrophages participate in both specific immunity via antigen presentation and IL-1 production and unspecific immunity by phagocytosis of bacterial, viral, fungal and neoplastic pathogens.

1.5.1.2. Monocytes and arteriogenesis

The attachment of monocytes to the collateral endothelium (Fig. 1) was observed for the first time in canine heart [32].

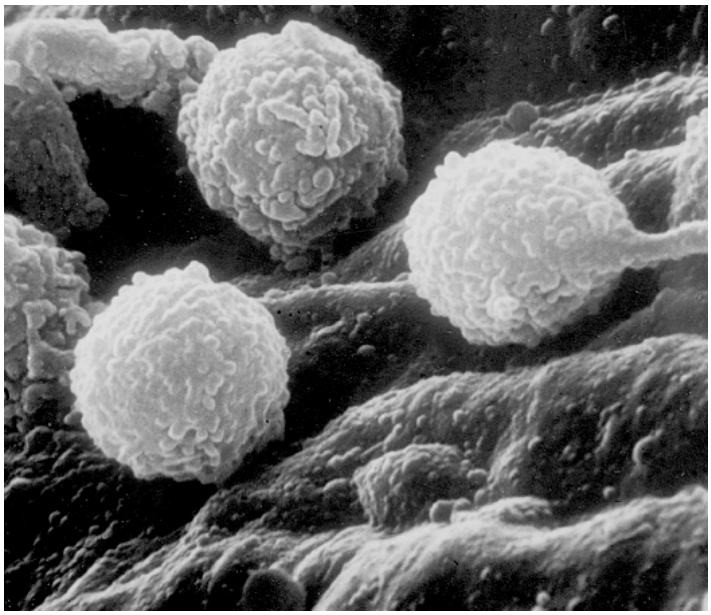


Fig. 1. Monocytes adhere to the endothelium of the growing collateral artery. (Schaper et al. 1976)

Later studies revealed that monocytes which accumulate in the perivascular space of growing collateral arteries [18] produce growth factors and cytokines like bFGF, TNF-α [33] and VEGF [34]. Conversion of monocytes into an active state occurs by binding of stimulatory factors such as cytokines and other cell-derived molecules. Monocytes are attracted to the collateral endothelium by cytokines like PlGF or MCP-1. The latter is expressed by shear stress-activated endothelial cells [35]. The expression of MCP-1 is regulated by growth factors (e.g. VEGF and FGF-2). In the rabbit ischemia hind limb model, a local administration of MCP-1 into the proximal stump of the occluded artery via an osmotic minipump resulted in

an increased density of collateral arteries in the thigh area and increased collateral conductance [35]. Our study showed that targeted disruption of the MCP-1 receptor, CCR-2, in mice almost abolished collateral artery growth [36] due to the impaired monocyte recruitment.
Monocyte adhesion to the endothelium is mediated by specific adhesion molecules on monocytes termed integrins and by both intercellular and vascular cell adhesion molecules (ICAM and VCAM) on endothelial cells. Integrins are heterodimeric cell surface receptors composed of two trans-membrane glycoproteins: a variable α-chain and a non-variable β-chain. The β_2-subfamily represents leukocyte-specific integrins such as LFA-1 (leukocyte function associated antigen-1) and Mac-1. Integrins mediate attachment of cells to the matrix, cell-to-cell connections, and regulate intracellular signal transduction [37]. Monocytes bind to the ICAM-1 molecules on endothelial cells by their Mac-1 receptor, invade the vascular wall and accumulate in clusters in the adventitia and the perivascular space of the growing artery. It was shown that an intravenous infusion of anti-ICAM-1 antibodies diminished monocyte attachment and arteriogenesis [38]. Monocytes also express VEGF receptor 1 (also known as Flt-1), which binds to VEGF A [39]. Incubation of monocytes with VEGF enhances surface expression of LFA-1 and Mac-1 [40] , thus enhances monocyte migration through the vessel wall.

1.5.2. Endothelial progenitor cells

Endothelial progenitor cells (EPC) were identified in the blood as a circulating cell population [41] which co-expresses both endothelial cell markers (VE-Cadherin, VEGFR-2) and hematopoietic stem cell markers (CD34, CD133). EPC are very rare in the blood but can be mobilized from the bone marrow into the circulation by vascular trauma, ischemia and administration of cytokines like G-CSF and GM-CSF. After mobilization with G-CSF, only 2 % of all CD34 positive cells also express CD133 and VEGFR-2 [6]. EPC have been shown to migrate to the place of physiological or pathological angiogenesis [42]. Their potential and mechanisms are controversially discussed.

1.5.3. Other cells involved in arteriogenesis

Mast cells were found in aggregates in the adventitia of growing collateral arteries. It has been shown that they produce growth factors like b-FGF, VEGF, platelet activating factor and cytokines [43]. Their role in arteriogenesis has yet not been completely elucidated.

Bone marrow-derived stem cells have been proposed to circulate in adult organisms and to be recruited to sites of pathological and physiological neovascularisation [41],[44]. Further promising studies revealed that transplantation of these cells could augment perfusion of an ischemic tissue [45],[46]. The suggested mechanism of perfusion improvement was due to the incorporation of bone marrow-derived cells into the adult vasculature. In contradiction, recent studies showed that these cells did not act by trans-differentiation into the organ-specific cells, but they produced angiogenic growth factors and cytokines, therefore described as “cytokine factories”, necessary for the collateral artery growth [47],[48].

1.6. Vascular growth factors and chemokines

1.6.1. Growth factors

More than three decades ago, a tumor growth factor was discovered which was found to be mitogenic for endothelial cells [10]. This factor was later identified as a member of the fibroblast growth factor family. Since that time, many angiogenic proteins have been discovered. Later studies on growth factors have shown that collaterals can develop only in the presence of growth- or angiogenic factors and when their receptors are expressed. Infusion of growth factors into healthy hearts did not induce angiogenesis probably because of the absence of their receptors in normal tissue [26]. Hence, the regulatory mechanism of vascular cell division is the availability of growth factor receptors and not the presence of a ligand. A list of the most important angiogenic proteins is in table 2.

On the one hand, growth factors are involved in the stimulation of functional repair processes, including collateral vessel formation. On the other hand, the role of growth factors in tumorgenesis and cardiovascular disorders, in particular atherogenesis and restenosis, have been recognized [49]. The modulation of growth factors-related signals was suggested to be used for the treatment of cardiovascular diseases.

Acidic fibroblast growth factor (aFGF)	Granulocyte colony-stimulating factor (G-CSF)
Angiogenin 1	Placental growth factor
Angiogenin 2	Platelet-derived growth factor (PDGF)
Angiopoietin-1	Proliferin
Angiopoietin-2	Thrombospondin (TSP)
Basic fibroblast growth factor (bFGF)	Transforming growth factor-α(TGF-α)
Fibroblast growth factor 3 (FGF-3)	Transforming growth factor-β (TGF-β)
Fibroblast growth factor 4 (FGF-4)	Tumor necrosis factor-α (TNF-α)
Fibroblast growth factor 5 (FGF-5)	Vascular endothelial growth factor 121 (VEGF-121)
Fibroblast growth factor 6 (FGF-6)	Vascular endothelial growth factor 145 (VEGF-145)
Fibroblast growth factor 7 (FGF-7)	Vascular endothelial growth factor 165 (VEGF-165)
Fibroblast growth factor 8 (FGF-8)	Vascular endothelial growth factor 189 (VEGF-189)
Fibroblast growth factor 9 (FGF-9)	Vascular endothelial growth factor 206 (VEGF-206)
Heparin	Vascular endothelial growth factor B (VEGF-B)
Heparin affinity regulatory peptide	Vascular endothelial growth factor C (VEGF-C)
Hepatocyte growth factor (HGF)	Vascular endothelial growth factor D (VEGF-D)
Insulin-like growth factor	Vascular endothelial growth factor E (VEGF-E)
Interleukin 8	Vascular endothelial growth factor F (VEGF-F)

Table 2. List of angiogenic proteins. Modified from Kutryk et al. 2003.

Growth factors regulate various cellular functions including proliferation, migration, differentiation and survival. They act through binding to their specific, membrane-bound cell surface receptors (e.g. tyrosine kinase type receptors). This activates a specific signal transduction cascade which can reach the nucleus and modulate proliferation and differentiation or it can directly modulate the function of cellular proteins. The majority of growth factors (e.g. PDGF, bFGF and VEGF) are positive regulators of the cell cycle and they stimulate cell proliferation and migration. A negative regulation of the cell cycle (e.g. by TGF-β) leads to a growth arrest. The interaction between growth factors and their receptors is increasingly complicated by the fact that different isoforms of a growth factor can bind to the same or different receptors. This has to be considered when a therapeutic strategy is aimed to block the interaction between a specific growth factor and its receptor.

Growth factors involved in collateral artery growth are fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and potentially also granulocyte-macrophage colony-stimulating factor (GM-CSF). FGF is involved in extracellular matrix modulation, cell replication and interactions related to capillary tubule formation. Transgenic overexpression of FGF-1 in the heart leads to increased arteriolar density and branching [50]. PDGF is a strong mitogen for mesenchymal cells such as vascular smooth muscle cells (VSMC) and fibroblasts. The PDGF-BB isoform is the most potent chemotactic agent for VSMC [51].

1.6.1.1. Granulocyte-macrophage colony-stimulating factor

Granulocyte-macrophage colony-stimulating factor (GM-CSF) was the first colony-stimulating factor being responsible for the mobilization of myeloid progenitor cells from the bone marrow. It belongs to the family of glycoprotein cytokines that act by stimulating proliferation, maturation and function of haematopoietic cells [52].

The human GM-CSF gene has been mapped to the long arm of chromosome 5, together with M-CSF, IL3, IL4 and IL5. GM-CSF can be synthesized by macrophages, T and B lymphocytes, fibroblasts, endothelial cells and mast cells in response to specific activating signals. T lymphocytes and macrophages are activated by immune or inflammatory stimuli. Fibroblasts and endothelial cells are activated by monokines such as IL-1 and TNF.

Human GM-CSF was identified in 1984. It is a glycoprotein with molecular weight of 22 kD. Human and murine GM-CSF cDNAs encode polypeptides of 144 and 141 amino acids, respectively. Two intra-chain disulfide chains are important for its biologic activity. Despite a 54 % identity at the amino acid level between murine and human GM-CSF, no cross-species reactivity was observed [52].

GM-CSF acts as a growth factor both in vitro and in vivo, stimulating proliferation and maturation of myeloid progenitor cells, eosinophils, neutrophils and monocytes. Furthermore, GM-CSF enhances the phagocytic activity of neutrophils, eosinophils and macrophages and increases antibody-dependent cell-mediated cytotoxicity towards tumor cells. The most important clinical application of GM-CSF is to counter the myelosuppressive side effects of chemotherapy and radiation therapy, to enhance host defense in immunocompromised states and to accelerate recovery after bone marrow transplantation. Moreover, a potential antineoplastic activity has been reported [53]. Proposed mechanisms include secretion of molecules that are cytotoxic to tumor cells. Recently, an anti-atherogenic effect of GM-CSF treatment was observed in a study performed on hyperlipidemic rabbits [54].

Histological studies showed reduced intimal thickening and areas of atherosclerotic lesions in the aortic arch of rabbits treated with GM-CSF compared to controls. Moreover, GM-CSF reduces both total and HDL cholesterol levels in patients [55].
Potential arteriogenic effects of GM-CSF could be associated with monocytes/macrophages. The suggested mechanisms of action of GM-CSF are by releasing immature monocytes from the bone marrow and by increasing life span of monocytes/macrophages [56].

1.6.2. Chemokines

Chemokines are a group of chemotactic cytokines responsible for attraction, migration and activation of leukocytes. Chemokines act locally in a paracrine and autocrine way via binding to specific cell surface receptors. There are about 50 human chemokines that are divided into the four major families according to their structure and functions. “CC-chemokines”, which contain two cysteine residues adjacent to each other, attract mononuclear cells to sites of inflammation. The best studied chemokines of this group are the monocyte chemoattractant proteins (MCPs) 1-5. MCP-1, also known as CCL2 [57], is a potent chemoattractant for monocytes, T lymphocytes and basophils [58]. MCP-1 mediates monocyte migration during collateral vessel growth [35]. Other members of the CC-chemokine family are macrophage inflammatory proteins (MIPs) α, β, γ and RANTES. Members of “CXC-chemokines”, e.g. interleukin-8, attract polymorphonuclear leukocytes. The third family, “CX3C-chemokines”, is represented by fractalkine which is a chemoattractant for monocytes, T lymphocytes and natural killer cells.

1.7. Prostacyclins

Prostacyclin (PGI_2) is the most abundant product of arachidonic acid in vascular tissues. Arachidonic acid is released from phospholipid pools by phospholipase A_2 and converted to prostaglandin H_2 (PGH_2) by the cyclooxygenase system (COX). PGH_2 is subsequently converted to PGI_2 by PGI_2 synthase. PGI_2 is labile and undergoes spontaneous inactivation within minutes in vivo. For clinical application, several stable synthetic analogues have been developed and are commercially available like Iloprost, Cicaprost and Carbaprostacyclin.

Prostacyclins exhibit vasoactive, mitogenic and differentiating properties. The important role in vascular physiology is based on their anticoagulatory and vasodilative activities. PGI_2 signaling pathway occurs through cell surface receptors (IPs) with a consequent increase of intracellular cAMP, and through peroxisome proliferator-activated receptors (PPARs), located in the nucleus [59]. The PGI_2 analogue Cicaprost acts only on IP receptors. Human monocytes express high levels of IP receptor mRNA. It has been proposed, that PGI_2 may induce VEGF production in human monocytic cell line via interaction with IP receptors on the cell membrane and thus may have a potential angiogenic effect [60]. Moreover, stimulation of monocytes with Cicaprost increases production of inducible NO synthase in coculture with lipopolysaccharide and may contribute to the increased NO production during inflammation [61]. In addition, PGI_2 was reported to modulate cell fate through PPARs by induction or suppression of apoptosis[62]. Whereas intracellular prostacyclin formed by prostacyclin synthase in human embryonic kidney cells promotes apoptosis, treatment of cells with extracellular prostacyclin reduces apoptosis.

Based on the fact that prostacyclins may delay the differentiation of monocytes into macrophages, we investigated the effect of monocyte priming with PGI_2 on their arteriogenic properties.

1.8. Study objectives

The objective of the present study was to investigate the role of monocytes for arteriogenesis. In our experimental settings, injections of monocytes were used to investigate the blood flow recovery after the experimental femoral artery occlusion in mice. An important focus was set on the concentration of blood monocytes. Furthermore, the influence of a systemic treatment with GM-CSF in a mouse ischemic hind limb model was tested. The present study was designed according to the several queries:

1. Is there a correlation between the concentration of circulating monocytes and the extent of arteriogenesis?
2. What is the origin of monocytes/macrophages which can be found around growing collateral vessels?
3. Can arteriogenesis be enhanced by manipulating with the differentiation process of monocytes?
4. Does GM-CSF play a role in arteriogenesis?

5. Does a synergistic effect arise from a combination of GM-CSF treatment and monocyte injections?

2. Material and methods

2.1. Overview

Murine monocytes were isolated from peripheral blood and after pre-treatment injected into mice with acute experimental femoral artery occlusion. Within the observation period of three weeks, blood flow recovery to the ischemic hind limb was monitored using laser Doppler Imaging (LDI) at certain time points. Three weeks after operation, animals were sacrificed, hind limb vessels were visualized using angiography and adductor muscles were sampled for histological analysis.

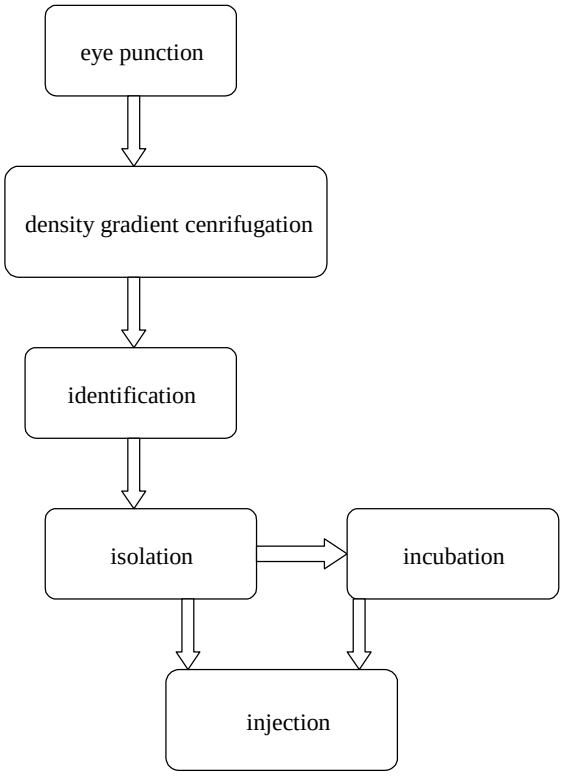


Fig.2. Scheme of the methods used for monocyte isolation and injection.

All animal experiments were performed with the approval of the bioethic committee of the Regierungspräsidium Darmstadt and according to Section 8 of the *German Law for the*

Protection of Animals, which conforms to the US National Institutes of Health (NIH) guidelines.

2.2. Animals

For all experiments, male BALB/cAnNCr1 mice were used which had been purchased from Charles River Laboratories, Germany. The animals were 9 to 14 weeks old, weighted between 20 and 25 grams and were free of pathogens. The mice had free access to water and chow. Housing and handling of animals was in accordance with the German Law for the protection of animals.

2.3. Isolation of murine blood monocytes

BALB/cAnNCr1 mice were used as blood donors. Mice were anaesthetized with an intraperitoneal injection of 10 % Ketamin (Medistar) and 2 % Xylazin (Medistar) in 0.9 % NaCl solution and heparinized with 0.1 ml of Heparin (500 IU; Roche). After 5 minutes, blood was obtained from orbital vessels by penetration with heparinized capillaries. These capillaries were slightly turned against the orbital bone. After collecting approximately 800 µl blood which was sampled in EDTA-coated tubes, mice were immediately sacrificed.

Narcosis : 1 ml Ketamin (10 %)
1 ml Xylazin (2 %)
2 ml NaCl (0.9 %)
0.08 ml of narcosis was used per mouse. If required, 0.02 ml of the same solution were injected at a later time point.

2.3.1. Density gradient centrifugation

The collected blood was diluted in an equal volume of 0.9 % NaCl. 10 ml of the diluted blood was layered over 3 ml Ficoll-Hypaque solution (Amersham Biosciences) in 15 ml centrifugation tubes. The density gradient centrifugation was performed for 20 min at 20 °C and 2300 rpm (1200 x g). After centrifugation, mononuclear cells which had accumulated

in a band above the gradient solution were removed using a pipette. Harvested mononuclear cells were diluted 1 : 2 with 0.9 % NaCl and centrifuged for 10 min at 4 °C and 1800 rpm (600 x g). The supernatant was removed and the pellet was resuspended in 0.9 % NaCl. Afterwards, the centrifugation was repeated as before.

2.3.2. Staining of monocytes using fluorochrome-conjugated monoclonal antibodies

To specifically identify the monocytes, mononuclear cells were incubated with 10 µl of a phycoerithrin (PE)-conjugated murine monoclonal antibody against CD 11b (Beckman-Coulter) for 30 min at 4 °C. Before sorting, remaining erythrocytes were lysed with 1 ml of Lysis-buffer for 1-5 min and washed with 0.9 % NaCl.

Lysis-buffer:

Ammoniumchloride	1.605 g
Potassiumhydrogencarbonate	500.6 mg
EDTA	20.2 mg
Distilled water	500 ml

pH was adjusted to 7.4

2.3.3. Flow cytometry-based monocyte sorting

Flow cytometry is used to analyse the distribution of the cells or cell surface molecules on the cells in a suspension. It serves, for example, for characterization of leukocytes or for identifying progenitor cells from the peripheral blood.

Cells of interest can be labeled with a monoclonal antibody specific to a cell antigen. The antibody is conjugated with a fluorochrome. Labeled cells can be further analyzed according to their forward and side scatter characteristics and – in case of monocytes - their CD 11b expression. Statistical analysis and counting of the labeled cells is performed by software. In order to isolate the cells of interest, flow cytometry-based sorting of suspension cells can be performed achieving high purity and yield of isolated cells.

In our experiments, a high speed cell sorter (EPICS Altra, Beckman-Coulter, USA) was used for isolation of monocytes from the mononuclear cell fraction. Prior to isolation, the sorter was set up and sheath fluid was equilibrated to a constant temperature of 8-10 °C using an

external cooling water bath. Sort settings were established as shown in table 3. To maximize cell viability, the system was run at a pressure of 12 psi using collection tubes coated with bovine serum albumin.

Sheath fluid :	0.9 % NaCl
Frequency :	28.9 kHz
Drive :	20-30 %
Delay :	33

Tab. 3. Sort settings used to isolate mouse monocytes using the EPICS Altra cell sorter.

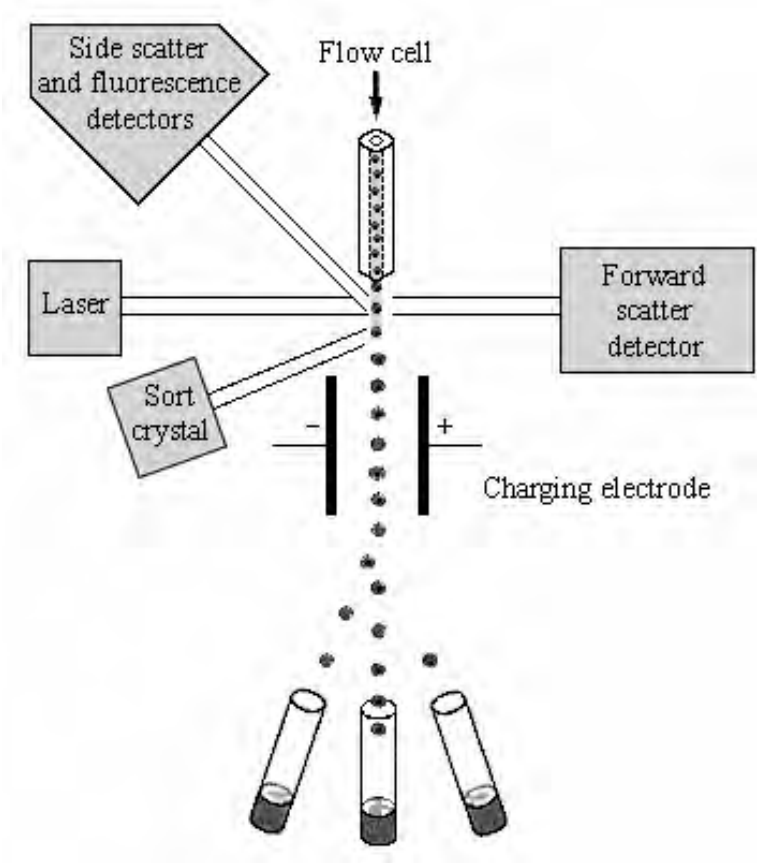


Fig. 3. Principle of flow cytometry and sorting.

As shown in figure 3, the cell suspension was directed into a sheath stream. The sample stream was disrupted into drops by a piezoelectric crystal at a frequency of 28.9 kHz. A laser beam was directed to the stream before it was disrupted into the drops. Emitted light

was recognized by electronic detectors. Drops which contained identified cells were deflected from the sample stream and collected in centrifuge tubes.

2.3.4. Incubation of monocytes

Isolated monocytes were centrifugated by 1800 rpm (600 x g) at 4 °C for 10 min and resuspended in 1 ml RPMI 1640 medium. After that, monocytes were incubated for 3 hours with 200 ng of rmGM-CSF (Schering) at 37 °C and 5 % CO_2.
After the incubation, monocytes were centrifuged at 1000 rpm (200 x g) for 8 min. Cells were resuspended in isotonic NaCl to a maximal volume of 150 µl for i.v. injection.

For the next experimental group, isolated monocytes were centrifuged as described above, resuspended in 1 ml RPMI 1640 and subsequently incubated with 100 ng of Cicaprost (Schering) for 2 hours at 37 °C and 5 % CO_2.

The following table provides an overview on the different groups included in the study.

Group	***Treatment***	***Dosis***	***N***
1	0.9 % NaCl (control)	150 µl; i.v.	24
2	Monocytes	2 x 10^5 cells/mouse; i.v.	11
3	Monocytes	3 x 10^5 cells/mouse; i.v.	9
4	rm GM-CSF	500 ng; i.p.	11
5	Monocytes and rm GM-CSF	2 x 10^5 cells/mouse i.v. + 1µg rm GM-CSF i.p. for 5 days	10
6	Monocytes and rm GM-CSF	3 x 10^5 cells/mouse i.v. + 1 µg rm GM-CSF i.p. for 5 days	7
7	Monocytes incubated with Cicaprost	2 x 10^5 cells/mouse + 100 ng Cicaprost; 2 h incubation; i.v.	11
8	Monocytes incubated with rm GM-CSF	2 x 10^5 cells/mouse + 200 ng rm GM-CSF; 3 h incubation; i.v.	9

Tab. 4. Overview on different groups included in the study.

2.4. Animal model

The study was conducted using a model of regional hind limb ischemia in mice. Mice were anaesthetized and pre-operative laser Doppler Imaging was carried out. The femoral artery was occluded and post-operative laser Doppler Imaging followed. Afterwards, isolated monocytes were injected into a tail vein as shown in figure 4.

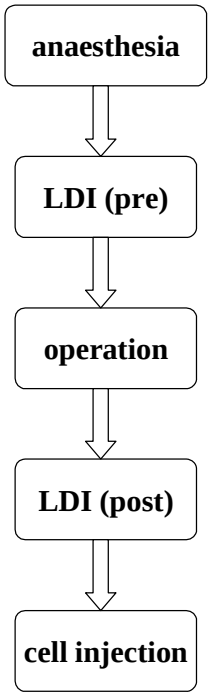


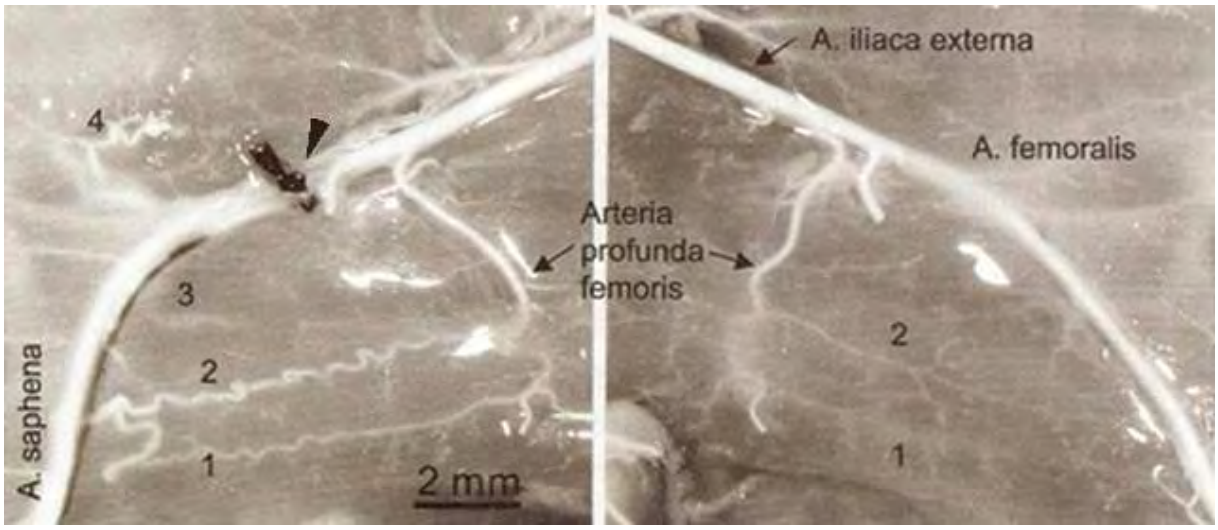
Fig.4. Schematic overview on the treatment included in the animal model.

2.4.1. Operation

Mice were anaesthetized with 0.08 ml of a solution containing 10 % Ketamin (2 mg/kg body weight) and 2 % Xylazin (13 mg/kg body weight) in 0.9 % NaCl solution. To protect the cornea against inflammation, eyes were covered with a panthenol containing cream. During operation mice were placed on a heated pad (37 °C) and limbs were fixed to it to ensure the immobilization and a good surgical access.

The skin was cut with one incision above the ligamentum inguinale. The A. femoralis was separated from the V. femoralis and the N. femoralis by a blunt preparation. The femoral

artery was ligated distally to the A. profunda femoris (as shown in figure 5) and proximally to the A. genus descendens by a surgical double knot using 6-0 metric non absorbable silk suture. The operation area was rinsed with 0.9 % NaCl to protect the tissue from drying. Finally, the skin was closed with the same 6-0 metric silk suture.



right leg left leg

Fig. 5. Vessels of the thigh filled with the contrast agent. Development of 4 corkscrew collateral arteries is visible in the occluded right leg. Arrowhead points at the ligature. Pre-existing collaterals are visible in the left leg. Modified from Ziegelhoeffer et al. 2003.

2.4.2. Injection of monocytes

Isolated monocytes were resuspended in 0.9 % NaCl to a concentration of 10^6 cells/ml. Mice were exposed to infrared light for 2 minutes to increase visibility of the tail veins due to vasodilation. After desinfection of the tail using alcoholic skin desinfection agent, monocytes (in 150 µl of saline solution) were injected into a tail vein. The injection was performed within 30 minutes after ligature of the femoral artery.

2.5. Laser Doppler Imaging

2.5.1. Principle of laser Doppler Imaging

The laser Doppler Imaging technique was developed to measure blood flow in the microcirculation. Since the laser penetration depth of the tissue is 1-2 mm, the method can be used for measurements of “fluxes” in capillaries close to the skin surface, in the underlying arterioles and venules and is also suitable for quantification of blood flow in mice feet.
According to the Doppler principle, the low power light from a monochromatic laser directed at a tissue is scattered by moving red blood cells. In consequence, the frequency of the reflected light correlates with the blood, motion which is used to quantify blood flow.

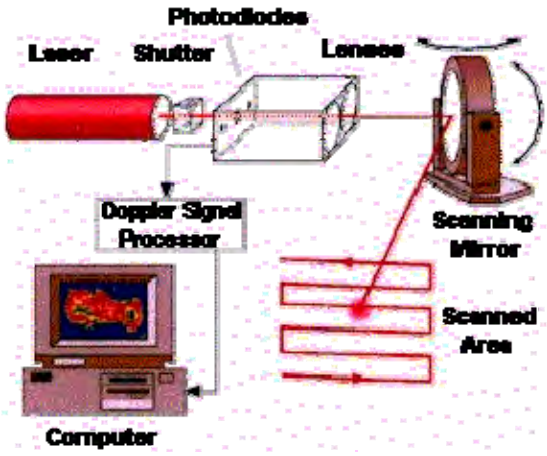


Fig. 6. Scheme of a Laser Doppler Imager.

The frequency range is typically 20 Hz to 20 kHz. Laser light can be directed to the tissue surface either via an optic fibre or as a light beam. In our laser Doppler blood flow Imager (LDI) the low intensity laser beam is used to scan across the tissue surface using a moving mirror (Fig. 6). There is no direct contact with the measured tissue. The scanned area can be defined and acquired data recorded and calculated. The laser Doppler Imager provides spatial information and has the ability to average blood flow measurements over large areas.
The term used to describe blood flow measured by the laser Doppler technique is “flux”. It is the quantity proportional to the product of the average speed of the blood cells and their number concentration. This is expressed in arbitrary “perfusion units” and is calculated using the first moment of the power spectral density.

2.5.2. Quantification of foot blood flow in the mouse model using LDI

Relative blood flow was measured in the feet of mice using a laser Doppler perfusion Imager (MLDI 5063; Moor Instruments, UK). Measurements were performed before operation, immediately after and on postoperative days 3, 7, 14 and 21.
To achieve standardized conditions, mice were anaesthetized and maintained in a climatized chamber at 37 °C for five minutes before every measurement. Mice were placed on the back and feet were taped in parallel to each other with the plantar side down on a pad so that the back of the foot was exposed to the laser beam. Afterwards, a scanning region was defined. The pixel resolution was set at 256 x 256 and the scan rate was 4 ms/pixel for 1.7 x 3 cm area. The background value was determined by performing post mortem measurements and was subtracted from every measured value. The right-to-left ratio (occluded-to-non-occluded leg) was quantified for each mouse. Results are expressed as mean ± SEM.

2.6. Hemoglobin oxygen saturation

2.6.1. General principle of spectrophotometry

Measurement of light absorption of substances for analyzing their optical properties and concentration can be performed using a *spectrophotometer*. The excitation light is focused by lenses and passes through a monochromator which separates it into a single-wavelength light. One particular wavelength is selected to pass into the sample. The light transmitted through the sample is detected by a photodetector and converted into the electric current measured by galvanometer. The electric current is translated into a readable signal on the digital display. The measurement is based on different intensities of the excited and transmitted light.

2.6.2. Assessment of hemoglobin oxygen saturation using spectrophotometry

Oxygen saturation of hemoglobin in the mice feet was determined using a tissue spectrophotometer AbTisSpec (LEA Medizintechnik, Germany) measuring the absorption

spectrum in a wavelength range of 500 to 620 nm. The optical sensor detected local values of both, the saturation and hemoglobin concentration.

Mice were placed for five minutes in a climatized chamber with a constant temperature of 37 °C to keep standardized conditions. The optical sensor was positioned above the mouse foot. Measurements were performed before the operation, immediately after and on postoperative days 3, 7, 14 and 21. The right-to-left ratio was calculated from each measurement. Results were expressed as mean ± SEM.

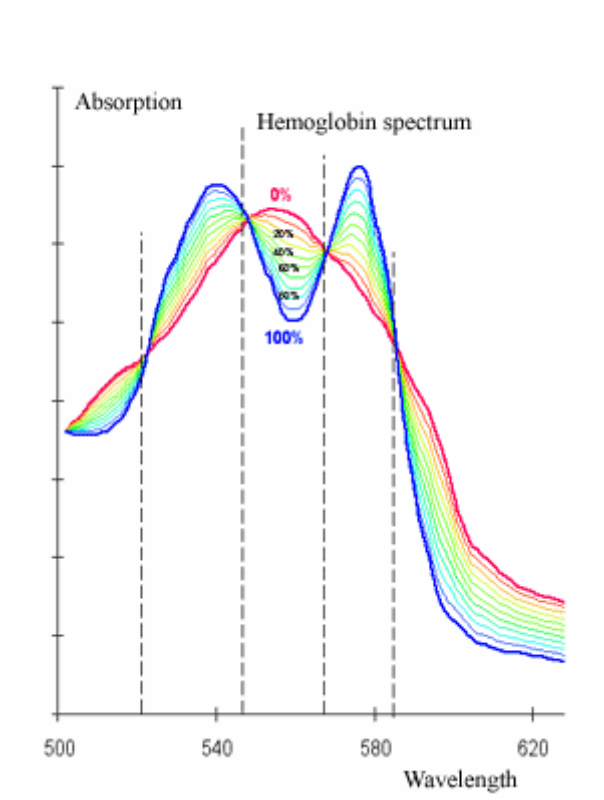


Fig. 7. Oxygen saturation of hemoglobin (0-100 %). Modified from www.cephalon.dk

2.7. Active foot movement

After ligature of the femoral artery, due to reduced pedal blood flow circulation, mice typically showed an impaired active foot movement. To assess the active foot movement as a functional parameter, a score system was developed to describe the functional recovery of the occluded hind limb (Tab.5).

0	No use of the foot, necrosis of the foot or toes
1	Passive movement, foot is dragged
2	Active movement without spreading the toes
3	Spreading of the toes is restricted, claudication
4	Unrestricted movement

Table 5. Active movement score.

Mice were placed into an empty cage and stimulated to move. The score zero was given to mice which did not use the foot at all. These mice often exhibited necrosis. Mice with a progressive necrosis were sacrificed. Mice with a passive foot movement received score one. The leg was not involved in movement and it was only pulled. Active movement was scored with two in the case, when mice could not spread toes but were already able to step with the occluded leg. Score three was given to mice which could spread the toes but only restricted. There was still an apparent claudication in the movement. Complete recovery of the foot movement was scored with four. In this case, no difference between occluded and non-occluded leg was visible.
The measurements were performed on postoperative days 3, 7, 14 and 21. Results are expressed as mean ± SEM.

2.8. Tissue perfusion and fixation

For tissue sampling which was performed three weeks after ligature of the femoral artery, all mice were heparinized with 0.1 ml of Heparin by an intraperitoneal injection and anaesthetized by injecting an overdose of Ketamin and Xylazin (10 % Ketamin, 2 % Xylazin) intraperitoneally. Mice were placed in a plastic dish laying on the back and limbs were spread and fixed. The chest and abdominal cavities were opened and the proximal part of the abdominal aorta was exposed, separated from the fat tissue and the vessel was cannulated. Subsequently, the inferior vena cava was cut. Catheters were made from a winged infusion set cutting off the tip of the needle and blunting the end of the needle. Hind limb vessels were perfused with dilatation buffer containing 0.1 % adenosine (Sigma) and 0.5 % bovine serum albumin (BSA; Sigma) in phosphate-buffered saline (PBS) with pH 7.4 for 4 minutes under the pressure of 100 cm H_2O. After reaching the maximal vasodilation, the perfusion was

switched to 3 % paraformaldehyde (pFA; Sigma) in PBS (pH 7.4) for 10 minutes. Afterwards, the vessels were again perfused with PBS for 3 minutes to remove pFA which reacts with the gelatine. Subsequently, the bismuth-based contrast agent was infused for 2 minutes under the constant pressure of 200 mm Hg for 2 minutes. During perfusion with the contrast agent, mice were immersed in 40 °C warm water. Finally, mice were covered with ice to enhance gelatination of the contrast agent.

Used solutions:

PBS (phosphate-buffered saline):

NaCl	7.948	g
KCl	0.2	g
KH_2PO_4	0.2	g
$NaHPO_4 * H_2O$	1.775	g
Distilled water	1	L

pH was adjusted with HCl or NaOH to 7.4

3 % pFA (paraformaldehyde):

30 g pFA powder were added to 1000 ml PBS. While stirring, pH was slightly increased by adding 2 or 3 NaOH crystals to the solution. After a complete dissolving of pFA, pH was adjusted to 7.4 and solution was stored at –20 °C until use.

Dilatation buffer:

Adenosin	1 g
Bovine serum albumin	5 g
Phosphate-buffered saline	1 L

pH was adjusted to 7.4

2.9. Angiograms

After one hour, mice were removed from the ice bath. Muscles of the lower limbs were exposed from the skin. The abdominal cavity organs were removed and the upper part of the body was cut off. Angiograms were taken using a Balteau radiography apparatus. For this, mice were placed at the bottom of a plexiglass box filled with cold water and fixed on the

bottom of it. A Roentgen film was placed underneath the box and was exposed for 2.5 minutes at 20 kV and 8 mA.

2.9.1. Preparation of contrast agent

100 g Bismuth chloride (Merck) was added to 800 ml 18 % NaCl solution. Afterwards, the solution was placed on a stirrer and 32 % HCl was added dropwise while stirring, until bismuth chloride was completely dissolved. Then, the solution was filtrated through a gaze. 5 L beakers were filled with cold water. Subsequently, 250 ml of the solution was added dropwise into the beaker while stirring. Bismuth was let to sediment for 40 minutes. After this, the supernatant was removed and the sediment was collected in an Erlenmeyer flask to finalize sedimentation. After this, the supernatant was removed and the bismuth was stored at –20 °C. Before using of the contrast agent, a 10 % gelatin solution in distilled water containing 0.01 % Sodiumazid was prepared. The bismuth solution was mixed with an equal amount of gelatin and incubated at 37 °C until the instillation.

Used solutions:

18 % NaCl: 180 g NaCl
1000 ml distilled water
10 % gelatin: 5 g gelatin
50 ml distilled water

2.9.2. Development of angiograms

Exposed Roentgen films were developed for four minutes in the darkroom by immersion in Kodak LX 24 developer and after washing the films were fixed for five minutes in AGE fixer. After 15 minutes of water bath, the films were immersed in the distilled water and finally dried for 30 minutes.
For analysis, films were scanned (Duoscan T1200, Agfa) and digitalized.

2.10. Tissue sampling and histology

2.10.1. Sample preparation

Adductor muscles of both hind limbs (ligated and non-ligated) were removed. The muscles were cut in triangle-like shape comprising the collateral vessel containing area. The basis of the triangle was cut proximally to the A. profunda femoris. The other side was cut laterally from the A. femoralis towards the knee and the third side connected knee with the medial side of the A. profunda femoris. Muscles were placed into a centrifuge tube filled with PBS. After 20 minutes, PBS was exchanged to 10 % sucrose in PBS and stored at 4 °C for 30 minutes. The muscles were dehydrated in this hyperosmotic solution and dropped down to the bottom of the tube. Then, the solution was replaced by 25 % sucrose in PBS and stored for five hours at 4 °C. Subsequently, samples were removed from the sucrose and placed upright positioned on cork squares. Whole muscles were covered in Tissue Tec and freezed immediately in - 150 °C cold Methyl-Butan. Subsequently, samples were shortly immersed in liquid nitrogen (-180 °C) and stored at the temperature of - 80 °C.

2.10.2. Preparation of tissue sections

For morphomethrical analysis, right and left adductor muscles were sectioned using the CM 3000 cryomicrotome (Leica, Germany). From every adductor, 6 sections were obtained; 2 from the upper part, 2 from the middle part and 2 from the lower part. The distances between the sections remained the same for every sample. Sections were 6 µm thick.

2.10.3. Immunohistological staining

After cutting, sections were fixed for 8 minutes in 4 % pFA. After this, they were washed twice for 5 minutes in PBS. Blocking solution containing 0.4 % glycin and 0.2 % bovine serum albumin (BSA) was used for 30 minutes to block unspecific binding sides.
For smooth muscle cells staining, a fluorescein-5-isothiocyanate (FITC) -conjugated monoclonal antibody against α-smooth muscle actin (Serotec) was used at a 1 : 500 dilution in

blocking solution. Sections were incubated for one hour at 37 °C. Afterwards, slides were washed twice for 5 minutes in PBS. To stain endothelial cells, sections were incubated with tetramethylrhodamine isothiocyanate (TRITC) -BS1 lectin (Sigma) at a 1 : 30 dilution in PBS for 45 minutes at 37 °C. Samples were again washed twice. Nuclei were stained using 4',6'-diamidino-2-phenylindole dilactate (DAPI; Molecular Probes) at a 1 : 1000 dilution in PBS. Incubation was performed at room temperature for 15 minutes. After this, slides were washed three times with PBS. After adding Mowiol to the slides, sections were covered with cover slips. After drying, slides were stored at 4 °C until analysis was performed.

Used solutions:

4 % pFA:

40 g PFA powder were added to 1000 ml PBS. While stirring, 2 or 3 NaOH crystals were added to the solution. After a complete dissolving of pFA, pH was adjusted to 7.4 and solution was stored at –20 °C until use.

Blocking solution:

0.4 g glycin and 0.2 g BSA were dissolved in 100 ml PBS.

2.10.4. Quantification of collateral artery growth

From each section, all arteries were photographed under the fluorescent light using a DMRB Leica microscope (Germany) and a Leica DMLD digital camera. Large arteries like A. femoralis and A. profunda femoris were excluded. The outer vessel wall perimeter was marked and arterial vessel area was determined using the computer assisted planimetry software Image J.

The vessel area of all arteries per muscle was counted to get the sum and the mean value. The sum reflected an increase in the number of collateral arteries which had grown after the ligature and the mean value described enlargement of the vascular perimeter. For every mouse, right-to-left ratio (R/L) was counted from sum and mean value, respectively. Finally, the mean R/L ratio was calculated for every experimental group of animals to compare the collateral vessel growth between controls and treated animals. Results were expressed as mean ± SEM.

2.11. Statistical analysis

Values of each experimental group are presented as mean ± SEM. To calculate a statistical significance, an ANOVA test with a Bonferoni post hoc analysis was used. A value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Isolation of blood monocytes

For injection into mice, monocytes were isolated from donor mouse blood using density gradient centrifugation. After staining with PE-conjugated murine monoclonal antibody against CD 11b, flow cytometric sorting of monocytes was performed. Monocytes subpopulation was identified according to their scatter characteristics and expression of CD 11b and gated as shown in the following figure:

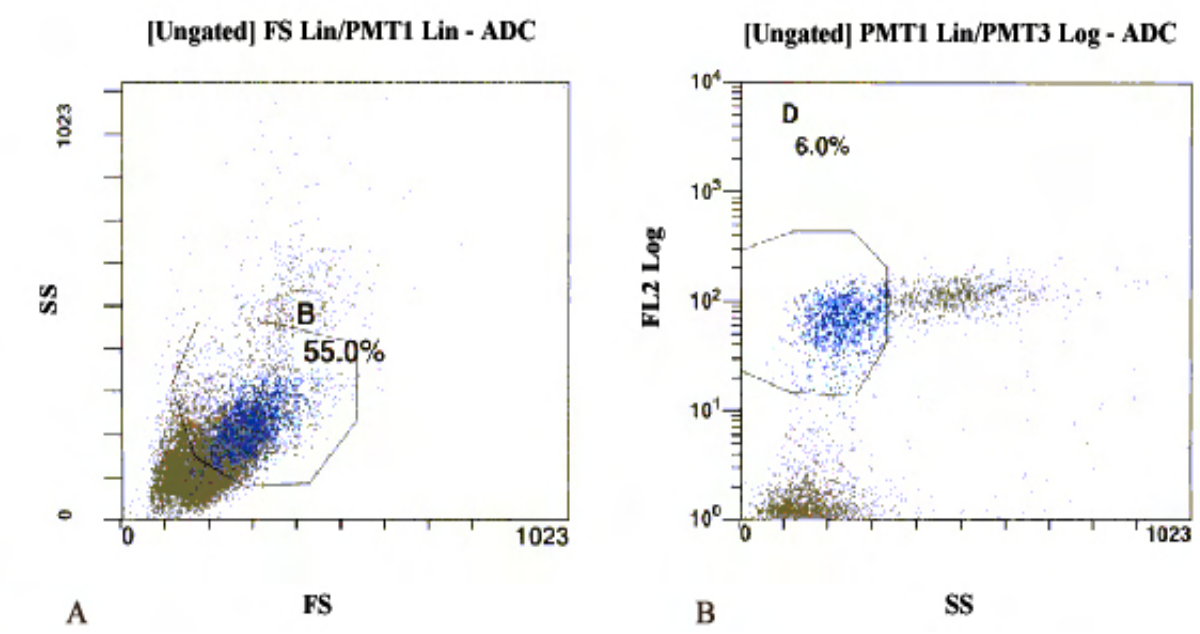


Fig. 8. Flow cytometry analysis of mouse leukocytes harvested from peripheral blood. Combination of regions B and D was used for sorting. A = Scatter light blot indicating measured blood cells. Cells from gate D reflecting monocytes are colored blue. B = CD11b expression of leukocyte subpopulations: Monocytes are gated, granulocytes (also CD11b positive) are distinguished from the monocytes by increased side scatter signal, lymphocytes (CD11b negative) are on the bottom (FL2Log: CD11b expression).

Monocytes were sorted using advanced mode with high selectivity. In order to estimate purity of sorted cells, flow cytometry analysis was performed. Almost 98 % of isolated cells were monocytes as shown in the figure 9B. Cell viability as assessed by propidium iodide staining

was > 98 % (Fig. 10). Propidium iodide is a nuclear marker used for visualization of necrotic cells.

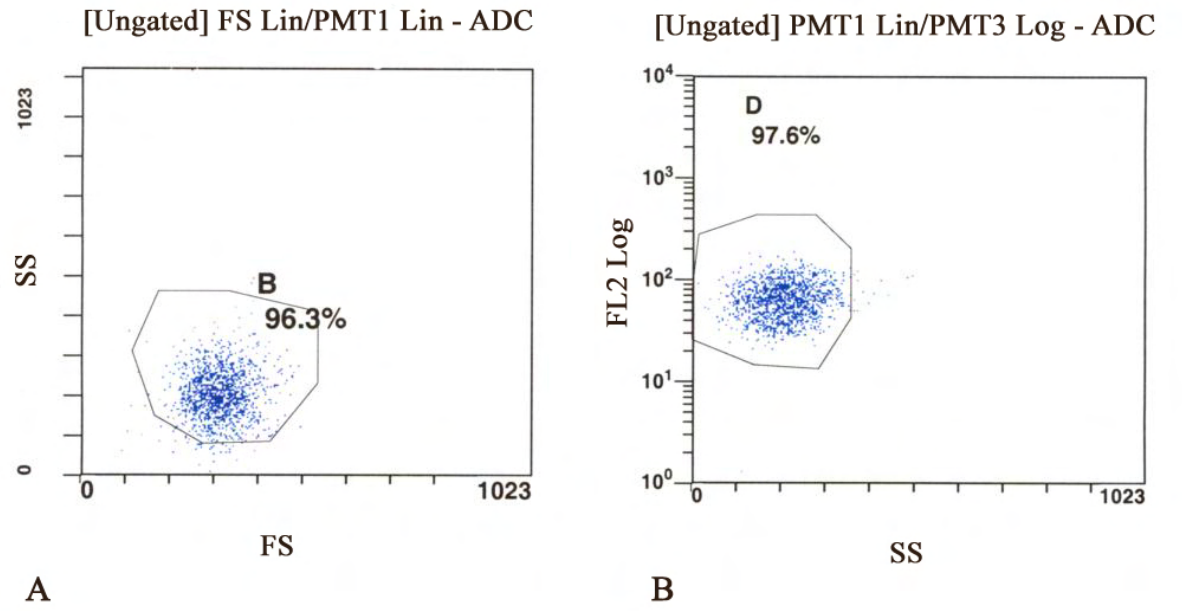


Fig. 9. Flow cytometry analysis of isolated cells. Regions B and D show percentage of CD 11b positive cells from all isolated cells.

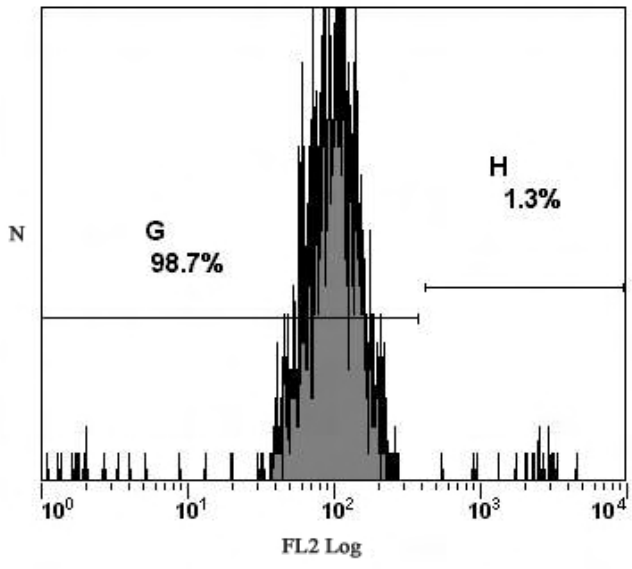


Fig. 10. Flow cytometry analysis of isolated monocytes after staining with propidium iodide. Region G shows the population of viable cells and region H shows dead cells.

3.2. Injection of isolated monocytes after femoral artery ligature of recipients

3.2.1. Hemodynamic evaluations using Laser Doppler Imaging

3.2.1.1. Pre- and post LDI measurements

Pedal blood flow to mice feet was quantified using LDI. Pre-operative measurements were performed immediately before the femoral artery ligature and post-operative measurements immediately after the operation. There were no significant differences in pre-operative and post-operative measurements between control group and the groups which had received injection of either 2 x 10^5 or 3 x 10^5 monocytes.

Group	**Pre-LDI**	**Post-LDI**	**N**
Control	1.04 ± 0.04	0.07 ± 0.003	24 (19*)
2x10^5 monocytes	1.01 ± 0.02	0.06 ± 0.006	11
3x10^5 monocytes	1.01 ± 0.02	0.08 ± 0.01	7 (5**)

Tab. 6. Overview on pre- and post LDI measurements in experimental groups.
* five animals were excluded at day 14 due to necrosis
** two animals died during experiment

3.2.1.2. LDI measurements at days 3, 7, 14 and 21

In all three groups, blood flow recovery was observed starting on day 3. From this time point, the group treated with 2 x 10^5 monocytes showed a significant improved blood flow recovery through the complete experiment. The maximal difference was noticed at day 7 (0.21 ± 0.02 in control mice vs. 0.30 ± 0.04 in 2 x 10^5 in monocyte-treated mice). Representative laser Doppler images of all groups are shown in figure 11.
At day 14, five mice from the control group had to be excluded due to progredient necrosis in the foot, obviously as a result of an insufficient blood supply in the foot. At day 7, these mice showed the lowest blood flow recoveries. From day 14, LDI ratios of control mice were probably false higher. In the monocyte-treated animals, no necrosis was observed.

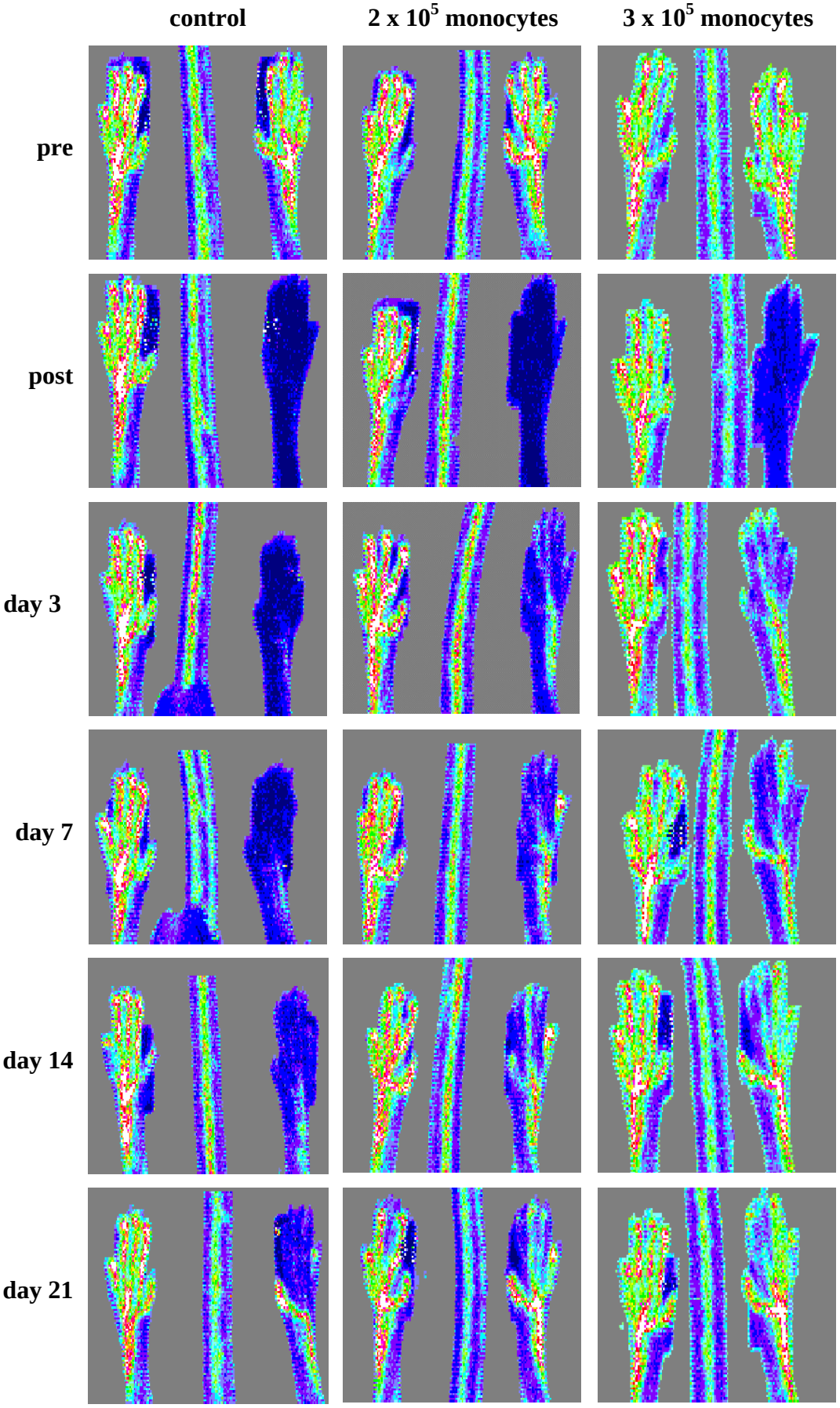


Fig. 11. Representative laser Doppler images of mice distal hind limbs. Ligature of the femoral artery had been processed on the right leg.

In order to evaluate if a correlation between the concentration of blood monocytes and the enhancement of arteriogenesis may exist, an increased number of monocytes (3 x 10^5 cells) was injected in mice. Mice which had received 3 x 10^5 monocytes showed superior blood flow recovery compared to controls and the group which had received 2 x 10^5 monocytes.

However, a significant difference between the group with 2 x 10^5 monocytes and the group with 3 x 10^5 monocytes could not be detected, despite the presence of a marked trend towards a concentration-dependent improvement of blood flow recovery (fig. 12).

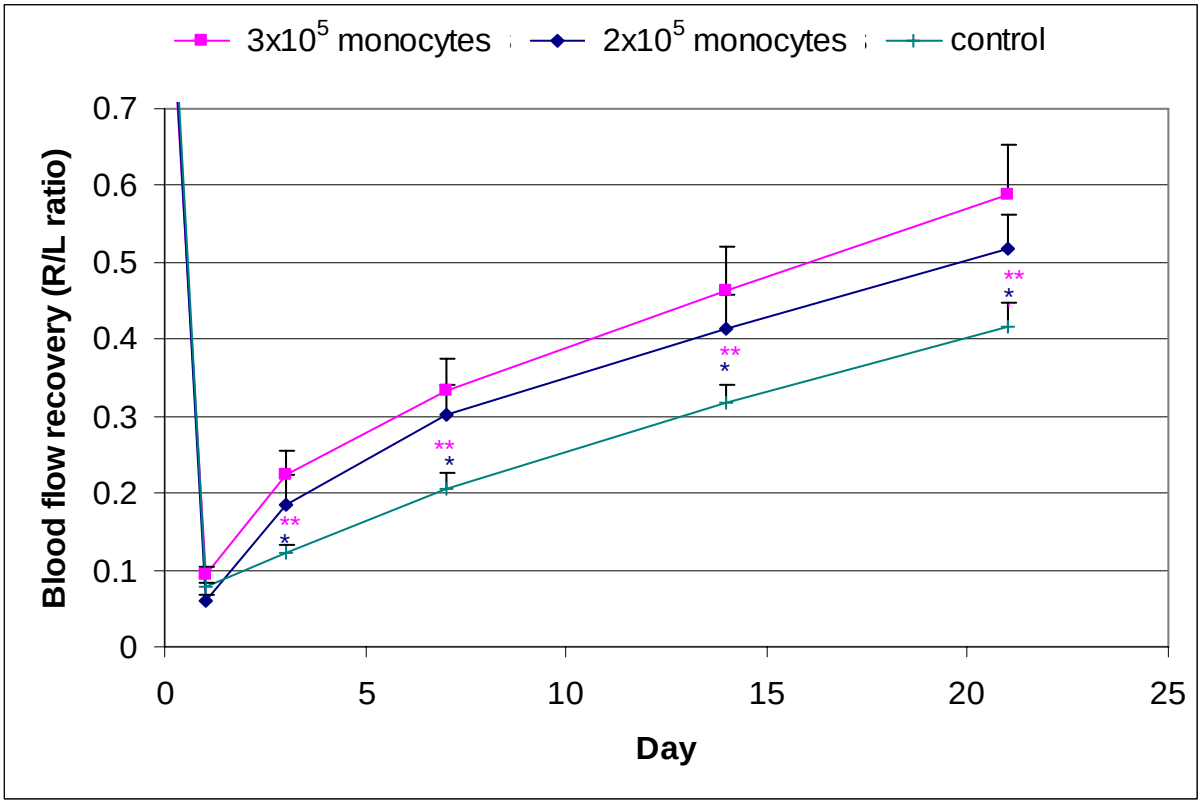


Fig. 12. Blood flow recovery in the foot after femoral artery ligature as assessed by LDI before operation, immediately after and on the postoperative day 3, 7, 14 and 21.

* = $p<0.05$; ** = $p<0.01$

3.2.2. Hemoglobin oxygen saturation measurements

3.2.2.1. Pre- and post measurements

The pedal hemoglobin oxygen saturation (HOS) was measured in anaesthetized mice immediately before and after femoral artery ligature. There were no differences between control and monocyte-treated groups in pre-operative and post-operative measurements of oxygen saturation detectable (tab. 7).

Group	**Pre-HOS**	**Post-HOS**
control	1.00 ± 0.009	0.09 ± 0.006
$2x10^5$ monocytes	1.00 ± 0.006	0.09 ± 0.007
$3x10^5$ monocytes	1.00 ± 0.007	0.10 ± 0.10

Tab. 7. Pre- and post HOS measurements in experimental groups**.**

3.2.2.2. Measurements at days 3, 7, 14 and 21

Starting on day 3, significantly increased HOS was observed in monocyte-treated mice in comparison to controls. This difference was persistent during the complete observation period. HOS values between groups after receiving 2 x 10^5 monocytes and 3 x 10^5 monocytes did not differ as shown in figure 13.
Monocyte-treated animals reached 90 % of the pre-operative values at the end of the observation period whereas control mice reached values only below 80 %.

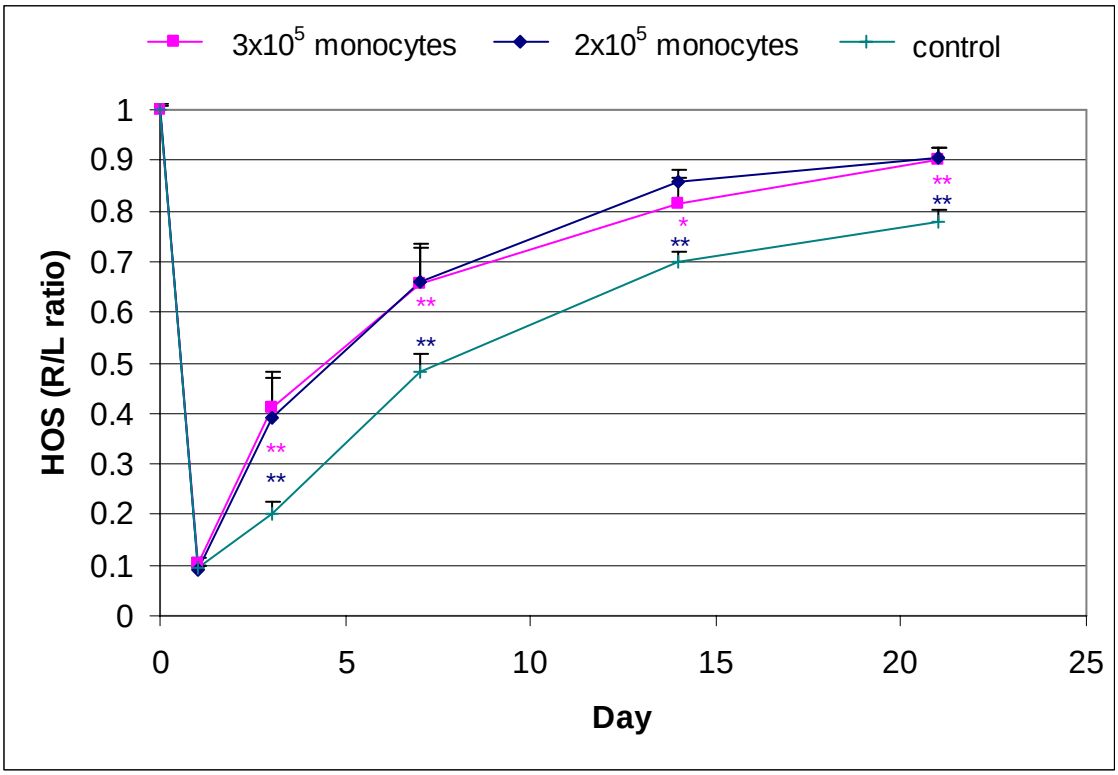


Fig. 13. Hemoglobin oxygen saturation measurements in control and monocyte-treated mice were performed before operation, immediately after and on post-operative days 3, 7, 14 and 21. * = $p<0.05$; ** = $p<0.01$

3.2.3. Histological findings

3.2.3.1. Morphology of collateral vessels

Adductor muscles of ligated and non-ligated hind limbs containing collateral arteries were harvested 21 days after femoral artery ligature. Cryosections were stained with primary antibodies (BS-1 lectin, anti-α-smooth muscle actin, DAPI) and analyzed.

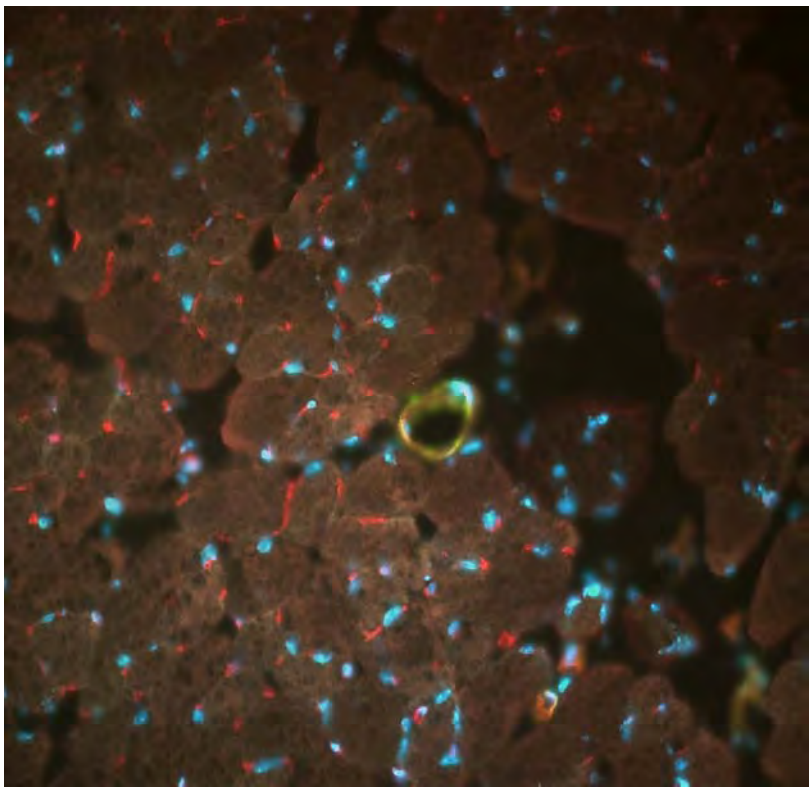
On the non-occluded side, pre-existing collateral arteries were characterized with smaller diameters and thinner vascular wall, in comparison to occluded side with grown collateral arteries. This was found in control group as well as in monocyte-treated groups (fig. 14). The left hind limb without occlusion served as control to ligated one and right-to-left ratios were calculated.

Pre-existing collateral arteries did not distinguish morphologically from normal vessels and were identified according to the typical localization in the superficial layers of the adductor

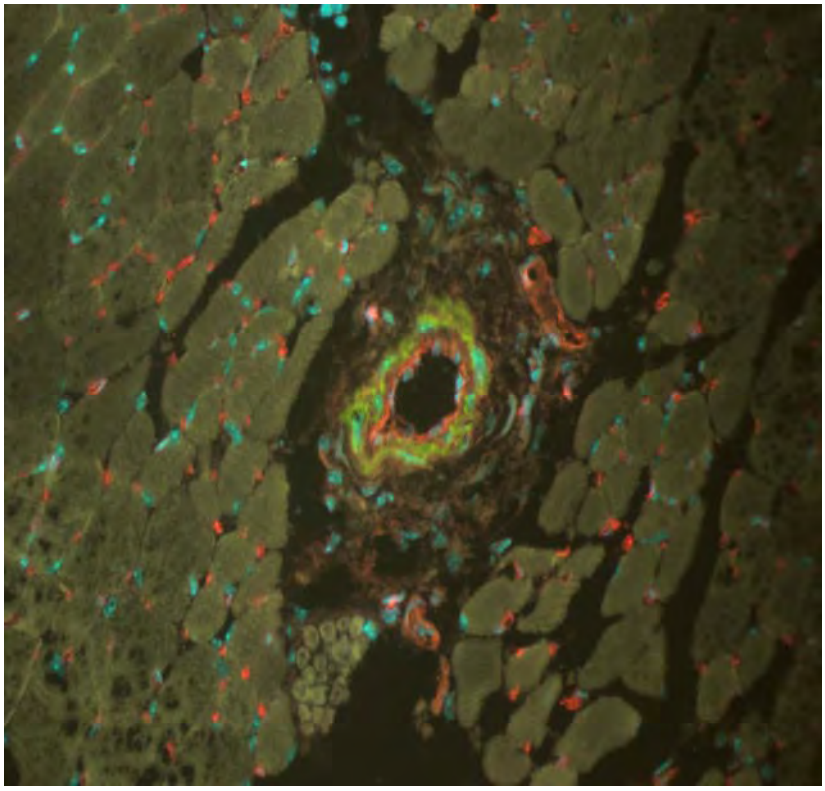
muscle. Growing collateral arteries were mostly round shaped with a thick tunica media and inflammatory cells were surrounded in the close perivascular space.

3.2.3.2. Injection of GFP-labeled monocytes in recipients

In order to assess if transplanted monocytes were involved in the collateral artery growth after the femoral artery occlusion, GFP (green fluorescent protein) transgenic mice were used as blood donors for monocytes injection. Littermates of transgenic mice underwent femoral artery ligature and immediately received an injection of GFP-labeled monocytes. On day 3 after the operation, animals were sacrificed, perfusion fixed and adductor muscles were cryopreserved. Moreover, tissue from lungs, heart, kidneys, liver and spleen was collected. All samples were sectioned and analyzed under a fluorescent microscope (fig. 15).
No GFP-positive cells were found in hearts, lungs and kidneys, some of them were detected in liver and many of them were found in spleen, which served as a positive control of monocyte transplantation (data not shown). Serial sections of adductor muscles were analyzed. In the non-occluded side, only few GFP-positive cells were found in the perivascular space.



A



B

Fig. 14. Collateral arteries of a mouse using immunofluorescent staining (x 40): green for tunica media, red for endothelium and blue for nuclei. A: pre-existing collateral artery from left adductor muscle. B: remodelled collateral artery from right adductor muscle on day 21 after right femoral artery ligature.

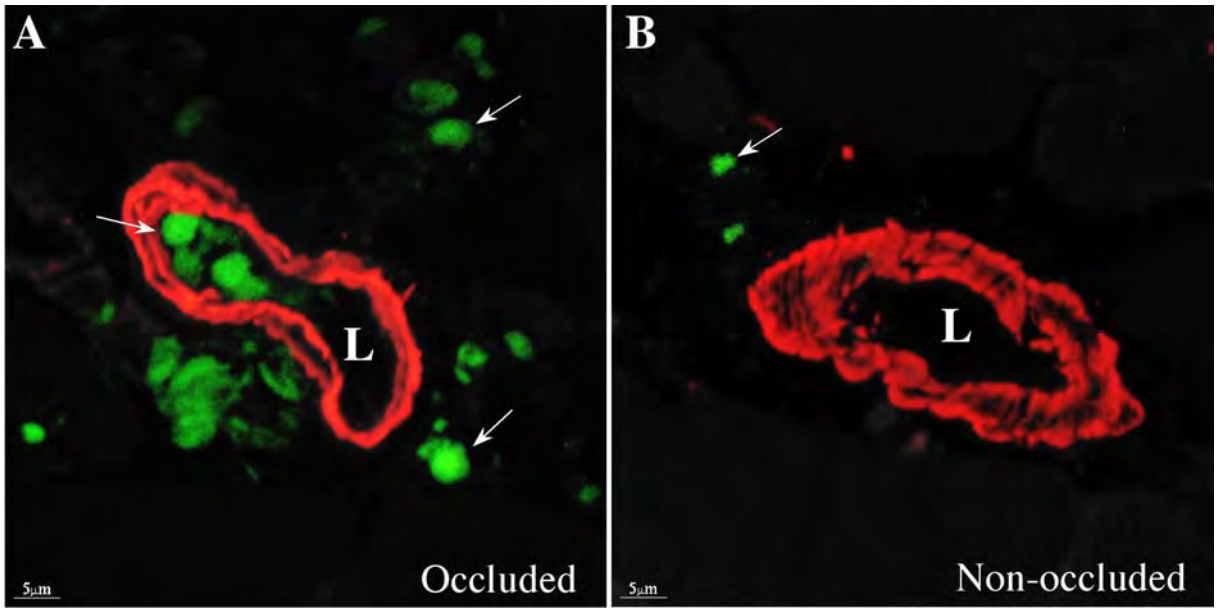


Fig. 15. Collateral artery of a mouse using confocal microscopy. A: in the right adductor, injected GFP-positive monocytes were observed in the lumen (L) of collateral arteries and in

the perivascular space. B: in the left adductor, only few GFP-positive monocytes were detected.

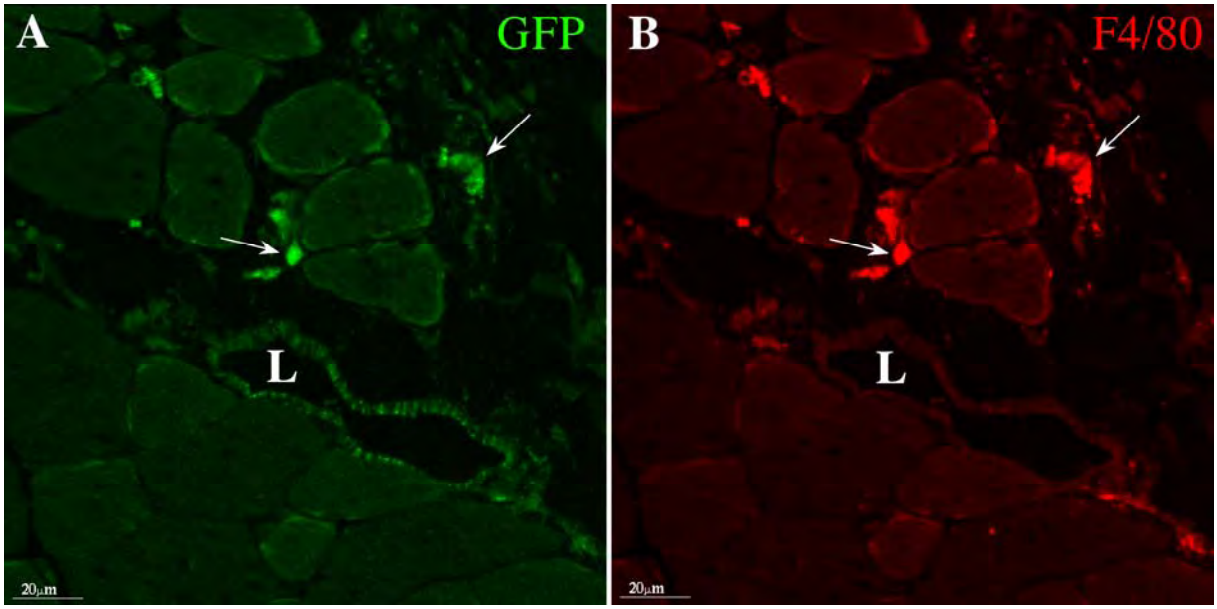


Fig. 16. Colocalization of GFP signal with F4/80 macrophage marker in the right adductor muscle using confocal microscopy. A shows GFP-positive cells and B shows specific F4/80 immunofluorescence. Arrows point to injected monocytes/macrophages. L = lumen of collateral artery.

After femoral artery ligature, a significantly increased number of GFP-positive cells was found in the lumen of growing collateral arteries and in the close perivascular space after transmigration through the vascular wall.
Subsequently, sections were stained for PE-conjugated F4/80 macrophage marker (Caltag). GFP-positive cells in the perivascular space also expressed macrophage marker as shown in figure 16. These findings indicate that GFP^+ monocytes which were injected via a tail vein were involved in the collateral artery growth.

3.2.3.3. Morphometrical evaluations

From every adductor muscle, 6 sections (2 from the proximal zone, 2 from the middle zone and 2 from the distal zone) were analyzed. Lumen of arteries was completely filled with a contrast agent, which helped to distinguish them from veins. In addition, they were mostly round shaped unlike veins which were collapsed and had a small lumen.

Mean and total arterial vessel area from every adductor muscle were calculated using Image J. R/L ratios were calculated for every mouse. Vessels on the occluded side had increased diameters and perimeters in control group as well as in monocytes-treated groups. Results are summarized in the following table:

Experimental group	***Mean collateral area (R/L ratio)***	***p***	***Total collateral area (R/L ratio)***	***p***
Controls	1.56 ± 0.07	-	1.88 ± 0.08	-
2 x 10^5 Monocytes	1.77 ± 0.07	< 0.05*	2.37 ± 0.12	< 0.01*
3 x 10^5 Monocytes	2.37 ± 0.11	< 0.01**	2.66 ± 0.12	n.s.**

Tab. 8. Morphometrical results.

* against controls

** against 2 x 10^5 monocytes

3.3. Treatment with GM-CSF alone and in combination with monocyte injection in the mouse ischemic hind limb model

3.3.1. Hemodynamic evaluations using Laser Doppler Imaging

To investigate whether GM-CSF therapy may enhance arteriogenesis, mice received five intraperitoneal injections of rmGM-CSF (0.5 µg in 150 µl NaCl per day) starting two days before femoral artery ligature. No differences in pre- and post measurements were observed between any of the groups. After administration of GM-CSF alone, no difference in blood flow recovery compared to control group was detected (fig. 17).

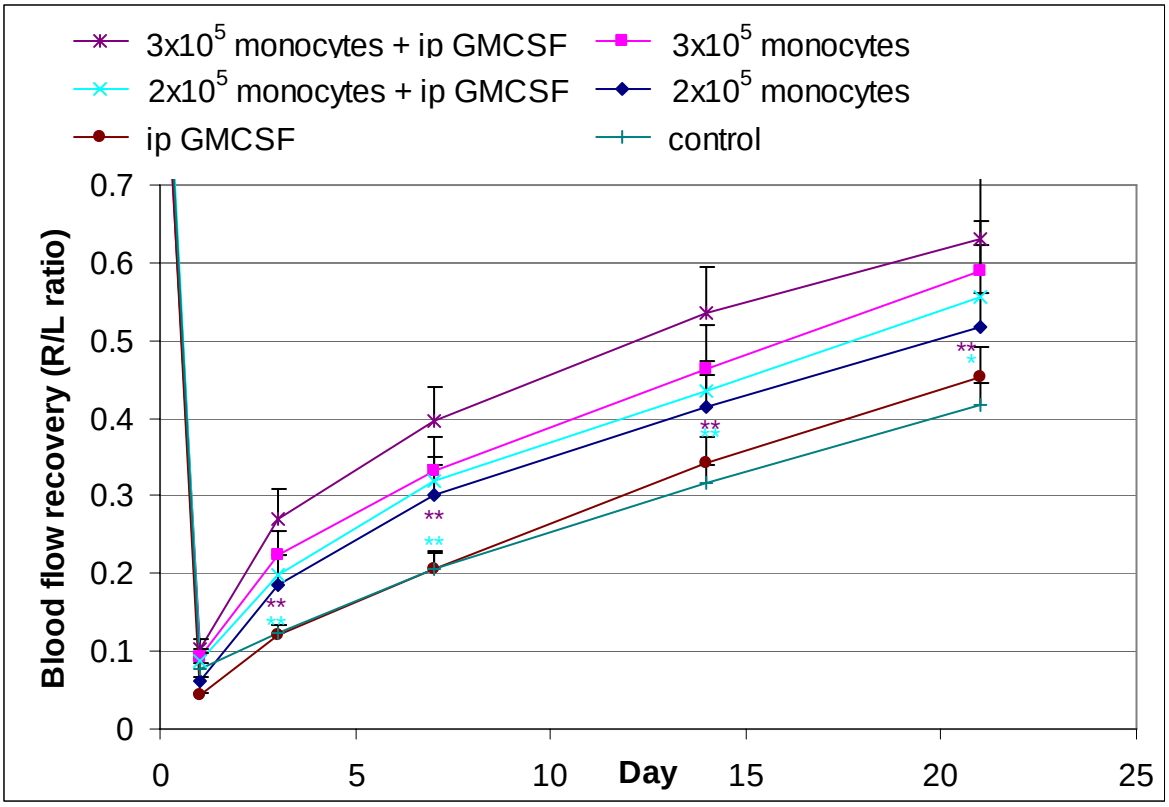


Fig. 17. Laser Doppler perfusion measurements of the foot in control, monocyte-treated animals, rmGM-CSF treated animals and combinations at several time points after femoral artery ligature. * = $p<0.05$; ** = $p<0.01$

Importantly, no necrosis in GM-CSF-treated animals was observed in comparison to controls. Overview on necrosis index is shown in the following table.

Experimental group	***Number of animals (N)***	***Number of animals with necrosis***
controls	24	5
i.p. GM-CSF	11	0

Tab. 9. Necrosis index.

A combination of GM-CSF injections (1 µg in 150 µl NaCl per day) and monocyte injection was performed to investigate a potential additive or synergistic effect of GM-CSF on monocyte therapy. An increase in improvement of blood flow recovery compared to 2 x 10^5 monocyte injections was detected in the combination group, but results did not reach

statistical significance. In our experiment, the group of animals treated with GM-CSF injections in combination with 3 x 10^5 monocyte injections showed the best blood flow recovery.

3.3.2. Hemoglobin oxygen saturation measurements

Pedal oxygen saturation of hemoglobin was measured in anaesthetized animals directly before operation, immediately after and on post-operative days 3, 7, 14 and 21. All pre-operative measurements did not differ and R/L ratios were about 1. Directly after the operation, HOS ratios dropped to 10 % of the starting point. From this time point on, R/L ratios of HOS continuously improved, however they did not reach pre-operative values at the end of the observation period. Mice which were treated with GM-CSF injections solely, did not differ in hemoglobin oxygen saturation values from control mice except from day 3 (fig. 18). Interestingly, mice treated with 2 x 10^5 monocytes and GM-CSF or 3 x 10^5 monocytes and GM-CSF did not differ in hemoglobin oxygen saturation values from mice which were treated either with 2 x 10^5 monocytes or 3 x 10^5 monocytes except from day 3.

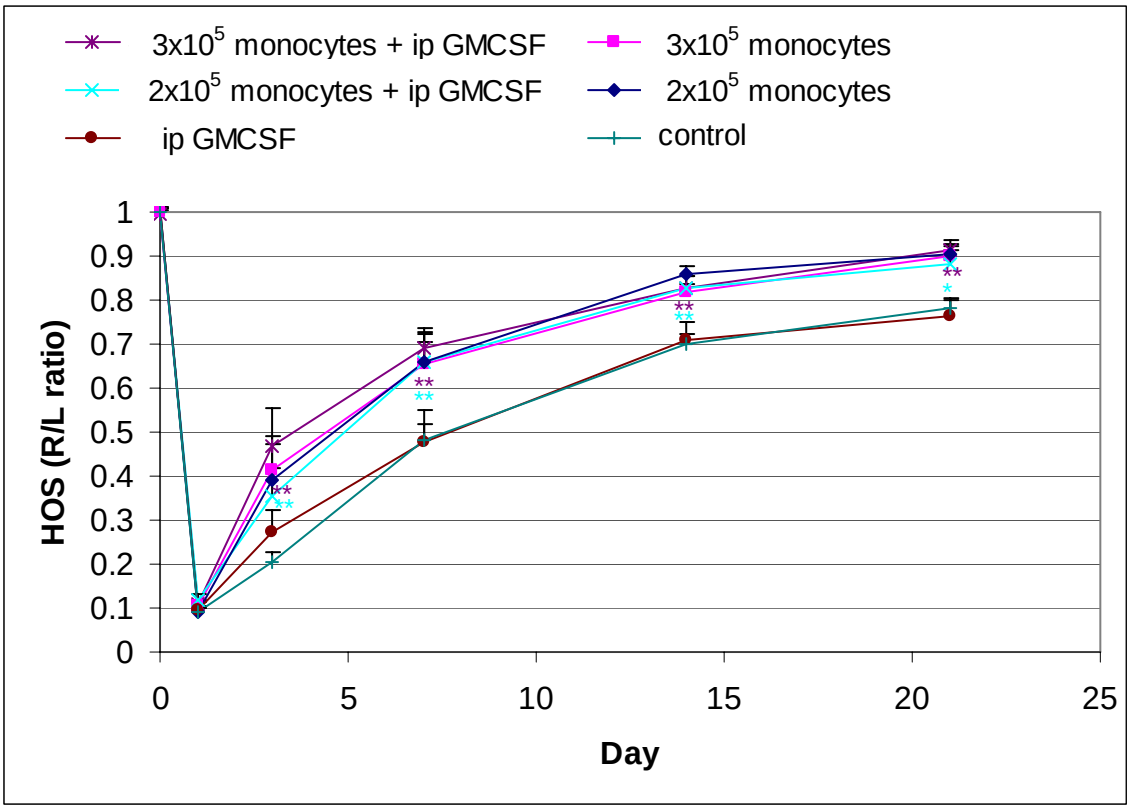


Fig. 18. Hemoglobin oxygen saturation measurements during the observation period in controls, in mice after monocyte injections (2 or 3 x 10^5 cells), in i.p. GM-CSF-treated mice

and in mice which received a combination of GM-CSF and monocytes. * = $p<0.05$; ** = $p<0.01$

3.3.3. Morphometrical findings

Adductor muscles were sectioned and stained for morphometrical analysis. Mice which were treated with intraperitoneal injections of rmGM-CSF had bigger vessel diameters and perimeters in comparison to controls. However, these differences were not statistical different which is in accordance with laser Doppler measurements (Tab. 10).

Experimental group	***Mean collateral area (R/L ratio)***	***p***	***Total collateral area (R/L ratio)***	***p***
i.p. GM-CSF	1.62 ± 0.06	n.s.*	1.91 ± 0.11	n.s.*
2 x 10^5 Monocytes + i.p. GM-CSF	2.29 ± 0.13	< 0.01**	2.66 ± 0.19	n.s.**
3 x 10^5 Monocytes + i.p. GM-CSF	2.96 ± 0.26	< 0.05***	3.32 ± 0.14	< 0.01***

* against controls ** against 2 x 10^5 monocytes *** against 3 x 10^5 monocytes

Tab. 10. Morphometrical results.

Morphometrical analysis of collateral arteries from mice treated with combination of i.p. GM-CSF and 2 x 10^5 monocytes revealed that the vessel diameters were significantly larger in comparison to mice which received only 2 x 10^5 monocytes. Mice treated with combination of i.p. GM-CSF and 3 x 10^5 monocytes had even larger collateral arteries and the difference was statistical significant in comparison to mice treated only with 3 x 10^5 monocytes. Moreover, total collateral vessel area was significantly increased in comparison to 3 x 10^5 monocytes-treated mice. These results suggest that a combination of GM-CSF treatment with monocyte injections was effective. Results are summarized in following diagrams:

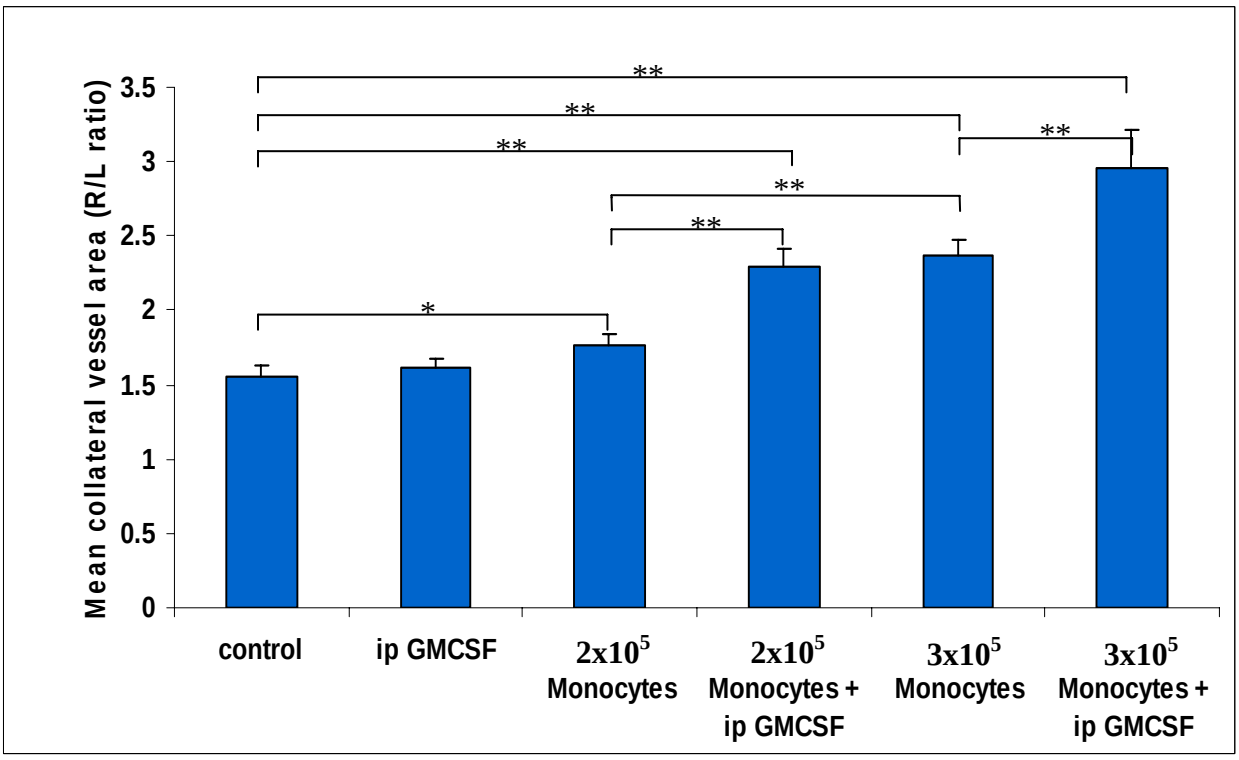


Fig. 19. Overview on mean collateral vessel areas in experimental groups. Largest mean collateral vessel area was observed in the group treated with combination of 3 x 10^5 monocyte injection and i.p. GM-CSF. * = $p<0.05$; ** = $p<0.01$

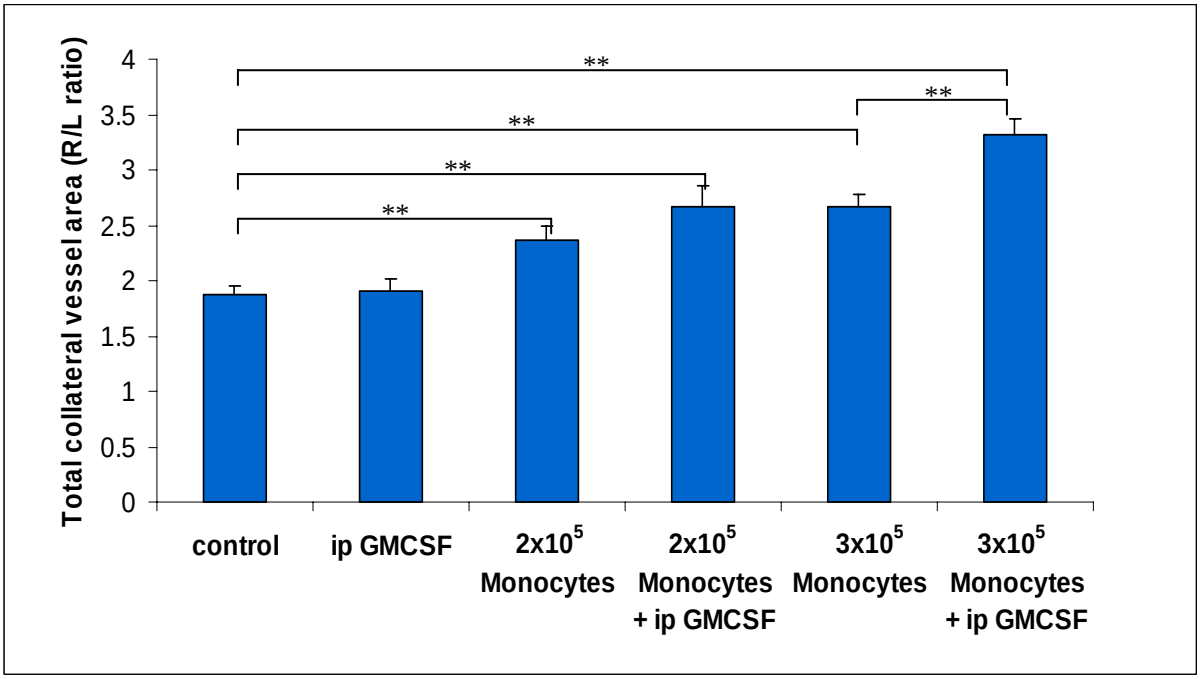


Fig. 20. Overview on total collateral vessel areas in experimental groups. Results correspond with mean collateral vessel areas. * = $p<0.05$; ** = $p<0.01$

3.4. Effect of Cicaprost and GM-CSF primed monocytes on blood flow recovery after femoral artery ligature

3.4.1. Hemodynamic evaluations

In order to test if the arteriogenic potential of monocytes may be enhanced by priming of isolated monocytes by incubation with a synthetic prostacyclin (Cicaprost, Schering, Germany), an additional experiment was performed. After 2 hours incubation with 100 ng/ml of Cicaprost under sterile conditions at 37 °C and 5 % CO_2, 2 x 10^5 monocytes were injected into a tail vein. Furthermore, we tested if an incubation of isolated monocytes with 200 ng/ml of rmGM-CSF for 3 hours might enhance the arteriogenesis. After incubation, conditioned monocytes were injected after femoral artery ligature into a tail vein of recipient.

Pre- and post measurements were performed before and directly after the operation. No differences in pre- and post measurements in comparison to control mice were noticed (fig. 21).

Mice treated with Cicaprost-incubated monocytes showed significantly higher blood flow on day 7 and 14 in comparison to controls. At the day 3, R/L ratio was comparable with control group. However, there was any improvement in blood flow recovery in comparison to mice treated only with 2 x 10^5 monocyte injection. On the contrary, there was a delay on the day 3.

Mice treated with GM-CSF primed monocytes did not show any improvement of blood flow recovery comparing to controls. Two mice from this group were excluded because of severe foot necrosis. Blood flow ratios were slightly below the control group suggesting that incubation with rmGM-CSF may have a negative effect on the arteriogenic properties of injected monocytes.

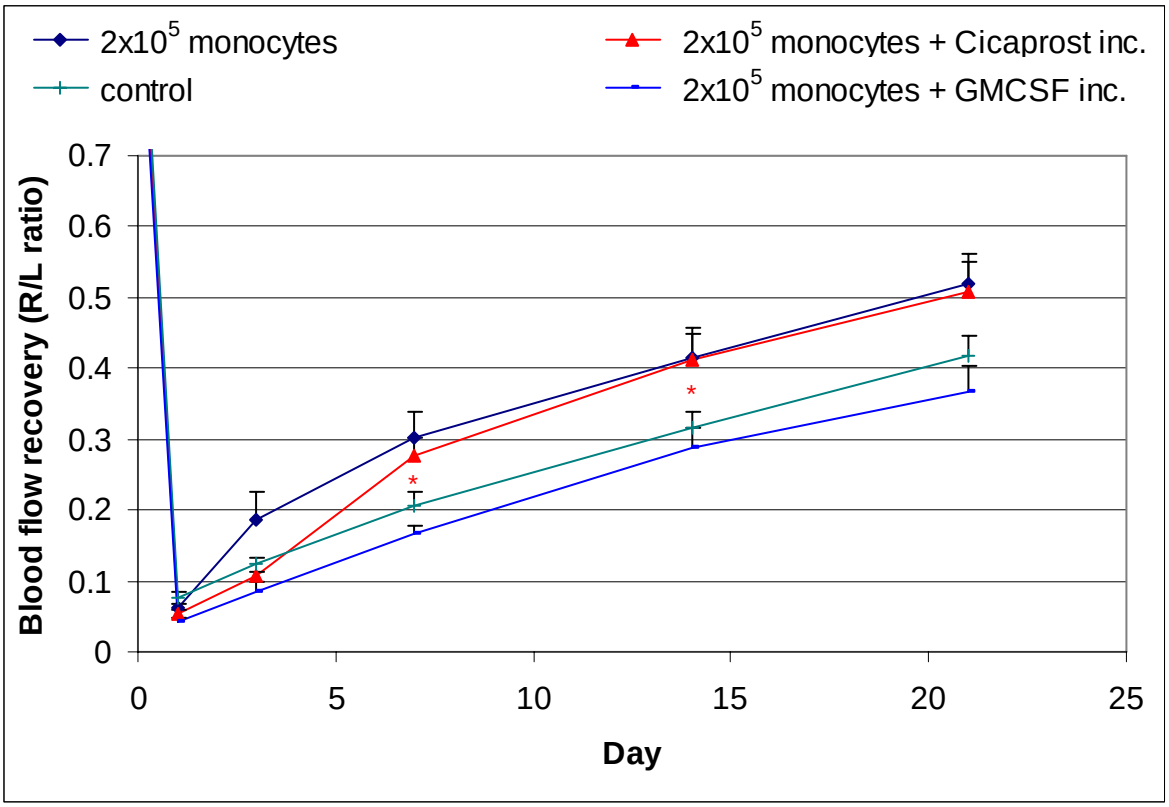


Fig. 21. Laser Doppler perfusion measurements of the foot in control, monocyte-treated mice, and mice treated with monocytes incubated with either Cicaprost or GM-CSF at several time points after femoral artery ligature. * = $p<0.05$

3.4.2. Hemoglobin oxygen saturation measurements

Pedal oxygen saturation of hemoglobin was measured in anaesthetized animals directly before operation, immediately after and on post-operative days 3, 7, 14 and 21. All pre- and post measurements in treated animals did not differ from controls. Mice which received an injection of monocytes after being incubated with GM-CSF had comparable hemoglobin oxygen saturation values with controls. This was in line with laser Doppler Imaging data. Mice treated with monocytes after being incubated with Cicaprost had significantly better hemoglobin oxygen saturation values than controls starting on day 7. However, similarly to laser Doppler measurements, there was a delay on day 3 in comparison with 2 x 10^5 monocyte-treated group. In general, these HOS measurements confirm laser Doppler measurements and show that incubation of monocytes with either Cicaprost or GM-CSF did not support the arteriogenesis.

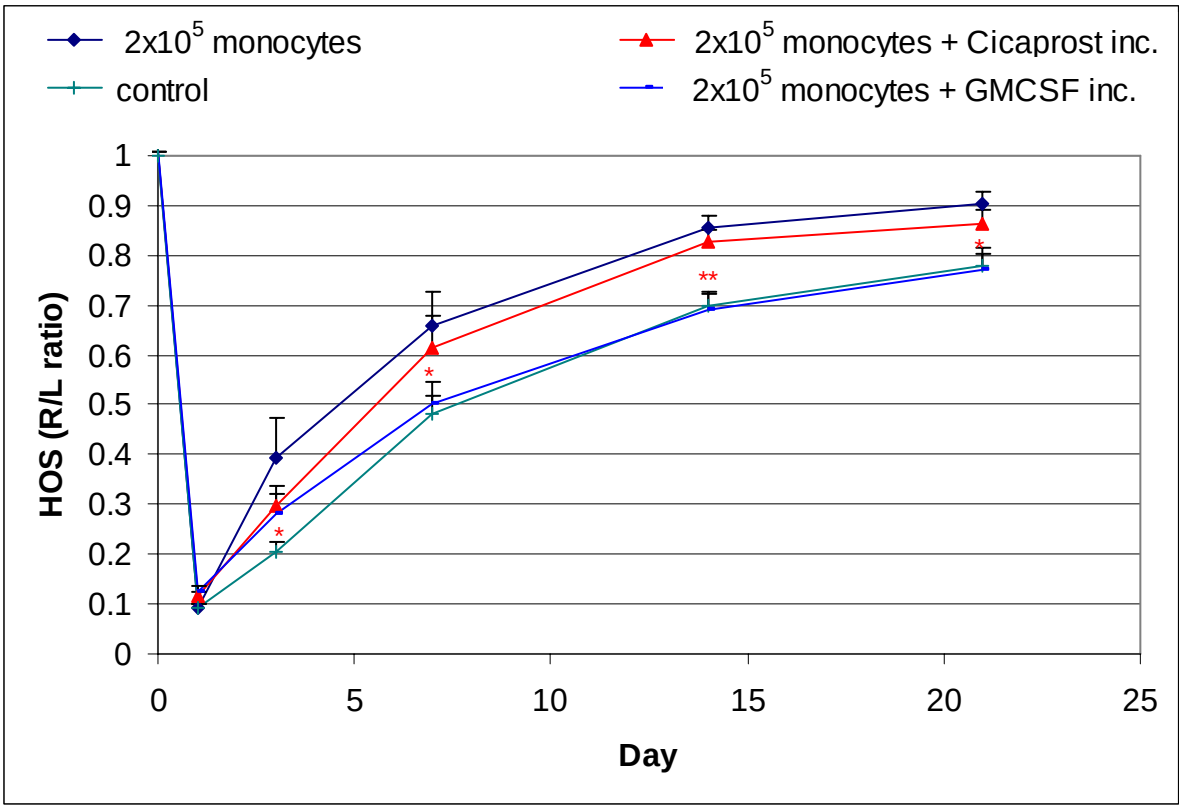


Fig. 22. Hemoglobin oxygen saturation measurements in control, monocyte-treated mice and mice treated with monocytes incubated with GM-CSF or Cicaprost at certain time points.* = $p<0.05$; ** = $p<0.01$

3.4.3. Histological evaluations

Adductor muscles were harvested on day 21 and stained for morphometrical analysis. Mice treated with 2 x 10^5 monocytes primed with GM-CSF had comparable mean arterial vessel area to control group. On the other hand, total arterial vessel area dropped down almost significantly. Again a negative effect of incubation with GM-CSF can be assumed.

Mice treated with 2 x 10^5 monocytes incubated with Cicaprost had the same mean arterial vessel area as mice which have received injection of 2 x 10^5 monocytes. However, total arterial area did not reach the values of 2 x 10^5 monocyte-treated mice. Results are summarized in table 11.

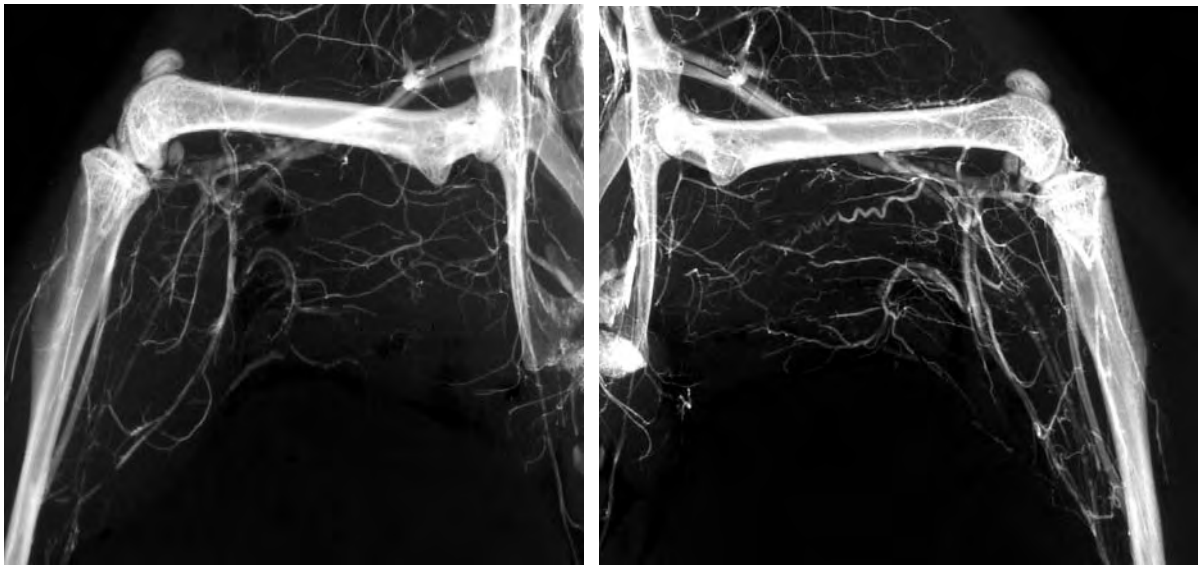
Experimental group	*Mean collateral area (R/L ratio)*	*p*	*Total collateral area (R/L ratio)*	*p*
2 x 10^5 Monocytes incubated with GM-CSF	1.62 ± 0.07	n.s.*	1.57 ± 0.06	n.s.*
2 x 10^5 Monocytes incubated with Cicaprost	1.77 ± 0.09	n.s.*	2.26 ± 0.13	< 0.05*

* against controls

Tab. 11. Morphometrical results.

3.5. Angiography

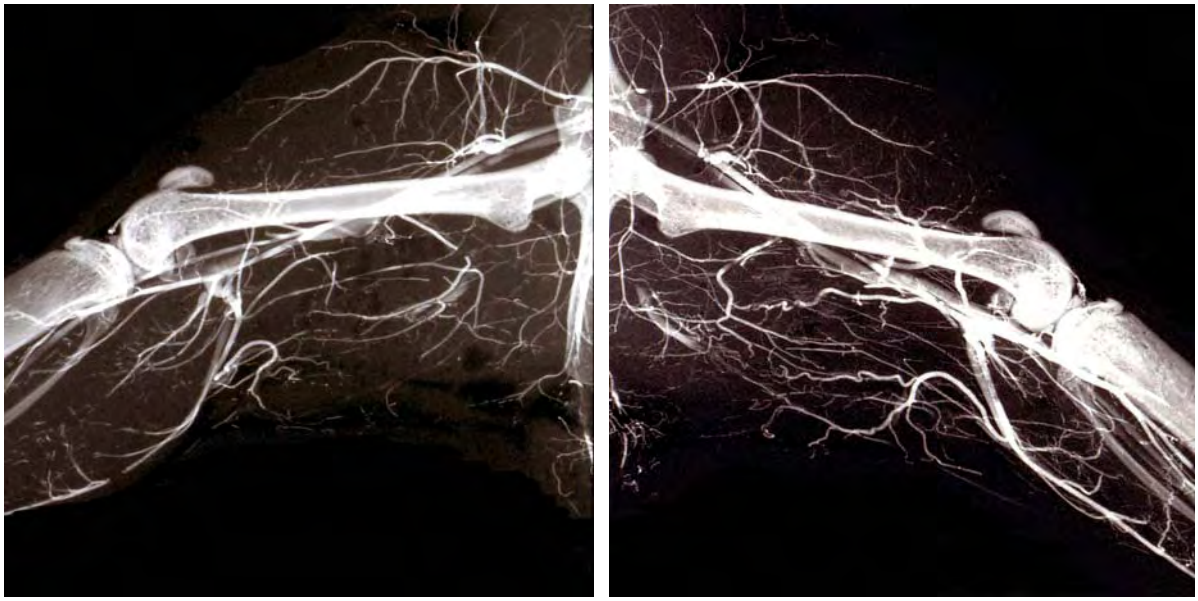
Post-mortem angiograms were performed 21 days after right femoral artery ligature. On the non-occluded sides, in every experimental group of animals, only thin and few pre-existing collaterals were found. Larger corkscrew-patterned vessels were identified in all animals on the occluded side. Although the number of visible collaterals was not quantified, marked differences between controls and monocyte-treated animals were noticed. Following representative angiograms show the differences in the collateral artery development on the occluded sides between experimental groups.



A B

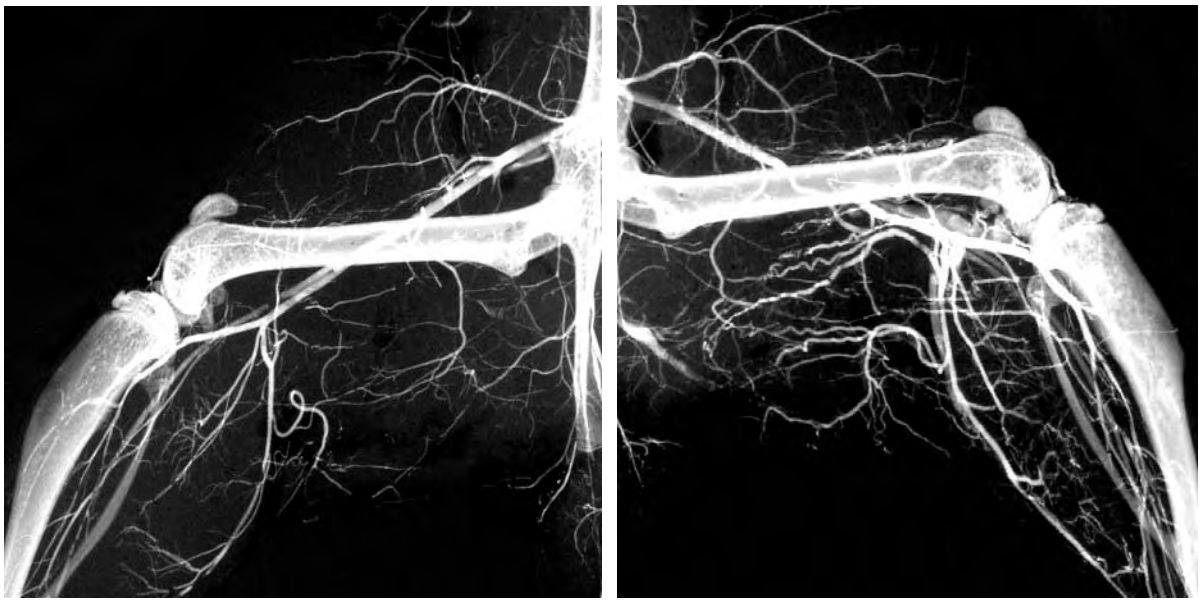
Fig. 23. Representative post-mortem angiograms of mouse hind limbs from a control group (no treatment). A: left B: right hind limb

In the control group of animals, only few collateral arteries have been developed. This corresponded well with the poor functional recovery of the occluded hind limb. Treatment with monocytes or combination with monocytes and GM-CSF caused except of better functional recovery of occluded hind limbs high density of well developed collateral network (Fig. 24; 25).



A B

Fig. 24. Representative post-mortem angiograms of mouse hind limb vessels after monocyte-Cicaprost treatment. A: left B: right hind limb



A B

Fig. 25. Representative post-mortem angiograms of mouse hind limb vessels after monocyte and GM-CSF combination treatment. A: left B: right hind limb

3.6. Active foot movement

Active foot movement was measured from the day 3 after the femoral artery ligature. In the control group, 5 from 24 mice have developed necrosis in the foot and were excluded from the study. Only in the control group has necrosis occurred. Mice which have received monocytes injection have recovered quicker than control mice. At the end of the measured period all the mice have reached almost complete recovery. The biggest differences in the recovery were noticed at the day 3. There were no significant differences between groups treated with monocytes and monocyte – GM-CSF combinations (Fig. 26). Between controls and monocyte–treated animals, there was a significant difference on days 3, 7 and 14 as shown in the following table:

Day	3	7	14	21
Controls	1.44 ± 0.10	1.88 ± 0.12	2.42 ± 0.11	3.34 ± 0.10
2 x 10^5 Monocytes	2.09 ± 0.17	2.59 ± 0.16	3.23 ± 0.25	3.59 ± 0.16
t-test	$p < 0.01$	$p < 0.01$	$p < 0.5$	n. s.

Tab. 12. Active foot movement score between control and monocyte-treated group.

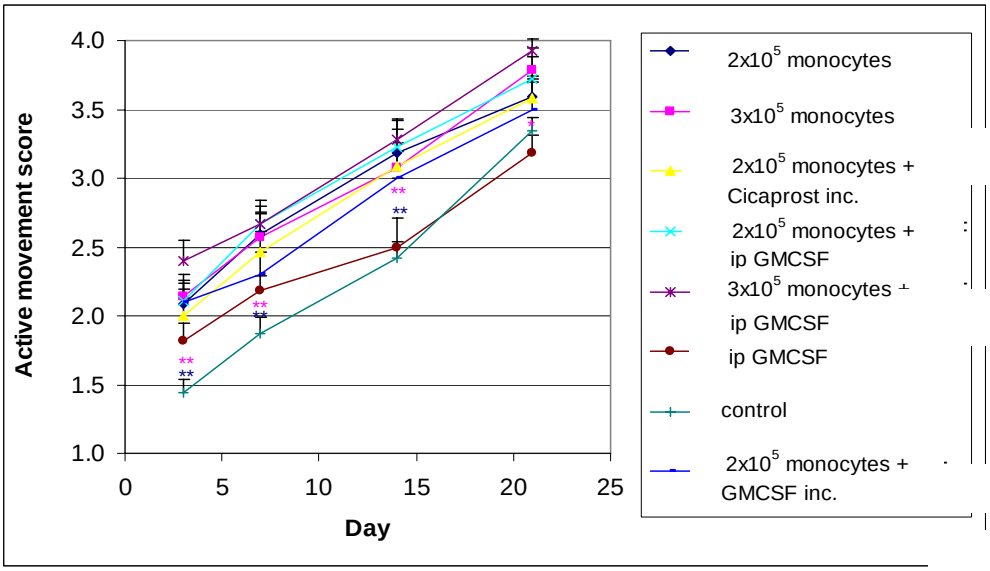


Fig. 26. Active foot movement score measurements performed on days 3, 7, 14 and 21 after the femoral artery ligature. * = $p<0.05$; ** = $p<0.01$.

4. Discussion

4.1. Introduction

Arteriogenesis was previously described as a process adaptive to arterial occlusion accompanied by growth of collateral arteries. This is in distinction to angiogenesis which is a de novo formation of capillary networks [21]. Positive remodeling of pre-existing collaterals is triggered by elevated fluid shear stress [63] and regulated by growth factors and cytokines which are secreted by activated endothelial cells, smooth muscle cells and by invading inflammatory cells, in particular monocytes and T-lymphocytes.

The objective of this study was to find out if monocytes can be used as cellular therapeutics in the treatment of cardiovascular occlusive diseases. Moreover, we tried to answer the question whether the arteriogenic potential of monocytes can be enhanced by priming with various substances. Furthermore, therapeutic implications of the use of GM-CSF as a potential growth factor for the treatment of peripheral occlusive diseases were tested. For this reason, the mouse ischemic hind limb model was used to simulate peripheral arterial occlusive disease.

Mechanisms by which monocytes contribute to collateral vessel growth have been previously described. The activated collateral endothelium expresses adhesion molecules (ICAM-1 and ICAM-2, VCAM-1) which are involved in adhesion and accumulation of blood monocytes. Activated monocytes bind to endothelial cell adhesion molecules via their integrin receptors LFA-1 and Mac-1 [64] and start to produce growth factors and matrix-metalloproteinases that facilitate their penetration of the intima. After entering the collateral vessel wall, monocytes differentiate into macrophages which then produce cytokines involved in vascular cell proliferation (e.g. TNF-α, bFGF and TGF-β).

4.2. Animal model

In numerous studies an animal model of acute hind limb ischemia was used to study arteriogenic mechanisms. Several variants of this model have been developed on different species like mouse, rat, dog, rabbit[65], [66], [20], [67].

A mouse model of arteriogenesis was first demonstrated by Couffinhal et al. 1998. The authors created unilateral hind limb ischemia by ligature of the proximal end of the femoral artery and the distal portion of the saphenous artery. Afterwards, the femoral artery including accompanying branches was excised. This extensive surgical intervention was performed in mice with the C57BL/6 genetic background that can tolerate ischemia well. This model is not suitable for the BALB/c strain of mice, as their genetically determined collateral network is poor [68] and they tend to develop foot necrosis more frequently (own observation). Because of strain differences in the extent and speed of blood flow recovery after the experimental arterial occlusion, it is reasonable to use BALB/c mice for studies in which the tested substances are expected to accelerate arteriogenesis.

The mouse model of hind limb ischemia was improved and modified by several research groups. The model as described in this dissertation was employed in the Max-Planck-Institut in Bad Nauheim. In distinction to previous mouse models, the femoral artery was ligated distally to the deep femoral artery and proximally to the branching of the popliteal artery. This surgical intervention allowed a development and growth of anastomoses between deep femoral artery and saphenous artery without excessive necrosis and self-amputation.

Compared to other models of arteriogenesis, i.e. in rabbits, pigs and dogs, the mouse model has several advantages. The main advantage compared to bigger animals is in the ability to operate and hemodynamically evaluate a set of mice sufficient for meeting statistical requirements per one day, due to the non-invasive blood flow measurements and easier manipulation of animals. Moreover, transgenic mice can be used to study the influence of disrupted gene expression and gene overexpression on physiological functions. Small animals are also suitable to test growth factors due to lower dosages and lower expenses.

However, a disadvantage of the mouse model is the impossibility to directly measure collateral conductance because of the small vessel parameters in mice. Instead, substitutional methods like blood flow recovery in the distal limb have to be used. Moreover, similarly to other animals, mice may have considerable interindividual differences in blood flow recovery in response to acute ischemia which can lead to increased variations in hemodynamical measurements.

In small animals like mice and rats, only few methods of in vivo blood flow measurements are available. The principle of microsphere technique is that regional blood flow to an organ is proportional to the number of microspheres trapped in that organ. Radioactive microsphere technique, previously reported to be a “gold standard” method for regional blood flow measurements, allows to measure blood flow in regions where the use of

probes or sensors is impractical, or where invasive surgical procedures may compromise the animal [69]. However, this method is depending upon correct placement of the reference blood-withdrawal catheter. The main disadvantage of microsphere technique, compared to flow probe methods, is in measuring of blood flow at a single time point at the termination of a study without the possibility of a continuous monitoring. In addition, microspheres are trapped in the capillaries and due to the small size of mouse capillaries they may embolize larger vessels and lead to the false interpretation of results. Furthermore, fluorescence and colored microspheres are only detectable after their isolation from tissues using complete tissue digestion [70]. Hence, further morphological analysis of tissue is impossible.

The laser Doppler technique for blood flow measurement is characterized as a flow probe method [69] which was employed to determine relative changes in regional blood flow. This technique allows the assessment of blood-vessel function at both, microcirculatory and macrocirculatory levels, depending on the penetration into the tissue. For blood flow recovery measurements in mice, the limited penetration of laser light was sufficient to obtain meaningful results.

The accurate application of LDI requires that several criteria for the measurement have to be respected. To avoid errors associated with temporary changes in vasomotion of measured vessels, mice have to be placed in a climatized chamber under standardized temperature immediately before and during every measurement. According to our observations, pre-warming of mice at 37 °C for five minutes is necessary to standardize the measuring conditions. Exact placement of mice on the pad with parallelly oriented hind limbs is required to achieve an equal penetration of laser light into both feet. Furthermore, in order to reach a higher resolution, the scanned area has to include only tissue or region of interest. In contrast, others have included complete distal parts of the body with losses in resolution [71].

The main disadvantage of LDI method is that it does not determine an absolute blood flow in a particular vessel. Despite of that, the laser Doppler technique is suitable for blood flow measurements in small experimental animals, because the blood vessels do not have to be isolated and exposed.

Very recently, a method based on magnetic resonance imaging (MRI) for blood flow measurements in mice has been developed [72]. The advantages of this method are the in vivo visualization of collateral arteries in the range of 100 µm without using a contrast agent injection. Moreover, an improved MRI method termed “Time-of-Flight” imaging allows for assessment of many mice in a short time period and the animals can be repetitively studied. This method provides a quantitative measurement of blood flow distally to the arterial

occlusion and shows the redistribution of the blood flow. Hence, in the future, improved assessment of data in the mouse model of hind limb ischemia can be used.

4.3. Morphometrical analysis

Histological analysis of adductor muscles was performed using conventional fluorescence microscopy. Vessels were filled with a bismuth-based contrast agent and stained with endothelial and smooth muscle cell markers. Arteries of the collateral region with the vessel area smaller than 100 μm^2 as well as branches of the deep femoral artery were not counted. The mean collateral vessel area was calculated to investigate whether the remodelation of collateral arteries of mice treated with monocytes was more extensive than of those of non-treated mice.

In this study, morphometrical analysis was performed as previously described by Heil et al. [36]. The advantage was the use of cryosections, which were easy to cut and allowed the use of fluorescent-labeled antibodies. On the other hand, it was not possible to measure the internal vessel diameters and vascular wall area because of the contrast agent filling the arteries.

Some other groups (Scholz et al. [73]) studied Epon-embedded semi-thin sections of two superficial collateral arteries stained with toluidin blue under light microscopy. Internal vessel diameters and the arterial wall area were evaluated. This method enables to measure vascular wall area by subtracting the luminal area from the area under the external border of the tunica media in order to differentiate between vessel dilatation and growth.

In our tissue perfusion model, a specific concentration of adenosine was added to avoid interindividual differences in vasodilation. Compared to previous studies where only two superficial collateral arteries per muscle were analyzed, we improved this method by including all collateral vessels in adductor muscles for morphometrical analysis, because all collateral vessels contribute to blood supply.

4.4. Active foot movement measurements

The active foot movement by operated mice was used as a supplementary measurement to obtain a functional parameter. However, it is influenced by the subjective evaluation of the evaluating person. Four score grades, as suggested and used before, seem to be insufficient to describe slight improvements and differences in using the ischemic leg.

In summary, we found a significant improvement in active foot movement in mice treated either with monocytes (2 x 10^5 or 3 x 10^5 cells) or with a combination of monocytes and GM-CSF compared to control mice, except from day 21 when no differences were detected. As five control mice had to be excluded on day 14, active foot movement values on day 14 and 21 were probably falsely higher in this group. In monocyte-treated groups and combination groups, the functional recovery of the hind limbs after femoral occlusion was very good and mice did not differ in active foot movement score. At the end of the observation period, these mice showed almost 100 % functional recovery of the occluded leg, which means that movement with the operated hind limb was unrestricted.

4.5. Angiographic evaluations

Angiographic measurements were performed post-tmortem via abdominal aorta cannulation on day 21 after the femoral artery ligature using a bismuth-based contrast agent. In the occluded hind limbs of control mice, only few collateral arteries were detected. In mice treated with monocytes (2 x 10^5 cells) or Cicaprost-incubated monocytes, high density of well developed collateral arteries could be identified compared to controls. The most developed collaterals were found in the groups treated with a combination of GM-CSF and monocyte injections.

As mice arteries are very small, it is impossible to detect differences in size by angiography. However, this method offers good qualitative evaluation of collateral vessel growth by comparing between experimental groups of animals.

4.6. Injection of isolated monocytes after femoral artery ligature

Monocytes were injected as a bolus directly after femoral artery ligature. Hence, monocytes were distributed through the circulation system into all tissues and not all injected cells contributed to the growth process after local accumulation. A concentration of 2 x 10^5 monocytes in 150 µl NaCl already had a significantly positive effect on blood flow recovery. Injection of 3 x 10^5 monocytes resulted in superior blood perfusion values of the foot. The highest well tolerated dose of monocytes was not established, however, two mice died several days after receiving an injection of 3 x 10^5 monocytes. Hence, we suppose that the highest tolerated single dose might be close to 3 x 10^5 monocytes. Application of 3 x 10^5 monocytes

did not show a significant improvement of blood flow recovery compared to dose of 2 x 10^5 monocytes although a distinct trend toward a further increase in blood flow recovery in mice which received 3 x 10^5 monocytes was observed. As the isolation of monocytes from mouse peripheral blood is very animal consuming and not trivial, we did not increase the number of animals in this experimental group (n=7). It can be speculated that the maximal effect in the BALB/c strain of mice after the monocyte injection was already achieved by injection of 3 x 10^5 monocytes. Furthermore, one can assume that several injections at different time points after femoral artery ligature could continuously increase the pool of monocytes and further enhance the blood flow recovery.

Hemoglobin oxygen saturation measurements showed no differences between the group, which received 2 x 10^5 monocytes and the group, which received 3 x 10^5 monocytes. This was unlike LDI measurements, where the application of 3 x 10^5 monocytes showed a distinct trend toward an increase in blood flow recovery. It can be assumed that mice treated with 2 x 10^5 monocytes already reached the maximum of hemoglobin oxygen saturation recovery measured under narcosis in the BALB/c strain of mice.

Our morphometrical results show that the diameters of collateral arteries and the collateral vessel area in the hind limb after femoral artery ligature in mice treated with 2 x 10^5 monocytes were significantly larger in comparison to non-treated mice [R/L ratio of mean collateral vessel area in 2 x 10^5 monocyte-treated (1.77 ± 0.09) vs. control mice (1.56 ± 0.07), $p < 0.05$]. Moreover, collateral vessel area R/L ratio in mice treated with 3 x 10^5 monocytes (2.37 ± 0.11) was significantly higher ($p < 0.01$) compared to mice treated with 2 x 10^5 monocytes. This result confirmed a trend from LDI measurements indicating toward increased blood flow recovery in mice treated with 3 x 10^5 monocytes. Hence, we conclude that the higher concentration of injected monocytes had indeed a positive effect on collateral artery growth.

Additionally, total collateral vessel area in mice which received 2 x 10^5 monocytes was significantly increased in comparison to non-treated mice (2.26 ± 0.13 vs.1.88 ± 0.08; $p < 0.01$). In mice treated with 3 x 10^5 monocytes, further enlargement of total collateral vessel area was detected (2.66 ± 0.19), although the difference did not reach statistical significance compared to mice treated with 2 x 10^5 monocytes. A possible explanation of this result can be derived from physiological mechanisms of the collateral blood vessel development. As previously described, large collateral arteries are maintained in order to conduct bulk flow, while smaller collateral vessels regress. Few large arteries are more effective for blood perfusion than many small vessels as can be affiliated from the Hagen-Poiseuille's law. This

can also explain why the collateral vessel area in mice treated with 3 x 10^5 monocytes did not increase significantly despite markedly enlarged vessel diameters.

Our results of monocyte-treatment after experimental artery occlusion correlate well with a previous study in our group, [19] in which the concentration of blood monocytes in mice and rabbits was manipulated by an injection of 5-fluorouracil. After femoral artery ligature, an increased collateral conductance and an increased number of visible collaterals in rabbits was observed during the rebound phase after monocyte depletion. In mice, pedal blood flow measured by laser Doppler was enhanced between days 7 and 21, if the femoral artery was ligated during the rebound period. In contrast, ligature during the depletion phase of monocytes resulted in impaired blood flow recovery.

The importance of monocytes in collateral artery growth was also reported in previous studies [32,74,33,19,75], in which it was observed that circulating blood monocytes adhere to the vascular wall during collateral vessel growth. Moreover, accumulated monocytes were positively stained for vascular growth factors (bFGF and TNF-α) which are necessary for collateral vessel growth [33]. These findings suggest that monocytes contribute to arteriogenesis as producers of growth stimulating substances, similarly to bone marrow stem cells which were reported to act through a paracrine mechanism by producing cytokines “cytokine factories” [76], Other studies investigated the molecular pathway of monocyte attraction and activation [35,77,36]. Monocyte chemoattractant protein-1 (MCP-1) was previously described to be upregulated by shear stress in collateral arteries in vitro. In the rabbit ischemic hind limb model, after local delivery of MCP-1 into the proximal branches of femoral artery via osmotic minipump, markedly increased collateral conductance was detected in comparison to rabbits, which received PBS.

In a mouse model of acute inflammation [77], resident tissue peritoneal macrophages were diminished using thioglycolate injection. In both, control and CC-chemokine receptor-2 (CCR2) knock out (k.o.) mice, the number of resident macrophages rapidly decreased as detected 4 hours after the injection. Within 24 hours, the number of peritoneal macrophages in the control group reached two times basal levels in contrast to CCR2 k.o. mice which did not even reach basal values. These results demonstrate that CCR2 receptor, which is the exclusive MCP-1 receptor in mice, is essential for monocyte/macrophage migration.

In our own study, we used CCR2 k.o. mice in the ischemic hind limb model to determine, whether the MCP-1-CCR2 signaling pathway is involved in collateral artery growth [36]. We performed a chemotaxis assay which revealed almost abolished mononuclear cell migration

towards MCP-1 in the blood of CCR2 k.o. mice. Additionally, blood flow recovery in BALB/c background of CCR2 k.o. mice as assessed by LDI was remarkably impaired in comparison to control mice. Moreover, the number of monocytes/macrophages around collateral arteries of CCR2 k.o. mice on day 4 after femoral artery ligature was significantly reduced in comparison to wild-type mice. Hence, these data also suggest that blood monocytes are necessary in early stages of collateral vessel growth.

4.7. Injection of GFP-labeled monocytes

To obtain further evidence for the pivotal role of circulating monocytes and evaluate the role of resident macrophages for arteriogenesis, we injected GFP-labeled monocytes into a tail vein in order to track the way of injected monocytes in recipients.

For this reason, GFP-transgenic mice were used as blood donors. This transgenic line shows ubiquitously expression of eGFP except erythrocytes and hair follicle cells [78]. Mononuclear cells from these transgenic mice were harvested via density gradient centrifugation of peripheral blood, stained with PE-conjugated murine monoclonal antibody against CD11b and isolated using a cell sorter according to their scatter characteristics and their CD 11b expression. GFP-monocytes were injected into a tail vein after femoral artery ligature of recipients. Animals were sacrificed on day 3 after the ligature, which was previously reported as a day of maximal monocyte/macrophage accumulation during the collateral artery growth [33].

Many GFP-positive cells were found in the spleen which served as a positive control of proper monocyte injection. Some GFP-positive monocytes were found in the liver and no GFP-labeled cells were found in the hearts, lungs, peritoneum and kidneys. In non-ligated hind limb adductor muscles, a marginal number of GFP-positive cells was detected as well which might be a consequence of the participation of the injected cells in physiological blood circulation. In ligated hind limb adductor muscles, a significantly increased number of GFP-labeled cells was observed in the collateral vessel region. This provides evidence that injected monocytes circulated in the blood, were attracted by collateral endothelium and migrated through the vessel wall into collateral artery surrounding tissues. Subsequent staining for specific macrophage marker F4/80 showed that accumulated GFP-labeled monocytes also expressed a specific macrophage marker and presumably secreted vascular growth factors.

However, some groups proposed that resident tissue macrophages rather than circulating blood cells play the most important role in arteriogenesis [79], [80]. In the study of Khmelewski,

Becker et al. 2004, fluorescence(CMFDA)-labeled blood cells were injected into the femoral artery of rats after its occlusion. The labeled cells included all leukocyte subpopulations and thrombocytes. It should be considered that the incubation of cells with a fluorescent cell membrane affecting marker might influence their arteriogenic activity, as no labeled cells were detected around collateral vessels. Moreover, no control rats were used to compare the collateral artery growth in non-treated and treated animals. In summary, the data presented by Khmelewski et al. are not very convincing because of these methodological shortcomings.
Our results are in line with another study of a unilateral hind limb ischemia model in rats [81]. The authors injected fluorescence-labeled human peripheral blood cells (mononuclear cells + platelets or mononuclear cells + platelets + polymorphonuclear leukocytes or polymorphonuclear leukocytes alone or platelets alone) intramuscularly into ischemic limbs of athymic rats after femoral artery ligature. LDI measurements revealed significantly increased blood flow in rats which received mononuclear cells + platelets in comparison to control rats, which received human umbilical vein endothelial cells (HUVEC). Interestingly, addition of polymorphonuclear leukocytes to mononuclear cells and platelets significantly attenuated blood flow compared to treatment with mononuclear cells and platelets alone. Infusion of platelets alone had a positive influence on blood flow recovery although the R/L ratios were markedly below those of the group treated with mononuclear cells + platelets. Infusion of polymorphonuclear leukocytes alone did not have any influence on blood flow recovery. In conclusion, mononuclear cells and platelets were involved in the collateral artery growth, whereas polymorphonuclear leukocytes did not have any effect on blood flow recovery. Furthermore, Iba et al. [79] proved that mononuclear cells and platelets migrated towards arterioles, accumulated in the perivascular region and secreted VEGF, bFGF, PDGF-AB and TGF-β. These findings suggest that monocytes contribute to the collateral vessel growth by a paracrine mechanism.

The importance of monocytes in the early stages of remodeling was confirmed after their experimental depletion from circulation using cytotoxic bisphosphonates [75]. It was followed by a complete abolishment of arteriogenesis in the rabbit ischemic hind limb model.
The role of other circulating cells in arteriogenesis has yet not been well elucidated. However, several kinds of blood cells seem to be involved in the growth process. After migration of their precursors from the blood stream, mast cells were found in a widened adventitia of [43] collateral vessel walls, secreting MMP-9 [82]. Moreover, mast cells were reported to produce VEGF, bFGF, TGF-β, TNF-α, IL-8 and other cytokines which stimulate cells (e.g. fibroblasts, macrophages) to secrete angiogenic factors [83]. Mast cells may contribute to arteriogenesis also

by chemotactic recruitment of monocytes/macrophages, lymphocytes and by activation of platelets which in turn could produce angiogenic factors.

The role of T lymphocytes in arteriogenesis was tested in CD4 gene-deficient mice in which the development of CD8+ T cells and myeloid components was unaltered, whereas the number of CD4+ T cells was markedly reduced [84]. After creation of unilateral hind limb ischemia, CD4 gene-deficient mice showed a significant reduction in collateral artery density in the thigh of the operated hind limb in comparison to control mice. Furthermore, a severe reduction in blood flow recovery in CD4 knockout mice was observed. Moreover, the suggested mediating mechanism of reduced collateral development was probably due to the decreased monocyte/macrophage recruitment.

Other circulating cells which contribute to postnatal neovascularization are endothelial progenitor cells (EPC) which originate from CD34+ mononuclear cells [41]. EPC mostly express monocyte/macrophage markers (CD14, Mac-1, CD11c) and lower percentage of cells express endothelial cell marker (VE-cadherin) and progenitor cell marker (AC133, c-kit) [85]. They contribute to collateral vessel formation by secretion of growth factors like VEGF, hepatocyte growth factor and GM-CSF rather than by proliferation and differentiation into endothelial cells. EPC were found in increased concentration in peripheral blood after acute myocardial infarction [86] or after stimulation with cytokines.

Studies on bone marrow stem cells and their contribution to tissue repair show contradictory conclusions. On the one hand, bone marrow cells were reported to be incorporated into tissue of injured organs (e.g. infarcted myocardium) and to differentiate into tissue-specific cells after their transplantation into ischemic myocardium [87,88] or ischemic hind limb tissue [89]. However, the mechanism by which these cells contribute to improved tissue perfusion has not been well elucidated. On the other hand, recent reports show that bone marrow-derived cells do not incorporate into the adult growing vasculature but accumulate in the area of growing vessels and produce a cocktail of growth factors and chemokines [47].

4.8. Combination of monocyte injection with GM-CSF treatment

A very important observation in almost all experimental settings of arteriogenesis is the lack of a complete recovery of physiological blood flow. This raises the idea to condition monocytes prior to injection for further stimulation of arteriogenesis. We tested the potential therapeutic efficacy of a combination of monocytic treatment with GM-CSF administration.

GM-CSF was previously reported to be a growth factor prolonging the life span of monocytes and their rate of differentiation [56].

In one approach, mice received 5 injections of rmGM-CSF starting two days before femoral artery ligature. On the third day, the femoral artery was occluded and monocytes were injected into a tail vein. LDI measurements revealed improvement in blood flow recovery starting on day 3 after the operation. Although a significant difference compared to mice which received 2 x 10^5 monocytes alone was not detected, a prominent trend toward a further enhancement in blood flow recovery was detected. A similar effect was noticed when a higher concentration of monocytes (3 x 10^5 cells) was combined with rmGM-CSF injections as described before. Blood flow recovery was improved in comparison to mice, which received 3 x 10^5 monocytes alone, but did not reach statistical significance.

Hemoglobin oxygen saturation (HOS) measurements of mice treated with a combination of rmGM-CSF and monocyte injection showed a significant improvement compared to controls. However, it seemed that mice treated with 2 x 10^5 monocytes already reached the maximal HOS values. That may explain why no further increase in HOS in mice which received a combination of either 2 x 10^5 monocytes or 3 x 10^5 monocytes and GM-CSF was observed.

Morphometrical analysis showed that collateral diameters and perimeters of the combination group (2 x 10^5 monocytes + GM-CSF) increased significantly compared to mice treated with 2 x 10^5 monocytes alone. Similar results were obtained from mice treated with 3 x 10^5 monocytes + GM-CSF, in which the mean collateral area was significantly larger than in mice treated with 3 x 10^5 monocytes alone. Moreover, the total collateral vessel area also was significantly larger.

4.9. Application of GM-CSF alone in the mouse ischemic hind limb model

In the second approach, mice received subcutaneous injections of GM-CSF to test if GM-CSF application alone can enhance the arteriogenesis. GM-CSF was previously described to be a potent arteriogenic growth factor [56], [55], [90]. After the local delivery of GM-CSF into the proximal stump of occluded femoral artery in rabbits via an osmotic minipump, an increased number and size of collateral arteries were detected on post-mortem angiograms as well as an increased collateral conductance [56]. The arteriogenic potential of GM-CSF was also tested in a rat cerebral hypoperfusion model [90]. After the occlusion of both vertebral and the left carotid artery in rats, GM-CSF or saline was injected into the non-occluded carotid artery and subcutaneous injections of GM-CSF or saline were administered every second day

for one week. GM-CSF treatment had no influence on the arterial architecture of the dorsal brain surface and on the diameters of Heubner's anastomoses in comparison to saline-treated rats. An increased diameter of the posterior cerebral artery (PCA) in saline- and GM-CSF-treated animals after one week (260 µm vs. 320 µm) was measured and both diameters were significantly larger than in non-operated animals without any ischemic events. It is not clear if the increase of the PCA diameter in GM-CSF-treated animals was also significant compared to saline-treated animals as this would be the result which may have shown the effectiveness of the GM-CSF treatment. However, an important question is whether these measurements really refer to arteriogenesis (development of collaterals from pre-existing arteriolar connections), considering that the PCA is an important supplying artery fully developed in healthy non-ischemic brain. Alternatively, this could be a vasodilation effect or an adaptation to altered flow conditions as can be frequently observed.

In our study, we showed that GM-CSF treatment alone does not improve blood flow recovery after experimental femoral artery occlusion. In our experiments, mice received five intraperitoneal injections of rmGM-CSF starting two days before femoral artery ligature. After 21 days, LDI measurements only showed a slight increase in blood flow recovery in GM-CSF treated mice. However, in contrast to control mice, no necrosis occurred in GM-CSF treated animals. HOS measurements confirmed that blood flow recovery between GM-CSF and control mice did not differ. At day 3, HOS of the GM-CSF group was slightly higher than in controls, probably because several control mice developed necrosis and had very low tissue oxygenation.

Histological evaluation of the adductor muscles revealed that the mean collateral area in GM-CSF-treated animals moderately increased compared to controls (1.62 ± 0.06 vs. 1.56 ± 0.07) as well as the total collateral area (1.91 ± 0.11 vs. 1.88 ± 0.08). However, these results did not reach statistical significance.

In conclusion, application of GM-CSF alone had a moderate effect on blood flow recovery which indicates that GM-CSF has only a supporting role in collateral vessel growth. Our results show that application of GM-CSF alone is not suitable for a treatment in the mouse ischemic hind limb model. Moreover, higher concentrations of GM-CSF were not well tolerated and it was difficult to find a proper concentration that would be effective and not toxic. Our data correspond with a study in a rabbit ischemic hind limb model where even local intraarterial delivery of GM-CSF did not significantly increase collateral conductance (Eitenmueller et al. data not published) but contrast with several previous studies under experimental and clinical settings as mentioned before.

However, pre-treatment with GM-CSF combined with monocyte injections has a synergistic effect and accelerates blood flow recovery after experimental femoral artery occlusion. The combination of monocyte injections and GM-CSF application allows to use smaller concentrations of monocytes with a high effect on blood flow recovery.

4.10. Effect of Cicaprost- and GM-CSF-primed monocytes on blood flow recovery after femoral artery ligature

The effect of a stable prostacyclin analogue (Cicaprost) on monocyte incubation has not yet been described. We tested, if Cicaprost can enhance their arteriogenic potential. Isolated monocytes (2 x 10^5 cells) were incubated with 100 ng/ml of Cicaprost for two hours and than injected into a tail vein of recipients after femoral artery occlusion.
In summary, LDI and HOS measurements did not support our hypothesis and the results were comparable to those from 2 x 10^5 monocyte-treated mice. In LDI measurements, a slight delay of blood flow recovery on day three was detected in mice, which received Cicaprost-incubated monocytes. However, it was described that Cicaprost decreases TNF-α production in activated monocytes [91] and TNF-α is responsible for adhesion and activation of additional monocytes. Furthermore, Cicaprost incubation with monocytes may have altered their activation status in a way that the transmigration process may have been negatively affected.

The effect of GM-CSF-priming on monocytes was again tested in our mouse ischemic hind limb model. GM-CSF is known to have a potent effect in stimulating proliferation, maturation and function of hematopoietic cells as well as the ability to enhance the function of fully differentiated cells [52]. Preliminary studies were performed to determine the optimal dose and incubation time for rmGM-CSF in coculture with peritoneal macrophages and the dose was established to be 200 ng/ml for two hours [53].
In our experiment, isolated monocytes (2 x 10^5 cells) were incubated with 200 ng/ml of rmGM-CSF for three hours. Immediately after incubation, cells were injected via a tail vein of a recipient after femoral artery ligature. Surprisingly, in two of the recipients a severe necrosis developed and they were excluded from the study. Such an incident has never been observed in mice treated with monocytes. Blood flow measurements revealed that mice which received monocytes incubated with GM-CSF did not show any improvement in comparison to non-treated animals. Hemoglobin oxygen saturation measurements confirmed these results.

Morphometrical measurements showed that collateral vessel perimeters did not increase compared to controls and the mean collateral vessel area dropped markedly.

In conclusion, priming of monocytes with GM-CSF may not enhance their arteriogenic potential. In contrast, the incubation of monocytes with GM-CSF prior to their injection appears to negatively influence arteriogenesis, most likely by decreasing of cytokine production. Another possible explanation could be that conditioned monocytes differentiated into macrophages and subsequently lost their ability to transmigrate through the vessel wall into the collateral area.

4.11. Limitations of arteriogenic therapy

Therapeutic arteriogenesis can be induced via administration of growth factors, cells or using artificially increased fluid shear stress [25].

The efficacy of growth factors, like VEGF, FGF2 and GM-CSF was already proven and/or disproven in clinical trials [92], [93], [53]. There are often discrepancies in results between animal studies and clinical trials. The reasons for this, among other things, may be that in animal studies, only young and healthy animals are used and they may show a different response to the treatment from the population used in clinical trials, which is mostly represented by elderly and polymorbid patients. An age-dependent impairment of collateral vessel development was previously reported in animal studies [92].

Furthermore, for optimal treatment, it is necessary to establish the proper dose and application route. On the one hand, systemic administration is often accompanied by several adverse events and results in a poor uptake by the targeted tissue. Furthermore, growth factors have very short plasma half-lives due to their degradation and binding to endothelial cells [93]. On the other hand, systemic application is more convenient for the patient and non-invasive. Local application as a single bolus may be insufficient and prolonged exposition of the growth factor to the targeted tissue is required. With local application of the drug, fewer side effects may be achieved. Most of the growth factors are effective only in very high dosages, which are close to toxic. There is a need to find an effective combination of growth factors instead of using only one.

We have shown that systemic application of monocytes effectively enhances collateral blood flow in the mouse ischemic hind limb model. Systemic application may not be suitable for patients with severe atherosclerosis because of a possible pro-atherogenic effect of the monocytes due to increased angiogenesis in the vasa vasorum of atherosclerotic plaques [58] [55].

Transplanted monocytes can as well contribute to impairment of diabetic retinopathy via pathological angiogenesis and accumulate in inflammation focuses.

4.12. Lessons from clinical trials on arteriogenesis

Stimulation of coronary arteriogenesis was tested in the VIVA trial [94] using a combination of intracoronary and intravenous administration of $rhVEGF_{165}$ in patients with stable exertional angina, unsuitable for standard revascularization. The results did not meet the expectations as by day 120 only a high dose of $rhVEGF_{165}$ (recombinant human vascular endothelial growth factor) significantly improved angina class compared to placebo-treated patients, and myocardial perfusion imaging measurements with angina frequency evaluations only showed non-significant trends.

In another trial the therapeutic efficacy of rFGF2 (recombinant fibroblast growth factor 2; FIRST trial) was tested by single-bolus intracoronary infusion over 10 minutes [95]. By day 90, exercise tolerance tests did not show any significant differences between FGF2- and placebo-treated patients as well as SPECT (single photon emission computed tomography) myocardial perfusion measurements. But the angina frequency score was significantly reduced in FGF2-treated patients. Possible explanations for these results are suboptimal dose or exposure time of applicated FGF2 growth factor and decreased therapy response in elderly patients or no arteriogenic functions of FGF2.

Therapeutic arteriogenesis in patients with peripheral artery disease was solely tested in the TRAFFIC trial [96] using intra-arterial administration of rhFGF2 in patients with inguinal atherosclerosis. The results showed significant improvement in the peak walking time on day 90 in patients treated with a single dose of rhFGF2 compared to those treated with placebo. Controversially, patients which received a double dose of rhFGF2 showed no significant improvement in the peak walking time compared to the placebo group. The study may have suffered from the lack of direct parameters like collateral conductance values.

Therapeutic implications of rhGM-CSF (recombinant human granulocyte-macrophage colony-stimulating actor; Molgramostim) was tested in patients with severe coronary artery disease which were unable to undergo coronary artery bypass surgery [55]. Patients received an intracoronary injection of 40 µg of GM-CSF or placebo followed by a subcutaneous administration of GM-CSF (10µg/kg) or placebo for two weeks. Collateral flow index (CFI) was measured after experimental PTCA occlusion for one minute. CFI in placebo-treated patients decreased but increased in GM-CSF-treated patients compared to pre- treatment

measurements after two week observation period. The difference between pre- and post-treatment CFI values was significant in the GM-CSF treated group of patients. However, the study was extensively criticized [97] because of methodological problems. The comments were concerning the relevancy of the short study time period (two weeks) and high intrinsic variability of the applied method for CFI measurements.
No clinical trial using GM-CSF in the treatment of peripheral occlusive disease has yet been performed.

There are several possibilities of explanation why previously described clinical trials did not meet the expectations. Results from animal studies suggest that the application of a single growth factor does not completely restore physiological blood. There is a need to find a combination of growth factors which allows to use lower doses and avoids the adverse events. Finally, as mentioned before, application route has to be chosen based on the hypothesized mechanism by which the tested substance could stimulate arteriogenesis. The consideration, which have to be done prior to a clinical approach can be summarized as followed: The right factor has to be at the right place at the right time in order to be supportive.

4.13. Conclusions

In conclusion, we showed that infusion of monocytes enhances blood flow recovery in the ischemic leg after experimental occlusion of the femoral artery. This improvement was related to the concentration of injected monocytes. Moreover, the combination of GM-CSF and monocyte treatment had an additive effect and resulted in superior blood flow recovery and well developed collaterals. Our results indicate that GM-CSF only has a supporting role in arteriogenesis and treatment with GM-CSF alone does not have the ability to improve blood flow recovery after arterial occlusions.
Finally, we found that priming of monocytes with Cicaprost or GM-CSF prior to injection does not support arteriogenesis, most likely because the migration process of these cells was negatively influenced.

5. Abbreviations

°C	degrees Centigrade
µg	microgram
µl	microliter
µm²	square micrometer
A.	arteria
BS1 lectin	bandeiraea simplicifolia 1 lectin
BSA	bovine serum albumin
CCR-2	CC-chemokine receptor-2
COX	cyclooxygenase
DAPI	4’,6-diamino-2-phenylindole dilactate
EPC	endothelial progenitor cells
FGF	fibroblast growth factor
Fig.	figure
FITC	fluorescein-5-isothyocyanate
FSS	fluid shear stress
g	acceleration due to gravity
g	gram
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
h	hour
HCl	hydrochloric acid
HIF	hypoxia inducible transription factor
HOS	hemoglobin oxygen saturation
Hz	hertz
i.p.	intraperitoneal
I.U.	international units
i.v.	intravenous
ICAM	intercellular cell adhesion molecule
IFN-γ	interferon-gamma
IL	interleukin
kd	kilodalton

kHz	kilohertz
kV	kilovolt
L	liter
LDI	Laser Doppler Imaging
mA	milliampere
MCP-1	monocyte chemoattractant protein-1
M-CSF	macrophage colony-stimulating factor
mg	milligram
min	minute
ml	milliliter
MMP	matrix metalloprotease
ms	millisecond
N	number of animals
N.	nervus
NaCl	sodium chloride
ng	nanogram
NO	nitric oxide
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PE	phycoerithrin
pFA	paraformaldehyde
PlGF	placental growth factor
psi	pounds per square inch
rh	recombinant human
rm	recombinant murine
rpm	rounds per minute
s.c.	subcutaneous
SEM	standard error of the mean
SMC	smooth muscle cells
Tab.	table
TGF-β	transforming growth factor-beta
TNF-α	tumour necrosis factor-alpha
t-PA	tissue-plasminogen activator
TRITC	tetramethylrhodamine isothiocyanate

u-PA	urokinase-plasminogen activator
V.	vena
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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7. Summary

Arteriogenesis, the growth of collateral arteries as a compensatory mechanism after occlusion of a major artery, is a complex process in which peripheral blood cells have been proposed to be involved. This study was concentrated on the role of monocytes, effects of different monocyte concentrations in the blood and effects of monocyte pre-treatment with specific substances on the enhancement of collateral artery growth.

For this purpose, a mouse ischemic hind limb model was established on the BALB/c genetic background. After acute femoral artery occlusion, mice received intravenous injections of monocytes in several concentrations, isolated from donor littermates using a cell sorter. Moreover, we pre-treated monocytes with a synthetic prostaglandin (Cicaprost) or with granulocyte-macrophage colony-stimulating factor (GM-CSF). We also evaluated if treatment of operated mice with GM-CSF alone could enhance collateral artery growth and if a combination of monocyte injections with GM-CSF had a synergistic effect. Pedal blood flow recovery in mice was measured using the laser Doppler imaging technique immediately after operation and on postoperative day 3, 7, 14 and 21. Moreover, pedal hemoglobin oxygen saturation was estimated at the same time points. Functional recovery of the foot movement was observed during the whole trial period. On the day 21 after the operation, post-mortem angiogramms were performed and adductor muscles were harvested for the morphometrical analysis.

The hemodynamic data showed that injection of monocytes had a concentration-dependent effect in enhancing of blood flow recovery in the operated foot. Seven days after femoral artery ligature, the blood flow recovery (right-to-left ratio) was only 0.21 ± 0.02 in control mice and significantly higher in mice which had obtained monocyte injections (0.30 ± 0.04 in mice with $2x10^5$ monocytes injected, $p<0.05$ and 0.33 ± 0.04 in mice with $3x10^5$ monocytes injected, $p<0.01$). Morphometrical analysis of adductor muscles of operated and non-operated hind limbs showed that total collateral area ratio, which was obtained as a parameter to estimate collateral artery size, increased markedly in monocyte-treated mice compared to controls (2.37 ± 0.12 in mice with $2x10^5$ monocytes injected; $p<0.01$ and 2.66 ± 0.12 in mice with $3x10^5$ monocytes injected; $p<0.01$ vs. 1.88 ± 0.08 in controls). Pre-treatment of monocytes with Cicaprost or GM-CSF did not enhance their arteriogenic properties. Administration of GM-CSF via intraperitoneal injections did not enhance the blood flow recovery, however the combination of GM-CSF and monocyte injections had a

moderate synergistic effect reflected in blood flow measurements (R/L ratio on day 7 was 0.40 ± 0.05 which was the highest ratio of all experimental groups of animals; $p<0.01$), as well as in morphometrical analysis of adductor muscles (total collateral area ratio was 3.32 ± 0.14; $p<0.01$)

Our results show that monocytes/macrophages play a crucial role in enhancement of collateral artery growth. In addition, a combination of GM-CSF treatment and monocyte injections allows administration of smaller concentrations of therapeutics and results in a superior blood flow recovery. Our study suggests a possibility of using cellular approaches for the treatment of vascular occlusive diseases.

8. Zusammenfassung

Als Arteriogenese wird das kompensatorische Wachstum von Kollateralgefäßen nach arteriellen Verschlüssen bzw. chronischen Stenosen bezeichnet. Aufgrund von Ergebnissen frühere Studien vermutete man, dass an den komplexen Prozessen, die dieses Wachstum charakterisieren, zirkulierende Blutzellen, vor allem Leukozyten, beteiligt sind. Zielsetzung dieser Studie war es deshalb, die Rolle der Monozyten, die Effekte unterschiedlicher Blutmonozytenkonzentrationen und den Einfluss einer Vorbehandlung der Monozyten mit spezifischen Substanzen auf die Arteriogenese zu untersuchen.

Hiezu wurde das Hinterlauf-Ischämien-Modell in der Maus (BALB/c) verwendet. Nach dem Verschluss der Arteria femoralis erhielten die Mäuse in unterschiedlichen Konzentrationen intravenöse Injektionen von Monozyten, die zuvor aus genetisch identischen Mäusen mittels cell sorter isoliert wurden. Weitere Versuchsgruppen wurden mit Monozyten behandelt, die zuvor mit einem synthetischen Prostaglandinen (Cicaprost) bzw. mit dem Granulozyten-Makrophagen-Kolonie-stimulierenden Faktor (GM-CSF) vorbehandelt wurden. Es wurde ebenfalls untersucht, ob Injektionen von GM-CSF das Kollateralgefäßwachstum nach Okklusion der Femoralarterie verstärken und ob eine Kombinationstherapie aus GM-CSF-Injektionen und Monozytentransplantationen einen zusätzlichen stimulativen Effekt haben. Sofort nach der Operation als auch an den Tagen 3, 7, 14 und 21 wurden Laserdoppler-Untersuchungen zur Bestimmung der Wiederherstellung der pedalen Durchblutung durchgeführt. Zusätzlich wurde die Sauerstoff-Sättigung im Hämoglobin in den distalen Gefäßen bestimmt. Als funktioneller Parameter während des Beobachtungszeitraumes wurde die aktive Bewegung der Hinterläufe quantifiziert. Drei Wochen nach Versuchsbeginn wurde das Kollateralgefäßsystem durch Angiographien post mortem dargestellt und Gewebeproben zur morphometrischen Analyse konserviert.

Die hämodynamischen Daten zeigen, dass die Monozyteninjektionen einen dosisabhängigen positiven Effekt auf das Kollateralgefäßwachstum hatten. Am siebten Tag nach dem Verschluss der Arteria femoralis war der pedale Blutfluss (rechts-zu-links Rate) in Kontrollmäusen nur 0,21 ± 0,02 und signifikant höher in Mäusen, die Monozyten injiziert bekommen haben (0,30 ± 0,04 in Mäusen, die $2x10^5$ Monozyten bekommen haben; $p<0,05$ und 0,33 ± 0,04 in Mäusen, die $3x10^5$ Monozyten bekommen haben; $p<0,01$). Morphometrische Analysen der Adduktoren von den operierten und nicht operierten Hinterläufen ergaben, dass die gesamte Kollateralflächenrate, die als Parameter zur Bewertung der Kollateralgefäßgröße diente, war in Monozyten behandelten Mäusen

verglichen mit den Kontrollen deutlich erhöht (2,37 ± 0,12 in Mäusen, die $2x10^5$ Monozyten bekommen haben; $p<0,01$ und 2,66 ± 0,12 in Mäusen, die $3x10^5$ Monozyten bekommen haben; $p<0,01$ vs. 1,88 ± 0,08 in Kontrollen). In Gegensatz hierzu hatten die Vorbehandlungen der Monozyten mit Cicaprost oder GM-CSF keinen weiteren arteriogenen Effekt. Des weiteren verstärkten die Injektionen von GM-CSF die Wiederherstellung der Durchblutung nicht, auch wenn die Kombinationstherapie von GM-CSF und Monozyten-Injektionen einen moderaten synergistischen Effekt hatte (R/L Rate am Tag 7 war 0,40 ± 0,05, der höchste Wert von allen Versuchsgruppen; $p<0,01$). Diese Ergebnisse wurden durch histologische Untersuchungen der Adduktorenmuskulatur von operierten und nicht operierten Hinterläufen erhärtet (die gesamte Kollateralflächenrate war 3,32 ± 0,14; $p<0,01$).

Die Ergebnisse zeigen, dass die Monozyten/Makrophagen eine wichtige Rolle in der Stimulierung des Kollateralgefäßwachstums spielen. Außerdem ist es möglich durch die Kombination von GM-CSF- mit Monozyten-Injektionen die Konzentrationen der Therapeutika gegenüber der Einzeldosierungen zu reduzieren, wobei trotzdem die besten Ergebnisse erzielt werden konnten. In dieser Studie wurden die Grundlagen zum Einsatz zellulärer Therapeutika in der Behandlung von Herz-Kreislauf-Erkrankungen geschaffen.

9. Lebenslauf

Persönliche Daten

Geburtsdatum:	21.03.1976
Geburtsort:	Bratislava, Slowakei
Familienstand:	verheiratet, Tochter Sophia geboren am 12.06.2005
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1982 – 1990	Grundschule Tilgnerova in Bratislava
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Studium und Berufserfahrung

1994 – 1995	Krankenpflegerin in 1. HNO Universitätsklinik in Bratislava
1995 - 2002	Studium der Medizin an der Commenius Universität in Bratislava
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10/2000 – 05/2001	wissenschaftliche studentische Hilfskraft am Max-Planck-Institut für Physiologische und Klinische Forschung, W. G. Kerckhoff Institut, Bad Nauheim
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08/1999	Praktikum – pädiatrische Chirurgie in Juiz de Fora, Brasilien
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Sprachkenntnisse

Deutsch, Englisch, Russisch

Interessen

Klavier spielen, joggen, fremde Sprachen

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„Ich erkläre: Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

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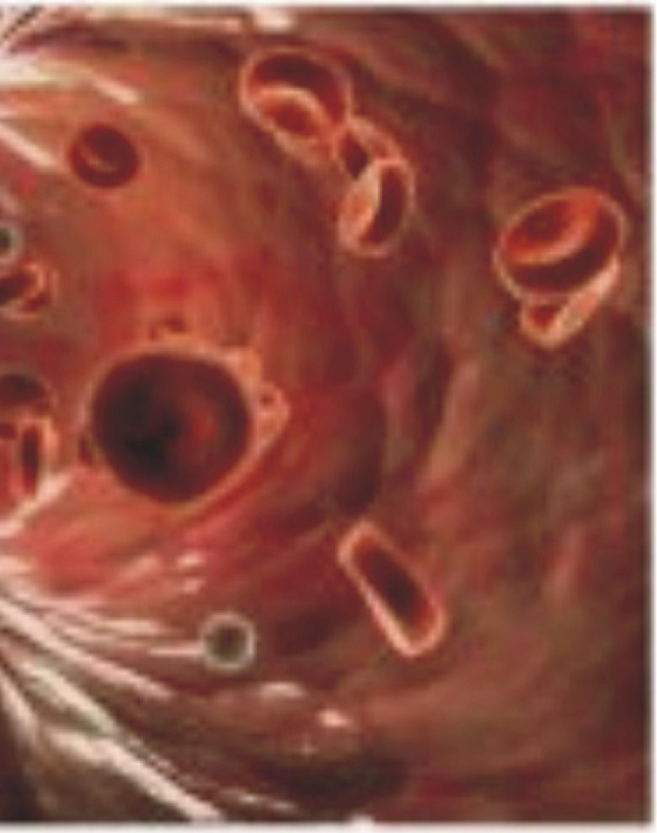

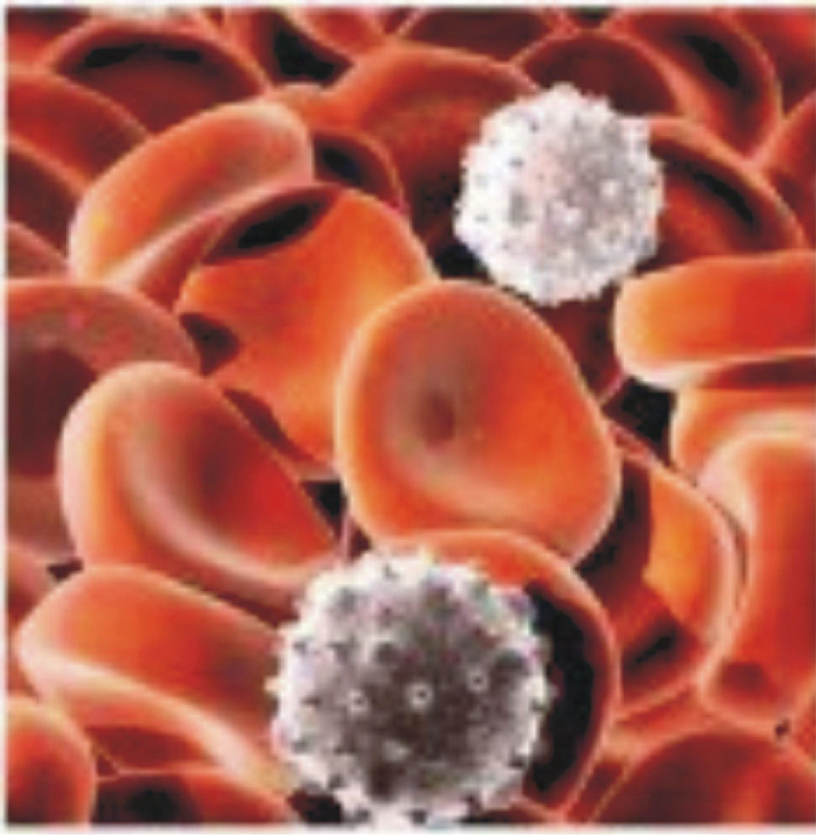

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