Macrophage heterogeneity in neovascularization

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Macrophage heterogeneity in neovascularization

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Macrophage heterogeneity in neovascularization

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Chapter 1

General Introduction

Cardiovascular diseases (CVD) are the leading cause of death worldwide. Stroke and myocardial infarction are the main acute manifestations of CVD and are mainly caused by atherothrombosis, the consequence of a ruptured atherosclerotic plaque ¹. Aside from smoking, stress and increased fat-consumption, a major recognized risk factor for the chronic state of atherosclerosis is inflammation ². By accumulation of inflammatory cells (especially macrophages) in the plaque, a mass production of pro-inflammatory cytokines contributes to the development and progression of atherosclerosis. Additionally, microvessel formation in the plaque is known to be associated with advanced lesions and risk for plaque rupture, ultimately leading to a cardiac event ³. The relation between macrophage accumulation and neovascularization is recognized in many pathologies and will be further evaluated in the sections below.

Neovascularization

The formation of new blood vessels, or neovascularization, is essential for the development of all vertebrate life. The cardiovascular system develops from the point of fertilization in order to support growth and development of all organs providing them with nutrients and oxygen. Embryonic blood vessels are built *de novo* through differentiation, migration and fusion of mesoderm-derived endothelial progenitor cells (angioblasts) creating a primitive vascular network (vasculogenesis) ^{4, 5}. Subsequently, this primitive network of progenitor cells develops complexity by sprouting of endothelial cells (angiogenesis). The fate of endothelial cells is genetically determined and already during vasculogenesis differentiation into arterial and venous endothelium takes place ⁶. Circumferential expansion of arteries and veins occurs to increase perfusion and maturation of the vasculature and is completed when endothelial cells are covered with smooth muscle cells (SMCs), pericytes and extracellular matrix (ECM) for stability (arteriogenesis) (Figure 1).

In parallel to the development of the vasculature, the lymphatic system arises from the same pool of endothelial progenitor cells and functions in draining all organs from waste products ⁷. Throughout life, both the vascular and lymphatic system are essential for homeostasis in the healthy organism.

Figure 1. Schematic representation of neovascularization. Angioblasts fuse together into immature vascular structures (vasculogenesis), which grow out into capillaries built of endothelial cells (red). Vascular networks gain complexity by formation of new capillaries out of the existing structures by sprouting, intussusception or bridging (angiogenesis). Enlargement and maturation of the vasculature (arteriogenesis) involves proliferation of endothelial cells and recruitment of smooth muscle cells (blue).

Neovascularization in health

Angiogenesis is described as the formation of new capillaries out of pre-existing blood vessels ⁸. This can occur through vessel splitting (intussusceptions) or bridging, but the main part of newly formed vessels is initiated by vascular sprouting. Some endothelial cells in the vessel wall, known as tip cells, are selected for sprouting and will react to pro-angiogenic signals while neighboring stalk cells do not respond initially, but follow the path of the tip cell ⁹. Endothelial sprouting is mediated by factors such as vascular endothelial growth factor (VEGF) and angiopoietin-2 (Ang2) secreted by for instance highly angiogenic tumor tissue. In order for angiogenic mediators to reach the endothelium the ECM surrounding the tip cells has to be degraded. Hereto, matrix metalloproteinase proteins (MMPs) are activated breaking down matrix and releasing matrix-bound angiogenic factors, which further

stimulate chemotactic migration and proliferation of endothelial cells. Stalk cells proliferate and migrate further along the path of the tip cell and produce platelet derived growth factor (PDGF) to attract pericytes and ensure stabilization of the sprout. Eventually, tip cells encounter other sprouting tip cells and by fusion and formation of strong adhesive cell-cell interactions a continuous lumen is realized.

In adult life, the vascular endothelium is quiescent and rarely forms new sprouts and branches. Pro-angiogenic and anti-angiogenic signals are balanced to control this quiescent state, however, there are some events in which this balance temporarily is disturbed. The female reproductive organs (e.g. ovaries, uterus and placenta) exhibit periodically dynamic growth and regression accompanied with rapid changes in blood flow. The main factors regulating this periodic stimulation of angiogenesis are VEGF, basic fibroblast growth factor (FGF-2), insulin-like growth factor (IGF-1) and transforming growth factor (TGF β)¹⁰. In response to fluctuations of the hormones estrogen and progesterone during the ovarian cycle, microvasculature formation is increased resulting in follicle growth, increased uterine blood flow and thickening of the endometrium, preparing it for implantation of a fertilized egg¹¹.

Another example of physiological angiogenesis is the process of wound healing. The healing process depends on cooperation of immune cells, connective tissue and vascular endothelial cells in order to succeed¹². Within minutes after injury platelets aggregate at the site of the wound and form a fibrin clot to stop the bleeding. Hereafter, the removal of bacteria and cell debris takes place during the inflammatory phase and is mainly facilitated by macrophages. In reaction to angiogenic growth factors (i.e. FGF-2, VEGF, Ang2, and TGF β), the fibrin clot, platelets and macrophages, angiogenesis starts in the proliferation phase. Key for the initiation of wound healing is the expression of $\alpha v \beta 3$, the integrin receptor for fibrin and fibronectin, by the endothelial tip cells of vascular sprouts. This $\alpha v \beta 3$ integrin empower the endothelial cells to enter the fibrin clot^{13, 14}. The rapid formation of new microvasculature stimulates the formation of granulation tissue and provides nutrition and oxygen to the growing tissue. Once tissue regeneration is completed, the newly formed microvasculature will regress. Physiological

angiogenesis, essential in the menstrual cycle and wound healing, is a very well regulated event of microvessel formation and regression in the adult life.

Neovascularization in disease

Angiogenesis

An imbalance of pro- and anti-angiogenic factors contributes to the development of multiple pathologies such as rheumatoid arthritis, diabetic retinopathy and cancer, which are driven by persistent and uncontrolled vessel formation. During the initiation, promotion and progression of a tumor, pro-angiogenic factors predominate the anti-angiogenic factors resulting in uncontrolled vessel growth, also known as the angiogenic switch ¹⁵. Tumor vessels develop predominantly by sprout formation or by intussusceptions from surrounding vasculature, however, circulating progenitor cells mobilized from the bone marrow can also contribute to tumor angiogenesis ¹⁶. Compared to normal vasculature, the structure and function of tumor vessels is abnormal. They have an immature and leaky phenotype as a result of malformed endothelial cells, widened intercellular junctions and the lack of surrounding basement membrane ¹⁷. Additionally, their appearance is highly disorganized, tortuous and with excessive branching which results in chaotic and variable blood flow. All these characteristics lead to increased influx of inflammatory cells in the tumor. Subsequently, hypoxia induces expression of multiple angiogenic factors by tumor cells ¹⁸, but also results in the death of tumor cells. The most important hypoxia regulated transcription factor is HIF-1 α , which regulates expression of VEGF, VEGFR1, Ang-2, inducible nitric oxide synthase (NOS2) and PDGF important for the development of tumor vasculature ¹⁹. By continuous release of these angiogenic factors, either produced by the tumor itself or by the attracted inflammatory cells, the ingrowth of microvessels is persistent and allows explosive growth of tumor mass.

Similar to tumor growth, the angiogenic switch also contributes to atherosclerosis, the stiffening of the arterial wall due to excessive accumulation of lipids, ECM and inflammatory cells in the vessel wall. Necrotic core formation, as a result of defective phagocytic clearance ²⁰, often coincides with hypoxia in the plaque. Expression of HIF-1 α correlates with increased number of plaque microvessels in both human ²¹ and experimental animal

plaques ²². Additionally, cellular density, matrix composition and leukocyte infiltrates also contribute to the development of plaque microvessels ²³(Figure 2). This microvasculature originates from the surrounding vasculature, described as vasa-vasorum, which supplies the outer layer of large vessels with nutrients and oxygen ²⁵. However, the leaky and unstable phenotype of plaque microvessels leads to the continuous supply of lipids and infiltration of inflammatory cells contributing to the progression into advanced, unstable (vulnerable) plaques that have increased risk of rupture and clinical complications ^{3, 26-28}. Preventing plaque progression and destabilization by anti-angiogenic therapy is one of the main focuses in modern medicine.

Figure 2. Cross section of human atherosclerotic plaque (left) with evidence of plaque neovascularization (right). Adapted from Galis ZS *et al.* ²⁴.

To study angiogenesis many *in vitro* and *in vivo* assays were designed in the past decades ^{29, 30}. *In vitro* assays to investigate endothelial proliferation, migration and differentiation are useful tools in which each step in the angiogenic process can be studied separately. To approach the *in vivo* situation even better, *ex vivo* organ cultures (e.g. the aortic ring assay) were optimized for routine use ³¹. A commonly used high throughput assay to examine the pro- or anti-angiogenic effects of new compounds is the tube formation assay. Here, endothelial cells are plated onto a layer of matrix or mixed within a matrix, stimulating migration and differentiation of endothelial cells in tube-like structures mimicking tube formation *in vivo* ³². The role of different angiogenic factors can be evaluated within 24h with the possibility to interfere at each desired time point. Although these assays are frequently used and easy to perform, their physiological relevance is debated. Therefore, simple *in vivo*

neovascularization assays have been developed. Historically, an early *in vivo* assay is the chorio-allantoic membrane (CAM) assay in which a small opening in the shell of chicken eggs is made exposing the membrane to test solutions and visualization^{29, 30}. Being highly vascularized, it is quite challenging to distinguish old from new vasculature in the CAM. New approaches using highly angiogenic implants in avascular areas (e.g. corneal pocket assay, sponge assay) were designed to circumvent the aforementioned problem³³. For a more realistic *in vivo* situation, the method of using implants was further elaborated which resulted in the evolution of Matrigel. Matrigel is an extract of basement membrane proteins from the Engelbreth-Holm-Swarm (EHS) tumor composed of laminin, collagen IV and growth factors^{34, 35}. This compound is liquid at 4°C and solidifies at 24-37°C which allows introduction of angiogenesis-inducing compounds or tumor cells by subcutaneous injection. Preferred injection sites are neck and flanks of the mice, since skinfolds at these sites allow the gel to form a plug. The plug gradually releases its angiogenic factors and attracts host cells that further stimulate vascularization in the Matrigel. Dependent on the potency of the angiogenic compound within the gel, the plugs can be removed after 7-14 days. Assessment of microvessel formation is achieved by examination of histological sections by staining for endothelial markers such as CD31 or von Willebrand factor (vWF). The Matrigel plug is an excellent method to study the formation of new microvessels in a non-hypoxic *in vivo* 3D situation.

Arteriogenesis

Arteriogenesis is the remodeling of capillaries into larger conducting vessels in response to acute vascular occlusion. Chronic arterial diseases such as perivascular artery disease (PAD) and coronary artery disease (CAD) are the result of atherosclerotic plaque formation and rupture accompanied by occluding thrombus formation. The outward remodeling of pre-existing collateral vessels contributes to restoration of blood flow and functions as a natural rescue mechanism to protect ischemic tissues from necrosis and death.

In contrast to angiogenesis, hypoxia or ischemia is not the initiating stimulus for arteriogenesis, but is merely the result of occlusion of the major conducting vessels leading to increased blood flow in the collaterals³⁶. Increased blood flow in the collaterals leads to increased shear stress on the

endothelial lining causing activation of endothelial cells ³⁷. In response, the activated endothelium expresses cell adhesion molecules (e.g. intercellular adhesion molecule-1 (ICAM-1) to recruit monocytes from the circulation and lead them into the vessel wall where they differentiate into macrophages and stimulate collateral remodeling by producing growth factors such as granulocyte macrophage-colony stimulating factor (GM-CSF), VEGF, FGF-2, PDGF and cytokines (e.g. monocyte chemotactic protein-1 (MCP-1) and TGFβ) ³⁸⁻⁴¹.

Another essential difference between angiogenesis and arteriogenesis is the key role of SMC in arteriogenesis. Whereas angiogenic vessel formation results in an immature vasculature consisting of simply endothelial cells which are very fragile and prone to rupture, collateral remodeling results in well-constructed and fully functional vasculature surrounded by SMC and pericytes. The remodeling of collateral arteries is studied by means of the *in vivo* hind limb ischemia model, which is extensively used in different animals. The essence of this model is to create ischemia in the hind leg by surgically occluding one or more conducting arteries (core protocol ⁴²). As a result, the perfusion of the tissue distal from the ligation site is significantly reduced whereas blood flow and shear stress in the collaterals is increased leading to outward remodeling in order to cope with the large amounts of blood and restore perfusion of the ischemic areas. Perfusion recovery is measured non-invasively over time by means of Laser Doppler Imaging (LDI), which gives insight in the remodeling process without requiring sacrifice of animals.

Macrophages in neovascularization

Neovascularization is enhanced by accumulation of inflammatory cells which was concluded from the massive leukocyte infiltrate observed inside tumor tissue ⁴³. Inflammatory cells (e.g. macrophages) directly stimulate tumor growth by the release of factors that stimulate tumor cell survival or indirectly by promoting endothelial sprouting and vessel formation leading in their turn to increased supply of nutrients and oxygen. Tumor associated macrophages (TAM) are recruited as monocytes from the circulation by tumor-derived chemoattractants such as M-CSF, VEGF, MCP-1, and CCL5 ⁴⁴. Activated macrophages are very effective in stimulating angiogenesis in tumors as was demonstrated by Polverini *et al.* ⁴⁵. Macrophages accumulate especially in

hypoxic areas of the tumor and in response to hypoxia secrete potent pro-angiogenic factors (e.g. VEGF, FGF-2, interleukin (IL)-8 and Angiopoietin) as well as modulating enzymes (e.g. MMP-2 and -9) that further release growth factors from the extracellular matrix⁴⁶⁻⁴⁸. Above all, Lin *et al.* demonstrated in a mouse model for breast cancer that macrophages play a key role in regulating the angiogenic switch, an essential step in the progression to tumor malignancy⁴⁹. Consequently, the number of TAMs strongly correlates with poor prognosis⁵⁰⁻⁵⁴.

Macrophages play a key role in the development of atherosclerosis and plaque progression. Macrophages contribute to this morphological change by plaque angiogenesis, necrotic core formation and thinning of the fibrous cap, resulting in a vulnerable plaque phenotype. In mice, it was demonstrated by Moulton *et al.* that microvessel density in the plaque correlates with the amount of inflammatory cells²². Treatment with inhibitors of angiogenesis resulted in reduced plaque angiogenesis and plaque size. Moreover, the number of macrophages was significantly reduced in plaques and around the vasa vasorum, yet, whether this was a cause or a consequence of the reduced plaque microvessels could not be clarified.

Aside from their adverse effects on the progression of disease macrophages also have wound healing and tissue regenerative capacities^{55, 56}. Macrophages contribute extensively to wound healing by interfering in all sequential steps of the healing process. First, macrophages exhibit an inflammatory phenotype secreting tumor necrosis factor (TNF α), nitric oxide (NO) and IL-1 which activate anti-microbial mechanisms. After bacterial phagocytosis and clearing the wound from cell remnants, macrophages switch to an anti-inflammatory, wound repair phenotype. It is thought that these macrophages stimulate granulation tissue formation by increasing the number of microvessels in the wound. Hunt and coworkers were the first to show that macrophages are associated with wound neovascularization demonstrating that wound-derived macrophages stimulated microvessel formation in the corneal pocket assay⁵⁷. Also in wounds, macrophages can change the composition of the ECM either by producing ECM components or by production of proteases that degrade ECM¹².

Not only angiogenesis but also arteriogenesis is positively influenced by macrophages. Heil *et al.* showed that when rabbits were treated with 5-

fluorouracil (5-FU), inducing monocytopenia, followed by femoral artery ligation, their monocyte population was temporarily depleted which resulted in hampered perfusion recovery⁵⁸. Interestingly, this could be restored by injection of isolated monocytes. Bergmann *et al.* showed in their study with *op/op* mice, which have a deficiency in their M-CSF coding gene leading to a defective monocyte and macrophage population, that arteriogenesis was inhibited⁵⁹. After femoral artery ligation, not only development of collateral vessels was significantly reduced in *op/op* mice but also a decline in vessel diameter was observed. These studies point out the importance of macrophages for developing collaterals in order to restore blood flow.

Thus, macrophage accumulation can lead on the one hand to a positive outcome for wound healing and tissue regeneration but on the other hand lead to adverse effects in the progression of a disease⁴. The eventual fate and function of the macrophage in the target tissue is determined by their molecular environment.

Macrophage heterogeneity

Macrophages are essential for tissue homeostasis and host defense, yet, they also contribute to the progression of several diseases amongst others by stimulating neovascularization⁶⁰. Over the years it has become clear that the macrophage population consists of a wide variety of subsets. Heterogeneity is a macrophage hallmark and since their phenotype is closely related to their function, specific subsets can exert a wide variety of functions. Analogous to the subdivision of T-lymphocytes into Th1 and Th2 cells, macrophages are subdivided into classically activated, pro-inflammatory M1 macrophages and alternatively activated, anti-inflammatory M2 macrophages⁶¹. M1 cells function primarily in host defense and are activated by cytokines such as interferon- γ (IFN- γ) and TNF α and/or microbial molecules such as lipopolysaccharide (LPS) of which the latter occurs via Toll-like receptor (TLR) signaling. In response to this activation, M1 macrophages have enhanced anti-microbial capacity and are characterized by increased production of pro-inflammatory cytokines IL-1 β , IL-6, TNF α and NO. Opposed to M1 macrophages, the characterization of M2 macrophages is not so straightforward. Alternatively activated macrophages are generally subdivided into three subsets defined by the polarization stimuli that create or silence

them. M2a macrophages are induced by IL-4 or IL-13, M2b macrophages are polarized by immune complexes in combination with LPS and M2c macrophages are deactivated by IL-10 or glucocorticoid hormones⁶². M2a macrophages are also designated as the “wound healing phenotype” since stimulation with granulocyte-derived IL-4 increases their arginase (Arg-1) activity. Arginase transforms arginine into ornithine, which is a precursor of collagen and thus contributes to the production of extracellular matrix⁶³. M2c macrophages are often described as the regulatory macrophage phenotype, referring to their induction by deactivating IL-10 and their role in dampening the immune response and limiting inflammation.

Macrophage phenotype is characterized by means of established M1 and M2 markers (Table 1). The M1 phenotype can be distinguished by increased expression of IL-12, NOS-2 and TNF α upon IFN γ stimulation, whereas IL-4 induced polarization leads to high expression of the mannose receptor (MRC), Arg-1, chitinase-3 like-3 (Ym-1) and Fizz-1⁶⁴⁻⁶⁶. Furthermore, the balance between the pro-inflammatory cytokine IL-12 and the anti-inflammatory IL-10 is still used as indication of macrophage polarization as is the NOS-2/Arg-1 balance and recently described SOCS-1/3 ratio⁶⁷.

The contribution of macrophages in the progression of different pathologies by stimulating neovascularization is acknowledged (as described above), but the role of each subset on neovascularization remains to be elucidated. In tumor biology, much effort has focused on clarifying the relationship between neovascularization and increased leukocyte infiltrate, leading to tumor growth. Tumor cells produce anti-inflammatory cytokines IL-4, IL-10 and TGF β , which skew infiltrated macrophages towards an anti-inflammatory phenotype creating an immunosuppressive environment in favor of tumor conservation⁶⁹. As a result, TAMs have poor antigen-presenting capacity, decreased ability to lyse tumor cells and they induce regulatory T-cells, thereby suppressing the activity of effector T-cells, promoting tumor growth⁷⁰. TAMs resemble an M2-like phenotype and accumulation of TAMs is associated with increased neovascularization⁴⁶. However, the cause-effect relationship of alternatively activated M2 macrophages on angiogenesis remains to be elucidated.

Table 1. Characteristics of macrophage subsets. Adapted from Murray *et al.* ⁶⁰ and Stoger *et al.* ⁶⁸.

Subset	M1	M2a	M2b	M2c
Inducers	IFN γ + LPS/TNF α	IL-4/IL-13	IC + IL-1 β /LPS	IL-10 (TGF β , GC)
Cytokines	IL-12 high	IL-10 high	IL-10 high	IL-10 high
	IL-23 high	IL-12 low	IL-12 low	IL-12 low
	IL-10 low	IL-23 low	IL-23 low	IL-23 low
	TNF α	IL-1Ra	TNF α	TGF β
	IL-1 β	TGF β	IL-1 β	IL-1Ra
	IL-6		IL-6	
Chemokines	IL-18			
	CCL2, CCL3, CCL4, CCL5	CCL-17, CCL18, CCL22, CCL24	CCL1	CCL18
Surface molecules	MHCII	Mrc1	CD86	Mrc1
	CD86	decoy IL-1RII		CD163
	CD80	SR		SR
	IL-1RI			
Markers	TLR2/4			
	Marco	Chi3l3 (YM1)		
	Socs3	Socs2		
	Nos2	Retnla (Fizz1)		
	NO	Arg-1		

Macrophage derived angiogenic factors

Activated macrophages produce not only a wide range of chemokines and cytokines to regulate inflammatory responses, but also pro- and anti-angiogenic growth factors influencing vascular sprouting and network formation. Macrophage secreted growth factors which are extensively studied and considered to be highly pro-angiogenic are the VEGFs and FGFs, yet PDGF-BB, IGF-1 and PlGF are also part of the macrophage secretome and involved in stimulating neovascularization ⁷¹. VEGFA and FGF-2 are described to stimulate vessel growth by promoting proliferation of endothelial cells directly ⁷² and facilitate migration by stimulating endothelial secretion of proteases to degrade the basal membrane. FGF-2 was the first described angiogenic factor ⁷³, but at present, the FGF family contains up to 23 factors and 4 different tyrosine kinase receptors (FGFR1-4) ⁷⁴. FGF-2 by itself is a potent promoter of angiogenesis but it also functions as a regulator of the VEGF signaling pathway. It was already known that angiogenic mediators can act synergistically ⁷⁵, as do

FGF and VEGF in a sprouting assay *in vitro* ⁷⁶. Recently, Murakami and coworkers showed that FGF-2 stimulates VEGF expression in endothelial cells and that inhibition of FGF signaling results in irresponsiveness to VEGF by downregulated expression of the VEGF receptor 2 (VEGFR2) ⁷⁷.

Furthermore, macrophage derived cytokines (e.g. TNF α , TGF β , MIF) are also described to exert angiogenic properties. Nicosia *et al.* described the angiogenic function of the pro-inflammatory cytokine TNF α in a study. Here, they show in a model of aortic injury that macrophage derived TNF α promotes angiogenesis by regulating VEGF expression ⁷⁸. Transforming growth factor (TGF β) is also involved in the proliferation and differentiation of endothelial cells, but, this effect is strongly dose-dependent. For both TNF α and TGF β it is known that low doses stimulate proliferation of endothelial cells and tube formation *in vitro*, whereas high doses have the opposite effect ⁷⁹. Macrophages contain large amounts of preformed macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine, which is stored in intracellular pools ready for fast secretion ⁸⁰ upon stimulation with LPS. MIF was demonstrated to be a potent pro-angiogenic factor as treatment with recombinant MIF induced tube formation and stimulated angiogenesis in Matrigel plugs ⁸¹.

Additionally, macrophages are rich sources of matrix metalloproteinases, essential for extracellular matrix breakdown. The MMP family consists of 23 members with high expression levels for the majority of the family in macrophage rich regions in human atherosclerotic plaques ⁸². The action of macrophage derived MMP production on angiogenesis is two-fold: by degrading the extracellular matrix space is created for migratory endothelial cells and matrix-bound growth factors are released, promoting proliferation and migration of tip cells and thus promoting angiogenesis.

Next to the production of pro-angiogenic factors, macrophages are capable of excreting a variety of anti-angiogenic factors. Macrophage derived anti-angiogenic factors include thrombospondin-1 (TSP-1) and IFN γ ⁸³. TSP-1 has angiostatic effects and induces apoptosis ⁸⁴ whereas downregulation of endothelial TSP-1 enhances angiogenesis *in vitro* ⁸⁵. The secretome of the macrophage is rather diverse and depends strongly on the environmental stimuli the cells receive. Whether the secretome varies between macrophage phenotypes remains to be investigated.

Inhibition of neovascularization

VEGF is a very potent angiogenic factor, which is highly related to tumor growth and therefore one of the most evident targets in cancer therapy. Many anti-VEGF strategies were designed, but only few of them have made it into the clinic ⁸⁶⁻⁸⁸. Bevacizumab is a human recombinant monoclonal antibody, which targets the VEGF ligand and prevents the interaction of VEGF with its receptors VEGFR-1 and -2 ^{89, 90}. In combination with standard chemotherapy, bevacizumab was shown to reduce tumor formation in colorectal ⁹¹, breast ⁹², non-small cell lung ⁹³ and renal cell carcinoma ⁹⁴ and hence became the first FDA-approved anti-VEGF therapy. The use of a soluble receptor to trap VEGF and PlGF (Aflibercept) and small molecules tyrosine kinase inhibitors (e.g. Sunitinib) to block VEGF signaling are other approaches to dampen the angiogenic response resulting in impaired cancer growth ⁸⁷.

Although proven to be effective in the clinic, resistance to VEGF-therapy after repeated treatments in combination with adverse side effects such as hypertension, bleeding and thromboembolic events demands further development of alternatives. Blocking VEGF signaling showed also promising results for the reduction of atherosclerotic plaque formation in rabbits. Gene therapy with sFlt-1 (the receptor for VEGF-A,-B and PlGF) significantly reduced intra-plaque neovascularization and possibly reduced plaque formation ⁹⁵ suggesting the suitability for sFlt-1 in atherosclerosis therapy. Moulton *et al.* investigated the effect of two potent inhibitors of endothelial cell proliferation on plaque neovascularization and plaque progression in ApoE^{-/-} mice ²³. Prolonged treatment with endostatin or TNP-470 reduced intimal neovascularization significantly and inhibited plaque growth by 85% and 70% respectively. Whether the reduced plaque neovascularization was a cause or a consequence of the inhibited plaque growth is not clarified.

Therapeutic neovascularization

Many strategies have been designed to restore blood perfusion after occlusion of a conducting artery as is the case in CAD and PAD. Thrombolytic therapy, stenting, balloon angioplasty and bypass surgery are interventions used in the clinic, however, not without risk ⁹⁶. Therapeutic angiogenesis or arteriogenesis is an alternative approach to these high risk interventions in order to stimulate

collateral formation and restore reperfusion to overcome ischemia⁹⁷. The idea of using angiogenic proteins (e.g. VEGF-A, FGF-2 and MCP-1) to stimulate neovascularization in ischemic tissues was investigated. Although VEGF-A and FGF-2 are proven to be efficient in animal models for hind limb ischemia^{98, 99}, continuous treatment due to short serum half-life of the proteins would be needed to reach the desired effect. Additionally, prolonged administration resulted in unwanted side effects which have been described for both factors^{100, 101}. MCP-1 treatment increased both collateral and peripheral conductance after femoral artery ligation¹⁰². In the thigh muscle an increase in collateral artery density after MCP-1 administration was observed while in the calf muscle (the ischemic part) the number of capillaries was significantly increased. Additionally, MCP-1 administration resulted in the attraction of monocytes which possibly led to further improvement of collateral remodeling by the production of mitogenic compounds.

As arteriogenesis shares numerous features with atherogenesis (e.g. expression of adhesion molecules and monocyte accumulation) possible side effects of pro-arteriogenic therapy must be considered. Since MCP-1 potentially contributes to plaque progression¹⁰³ the effect on arteriogenesis was tested under hyperlipidemic conditions. In ApoE^{-/-} mice local treatment with MCP-1 improved collateral conductance significantly, however, plaque formation was also increased¹⁰⁴. This pro-atherogenic character confirms earlier reports of pro-atherogenic properties of VEGF and FGF-2¹⁰⁵ and questions the safety of protein treatment.

Cell-based therapy

Despite the success of protein therapy in animals, contradictory results in clinical trials about the role of VEGFA and FGF-2¹⁰⁶ stimulate the pursuit for novel therapies based on different principles. The potential of cell based therapy to enhance cardiac function and repair after stroke or acute myocardial infarction is investigated in several studies. Suzuki *et al.* induced myocardial infarction in rats and transplanted VEGF-expressing skeletal myoblasts into the area of infarction. After 28 days, the size of the infarction area is significantly reduced upon VEGF-expressing myoblast transplantation, which resulted in reduced mortality. Alongside this observation angiogenesis is significantly enhanced by treatment with skeletal myoblast¹⁰⁷. Bone-marrow

injection in the ischemic myocardium of minipigs resulted in improved blood flow and cardiac function and reduced infarction size, accompanied by increased vessel density, after coronary artery ligation¹⁰⁸. Moreover, it is demonstrated that bone-marrow mononuclear cells (BM-MNCs) produce angiogenic growth factors (e.g. FGF-2, VEGF, Ang-1) and cytokines such as TNF α and IL-1 β . The potential of BM-MNCs is also confirmed in a hind limb ischemia model in the rabbit¹⁰⁹ and in mice¹¹⁰. Iba *et al.* investigated the possibility of stimulating arteriogenesis by implanting peripheral blood mononuclear cells (PBMNCs), polymorphonuclear leukocytes (PMNs) and platelets as they are known to be a source of several angiogenic growth factors^{53, 111}. From human blood all three cell types were isolated and injected in the thigh muscle of rats after the induction of hind limb ischemia. Perfusion recovery was significantly increased after treatment with PBMNCs and platelets, which could be ascribed to the enhanced formation of collateral vessels most probably promoted by excreted VEGF¹¹². These studies show that neovascularization can be stimulated by local delivery of different cell types from the myeloid lineage providing novel insights for therapeutic angiogenesis in modern medicine.

Hypothesis and outline of this thesis

Macrophages are associated with neovascularization, however, differences between subsets regarding their angiogenic potential have not been clearly investigated. We hypothesize that the alternatively activated M2 macrophages are both pro-angiogenic and pro-arteriogenic and stimulate neovascularization in health and disease. To investigate the role of different macrophage subsets in neovascularization we performed several studies in experimental models of neovascularization. The focus of **chapter 2** is on the effect of macrophage subsets on angiogenesis using an *in vitro* tube formation assay and an *in vivo* Matrigel plug assay. In addition, we investigated the possible underlying mechanism as an explanation for macrophage function and phenotype. Next, **chapter 3** will address the role of macrophage subsets in a hind limb ischemia model of arteriogenesis in mice. In this model, we aimed to gain insight in the effect of local treatment with exogenously polarized macrophages on collateral remodeling. Additionally, we investigated the contribution of endogenously polarized macrophages in IL10R^{fl/fl}/LysMCre mice. In **chapter 4** we discuss the role of MIF as therapeutic agent for stimulating arteriogenesis and its possible function in macrophage polarization. In **chapter 5** the role of macrophage subsets in wound healing is investigated in both (non-) diabetic mice. Finally, the whole content of this thesis is discussed in **chapter 6** putting the outcome in perspective.

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Chapter 2

Anti-inflammatory M2, but not pro-inflammatory M1 macrophages promote angiogenesis in vivo

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Abstract

Objective: Macrophages show extreme heterogeneity and different subsets have been characterized by their activation route and their function. For instance, macrophage subsets are distinct by acting differently under pathophysiological conditions such as inflammation and cancer. Macrophages also contribute to angiogenesis, but the role of various specific subsets in angiogenesis has not been thoroughly investigated.

Methods and Results: Matrigel supplemented with macrophage subsets (induced by IFN γ (M1), IL-4 (M2a) or IL-10 (M2c)) was injected subcutaneously in C57BL/6J mice and analyzed by CD31 staining after 14 days. Increased numbers of endothelial cells and tubular structures were observed in M2-enriched plugs compared to control and other subsets. Additionally, more tubular structures formed *in vitro* in the presence of M2 macrophages or their conditioned medium. To identify a mechanism for the pro-angiogenic effect, gene expression of angiogenic growth factors was analyzed. Induced expression of basic fibroblast growth factor (*Fgf2*), insulin-like growth factor-1 (*Igf1*), chemokine (C-C motif) ligand 2 (*Ccl2*) and placental growth factor (*Pgf*) was observed in M2 macrophages. Using a blocking antibody of PlGF to inhibit M2c induced angiogenesis resulted in mildly reduced (40%) tube formation whereas neutralization of FGF-2 (M2a) signaling by sFGFR1-IIIc affected tube formation by nearly 75%.

Conclusions: These results indicate that macrophages polarized towards an M2 phenotype have a higher angiogenic potential compared to other subsets. Furthermore, we propose FGF signaling for M2a- and PlGF signaling for M2c-induced angiogenesis as possible working mechanisms, yet, further research should elucidate the exact mechanism for M2-induced angiogenesis.

Introduction

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, is essential in physiological processes such as embryonic development and wound healing. However, in pathophysiological conditions involving inflammation, such as tumor growth and atherosclerosis, the growth of new blood vessels is often associated with a negative clinical outcome since these newly formed blood vessels are leaky, unstable and prone to hemorrhage. Tumor tissue is dependent on the supply of nutrients and oxygen-rich blood by this immature vasculature in order to grow excessively. In atherosclerosis, micro-vessels originating from the surrounding vasculature can invade the lesion serving as an entry point for infiltrating leukocytes thus contributing to expansion, destabilization and eventually rupture of the plaque ¹⁻⁴.

Macrophages are key players in inflammatory diseases. Besides their protective immunological function they also promote angiogenesis by producing pro-angiogenic cytokines and growth factors such as vascular endothelial growth factor (VEGF-A) and basic fibroblast growth factor (FGF-2). Both growth factors are known for their dose-dependent angiogenic effect and have shown to act synergistically *in vitro* ⁵. Being a pivotal regulator of angiogenesis, VEGF-A is constitutively expressed by macrophages yet strongly increased upon macrophage activation ⁶. Macrophages also express FGF-2, which functions as a very potent pro-angiogenic mitogen and growth factor ⁷. Their expression of pro-angiogenic growth factors enables macrophages to interfere with each phase of the angiogenic cascade, including proliferation and migration of endothelial cells and formation of vascular sprouts ⁸⁻¹⁰.

Macrophages have a remarkable plasticity in order to effectively respond to environmental changes. They can switch phenotype and exert different functions depending on surrounding signals ¹¹⁻¹³. Based on the activation route, macrophages can generally be divided into two major phenotypes: M1 or classically activated macrophages that are induced by microbial agents e.g. LPS and/or IFN γ , characterized as pro-inflammatory and lead to a type 1 response and IL-4, IL-13 or IL-10-induced M2 macrophages, which are characterized as anti-inflammatory and elicit a type 2 response. Being a more heterogeneous population, M2 macrophages can be further subdivided based on their functionality: wound healing macrophages activated by IL-4 belong to the M2a subset also known as alternatively activated

macrophages, whereas regulatory macrophages activated by many stimuli such as immune complexes, glucocorticoids, prostaglandins and IL-10 are also called M2c macrophages¹⁴⁻¹⁷. Furthermore, characteristics of subsets may overlap depending on cell-fate in different diseases¹⁷.

Pro-angiogenic properties are not shared between all subsets as it was shown *in vitro* that angiogenic growth factors and cytokines are higher expressed by M2- than by M1-macrophages¹⁸. Several studies demonstrated the association of pro-angiogenic macrophages (TAMs) resembling an M2 phenotype, with tumor progression and neovascularization¹⁹⁻²². Moreover, Tie-2 expressing monocytes (TEMs) are considered to have an even stronger pro-angiogenic capacity compared to TAMs. Depletion of TEMs in mice reduced tumor growth extensively suggesting TEMs to be a therapeutic target for drug development²³. However, the main focus of these studies has been on the function of macrophages in a tumor environment whereas the focus of the current study is to elucidate the effect of macrophage subsets induced by T-cell derived cytokines.

We demonstrate for the first time that IL-4 induced M2a and IL-10 M2c macrophages promote angiogenesis *in vivo* as well as *in vitro*. Additionally, we observed significant differences in gene expression levels of pro-angiogenic growth factors between the subsets. Conclusively, we demonstrate that M2c-induced angiogenesis depends on PlGF signaling and that M2a-induced angiogenesis might be regulated by FGF signaling. This study gives us more information about the angiogenic profile of macrophage subsets and their functions in physiology and disease.

Methods

Bone marrow-derived macrophages

Bone marrow was isolated from the femur and tibia of C57BL/6 mice 10-14 weeks of age as described previously²⁴. Cells were differentiated into bone marrow derived macrophages by culture in R10 medium (RPMI-1640 medium (Gibco Invitrogen, Breda, The Netherlands), 10% heat-inactivated Fetal Calf Serum (FCS) (Bodinko BV, Alkmaar, The Netherlands), L-glutamine 2mM, 100 U/ml penicillin, 100ug/ml streptomycin and 10mM HEPES (all Gibco Invitrogen, Breda, The Netherlands)) supplemented with 15% L929-conditioned medium (LCM) for 8 days.

Macrophage polarization

Differentiated macrophages were harvested, counted and suspended in R10 + 15% LCM. Cells (3.5×10^5 cells/500 μ l) were seeded in 24-wells plates for overnight adhesion. Macrophages were then polarized for 24h with interferon- γ (IFN γ) (100U/ml, Hycult biotech) for M1, interleukin (IL)-4 (20ng/ml, Peprotech) for M2a or IL-10 (10ng/ml, R&D Systems) for M2c macrophages. Non-stimulated or -activated cells are referred to as M0 macrophages. Polarization was confirmed by qPCR analysis of gene expression of established polarization markers (Supplemental Figure 1A-C).

Endothelial cells

For all *in vitro* experiments Simian virus 40 immunized murine endothelial cells (SVEC) were used which were kindly provided by Prof. J. Bernhagen (UK Aachen, Germany). Cells were cultured in DMEM/F-12 supplemented with 10% FCS, 100 U/ml penicillin and 100ug/ml streptomycin.

Matrigel plug assay

Female C57BL/6 mice (Charles River, France) (n=48), 10-12 weeks old, were held under the guidelines of the animal care facility (Maastricht University) and were fed and supplied with drinking water *ad libitum*. Prior to injection, macrophage subsets (5×10^5 cells/500 μ l) were gently mixed in Matrigel supplemented with basic fibroblast growth factor (FGF-2) (150ng/ml, Peprotech) and kept on ice. Mice were sedated with isoflurane (2-2.5 %) and s.c. injected with 500 μ l Matrigel (BD Biosciences) at the lower left quadrant of the abdomen. After 14 days, mice were euthanized and plugs were removed and prepared for immunohistochemistry. All animal experiments were approved by the Committee for Animal Welfare of the Maastricht University.

Immunohistochemistry

Tissues were prepared for immunohistochemical analysis and stained for endothelial marker CD31 (PECAM-1) (BD biosciences) and macrophage marker MOMA-2 (a gift from G. Kraal). Matrigel plugs were embedded in O.C.T. Tissue tek (Sakura Finetek), snap frozen in pre-cooled isopentane and stored at -80°C until use. Frozen sections (10 μ m) were fixed in dry acetone, air dried and rehydrated in PBS. Sections were pre-incubated with a biotin blocking system

(VectorLabs) to prevent non-specific binding. After incubation with the primary and secondary antibody, staining was amplified by incubating sections with ABC reagent (VectorLabs). Sections were developed using an AEC substrate kit (VectorLabs) for 15 minutes followed by haematoxylin counterstaining. Ten randomly distributed pictures at 200x magnification were made of each plug. The total area of endothelial cells and macrophages per high power field (HPF) was measured using Photoshop CS2.

Tube formation assay

96-wells plates were filled with 50µl Matrigel (BD Biosciences) and allowed to solidify at 37°C after which SVECs (2×10^4 cells/100µl) were gently seeded on top of the gel. Subsequently, macrophage subsets (4×10^4 cells/100µl) were co-incubated with endothelial cells. After 24h, network structures were analyzed at 4x magnification (Carl Zeiss Axio Vision, Germany) and photographed with a digital camera (Nikon). The number of tubes per picture was counted using ImageJ.

Conditioned medium

After polarization, macrophages were washed thoroughly and provided with new R10 + 15% LCM and incubated at 37°C. This conditioned medium of polarized macrophages was collected after 24h and used in the tube formation assay as described before. The control group was incubated with fresh R10 + 15% LCM.

PKH Labeling

In order to distinguish between endothelial cells and the different macrophage subsets in the tube formation assay, cells were fluorescently labeled prior to co-culture. Endothelial cells were labeled with PKH67 (green) and macrophages were labeled with PKH26 (red) according to the manufacturer's protocol (Sigma Aldrich).

Transfection of SVECs with sFGFR1-IIIc plasmid

To inhibit FGF-2 signaling *in vitro*, we transfected SVECs with a plasmid expressing a splicing isoform of the soluble fibroblast growth factor receptor-1 (sFGFR1-IIIc), which was kindly provided by Dr. M. Murakami (Yale University, New Haven, USA). Two days before transfection, SVECs ($10.000 \text{ cells/cm}^2$)

were plated in 6-wells plates in complete culture medium. Transfection was performed using 5µl Lipofectamine-LTX (Invitrogen), 4µl PLUS reagentia (Invitrogen) and 2000ng plasmid sFGFR1-IIIc or the empty vector pCEP4 (Invitrogen) diluted in Opti-MEM (Invitrogen). Transfection efficiency was in all experiments > 80%. After 24h of incubation, we performed tube formation assays with the transfected SVECs alone, and in co-culture with M0 (control) or M2a macrophages.

Inhibition of PlGF signaling via sFlt-1 and PlGF blocking antibody

To inhibit PlGF signaling *in vitro*, we used 25ng/ml recombinant soluble Flt-1 (Reliatech). Tube formation assays were performed with SVECs alone, and in co-culture with M0 (control) or M2c macrophages. Because Flt-1 is also involved in VEGF signaling we used a blocking antibody of mouse PlGF (20µg/ml anti-mouse PlGF, Reliatech) to specifically inhibit PlGF-induced tube formation. Combined inhibition of the FGF and PlGF pathway was studied by using anti-PlGF on cells transfected with sFGFR1-IIIc in the presence of M2a macrophages. Tube formation assays were performed in co-culture with M0 (control), M2a or M2c macrophages.

RNA isolation and qPCR analysis of gene expression

RNA was isolated using a High Pure RNA Isolation Kit (Roche, Basel, Switzerland). 500ng total RNA was subjected to reverse transcription using iScript cDNA Synthesis Kit (BioRad, Veenendaal, The Netherlands). Quantitative Real-time Polymerase Chain Reaction (qPCR) was performed using Sensimix SYBR & Fluorescein kit (Bioline) and a primer concentration of 300nM. Primers for angiogenic growth factors and cytokines were designed using Primer Express (Table 1).

Table 1. Primer sequences of M1/M2 markers and angiogenic factors.

Gene	Forward	Reverse
<i>Arg1</i>	CATGGGCAACCTGTGTCCTT	CGATGTCTTTGGCAGATATGCA
<i>Ccl2</i>	GCTGGAGAGCTACAAGAGGATCA	TCTCTCTTGAGCTTGGTGACAAAA
<i>Chi3l3</i>	TGGCCCACCAGGAAAGTACA	CAGTGGCTCCTTCATTCAGAAA
<i>Fgf2</i>	CAAGGGAGTGTGTGCCAACC	AGATTCCAGTCGTTCAAAGAAGAAA
<i>Igf1</i>	CTGGACCAGAGACCCTTTGC	GGTGCCCTCCGAATGCT
<i>Il10</i>	TTTGAATTCCTGGGTGAGAA	CTCCACTGCCTTGCTCTTATTTTC
<i>Mif</i>	CCAGAACCGCAACTACAGTAAGCT	GGCAGCGTTCATGTCGTAATAG
<i>Mrc1</i>	TGCCAAAAATTATTGATCCTGTAAC	CGCCGGCACCTATCACA
<i>Nos2</i>	TTGCAAGCTGATGGTCAAGATC	CAACCCGAGCTCCTGGAA
<i>Pgf</i>	GTCTGCTGGGAACAACCAACA	CACCTCATCAGGGTATTCATCCA
<i>Thbs1</i>	AAGCGGCCTCCCTCTG	GGGCATGATGGGACAGGAC
<i>Tnf</i>	CATCTTCTCAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
<i>Vegfa</i>	GCTTTACTGCTGTACCTCCACCA	GGGACTTCTGCTCTCCTTCTGTC

FGF-2 and PlGF ELISA

We performed ELISA to determine the protein levels of FGF-2 (Abcam) and PlGF (USCNK Life science) in conditioned medium of macrophage subsets. Macrophages were polarized for 24h and 48h as described above in serum free medium after which medium was collected and 10x concentrated by using Vivaspın 2 columns (Sartorius). At both time points, cells were lysed in Ripa buffer and analysed with ELISA.

Statistical analysis

Data are represented as mean $\pm$ SEM. Matrigel plug results were compared by Student's t-test and tube formation and gene expression data were analyzed using one-way ANOVA with Bonferroni's post hoc test for multiple comparison. $P \leq 0.05$ was considered to be statistically significant.

Results

M2 macrophages promote angiogenesis *in vivo*

We studied the role of different macrophage subsets in angiogenesis using an *in vivo* Matrigel plug assay. To confirm the macrophage phenotype after polarization, gene expression was analyzed using established M1 and M2 markers. Macrophages polarized with IFN γ towards an M1 phenotype showed increased expression of Tumor Necrosis Factor (*Tnf*), inducible Nitric Oxide Synthase (*Nos2*) (Supplemental Figure 1A) and *Il10* (Supplemental Figure 1C). When polarized with IL-4, expression of the distinctive M2a markers Arginase-1 (*Arg1*), chitinase 3-like 3 (*Chi3l3*) (Supplemental Figure 1B) and *Mrc1* (Supplemental Figure 1C) was increased. Polarization with IL-10 (M2c subset) resulted in increased expression of *Il10* and *Mrc1* (Supplemental Figure 1C).

To study the role of macrophage subsets in angiogenesis *in vivo*, Matrigel enriched with FGF-2 was mixed with M0, M1, M2a and M2c macrophages and s.c. injected in mice. After 14 days angiogenesis was analyzed by quantification of CD31 staining. As expected, we observed enhanced accumulation of endothelial cells in FGF-2-enriched plugs (-M) compared to the control situation ($p=0.02$). However, there was no additional effect on endothelial influx observed in the presence of M0 or M1 macrophages. Interestingly, in Matrigel plugs supplemented with M2a and M2c macrophages, more endothelial cells and tube-like structures were observed (M2a, 69.5% and M2c, 107%) compared to plugs supplemented with M0 (Figure 1A, B. $p<0.05$).

Figure 1. Effect of macrophage subsets on angiogenesis *in vivo*. Pictures of CD31 (A) and MOMA-2 (C) staining in Matrigel plugs after 14 days (n=8, scale bar = 200μm). B, M2 macrophages increase the number of endothelial cells per HPF ($p<0.05$). D, macrophage subsets do not affect macrophage chemotaxis into Matrigel plug. The data represent the mean $\pm$ SEM. The statistical significance was determined by student's t-test * $p<0.05$.

To exclude the possibility that differences in endothelial cell numbers and number of tube-like structures was the result of different recruitment of endogenous macrophages or even different numbers of injected macrophages, we performed MOMA-2 staining to quantify the number of macrophages (Figure 1C, D). As previously described²⁵, FGF-2 facilitated recruitment of macrophages into the plug ($p=0.01$). Polarized macrophages did not affect macrophage accumulation in the plug. Interestingly, the spatial accumulation of macrophages in the plug strongly correlated with the localization of endothelial cells, indicating close contact of these cells (Supplemental Figure 2).

M2 macrophages promote tube formation *in vitro*

To study the role of macrophage subsets in angiogenesis in more detail we performed *in vitro* tube formation assays using a co-culture of endothelial cells and macrophage subsets on a Matrigel base. Culturing endothelial cells in these settings led to the formation of tubular structures after 24h. Adding M0 macrophages to endothelial cells did not enhance the number of tubes compared to the control situation. Remarkably, tube formation was inhibited by the presence of M1 macrophages in the assay (56%, $p < 0.05$). When endothelial cells were co-cultured with M2a and M2c macrophages we observed a significant increase in the number of tubes (respectively 155% and 91.7%, both $p < 0.01$) (Figure 2A, B).

Figure 2. Effect of macrophage subsets on *in vitro* tube formation. **A**, Representative pictures of tube formation assay of co-cultures of SVEC and macrophage subsets. **B**, M2a and M2c macrophages stimulate tube formation, whereas M1 macrophages show an inhibition ($n=5$). **C**, Conditioned medium of macrophages promotes tube formation compared to control. **D**, Co-localization of fluorescently labeled SVECs (green) and macrophages (red). Arrows indicate macrophages merging into the tubular structures. Three independent experiments were performed ($n=5$) and the data represent the mean $\pm$ SEM. The statistical significance was determined by one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, # $p < 0.01$.

To establish if this pro-angiogenic effect of M2 macrophages was caused by secreted growth factors, we performed a tube formation assay with conditioned media of different macrophage subsets. We could confirm the angiogenic potential of M2 macrophages using conditioned medium of M2a and M2c macrophages ($p < 0.01$) (Figure 2C). Interestingly, in contrast to the co-culture of M0 and M1 macrophages with endothelial cells, conditioned medium of M0 and M1 macrophages also stimulated tube formation, suggesting that cell-cell contact of these macrophage subsets with endothelial cells inhibits tube formation (Figure 2B).

Upon close examination of the networks, we observed a strong association in location between endothelial cells and macrophages similar to the *in vivo* observations. Prior to the assay, both endothelial cells and macrophages were fluorescently labeled in order to discriminate between the two cell types. Interestingly, macrophages co-localized with endothelial cells at the branching points and also incidentally merged into the tubes becoming part of the tubular network (Figure 2D, arrows).

Differential growth factor expression by macrophage subsets

To investigate the functional differences between macrophage subsets, gene expression of angiogenic growth factors and cytokines was analyzed by means of qPCR. As Figure 3A shows, the expression of basic fibroblast growth factor (*Fgf2*), insulin-like growth factor-1 (*Igf1*), placental growth factor (*Pgf*) and monocyte chemotactic protein-1 (*Ccl2*) differed significantly between macrophage subsets after 24h polarization. Increased expression of *Fgf2*, *Igf1*, and *Ccl2* was a distinctive M2a cell feature, whereas *Pgf* expression was upregulated in both M2a and M2c macrophages ($p < 0.01$). Moreover, M2a macrophages expressed much less thrombospondin-1 (*Thbs1*), which is the most abundant natural angiogenesis inhibitor. On the other hand, M1 macrophages showed an increased expression of vascular endothelial growth factor (*Vegf*), *Thbs1* and macrophage migration inhibitory factor (*Mif*) (Figure 3B).

Figure 3. Pro-angiogenic gene expression by macrophage subsets. **A**, Expression of *Fgf2*, *Ccl2* and *Igf1* is increased in M2a macrophages and *Pgf* expression is increased in both M2a and M2c macrophages (n=3). **B**, M1 macrophages express pro- as well as anti-angiogenic factors. The data represent the mean $\pm$ SEM. The statistical significance was determined by one-way ANOVA. *p<0.05, **p<0.01.

Neutralizing FGF-2 signaling impairs M2a induced angiogenesis

Since *Fgf2* was upregulated up to ~1000 fold in M2a macrophages, we investigated the role of this growth factor in M2a induced angiogenesis. In order to neutralize FGF activity, we used transfected SVECs with a plasmid for sFGFR1-IIIc. This splicing isoform is a soluble receptor which binds FGF1, -2, -4, -5, -6 and -8²⁶ and functions as a highly specific FGF trap. Transfection of SVECs with the empty vector (pCEP4) had no effect on tube formation. However, transfection with the sFGFR1-IIIc plasmid (-M) resulted in a repression of the tube forming capacity by endothelial cells with 55.2% (Figure 4).

When co-cultured with M0 macrophages, the number of tubes was reduced by 37%, suggesting that basal tube formation is also FGF dependent. Importantly, the inhibition of FGF signaling was even more apparent in co-cultures with M2a macrophages (74.7%). We show here that FGF signaling is involved in M2a induced angiogenesis, however, this is most likely not mediated via FGF-2 specifically.

Figure 4. Inhibition of FGF signaling by use of sFGFR1-IIIc plasmid. Transfection of endothelial cells with the FGFR1-IIIc reduced tube formation by 55.2% compared to the empty vector control. Tube formation was repressed by 37% and even 74.7% when co-cultured with M0 macrophages and M2a macrophages respectively. Data are representative of three independent experiments (n=5). The data represent the mean $\pm$ SEM. The statistical significance was determined by one-way ANOVA. * $p < 0.05$, ** $p < 0.01$.

In vitro, macrophages did not produce detectable levels of FGF-2 (data not shown), although this was not measured in co-culture with endothelial cells and/or matrigel. This suggests that M2a-induced angiogenesis is mediated by other FGF isoforms or a combination of them, not solely by FGF-2.

Blocking PlGF signaling reduces M2c driven angiogenesis

To determine whether the upregulation of PlGF in M2c macrophages is responsible for the induction of tube formation by this phenotype, we used soluble Flt-1 (VEGFR-1) to inhibit PlGF signaling. Soluble Flt-1 reduced tube formation of endothelial cells alone (-M, 34.7% $\pm$ 24.7, $p < 0.001$) as well as endothelial cells co-cultured with either M0 macrophages (27.4% $\pm$ 33.6, $p < 0.05$) or M2c macrophages (44.5% $\pm$ 29.5, $p < 0.01$) (Supplemental Figure 3), yet, the effect between macrophage subsets was not significantly different ($p = 0.34$). Protein levels of PlGF were elevated in the M2c subset compared to M0 macrophages, suggesting that M2c macrophages can promote angiogenesis via PlGF signaling (Supplemental Figure 4).

Since PlGF expression was also upregulated in M2a macrophages we aimed to investigate the contribution of PlGF in M2a-induced tube formation. Therefore, we used sFGFR1-IIIc transfected SVECs in combination with a

blocking antibody for PlGF in co-culture with macrophage subsets. In the presence of sFGFR1-IIIc, the PlGF blocking antibody more specifically inhibited M2c-induced tube formation. Anti-mouse PlGF reduced tube formation by M2c macrophages significantly ($40\% \pm 12.8$, $p < 0.05$), whereas tube formation in M0 and M2a co-cultures remained unaffected (Figure 5).

Figure 5. Inhibition of PlGF signaling in sFGFR1-IIIc transfected SVECs in co-culture with macrophage subsets. M2c-induced tube formation was reduced by 40% upon treatment with 20 μ g/ml anti-PlGF. Tube formation in co-culture with M2a macrophages was not affected by anti-PlGF treatment. Data represent the mean $\pm$ SEM. The statistical significance was determined by one-way ANOVA. * $p < 0.05$.

From this we can conclude PlGF signaling is a driver of M2c-induced tube formation, yet, does not contribute significantly to M2a-induced angiogenesis.

Discussion

In this study we show that M2 macrophages promote angiogenesis *in vivo* as well as *in vitro*. Macrophage accumulation *in vivo* was similar between different macrophage subsets, ruling out an effect of macrophage cell number. Therefore, functional differences between macrophage subsets must be decisive for the pro-angiogenic effect.

In vitro tube formation was induced by conditioned medium of M2 macrophages, which confirmed the pro-angiogenic phenotype of this subset. Despite the angiogenic potential of conditioned medium of M0 and M1 macrophages, tube formation was inhibited in the co-culture setting. This

suggests that cell-cell contact of endothelial cells and these macrophages inhibits tube formation and counteracts the production of pro-angiogenic factors by macrophages. The importance of endothelial-macrophage contacts has also been demonstrated by He *et al.* who showed the requirement of membrane-bound Csf1 in endothelial cells to stimulate macrophage differentiation and colony formation ²⁷. Vice versa, cell-cell contact was required for the induction of ICAM-1 and VE-cadherin expression in endothelial cells by macrophages. Future research should aim to elucidate which membrane bound factors are responsible for the anti-angiogenic effect of M0 and M1 macrophage subsets.

This study provides evidence for a role of FGF and PlGF signaling in M2a- and M2c-induced angiogenesis respectively. Kodelja *et al.* observed a difference in gene expression levels between classically and alternatively activated macrophages. They cultured human monocytes with IFN γ or IL-4/dexamethasone and showed increased expression levels of IGF-1 and TGF- α in alternatively activated macrophages after 6 days ¹⁸. We showed that M2a macrophages significantly upregulated *Fgf2*, *Igf1*, *Ccl2* and *Pgf* while M2c macrophages only upregulated *Pgf*. To demonstrate the significant contribution of these growth factors on angiogenesis we blocked the signaling pathways of FGF (M2a) and PlGF (M2c) *in vitro*. Because of the massive induction of *Fgf2* in M2a macrophages we focused on FGF signaling using transfected endothelial cells in a tube formation assay expressing a soluble FGF-trap (sFGFR1-IIIc). A highly significant reduction in tube formation was detected when FGF was trapped, showing that FGF signaling indeed contributes to M2-induced angiogenesis. However, the approach we used is not specific for FGF-2 signaling, as many other FGF isoforms (FGF-1, -4, -5, -6 and -8) bind to the FGFR1-IIIc ²⁸, which could be of equal importance in M2a-induced angiogenesis. Further studies should point out which specific isoform or what combination of FGFs regulates M2a-induced angiogenesis. Blocking PlGF signaling with soluble Flt-1, the main receptor for PlGF, did not reduce M2c-induced tube formation whereas specific blocking of PlGF with the antibody showed a significant inhibition (40%) of tube formation compared to other macrophage subsets. However, M2a-induced tube formation was not inhibited by treatment with the PlGF blocking antibody. This indicates that PlGF is mainly involved in M2c-induced angiogenesis and that PlGF signaling does not contribute to M2a-induced angiogenesis.

FGFs function through interaction with endothelial surface receptors (e.g. tyrosine kinases, heparan sulphate proteoglycans and integrins) and can influence angiogenesis from the very early invasion phase of matrix degradation, endothelial cell proliferation and migration, to the late phase of vessel maturation by matrix deposition and pericyte recruitment²⁹⁻³¹. Aside from promoting angiogenesis by secreting growth factors, macrophages are key players in facilitating FGF signaling by producing heparinases and plasmin that degrade the extracellular matrix in order for FGF to reach its receptors on endothelial cells¹⁰. Interestingly, macrophages can degrade the extracellular matrix creating tunnels as a guiding path for proliferating and migrating endothelial cells, indirectly promoting sprout formation³². Furthermore, it has recently been described that macrophages can function in a most important stage of vessel formation, which is the fusion of two sprouts. They can act as chaperones and facilitate anastomosis by forming bridges between two tip cells resulting in a complex vasculature³³. Indeed, we showed that macrophages not only co-localize with endothelial cells but also fuse into the tubular network. Future research should point out if specific macrophage subsets show differences in fusion capacity of endothelial cells. We demonstrated here the involvement of PlGF and FGF signaling in M2-induced angiogenesis. However, since several candidate-genes for the pro-angiogenic capacity of M2 macrophages emerged from the expression profile it is likely that a combination of these factors is responsible for their strong angiogenic character. *In vivo* we showed that M2 macrophages in combination with a supplement of exogenous FGF-2 resulted in increased angiogenesis in the Matrigel plug model. However, without the addition of exogenous FGF-2 the induced angiogenesis by the M2 subset was no longer detected (data not shown), suggesting that macrophages are not sufficient to initiate angiogenesis in this model. Although Matrigel by itself already contains a mixture of different growth factors there is high dependency of exogenous FGF-2, which hampers mechanistic studies on the role of endogenous FGF-2 in this model.

Macrophage polarization has been associated with angiogenesis in tumor growth. Tumor associated macrophages (TAMs) resemble the phenotype of an M2 macrophage, as evidenced by a poor production of nitric oxide (NO) in response to LPS and reduced expression of inflammatory cytokines such as IL-12, TNF and IL1 β . In addition they highly express the immunosuppressive cytokines IL-10 and TGF β ¹⁶. Tumor cells attract

monocytes from the circulation into the tumor-tissue, by secreting CCL2 and CSF1. Inside the tumor, the monocytes differentiate into TAMs^{22, 25, 34}. Rolny *et al.* showed that host-produced histidine-rich glycoprotein (HRG) skews TAMs away from their M2-like phenotype towards a tumor-inhibiting M1 phenotype³⁵. This depended largely on the down-regulation of PlGF by HRG and resulted in normalization of tumor vessels, suggesting that the phenotypic switch of TAMs is decisive for the tumor development. TAMs especially accumulate in poorly vascularized, hypoxic areas and enhance tumor progression directly by promoting proliferation of tumor cells. They indirectly facilitate tumor development by producing proteases such as metalloproteinase-9 (MMP-9) to degrade the extracellular matrix and release growth factors (e.g. VEGF-A, FGF-2 and PDGF) for proliferation of endothelial cells and microvessel formation^{34, 36}. Furthermore, TAMs function in recruiting massive amounts of leukocytes from the circulation and they facilitate the formation of metastases²⁵. Thus, high numbers of TAMs in tumors are associated with increased microvessel density and poor prognosis in patients³⁷. Also, a study of Lin *et al.* showed a close association between macrophage infiltration, an angiogenic switch and malignancy of the tumor¹⁹. Another pro-angiogenic cell-population from the myeloid lineage is the Tie2-expressing monocyte (TEMs), which is recruited by the tumor tissue in response to angiopoietin-2 (Ang-2)³⁸. These cells accumulate also in hypoxic areas, but are mainly located around the vasculature of the tumor mass in response to Ang-2 secreted by the tumor cells³⁹. De Palma *et al.* showed that depletion of TEMs reduced the size of tumors and their vascularization significantly, whereas co-injection of TEMs with tumor cells increased the vascular area of the tumor 4-fold. Furthermore, they observed that TEMs in brain tumors widely expressed FGF-2, suggesting involvement of FGF-2 in TEM stimulated angiogenesis⁴⁰. Overall, the pro-angiogenic character of TAMs and TEMs has been established only for the tumor environment, which by itself is already highly angiogenic and hypoxic. Our current study provides evidence for an *in vivo* pro-angiogenic capacity of M2 macrophages in a non-tumor environment.

The paradox of macrophage heterogeneity in disease remains. Depending on the local environment, macrophages will obtain a phenotype suited for executing their specialized task. In an inflammatory environment such as an atherosclerotic lesion, accumulation of M1 macrophages is related to plaque growth. In contrast, lesion progression is inhibited by macrophages

of the M2 phenotype that expresses anti-inflammatory factors^{41, 42}. However, it is the M2 phenotype that is pro-angiogenic, which could result in destabilization of the plaque by the formation of neovessels. In tumor development, infiltrated macrophages act as *M2-like* immune suppressor cells and stimulate tumor growth through their pro-angiogenic character^{20, 34}. Macrophage phenotypes should be considered part of a spectrum of different macrophage populations executing specialized functions¹⁷. For the development of new therapies in cardiovascular disease or cancer development, this plasticity of macrophages should be taken into account, certainly since macrophages undergo a phenotypic switch from pro- into anti-inflammatory and vice versa depending on the environmental trigger^{11, 13, 43}.

Our current study provides more background on the possible mechanisms involved in the pro-angiogenic capacity of M2 macrophages. However, the exact mechanism behind macrophage-stimulated angiogenesis and cell-cell interactions of endothelial cells and macrophage subsets is yet to be elucidated. Classification of the macrophage into distinct subsets oversimplifies the complex functional activity of these cells *in vivo*. Future research is needed to identify what therapeutic applications can be designed to regulate macrophage phenotype switches *in vivo*, depending on the type of disease.

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Conflict of interest

The authors declare that they have no conflict of interest.

Supplemental Figures

Supplemental Figure 1. Gene expression profile of macrophage subsets after 24h polarization. After 8 days of culturing, macrophages are polarized with IFN γ , IL-4 or IL-10 (n=3). **A**, IFN γ stimulation results in increased expression of *Nos2*, *Tnfa* and *Il10* (**C**). **B**, IL-4 stimulation results in upregulation of *Chi3l3*, *Arg1* and *Mrc1* (**C**) whereas IL-10 stimulates expression of *Il10* and *Mrc1*(**C**). The statistical significance was determined by one-way ANOVA. **p<0.01.

A **B**

Supplemental Figure 2. Fluorescently labeled macrophages (red) and endothelial cells (green) in a Matrigel plug. **A**, Co-localization of macrophages and endothelial cells is apparent *in vivo*. **B**, Microvessels are surrounded by macrophages.

Supplemental Figure 3. Inhibition of PlGF signaling using soluble Flt-1 in a tube formation assay. sFlt-1 reduced tube formation of endothelial cells alone (-M, 34.7%), endothelial cells co-cultured with M0 macrophages (27.4%) or M2c macrophages (44.5%) compared to control. The data represent the mean $\pm$ SEM. The statistical significance was determined by one-way ANOVA.

Supplemental Figure 4. ELISA for PIGF. Concentration of PIGF protein was measured in concentrated conditioned medium of macrophage subsets. A trend could be observed towards more PIGF production in M2c macrophages. The data represent the mean $\pm$ SEM.

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Chapter 3

Local delivery of polarized macrophages improves reperfusion recovery in a mouse hind limb ischemia model

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Abstract

Aims: Enhancement of collateral development in coronary or peripheral artery disease is a therapeutic target, but it has proven difficult to achieve. Macrophages are key players in collateral remodeling, yet the effect of different macrophage subsets on arteriogenesis has not been investigated.

Methods and Results: Murine macrophages were cultured from bone marrow and polarized into M1 (IFN γ), M2a (IL-4) or M2c (IL-10) subsets. C57BL/6 mice underwent femoral artery ligation followed by intramuscular injection of macrophage subsets. Using eGFP expressing macrophages, cells could be detected at least 6 days after ligation and were located in the perivascular space of collateral vessels. After 14 days, perfusion ratio was increased in animals treated with M1 as well as M2a and M2c macrophages compared to control. Depletion of circulating monocytes by clodronate liposome injections did not hamper reperfusion recovery, however, treatment with exogenous polarized macrophages improved perfusion ratio after 14 days again. We used IL10R^{fl/fl}/LysMCre⁺ mice to study the effect of inhibition of endogenous polarization towards specifically M2c macrophages on arteriogenesis. Deletion of the IL10-receptor (IL10R) in the myeloid lineage did not affect reperfusion recovery, yet the pro-arteriogenic effect of exogenously injected M2c macrophages was still present.

Conclusions: Local injection of polarized macrophages promotes reperfusion recovery after femoral artery ligation and is not influenced by depletion of circulatory monocytes. Preventing endogenous M2c polarization did not affect reperfusion recovery suggesting that M2c's are not required for collateralization, but are sufficient to induce collateral formation upon exogenous administration. This is the first study using local injection of macrophage subsets showing the pro-arteriogenic effect of polarized macrophages.

Introduction

Many people worldwide suffer from the consequences of arterial occlusion common for coronary artery disease or peripheral artery disease (CAD and PAD). Drug therapies have been designed to restore blood flow artificially, but until now without robust clinical results ¹. Stimulating natural reperfusion recovery by promoting the transformation of pre-existing arterioles into larger conductance arteries, also termed arteriogenesis, is a very attractive target for designing new treatment strategies.

Acute occlusion of a major conductance artery, due to plaque rupture and thrombosis formation, decreases the blood perfusion rate dramatically and will eventually lead to post-occlusive ischemic tissues. Consequently, a steep pressure gradient develops over pre-existing collateral anastomoses leading to an increased fluid shear stress, which activates the endothelium and triggers the initiation of the remodeling process ^{2, 3}. Decades ago, it was already demonstrated that collateral growth is highly associated with macrophage accumulation around the collateral vasculature ⁴. Monocytes from the circulation adhere to the activated endothelium, expressing adhesion molecules, such as ICAM-1 ⁵, VCAM-1 and MCP-1 ⁶, and subsequently transmigrate into the perivascular tissue where they mature into macrophages ⁷. Being a rich source of cytokines and growth factors, these macrophages can stimulate the proliferation of endothelial cells and smooth muscle cells ⁸. In addition, the prototypic monocyte recruiting cytokines, MCP-1 ⁹ and GM-CSF, increase collateral formation on their own but also synergistically by prolonging life of monocytes and residence time of macrophages ¹⁰.

The importance of monocytes and macrophages on the progression of arteriogenesis was demonstrated by using monocyte-deficient op/op mice ¹¹. Femoral artery ligation resulted in more than 40% impaired reperfusion recovery and in a significant decrease in collateral arteries. Likewise, depleting circulating monocytes by 5-fluorouracil (5-FU) treatment attenuated flow recovery, which could be rescued by intravenous injections of blood-isolated monocytes ¹². Finally, when monocyte recruitment was inhibited by knocking out the MCP-1 receptor (CCR2), flow recovery was significantly decreased ¹³.

For several years now it is known that different subsets of macrophages coexist *in vivo* and exert different functions depending on their environmental stimulus. We define pro-inflammatory M1 macrophages

(induced by LPS and/or IFN γ) and anti-inflammatory M2 macrophages (induced by IL-4/IL-13 or IL10)^{14, 15}. M2 macrophages are pro-angiogenic as was already demonstrated *in vitro*¹⁶ and *in vivo* by our previous data, indicating a pro-angiogenic effect of M2 macrophages by increased production of FGF-2 and PlGF¹⁷. Since angiogenic factors are considered to stimulate arteriogenesis¹⁸, we hypothesize that M2 macrophages also have pro-arteriogenic capacities. Though some knowledge on macrophage subsets in relation to arteriogenesis is gained¹⁹, intervention studies on this particular process have not been performed.

In this study we investigated the effect of macrophage subsets on reperfusion recovery and collateral remodeling *in vivo*. We demonstrated for the first time that local injection of polarized macrophages improves reperfusion recovery regardless of the subset. Polarization of macrophages therefore is important for collateral formation, but the effect is independent of the direction of polarization.

Methods

Ethics statement

Animals were held under the guidelines of the animal care facility (Maastricht University) with unlimited access to food and drinking water. All animal experiments were approved by the Committee for Animal Welfare of the Maastricht University and conforms the Directive 2010/63/EU of the European Parliament.

Bone marrow isolation

Bone marrow was isolated from the femur and tibia of C57BL/6 mice 10-14 weeks of age as described previously²⁰. Cells were differentiated into bone marrow derived macrophages by culture in R10 medium for 8 days (RPMI-1640 medium (Gibco Invitrogen, Breda, The Netherlands), 10% heat-inactivated Fetal Calf Serum (FCS) (Bodinko BV, Alkmaar, The Netherlands), L-glutamine 2mM, 100 U/ml penicillin, 100ug/ml streptomycin and 10mM HEPES (all Gibco Invitrogen, Breda, The Netherlands)) supplemented with 15% L929-conditioned medium (LCM) containing M-CSF.

Macrophage polarization

After 8 days, differentiated macrophages were harvested, counted and suspended in R10 + 15% LCM. Cells (3.5×10^5 cells/well) were plated in 24-wells plates and kept overnight in order to adhere again. The next day, differentiated macrophages were polarized for 24h with interferon- γ (IFN γ) (100U/ml, Hycult biotech) for M1, interleukin (IL)-4 (20ng/ml, Peprotech) for M2a or IL-10 (10ng/ml, R&D Systems) for M2c macrophages. Non-polarized cells are referred to as M0 macrophages.

Animal models

Forty-five (n=9/group) female C57BL/6 mice (10-12 weeks old, Charles River, France) underwent surgical procedures to induce hind limb ischemia (as described below). Directly after ligation, animals were treated with NaCl or cell suspension containing macrophage subsets (M0, M1, M2a or M2c) and by Laser Doppler Imaging the reperfusion recovery was measured over time.

Clodronate liposomes

For a macrophage depletion experiment, 45 female C57BL/6 mice (10-12 weeks old, Charles River, France) were subjected to i.v. injection of clodronate loaded liposomes (200ul) (Roche, Mannheim, Germany) into the lateral tail vein ²¹. First injection was provided 24h prior to femoral artery ligation followed by a second injection (50ul) post-operatively.

eGFP macrophages

Bone marrow of RA/EG mice (n=4), kindly provided by C. van 't Veer (Academic Medical Centre Amsterdam), was isolated and differentiated into bone marrow derived macrophages. Cells of the myeloid lineage of these mice express enhanced green fluorescent protein (eGFP) ²². Forty female C57BL/6 mice (10-12 weeks old, Charles River, France) underwent femoral artery ligation, followed by injection of eGFP macrophage subsets (M0, M1, M2a or M2c). Animals were subsequently sacrificed at day 2 (n=6), 4 (n=2) or 6 (n=2) after surgery for histological analysis.

IL10R1^{fl/fl}/LysMCre mice

Femoral arteries of 27 IL10R1^{fl/fl}/LysMCre mice ²³ (n=9, IL10R1^{fl/fl}/LysMCre⁻; n=18, IL10R1^{fl/fl}/LysMCre⁺) were ligated and reperfusion recovery was

measured. These mice have a myeloid cell specific deficiency of the interleukin-10 receptor-1 (IL10R1), leading to strongly reduced sensitivity of these cells to IL-10²⁴ and subsequently lack of *in vivo* IL-10 induced M2c macrophages. One group (n=9, IL10R1^{fl/fl}/LysMCre⁺) was treated with exogenous M2c macrophages directly after the surgical procedure.

Femoral artery ligation

Surgical procedures to induce hind limb ischemia were performed as described before²⁵. During the whole procedure mice were anaesthetized with isoflurane (1.5-2%) and sedated 30 minutes before and 1 hour after the procedure with Temgesic (0.5 mg/BW). Ligations were placed at the site of bifurcation of the femoral artery and the epigastric artery, on the epigastric artery itself and at the bifurcation of the femoral artery and the popliteal artery. Directly after ligation, NaCl or cell suspension of macrophage subsets was injected via the open wound into the adductor muscle. In the region between the profunda artery, the saphenous artery and the femoral artery, 5x10⁵ cells (total volume of 60ul) were injected divided over three injection sites in the area where collateral vessels are located.

Laser Doppler Imaging

Under standardized conditions, blood flow in the hind legs of all animals was measured using Laser Doppler Imaging (LDI) (Perimed, Sweden). Measurements were performed before and directly after the surgical procedure and at post-operative days 3, 7 and 14. During the measurements mice were placed on a heating pad in a climate controlled chamber (37°C) to ensure minimal variation between flow measurements. The perfusion ratio per time point was calculated by dividing the perfusion of the ligated by the non-ligated leg.

FACS

Blood was collected at day 1 and 3 after PBS- or clodronate-liposome injection and analyzed with flow cytometry to study the effect of clodronate treatment on blood leukocyte populations. After lysis of red blood cells, leukocytes were stained with antibodies against CD3 (Ebioscience, clone 145-C11), CD45R/B220 (Ebioscience, clone RA3-6B2), Ly6G (BD, clone 1A8) and CD11b (BD, clone M1/70) to discriminate between T-cells (CD3⁺ B220⁻), B-cells (CD3⁻ B220⁺),

granulocytes (CD3⁻ B220⁻ Ly6G⁺ CD11b⁺) and monocytes (CD3⁻ B220⁻ Ly6G⁻ CD11b⁺) respectively. Leukocyte populations were measured using a FACS CANTO II and data were analyzed by using the FACSDiva software (BD Biosciences, CA, USA).

Immunohistochemistry

Tissues were prepared for immunohistochemical analysis and stained for endothelial marker CD31 (PECAM-1) (BD biosciences). Adductor muscles were isolated and embedded in O.C.T. Tissue Tek (Sakura Finetek), snap-frozen in isopentane and stored at -80°C. Frozen sections (7µm) were fixed in acetone, air dried and rehydrated in PBS. Sections were pre-incubated with a biotin blocking system (VectorLabs) to prevent non-specific binding. After incubation with the primary (CD31, [1:200]) and secondary mouse-anti-rat antibody (Dako), staining was amplified by incubating sections with ABC reagent (VectorLabs). Sections were developed using an AEC substrate kit (VectorLabs) for 15 minutes followed by hematoxylin counterstaining. Analysis of vessel number/mm² and size was performed using a morphometry program Qwin version 3.5.1 (Leica, Cambridge, UK), where after vessels were categorized according to their diameter.

Sections of adductor muscles of eGFP-macrophage injected animals were stained with a monoclonal antibody against GFP (Invitrogen). Sections were fixed in acetone supplemented with 0.5% H₂O₂, air dried and rehydrated in PBS. Anti-GFP [1:50] was incubated overnight with blocking fetal calf serum (FCS). Bright vision (Immunologic) was used to amplify the staining and sections were developed using AEC substrate for 15 minutes followed by hematoxylin counterstaining.

Statistical analysis

All data are presented as mean ± SEM. Laser Doppler measurements were analyzed using SPSS 17.0 repeated measures ANOVA and LSD post-hoc test. In all cases, p-value ≤0.05 was considered to be significant.

Results

Exogenously injected macrophages remain located in the muscle up to 6 days after ligation

Localization of exogenous macrophages during the reperfusion recovery period was visualized using eGFP expressing macrophages in a hind limb ischemia model. Directly after femoral artery ligation mice were injected with eGFP-macrophages and sacrificed at day 2, 4 or 6. After 2 days, injected eGFP-macrophages could be clearly visualized within the adductor muscle. Overall, cells are clustered at the injection site in between the muscle fibers (Figure 1A-C).

Figure 1. Visualization of eGFP-macrophages in muscle tissue (n=4). **A**, Overview of muscle tissue after ligation and macrophage injection. **B**, eGFP-macrophages located in the injection site at post-operative day 2 (scale bar = 100µm). **C**, Negative control eGFP staining. Injected macrophages could be detected in the perivascular space of collateral vessels at day 2 (**D**), day 4 (**E**) and day 6 (**F**) (scale bar = 20µm).

Already at this time point some eGFP-macrophages were located in the perivascular space of collateral vessels (Figure 1D). Moreover, injected macrophages were found in a more dispersed distribution throughout the muscle after 4 and 6 days after ligation. Cells migrated out of the injection site towards the site of ligation and most interestingly towards the perivascular space of collateral vessels (Figure 1E,F).

Treatment with polarized macrophages results in improvement of reperfusion recovery

Our previous data show that M2 macrophages increase angiogenesis *in vitro* and *in vivo* by inducing the expression of the pro-angiogenic growth factors FGF-2 and PlGF¹⁷. Arteriogenesis however is a distinct mode of neovascularization and is triggered by hemodynamic stimuli rather than by ischemia. Since angiogenesis and arteriogenesis are considered to encompass similar mechanisms, we examined the effect of different macrophage subsets on arteriogenesis by local injection of M0, M1, M2a and M2c macrophages i.m. directly after femoral artery ligation and measured reperfusion recovery. As a control for cell injections we injected the same volume of NaCl into the muscle. By means of LDI the reperfusion recovery over time was measured and perfusion ratio (ligated/non-ligated) was analyzed (Figure 2A).

Figure 2. Reperfusion recovery after femoral artery ligation and macrophage treatment (n=9). **A**, Representative Laser Doppler images of each group post-operatively and at day 14. **B**, Treatment with M1, M2a and M2c macrophages increases the reperfusion recovery after 14 days. **C**, A trend towards an increase in vessel number could be detected in the category '50-60µm diameter' in animals treated with M2a and M2c macrophages.

Directly after the ligation procedure the average perfusion ratio dropped to ~20% of baseline. The initial reperfusion recovery did not differ between groups and reached an average ratio of $36.5\% \pm 0.01$ 3 days after the operation procedure. After 14 days, we observed a trend in reperfusion recovery in animals treated with the M1 ($40.7\% \pm 0.1$, $p=0.07$) and the M2a subset ($39.9\% \pm 0.1$, $p=0.07$) and a significant improvement in animals treated with the M2c subset ($42.1\% \pm 0.1$, $p=0.05$) compared to treatment with M0 macrophages (Figure 2B). The perfusion ratio levels of NaCl and M0 treated animals did not rise above $37.3\% \pm 0.1$ and $32.8\% \pm 0.1$ respectively. After quantitative immunohistochemical analysis we could detect a trend towards an increase in number of vessels of 50-60 μ m diameter in the M2a and M2c treated animals.

Polarized exogenous macrophages improve reperfusion recovery regardless of endogenous macrophages depletion

To study the contribution of the endogenous macrophage population on effect of polarized macrophages we repeated the treatment experiments after pretreatment with clodronate liposomes. After a one-time injection of clodronate liposomes we observed a reduction in the blood monocyte population of $53\% \pm 0.35$ ($p<0.001$) compared to injection with PBS loaded liposomes (Figure 3A). When treated a second time with clodronate directly after the surgical procedure we observed a reduction of $68\% \pm 0.59$ ($p<0.001$) that was sustained for at least three days ($62\% \pm 1.29$; $p<0.001$) after surgery. Relative increase (60%) in the granulocyte population in response to the clodronate liposome treatment could be observed which was increased even more in response to the second injection (129%) (Figure 3B).

Figure 3. Effect of clodronate liposomes on blood monocyte and granulocyte population (n=6). Clodronate liposome treatment reduces the endogenous monocyte population significantly compared to PBS liposome treatment (***) $p<0.001$) and compared to single clodronate injection (# $p<0.05$). **A**, Single injection with clodronate reduces the monocyte population with 53%, whereas a second injection results in 68% reduction. **B**, Granulocyte population increases upon clodronate treatment after double injections with clodronate compared to PBS-liposome treatment (***) $p<0.001$) and single clodronate injection (# $p<0.001$), most probably due the depletion of the monocyte population.

Surprisingly, reperfusion recovery was not impaired by clodronate liposome injection when compared to animals that underwent the same procedure without the clodronate treatment up to day 7, suggesting that depletion of endogenous macrophages does not affect collateral remodeling. No differences in vessel number and diameter could be detected. However, consistent with our previous results we observed an increased perfusion ratio after 14 days in mice treated with M1 ($71.5\% \pm 0.2$, $p<0.001$), M2a ($71.8\% \pm 0.2$, $p<0.001$) or M2c macrophages ($68.3\% \pm 0.2$, $p<0.001$) compared to M0 ($56.8\% \pm 0.1$) and NaCl treatment ($46.3\% \pm 0.1$) (Figure 4).

In conclusion, administration of exogenously polarized macrophages improves reperfusion recovery after induction of hind limb ischemia, irrespective of their polarization status and regardless of depletion of the endogenous macrophage pool.

Figure 4. Reperfusion recovery after clodronate liposome treatment (n=9). Reperfusion recovery is increased at day 14 in animals treated with either M1, M2a or M2c macrophages compared to NaCl treatment (***)p<0.001). Depletion of the circulating monocytes/macrophages did not inhibit reperfusion recovery and did not alter the effect of exogenously injected polarized macrophages.

Exogenously, not endogenously polarized macrophages improve reperfusion recovery

In order to study the effect of endogenously polarized M2c macrophages we used mice with a conditional deletion for the IL10 receptor ($IL10R1^{fl/fl}/LysMCre^{+}$) on myeloid cells. Consequently, macrophages of these mice are not responsive to IL10 and cannot polarize towards an M2c phenotype. After 14 days, a slight delay in reperfusion recovery could be observed in $IL10R1^{fl/fl}/LysMCre^{+}$ ($57.9\% \pm 0.195$) compared to $IL10R1^{fl/fl}/LysMCre^{-}$ animals ($67.5\% \pm 0.146$), although the effect was not significant. When exogenous M2c macrophages were injected into the adductor muscle of $IL10R1^{fl/fl}/LysMCre^{+}$ mice, reperfusion recovery improved significantly ($76.2\% \pm 0.217$, $p<0.05$) compared to the $IL10R1^{fl/fl}/LysMCre^{+}$ without macrophage treatment (Figure 5).

Figure 5. Effect of inhibiting endogenous macrophage polarization (n=9). Reperfusion recovery in IL10R1^{fl/fl}/LysMCre⁻ (WT) and IL10R1^{fl/fl}/LysMCre⁺ (KO) mice. Treatment with M2c macrophages improves reperfusion recovery in IL10R1^{fl/fl}/LysMCre⁺ mice after 14 days compared to untreated animals (*p<0.05).

This indicates that reperfusion recovery is not dependent on endogenous polarization of M2c macrophages and that treatment with exogenously polarized cells positively influences arteriogenesis.

Discussion

In this study we investigated the effect of exogenous macrophage subsets on reperfusion recovery and collateral remodeling *in vivo*. We showed that local injection of exogenously polarized macrophages of the M1 as well as the M2 subset improves reperfusion recovery. The effect was not ablated by depletion of endogenous circulating monocytes and macrophages by clodronate liposome treatment, which emphasizes the pro-arteriogenic effect of the exogenous cells. M2c macrophages are not required for arteriogenesis as the IL10R1^{fl/fl}/LysMCre⁺ myeloid cells allowed normal reperfusion recovery. In the IL10R1^{fl/fl}/LysMCre⁺ mice however, exogenously administered M2c macrophages were still able to induce arteriogenesis. Polarization of macrophages therefore is sufficient to induce collateral formation and it seems independent on the direction of polarization.

The effect of macrophage heterogeneity on arteriogenesis was investigated in a study performed by Takeda *et al.*¹⁹. Contrary to our own study in which we demonstrated the effect of local administration of exogenously polarized M1 and M2 macrophage subsets, they showed

improved arteriogenesis after endogenous M2-like polarization by haploinsufficiency of the oxygen sensor Phd2. Macrophages were characterized by increased gene expression levels of different M2 markers, but mainly arginase-1, which resembles an IL-4 induced polarization as was described by Loke *et al.* ²⁶. We showed that endogenous polarization of macrophages specifically into the M2c subset using the IL10R1^{fl/fl}/LysMCre⁺ mice did not improve reperfusion recovery. Though endogenous IL-10 may be essential for reperfusion recovery, it probably does not rely solely on responsiveness of myeloid cells but also of endothelial cells and smooth muscle cells (SMCs).

Schaper *et al.* demonstrated that macrophage accumulation in the vessel wall is correlated with increased collateral remodeling ⁴. Since macrophage accumulation is reported to be maximal 3 days after femoral artery ligation, restraining the endogenous monocyte population in this critical phase should increase the opportunity for the exogenous cells to exert their function. To induce a sustained suppression of monocytes during the first three days after ligation multiple doses of clodronate liposomes were administered ^{27, 28}. Although the monocyte population was severely reduced during this period, we did not observe an effect on reperfusion recovery. It was described that by i.v. injection of clodronate liposomes only circulating cells are depleted ²¹, leaving the tissue resident macrophages unaffected which could still support collateral remodeling. Khmelewski *et al.* showed that depletion of bone marrow cells neither affects collateral growth nor macrophage accumulation, drawing the conclusion that in the early phase of collateral proliferation resident macrophages, rather than circulating monocytes, are essential ²⁹. We observed a large increase of the granulocyte population upon multiple clodronate injections, a phenomenon which was also reported in other depletion studies ^{11, 12}. Hoefer *et al.* investigated the effect of different leukocyte populations on arteriogenesis and concluded that attraction of leukocyte populations other than monocytes failed to induce arteriogenesis directly ³⁰.

Our results on the stimulatory effect of locally injected cells were supported by a study in which marrow-derived stromal cells (MSCs) were injected in the adductor muscle 24h after femoral artery ligation ³¹. Treatment with the MSCs resulted in enhanced reperfusion recovery and improvement of collateral remodeling and limb function without incorporation of these cells into collateral vessels. It was already described that bone-marrow cells do not

incorporate into the growing vasculature, but function as supporting cells ³². This suggests a paracrine effect, which was demonstrated by increased expression of FGF-2 and VEGF in MSC treated limbs. Furthermore, implantation of a combination of peripheral blood mononuclear cells (PBMNCs) and platelets, both being producers of angiogenic growth factors, into the thigh muscle was successful in stimulating vessel formation and collateral remodeling ³³. These studies confirm our findings on the improvement of arteriogenesis by local administration of exogenous cells. By injection of eGFP expressing macrophages we could localize exogenously injected macrophages for at least 6 days after surgical procedure. Moreover, we could observe their presence in the perivascular space of collateral vessels at different time points. Herewith, we validate the prevalence of macrophages during the early phase of arteriogenesis that might be recruited from local sites rather than from the circulation.

Unexpectedly, treatment with M1 macrophages resulted in equal improvement in blood perfusion as treatment with M2a or M2c macrophages. Arras *et al.* already showed that accumulated monocytes around collateral vessels are the main producers of TNF α and FGF-2 ³². By double staining, they nicely showed co-localization of the rabbit macrophage marker RAM-11 and either FGF-2 or TNF α expression. Pro-angiogenic cytokines and growth factors also share pro-arteriogenic properties. The pro-angiogenic effect of TNF α produced by M1 macrophages was demonstrated *in vitro* ³⁴. In rabbits, the effect of TNF α on arteriogenesis was investigated by treating animals with different TNF α antagonists after femoral artery occlusion. Here, both collateral growth and conductance was significantly reduced in animals treated with TNF α antagonist of either kind, correlating with less macrophage accumulation around collateral arteries ³⁵. Inducible nitric oxide synthase (NOS2), being highly expressed by M1 macrophages as well, plays an essential role in tumor development and angiogenesis and inhibition of NOS2 resulted in less collateral vessel remodeling after femoral artery occlusion ^{36, 37}. FGF-2 also has pro-arteriogenic properties as was demonstrated by a study in rats which underwent femoral artery ligation followed by daily infusion of FGF-2 by osmotic pumps. As a result, collateral blood flow was significantly increased in the first two weeks after occlusion ³⁸. Furthermore, it was demonstrated that during the early phase of arteriogenesis FGFR-1 expression was increased, in particular in SMCs, suggesting that collateral remodeling is mediated by

FGFR-1 availability³⁹. Since TNF α is expressed by classically activated M1 macrophages⁴⁰ and FGF-2 expression is highly increased in M2a macrophages¹⁷, the observation of Arras *et al.* suggests that M1 and M2 macrophages co-exist during the early phase of collateral remodeling and both invest in promoting collateral growth. This was confirmed by a recent study where the distribution of macrophage subsets, in both space and time, was investigated after femoral artery ligation. A strong increase in M2 macrophages was observed shortly after occlusion followed by increased numbers of M1 macrophages on day 3, after which both populations continue to increase over time. Whereas M1 macrophages were detected adjacent to the media, M2 macrophages were positioned in the outer perivascular area. Speculations about the function of this spatial distribution were made by the authors, suggesting a monocyte attracting function for the M1 subset close to the lumen and a vascular remodeling function for the M2 subset in the perivascular space⁴¹. Co-existence of both macrophage populations during collateral remodeling together with our current findings proposes complementary mechanisms (including TNF α and FGF-2 production by M1 and M2 macrophages respectively) of equal importance for each subtype during vascular remodeling.

In this study we showed that local infusion of polarized macrophages stimulated arteriogenesis. The pro-arteriogenic effect of macrophage subsets is most likely the result of a concerted action of macrophage-derived cytokines and growth factors, which have proven pro-angiogenic and pro-arteriogenic properties. We did not find enhancement of collateral flow by non-polarized macrophages, so the paracrine stimulation of specific cytokines and growth factors produced by polarized macrophages likely stimulates collateral formation. Polarization might lead to complementary actions of macrophages in the arteriogenic process rather than specialization of one macrophage subset.

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Chapter 4

Macrophage migration Inhibition Factor, a CXCR ligand with potential function in arteriogenesis

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In preparation

Abstract

Aims: Monocytes are recognized stimulators of arteriogenesis as they produce a variety of growth factors and cytokines. Being associated with monocyte recruitment, the pro-inflammatory macrophage migration inhibitory factor (MIF) is involved in stimulating atherosclerosis. Here, we analyzed the role of MIF in arteriogenesis in a murine hind limb ischemia model.

Methods and Results: Male C57BL/6 mice underwent femoral artery ligation to induce hind limb ischemia and were i.p. administered with either recombinant-mouse (rm)MIF (10µg), anti-MIF (100µg), or PBS. Reperfusion recovery was diminished after rmMIF stimulation, whereas blocking MIF enhanced reperfusion recovery and collateral formation. Furthermore, the number of tissue macrophages (Mac2) and CXCR2 positive cells in the ischemic skeletal muscle was significantly reduced after MIF treatment, but increased after anti-MIF administration. By FACS analysis we observed a decrease in Ly6C^{lo} and an increase in Ly6C^{hi} monocytes. *In vitro*, a possible polarizing effect of MIF could not be determined in bone marrow derived macrophages. Additionally, MIF treatment resulted in increased adhesion, but a decreased transmigration of macrophages.

Conclusions: Blocking MIF enhanced arteriogenesis, likely by affecting circulating monocyte subpopulations and subsequent infiltration into the tissue surrounding nascent collaterals. MIF blocks recruitment of Ly6C^{lo} monocytes and stimulates recruitment of Ly6C^{hi} from the hematopoietic compartment.

Introduction

Patients suffering from peripheral arterial disease (PAD) or coronary artery disease (CAD) have an increased risk of secondary events and may require adjunct therapy to revascularize ischemic tissue ¹. Arteriogenesis represents the rapid outward remodeling of collateral arteries which is induced by an increase in blood flow through the pre-existent interconnecting blood vessels resulting in enhanced shear stress and hence to the induction of collateral remodeling ².

Arteriogenesis is not only mediated by physical forces, but different cell derived factors (i.e. cytokines, chemokines and growth factors) also contribute to the remodeling process. Being a rich source of these factors, macrophages are recognized to be essential for collateral artery growth for several decades ³. The functional link between monocyte concentration in the peripheral blood and improvement of arteriogenesis was shown in a model of acute femoral artery ligation ^{4, 5}. The number of accumulating monocytes was shown to correlate with the collateral conductance and the number of collateral arteries that had remodeled. On the other hand, depletion of monocytes resulted in severe abolished growth of the collaterals ⁴. Monocytes are known to produce a variety of growth factors, cytokines and chemokines and therefore create a pro-inflammatory and mitogenic environment ^{6, 7}.

Macrophage migration inhibitory factor (MIF) is a known inflammatory cytokine with chemokine-like function. MIF exerts its effect via chemokine receptors CXCR2 and CXCR4 ⁸, but also through interaction with CD74, a type II transmembrane protein ^{9, 10}. MIF has been associated with pro-inflammatory macrophage functions related to pathologies such as atherosclerosis by increasing the recruitment of leukocytes into atherosclerotic lesions ¹¹. It was already demonstrated that overexpression of MIF in advanced human atherosclerotic plaque resulted in plaque destabilization ¹², while blocking of MIF led to regression of pre-existing plaque ⁸. Moreover, MIF also has pro-angiogenic capacity as it mediated endothelial migration and tube formation *in vitro* and induced angiogenesis in Matrigel plugs *in vivo* ¹³.

For several years macrophage heterogeneity is recognized as a true hallmark of the macrophage as different subsets are characterized by their environmental stimuli. We define pro-inflammatory M1 (induced by LPS

and/or IFN γ) and anti-inflammatory M2 macrophages (induced by IL-4/IL-13 or IL-10). Aside from producing cytokines to regulate inflammatory responses, macrophages (anti-inflammatory M2 macrophages in particular) express a scala of pro-angiogenic growth factors (described in chapter 2). It is recognized that pro-angiogenic growth factors also have pro-arteriogenic properties ¹⁴, suggesting that M2 macrophages promote arteriogenesis. However, we showed that both macrophages of the M1 as of the M2 subset stimulate collateral remodeling to improve reperfusion recovery (chapter 3) as was confirmed by the accumulation of both TNF expressing macrophages (M1) and FGF-2 expressing macrophages (M2) around collateral arteries (Arras et al). Additionally, it was shown that MIF plays an essential role in controlling TAM activation and polarization as it promotes an immunosuppressive and pro-angiogenic phenotype in TAMs. So, MIF has polarizing activities in a tumor environment, but its polarizing effect on macrophages is not known thus far.

A role for MIF or related signaling in arteriogenesis remains unknown. From previous gene expression analysis of collateral tissue in the rat we found upregulation of MIF-binding protein CD74 during the early phase of arteriogenesis, overlapping with the profiles of macrophage maker CD68 and MCP-1/CCL2 and CXCR4, suggesting a potential role for MIF in collateral artery growth. The aim of this study was to analyze the role of MIF in arteriogenesis in a murine hind limb ischemia model.

Methods

Animals

Male C57BL/6 mice underwent surgical procedures to induce hind limb ischemia (as described below) and were blinded and randomly assigned to receive either recombinant-mouse (rm)MIF (10 μ g, i.p.) for five consecutive days starting 24h before ligation, anti-MIF (100 μ g, i.p.) 24h before ligation, or PBS. For analysis of perfusion recovery, animals (n=6 per treatment) were sacrificed 14 days after the procedure. Animals that were assigned to histological evaluation (n=3 per treatment) were sacrificed 48h after ligation and hind limbs were fixated in 2% PFA. To examine the circulating blood monocyte population, animals were sacrificed 24h (n=12) or 48h (n=12) after surgery where after blood withdrawal from the hearts was performed and

collected in EDTA tubes. All experiments were approved by the Maastricht University Institutional animal care and usage committee.

Femoral artery ligation

Surgical procedures to induce hind limb ischemia were performed as described before ¹⁵. During the whole procedure mice were anaesthetized with isoflurane (1.5-2%) and sedated 30 minutes before and 1 hour after the procedure with Temgesic (0.5 mg/BW). Ligations were placed at the site of bifurcation of the femoral artery and the epigastric artery, on the epigastric artery itself and at the bifurcation of the femoral artery and the popliteal artery.

Laser Doppler Imaging

Under standardized conditions, blood flow in the hind legs of all animals was measured using Laser Doppler Imaging (LDI) (MoorLDI, Moor Instruments, Axminster, UK). Measurements were performed before and directly after the surgical procedure and at post-operative days 3, 6, 9 and 14. During the measurements mice were placed on a heating pad in a climate controlled chamber (37°C) to ensure minimal variation between flow measurements. The perfusion ratio per time point was calculated by dividing the perfusion of the ligated by the non-ligated leg.

Cell lines

Simian virus 40 immortalized murine endothelial cells (SVECs) were cultured in Dulbecco's modified eagle's medium/Ham's F-12 supplemented with 5% fetal bovine serum (FBS) and gentamycin (1:1000). The mouse monocytic cell line, WEHI 274.1 was obtained from the American Type Culture Collection (Rockville, MD, USA) and was grown in RPMI 1640 medium with L-glutamine (PAA, E15-840) supplemented with 10% FBS, 100 U/ml penicillin and 100µg/ml streptomycin.

Bone marrow derived macrophages

Bone marrow was isolated from the femur and tibia of C57BL/6 mice as described previously ¹⁶. Cells were differentiated into bone marrow derived macrophages by culture in R10 medium for 8 days (RPMI-1640 medium (Gibco

Invitrogen, Breda, The Netherlands), 10% heat-inactivated Fetal Calf Serum (FCS) (Bodinko BV, Alkmaar, The Netherlands), L-glutamine 2mM, 100 U/ml penicillin, 100ug/ml streptomycin and 10mM HEPES (all Gibco Invitrogen, Breda, The Netherlands)) supplemented with 15% L929-conditioned medium (LCM) containing M-CSF. After differentiation, macrophages were incubated with rmMIF (5, 10, 50, 100, 200, 400 and 500 ng/ml) for 24h. The supernatant was collected for ELISA analysis and macrophages were lysed for RNA isolation and gene expression analysis.

RNA isolation and qPCR analysis

RNA was isolated from macrophages using ZR RNA MicroPrep™ of which 1µg total RNA was subjected to reverse transcription. Quantitative Real-time Polymerase Chain Reaction (qPCR) was performed using the Maxima™ SYBR Green qPCR Master Mix and a primer concentration of 10µM. Primers for specific macrophage markers and MIF receptors were used to measure gene expression (Table 1).

Table 1. Primer sequences for quantitative PCR analysis

Gene	Forward	Reverse
<i>Cd74</i>	CCCATTCTGACCCATTAGT	TGTCCAGCCTAGGTTAAGGGT
<i>Chi3l3</i>	TGGCCACCAAGGAAAGTACA	CAGTGGCTCCTTCATTGAGAAA
<i>Cxcr2</i>	GCCTTGAGTCACAGAGAGTTG	CAAGGCTCAGCAGAGTCAC
<i>Cxcr4</i>	GGCTGTAGAGCGAGTGTTG	CAGAAGGGGAGTGATGAC
<i>Gapdh</i>	ATTGTCAGCAATGCATCCTG	ATGGACTGTGGTCATGAGCC
<i>Il10</i>	TTTGAATCCCTGGGTGAGAA	CTCCACTGCCTTGCTCTTATTTTC
<i>Il12</i>	GGTGCAAAGAAACATGGACTTG	CACATGTCACTGCCCCGAGAGT
<i>Mif</i>	TTTAGCGGCACGAACGATCC	CGTTGGCAGCGTTTCATGTC
<i>Nos2</i>	TTGCAAGCTGATGGTCAAGATC	CAACCCGAGCTCCTGGAA
<i>Retnla</i>	GGAAGTCTTGCCTCAATCCAG	ACACCCAGTAGCAGTCATCCC
<i>Tnf</i>	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC

Flow Cytometry

Whole blood was drawn from the heart of the animals and processed for fluorescence-activated cell sorting (FACS) analysis. After erythrocyte lysis, the cells were stained with anti-CD11b-PE-Cy7 (BD Biosciences), anti-Ly6C-PE (Miltenyi Biotec), anti-Ly6C-FITC (Miltenyi Biotec), anti-CXCR2-PE (R&D Systems), anti-CXCR4-FITC (BD Biosciences) and anti-CD74-FITC (BD Biosciences) in three different antibody mixes per animal. Cell sorting was performed with the Beckman Coulter FC500 cell sorter (Beckman Coulter, Inc., Fullerton, CA, USA) and results were analyzed with Kaluza® Analysis Software (Beckman Coulter, Inc., Fullerton, CA, USA).

Immunohistochemistry

Serial sections (5µm) of the adductor muscle were stained to analyze the number of macrophages (MAC-2, CEDARLANE) and the expression of CXCR2 (GeneTex), CXCR4 (lifespan Biosciences), CD74 (Santa Cruz), and MIF (Invitrogen). Positive cells were counted and tissue surface was measured using Diskus software (Hilgers) and expressed as numbers of cells/field, percentage of positive tissue of total tissue, or pixels/field.

Flow chamber adhesion Assay

Monocyte arrest on an endothelial monolayer (SVECs) was analyzed under flow conditions as described ^{8, 17}. SVECs were grown to confluence in 35mm dishes coated with collagen. Calcein-AM labeled WEHs were pre-incubated with rmMIF (100ng/ml), anti-MIF (10µg/ml) or PBS for 30 min and perfused at 1.5 dynes/cm². Adhesion of the monocytes was recorded and the number of adherent cells was counted in multiple fields.

Transmigration Assay

Using a transwell sytem, we compared the effect of rmMIF and anti-MIF on migration ⁸. Transmigration assay was performed using a 24-wells transwell cell culture chamber with Falcon® HTS FluoroBlok™ inserts. BrdU marked SVECs were placed on top of fibronectin-coated filters and incubated for 24h at 37°C for the formation of an endothelial monolayer. Test factors (50ng/ml MIF, 10µg/ml anti-MIF) were added in new medium and placed in the lower chamber. Calcein-labeled WEHs were suspended in assay medium and applied

on top of the insert filters. After 2h incubation, transmigrated WEHIs on the bottom side of the filter were stained with DAPI and counted using a fluorescence microscope.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. To compare data, we either used unpaired Student's t-test or one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test. P-values ≤ 0.05 were considered statistically significant.

Results

Reperfusion recovery is impaired by rmMIF treatment

To determine whether MIF can affect arteriogenesis, paw reperfusion was examined as a reflection of collateral remodeling in the hind limb. Reperfusion ratio after treatment with PBS, rmMIF, or anti-MIF Ab was measured by LDI pre-, post-ligation and at follow up days 3, 6, 9, and 14. In all experimental groups increased reperfusion recovery was observed after 14 days compared to the post-ligation time point, although differences per group were apparent.

Figure 1. A, Laser Doppler Images of the experimental groups at day 9 and 14 post ligation. Blue represents low and red represents high reperfusion rate. Clear recovery was observed after control and anti-MIF Ab treatment (area 2). **B,** Reperfusion data presented as the ratio of the ligated (right) to the non-ligated (left) hindlimb. Recovery after rmMIF treatment was inhibited, while treatment with anti-MIF Ab resulted in significantly increased recovery.

Mice treated with rmMIF showed depressed reperfusion rates compared to control, whereas mice treated with the anti-MIF Ab resulted in increased recovery. Significant differences in reperfusion rate were observed between anti-MIF Ab and rmMIF treatment at day 6 (0.49 ± 0.04 vs 0.39 ± 0.02 , $p < 0.05$), day 9 (0.55 ± 0.03 vs 0.42 ± 0.03 , $p < 0.05$) and day 14 post-ligation (0.55 ± 0.02 vs 0.37 ± 0.4 , $p < 0.05$) and at day 14 between control and rmMIF treatment (0.50 ± 0.02 vs 0.37 ± 0.04 , $p < 0.05$) (Figure 1).

MIF treatment reduces the number of CXCR2 and Mac2 positive cells

Previous studies suggest that macrophages express high levels of the functional MIF receptor CXCR2^{18, 19}. Immunohistochemical staining for CXCR2 illustrated that the number of CXCR2+ macrophages was significantly lower in the rmMIF treated group compared to the anti-MIF Ab treated group 2 days post-ligation ($13,69 \pm 1,25$ cells/field vs. $36,16 \pm 1,8$ cells/field). Mac2 staining showed the same tendency with a significant lower number of positive cells in the rmMIF group (16.54 ± 0.69 cells/field vs. 36.25 ± 1.68 cells/field in controls) and a significant higher number in the anti-MIF Ab group (44.81 ± 2.49 cells/field) (Figure 2). Accordingly, CXCR2/Mac2 double staining showed that macrophages in the ischemic skeletal muscle express CXCR2 (data not shown).

Macrophage polarization is not induced by rmMIF treatment

As the differences in arteriogenesis for the rmMIF group can relate to altered macrophage polarization, we tested the effect of rmMIF on isolated mouse BMDMs. Macrophages were stimulated with different concentrations of rmMIF. In contrast to classic inducers of macrophage polarization IFN γ (M1) and IL-10 (M2), rmMIF did not induce macrophage polarization to either the M1 or to the M2 phenotype (Figure 3).

Figure 2. rmMIF treatment reduces the number of CXCR2 positive cells and macrophages in the ischemic skeletal muscle (* $p < 0.05$ vs. control). Violet (A) and red (B) arrows indicate the positive stained cells.

Figure 3. Expression of M1 (TNF α) and M2 (IL-10, YM-1) markers after rmMIF stimulation. Bone marrow derived macrophages were stimulated with different concentrations of rmMIF and gene expression of polarization markers was measured by real-time qPCR analysis after 24h. No significant changes in expression of polarization markers were observed.

However, rmMIF exposure down-regulated the CXCR2 mRNA expression of BMDMs, whereas the expression of CD74 was enhanced (Figure 4). In addition, the expression of CXCR4 is dependent on the rmMIF concentration: 5ng/ml rmMIF elevated CXCR4 expression, whereas 10 and 50ng/ml decreased the CXCR4 expression (Figure 4B), showing the biphasic effect of rmMIF stimulation on BMDMs.

Figure 4. Gene expression data of CXCR2, CXCR4, MIF and its receptor CD74 in BMDMs after rmMIF stimulation. Cultured BMDMs were stimulated for 24h with different rmMIF concentrations. Real-time qPCR defined expression levels of CXCR2 (A), CXCR4 (B), MIF (C) and CD74 (D) (* $p < 0.05$ vs. control, $n = 3$).

Blocking MIF signaling raises CD11b⁺ population in the blood

Flow cytometry analysis on circulating blood monocytes 24h and 48h after hind limb ischemia revealed differences in monocyte subpopulations after treatment with rmMIF and anti-MIF Ab. rmMIF administration resulted in reduced levels of circulating CD11b⁺ monocytes and CXCR2⁺ monocytes (Figure 5), but had no effect on pro-arteriogenic Ly6C^{lo} monocytes. In contrast,

blocking MIF signaling resulted in an increase of CD11b⁺ monocytes. For CXCR2⁺ and Ly6C^{lo} monocytes the effect of blocking MIF signaling was time dependent. Ly6C^{lo} monocytes were induced at 24h but had returned to base line levels 48h after antibody treatment. CXCR2⁺ monocyte levels were elevated at 24h and reduced at 48h.

Figure 5. FACS analysis of circulating blood monocytes after hindlimb ischemia. **A**, The number of CD11b⁺ monocytes in peripheral blood was reduced after rmMIF treatment and increased when MIF signaling was blocked (**p*<0.05). **B**, After 24h, CXCR2⁺ monocytes are not significantly reduced after rmMIF, but almost tripled after anti-MIF Ab treatment (**p*<0.05 vs. ctrl, #*p*<0.05 vs. 24h rmMIF) and returned to lower levels after 48h (§*p*<0.05 vs. 24h anti-MIF mAb). **C**, Ly6C^{hi} monocytes were reduced after 24h (**p*<0.05 vs. ctrl, #*p*<0.05 vs. 24h rmMIF) and doubled after 48h (**p*<0.05 vs. ctrl, #*p*<0.05 vs. 48h rmMIF, §*p*<0.05 vs. 24h anti-MIF Ab). Furthermore, a strong increase in Ly6C^{lo} monocytes after anti-MIF Ab treatment was observed (**p*<0.05 vs. ctrl, #*p*<0.05 vs. 24h rmMIF) with a drop to the levels of rmMIF and control group (§*p*<0.05 vs. 24h anti-MIF Ab).

MIF treatment reduces transmigration of monocytes

To further investigate the mechanism of reduced macrophage recruitment to the ischemic hind limb, we performed an adhesion and transmigration assay of a monocytic cell line (WEHI) on an endothelial monolayer under flow condition after rmMIF and anti-MIF incubation. Surprisingly, both treatments induced and enhanced the adhesion of monocytes (ctrl 41.00 ± 8.04 vs. MIF 63.55 ± 5.55 vs. anti-MIF 84.77 ± 6.73 , $p < 0.05$) (Figure 6).

Figure 6. A, Anti-MIF triggered WEHIs adhesion on SVEC monolayers. SVEC monolayers were perfused with rmMIF or anti-MIF stimulated WEHIs. Firmly adherent monocytes were counted (* $p < 0.05$ vs. control, # $p < 0.05$ vs. rmMIF). **B,** rmMIF reduces the transmigration capacity of WEHIs through an endothelial monolayer (* $p < 0.05$ vs. control, # $p < 0.05$ vs. anti-MIF ab)

Consistent with reduced macrophage numbers in the hind limb, a significant reduction in the number of transmigrated cells through an endothelial monolayer was observed after MIF stimulation. The number of transmigrated cells was slightly increased after blocking MIF, but did not reach statistical significance (rmMIF 10.62 ± 0.74 vs. anti-MIF 22.80 ± 3.0 , $p < 0.05$; ctrl 21.23 ± 2.3 vs. rmMIF 10.62 ± 0.74 , $p < 0.05$).

Discussion

Cytokines such as MIF regulate leukocyte functions and may therefore be operative in monocyte/macrophage dependent collateral remodeling after femoral artery obstruction. Our results demonstrate that reperfusion recovery was diminished after MIF stimulation, whereas blocking MIF enhanced reperfusion recovery and therefore collateral formation. Moreover, exogenous MIF reduced the number of Mac2 positive tissue macrophages and CXCR2 positive cells in the ischemic skeletal muscle whereas blocking endogenous MIF resulted in an increase of macrophage numbers.

Recruited monocytes turning into tissue resident macrophages in the perivascular space of nascent collaterals determines remodeling of the artery towards a functional conduit ⁴. Increasing numbers of CD11b+ cells enhanced reperfusion in a murine model of acute hind limb ischemia ²⁰. Here, we demonstrate that blocking MIF raises the circulating CD11b+ monocytes. Additionally, Ly6C^{hi} monocytes were strongly decreased 24 hours after ligation, whereas the pro-arteriogenic Ly6C^{lo} monocytes were up-regulated 24 hours after ligation with an enhanced ability to infiltrate the ischemic muscle observed in a drop of these cells after 48 hours post-occluded. MIF suppressed CXCR2 expression on macrophages *in vitro*, and hinders monocytes to transmigrate into the tissue.

MIF plays an essential role in the formation of new blood vessel through angiogenesis. In mice, the administration of neutralizing anti-MIF antibodies delayed the growth of B lymphoma cells and dampened tumor growth, which was accompanied by a strong reduction in tumor angiogenesis. Tumor endothelial cells express MIF *in vivo*, and *in vitro*, microvascular endothelial cells secrete MIF needed for proliferation ²¹. Similarly, recombinant human MIF induces tube formation in Matrigel of human dermal microvascular endothelial cells *in vitro* and angiogenesis in Matrigel plugs and in the corneal bioassay *in vivo*, further support the pro-angiogenic function of MIF ¹³.

Although angiogenesis and arteriogenesis can be observed after an experimental occlusion of a peripheral artery, the relationship between these two modes of neovascularization remains undefined. Hypoxia is the principal trigger for angiogenesis whereas arteriogenesis is mainly initiated by local changes in shear stress as a result of altered pressure gradients along the

microvessels that later grow out to collaterals ²². On the other hand, most growth factors that have been studied in detail have pleiotropic effects and induce angiogenesis and arteriogenesis ²³. Therefore, the inhibitory effect of MIF on arteriogenesis is surprising and should be subject of further research. Inhibition of MIF might also lead to reduced atherogenesis and the combination of pro-arteriogenesis and anti-atherogenesis might be of particular therapeutic value.

Monocytes likely stimulate arteriogenesis by stimulating migration and proliferation of endothelial cells and smooth muscle cells through the production and secretion of a cocktail of survival factors, growth factors and cytokines. Monocytes/macrophages are a heterogeneous cell population polarized by chemokines and cytokines into so called M1 and M2 macrophages with potentially differential effects on arteriogenesis and differences in receptor expression, cytokine profile, and chemotactic properties ²⁴. Within this context, M1 macrophages are considered pro-inflammatory whereas M2 macrophages are pro-angiogenic. In this study, neither the polarization to M1 nor to M2 macrophages was observed after MIF stimulation indicating that MIF does not affect the phenotype of tissue resident macrophages.

However, blocking endogenous MIF does affect the circulating blood monocytes including Ly6C^{lo} and Ly6C^{hi}. Ly6C^{hi} monocytes exhibit an inflammatory profile and digest damaged tissue, whereas Ly6C^{lo} monocytes show an anti-inflammatory profile, tissue repair activities and promote healing ²⁴⁻²⁷. More specifically, Ly6C^{lo} monocytes are reported to be pro-arteriogenic ²⁸ and circulate in higher numbers upon blocking MIF, suggesting that endogenous MIF keeps these monocytes in check. A similar response is shown for CXCR2 positive cells and in fact these cells may in part or even completely overlap the Ly6C^{lo} population of monocytes.

The anti-MIF induced drop in Ly6C^{hi} and rise in Ly6C^{lo} population at 24h, with a drop at 48h after ligation could be the result of induced recruitment from the bone marrow, followed by migration into the vascular wall. Indeed, in our hands, blocking MIF enhances monocyte adhesion, although it did not affect migration. We hypothesize that blocking MIF increases the recruitment of monocytes and specific subsets such as Ly6C^{lo} monocytes as well as the infiltration into the tissue.

In conclusion, we showed that blocking MIF enhanced arteriogenesis likely by affecting circulating monocyte subpopulations and subsequent infiltration into the tissue surrounding nascent collaterals. MIF blocks recruitment of Ly6C^{lo} monocytes and stimulates recruitment of Ly6C^{hi} from the hematopoietic compartment. The divergent effect of MIF blockade on atherogenesis and arteriogenesis offers opportunities for selective therapeutic intervention in peripheral artery disease.

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Chapter 5

M2 macrophage treatment hampers cutaneous wound healing in diabetic mice

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Abstract

Objective: Diabetic patients suffer from impaired wound healing, driven by chronic inflammation, leading to serious complications if left untreated. The phenotype of macrophages is influenced by the wound microenvironment and evolves during healing from a pro-inflammatory (M1) profile in early stages to a less inflammatory, pro-healing (M2) phenotype in later stages of repair. The aim of the current study was to investigate the potential of exogenous administration of M2 macrophages to promote wound healing in an experimental mouse model of cutaneous injury.

Methods and Results: Bone marrow derived macrophages were stimulated *in vitro* with IL-4 or IL-10 to obtain two different subsets of M2 polarized cells, M2a and M2c respectively. Polarized macrophages were injected in full-thickness excisional skin wounds of either C57BL/6 or diabetic *db/db* mice. Control groups were injected with non-polarized (M0) macrophages or culture medium. Wound closure was not positively influenced by macrophage treatment as all wounds closed very rapidly. In diabetic mice (*db/db*) which suffer from an impaired healing response, treatment with either M2a or M2c macrophages deteriorated wound closure. Additionally, a heavily sustained influx of neutrophils (NIMP-1) confirmed the delay in wound healing influenced by M2 macrophages.

Conclusions: Treatment with exogenously activated M2 macrophages does not improve wound healing in C57BL/6 mice. However, in diabetic mice similar treatment resulted in aggravation of wound phenotype and in delay of the healing process.

Introduction

Wound healing is a very dynamic process that involves complex interactions between different cell types. It is a well-orchestrated series of events in which we can discriminate four overlapping phases. First, the coagulation cascade is initiated and platelets aggregate in the wound to prevent blood loss. Subsequently, neutrophils and macrophages have a leading role in the inflammation phase by killing pathogens and clearing the wound from matrix and cell debris ¹. Granulation tissue formation characterizes the proliferation phase of the repair response coinciding with angiogenesis and the influx of fibroblasts, which differentiate into myofibroblasts being responsible for the production of fibronectin and extracellular matrix (ECM). Finally, in the remodeling phase tissue maturation is completed by the degradation of capillaries, collagen deposition and scar tissue formation ²⁻⁴.

Macrophages contribute significantly to each phase of the wound healing process. Their number increases during the inflammation phase, peaks during the proliferation phase and gradually declines during the maturation phase ¹. Not only do they have a phagocytic function, they also produce numerous cytokines and (angiogenic) growth factors promoting proliferation of endothelial cells and fibroblasts. Depletion of macrophages results in delayed re-epithelialization, reduced collagen deposition, impaired angiogenesis and decreased cell proliferation due to decreased levels of TGF β and VEGF ⁵. In a study by Lucas *et al.* the role of macrophages in all different phases of wound healing was investigated ⁶. Deletion of macrophages in the early inflammatory phase hampered myofibroblast differentiation, reducing wound contraction, and the formation of granulation tissue. In the proliferation phase, deletion of macrophages impeded stabilization of vascular structures leading to hemorrhages in the wound and inhibited the transition into tissue maturation. However, absence of macrophages in the remodeling phase did not affect the healing response at all. Though macrophages have specialized functions in each separate phase of the healing process, the evolution of wound healing and the transition from one phase to the next is strongly dependent on macrophage presence in general.

Macrophages can be divided in different subsets dependent on the stimuli they receive. We define pro-inflammatory M1 (induced by LPS and/or IFN γ) and anti-inflammatory M2 macrophages, which can be further subdivided into M2a (induced by IL-4/IL-13) or M2c macrophages (induced by IL10) ^{7, 8}. Granulocytes are highly represented at the wound site and are considered the main producers of IL-4, which skews wound macrophages towards an M2a phenotype ⁸. In response to IL-4 these cells have increased arginase activity which converts arginine into ornithine, a precursor for collagen, contributing to the production of extracellular matrix and the healing of the wound ⁹. Furthermore, they display high levels of scavenger receptor activity clearing the wound from apoptotic neutrophils and cellular debris ¹⁰. Macrophages of the M2c subset are described to be regulatory macrophages, being different from the M2a population in the fact that they can present antigens to T-cells and do not contribute to extracellular matrix deposition.

The phenotype of murine wound macrophages was already studied by Daley and coworkers ¹¹. Poly-vinyl alcohol (PVA) sponges were subcutaneously implemented and cellular infiltrate was analyzed at post-wounding day 1, 3 and 7. Wound macrophages expressed pro-inflammatory Tumor Necrosis Factor (TNF) and interleukin (IL)-6 to a higher extent at day 1 (M1-like), whereas at a later stage (day 7) the expression pattern shifted towards elevated expression of anti-inflammatory arginase-1 (Arg-1) and Mannose Receptor (CD206) (M2-like). This study nicely shows the heterogeneity of macrophages during the course of wound healing in a healthy situation.

Wounds of diabetic patients fail to progress through the normal phases of wound healing resulting in chronic venous leg ulcers with increased risk of recurrent infections, tissue necrosis and leading eventually to limb amputation ¹². In order to find new strategies to stimulate wound healing we investigated here the therapeutic effect of alternatively activated M2 macrophages on cutaneous wound healing in mice.

Methods

Bone marrow isolation

Bone marrow was isolated from the femur and tibia of C57BL/6 and *db/+* mice, 10-14 weeks of age as described previously ¹³. Cells were differentiated into

bone marrow derived macrophages by culture in R10 medium (RPMI-1640 medium, 10% heat-inactivated Fetal Calf Serum (FCS) (Bodinko BV, Alkmaar, The Netherlands), L-glutamine 2mM, 100 U/ml penicillin, 100ug/ml streptomycin and 10mM HEPES (all Gibco Invitrogen, Breda, The Netherlands)) supplemented with 15% L929-conditioned medium (LCM) containing M-CSF for 8 days.

Macrophage polarization

After 8 days, differentiated macrophages were harvested, counted and suspended in R10 + 15% LCM. Cells (3.5×10^5 cells/well) were seeded in 24-wells plates and kept overnight in order to adhere again. The next day, differentiated macrophages were polarized for 24h with interferon- γ (IFN γ) (100U/ml, Hycult biotech) for M1, interleukin (IL)-4 (20ng/ml, Peprotech) for M2a or IL-10 (10ng/ml, R&D Systems) for M2c macrophages. Non-polarized cells are referred to as M0 macrophages. For *in-vivo* injections, cells were harvested after 24h of polarization, counted and resuspended in RPMI medium alone (no additional components added).

Animals

Twenty four female C57BL/6 mice (10-12 weeks) and 24 female *db/db* mice (8-10 weeks) were included and underwent the cutaneous wounding procedure as described below. ROSA26-EGFP transgenic mice ¹⁴ (a generous gift from Prof. Rene H. Tolba; RWTH Aachen) were used for localization experiments. All animals were held under standard pathogen-free conditions and were housed in special paper bedding material (7089 Harlan Teklad Diamond Soft Bedding) with unlimited access to food and drinking water. All animal experiments were approved by the Committee for Animal Welfare of the Maastricht University. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Wounding model

During the entire procedure mice were anesthetized by isoflurane inhalation. Dorsal hair was removed and skin was cleaned with gauze and 70% ethanol. Two (C57BL/6) or four (*db/db*) full-thickness excisional skin wounds were generated equidistant from the dorsal midline using 4mm surgical biopsy punches (KAI industries, Japan). The procedure was repeated about 1cm distally from the first wounds and wounds were allowed to form a scab. We injected cell suspension with macrophage subsets or culture medium s.c. into the wound edges. Per wound 3 injections of 20µl solution (5×10^5 cells per wound area) were applied at post-wounding days 1 and 3. Wounds were photographed every two days and wound area was quantified using Image J. Macroscopic wound closure was determined by measuring the wound area until day 9 (C57BL/6) and 13 (*db/db*). The percentage of wound closure was calculated using the following formula:

$$\text{Wound closure (\%)} = \frac{(\text{wound area day 0} - \text{wound area indicated day})}{\text{wound area day 0}} \times 100$$

At sacrifice, wounds were excised and snap frozen in liquid nitrogen or processed for generation of paraffin or cryosections.

RNA isolation and qPCR analysis of gene expression

Total RNA was isolated from frozen tissues homogenized in TriReagent (T9424, Sigma Aldrich, Zwijndrecht, The Netherlands) with the MiniBeadBeater (3110BXEUR, Biospec Products, Bartlesville, USA). 500 ng total RNA was subjected to reverse transcription using iScript cDNA Synthesis Kit (BioRad, Veenendaal, The Netherlands). Quantitative Real-time Polymerase Chain Reaction (qPCR) was performed using Sensimix SYBR & Fluorescein kit (Bioline) and a primer concentration of 300nM. Primers were designed using Primer Express (Table 1).

Table 1: Primers for quantitative real-time PCR

Gene	Forward	Reverse
<i>Actb</i>	GACAGGATGCAGAAGGAGATTACTG	CCACCGATCCACAGAGTACTT
<i>Arg1</i>	CATGGGCAACCTGTGTCCTT	CGATGTCTTTGGCAGATATGCA
<i>Chi3l3</i>	TGGCCCAACAGGAAAGTACA	CAGTGGCTCCTTCATTGAGAA
<i>Cyclophilin</i>	TTCCTCCTTTACAGAATTATTCCA	CCGCCAGTGCCATTATGG
<i>Hmox1</i>	CCGCCTTCCTGCTCAACAT	ATCTGTGAGGGACTCTGGTCTTTG
<i>Il1b</i>	AAAGAATCTATACCTGTCCTGTGAATGAAA	GGTATTGCTTGGGATCCACACT
<i>Il6</i>	GCTACCAAACTGGATATAATCAGGAAA	CTTGTTATCTTTAAGTTGTTCTTCATGTACTC
<i>Il10</i>	TTTGAATTCCTGGGTGAGAA	CTCCACTGCCTTGCTCTTATTTTC
<i>Il12</i>	GGTGCAAAGAAACATGGACTTG	CACATGTCACTGCCCGAGAGT
<i>Nfkbia</i>	TGGAAGTCATTGGTCAGGTGAA	CAGAAGTGCCTCAGCAATTCCT
<i>Retnla</i>	GGAACCTCTTGCCAATCCAG	ACACCCAGTAGCAGTCATCCC
<i>Tnf</i>	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC

Immunohistochemistry and immunofluorescence

For immunofluorescent detection of eGFP macrophages, wounds were isolated and fixated in 4% paraformaldehyde with 10% sucrose in PBS for 6h. Fixated wounds were cut in half and embedded in O.C.T. Tissue tek (Sakura Finetek) with cut surface facing down. Frozen sections (7µm) were fixated for 5 minutes in acetone, washed with PBS and counterstained with 4',6-diamino-2-phenyl indole (DAPI; Molecular Probes). For immunohistochemical detection, wounds were cut in half and embedded in O.C.T. Tissue Tek with cut surface facing down. Sections were fixed in acetone, air dried and rehydrated in PBS. After pre-incubation with a biotin blocking system (VectorLabs), tissues were stained for endothelial marker CD31 (PECAM-1) (BD Biosciences), macrophage marker F4/80 (AbD, Serotec) and neutrophil marker NIMP-1 (BD Pharmingen) and counterstained with haematoxylin. After incubation with the primary and secondary antibody, staining was amplified by incubating sections with ABC reagent (VectorLabs). Sections were developed using an AEC substrate kit (VectorLabs) for 15 minutes followed by haematoxylin counterstaining. Ten pictures were made randomly distributed throughout each wound at a 200x magnification. The total area of endothelial cells and macrophages per high power field (HPF) was measured using Photoshop CS2.

Statistical analysis

Statistical analyses were performed using Graphpad Prism or SigmaPlot t-tests. All data are presented as mean $\pm$ SEM. Wound area measurements were analyzed using two-way ANOVA and Bonferroni post-hoc test. In all cases, p-values ≤ 0.05 were considered to be significant.

Results

Exogenously administered macrophages localize in wounds for several days

To determine the fate of exogenously injected macrophages, bone marrow derived macrophages were differentiated from ROSA26-EGFP transgenic mice¹⁴ and 0.5×10^6 cells were injected intradermally around the wound at 3 injection sites 1 day after wounding. Wounds were isolated 3, 4, 6 and 8 days later and analyzed for the presence and localization of eGFP expressing macrophages. As shown in Figure 1, macrophages are clearly present in the wound bed 3 and 4 days after injection and they continue to be present, albeit in lower numbers and exhibiting less intense fluorescence at approximately 8 days after injection.

Figure 1. Exogenously injected eGFP macrophages in the wound bed after 6 days (A) and after 8 days (B) at a 400x magnification.

M2 macrophages retain the M2 polarization profile 15 days post injury

At 15 days post injury, mice were sacrificed and wounds were isolated for analysis. Real time PCR on RNA isolated from wound tissue indicated that M2 polarization markers as Arginase-1 and Ym-1 were significantly upregulated in M2 macrophage injected wounds compared to control (Figure 2B). Since

differentiation of macrophages from bone marrow stem cells requires culturing cells in the presence of 15% L-929 cell conditioned medium (LCM) containing M-CSF for 8 days, it is not surprising that M0 cells are also exhibiting some traits of M2 differentiation as well. Analysis of inflammatory cytokines indicated no significant differences between the groups at day 15 post injury (Figure 2A), however at this time point peak inflammatory responses have largely subsided.

Figure 2. Gene expression of diabetic wounds at day 15. **A**, Expression of inflammatory cytokines was not different between the wounds of M0, M2a or M2c treated animals. **B-C**, Anti-inflammatory markers were slightly upregulated in wounds treated with M2a, but not M2c macrophages (* $p < 0.05$).

Treatment with M2 macrophages does not improve wound healing in C57BL/6 mice

To study the effect of wound healing (M2) macrophage treatment we injected non-polarized M0, M2a or M2c macrophages subcutaneously in wound edges of C57BL/6 mice at post-wounding day 1 and 3. In wound edges of control mice we injected a similar volume of RPMI medium. Figure 3A shows representative pictures of wounds at several post-wounding days up to day 9. The wound area was measured every other day and is expressed in percentages compared to the original wound area (Figure 3B).

Figure 3. The effect of macrophage subsets on wound healing in C57BL/6 mice (n=6). **A**, Representative wound healing pictures of post-wounding days 1, 3, 5, 7 and 9. **B**, Wound area per time point as percentage of the original wound area representing healing in time. Already at day 3 the average wound area was decreased to about 31%. The data represent the mean $\pm$ SEM.

Wound healing in C57BL/6 mice progressed very rapidly since the average wound area decreased to about $74.37\% \pm 17.17$ at day 1 and already to $31.03\% \pm 6.95$ of the original size ($p < 0.0001$ compared to day 1) at day 3 post-wounding. We were unable to detect an additive effect of macrophages on wound healing. Moreover, we could not observe a difference in wound healing between mice treated with different macrophage subsets.

Wound healing in *db/db* mice is significantly impaired by treatment with M2 macrophages

Next, we investigated the effect of M2 macrophage treatment in a delayed wound healing response using diabetic *db/db* mice. These mice have a defect in their leptin signaling leading to increased food intake and therefore elevated

insulin levels which results in obesity already at 3-4 weeks of age¹⁵. To first investigate the polarization capacity of macrophages of *db/db* mice we measured gene expression of established polarization markers. We could not define any differences between macrophages of *db/+* animals and *db/db* animals (data not shown), suggesting no difference in polarization ability of macrophages *in vivo* between the genotypes. If we compared the course of wound healing of *db/db* mice with C57BL/6 mice a delay in wound healing was already evident at day 3. Where the average wound area of C57BL/6 mice is $31.03\% \pm 6.95$ the wounds of *db/db* mice have an average open area of $68.49\% \pm 10.33$. Although wound closure in C57BL/6 mice is completed after 9 days, wounds of *db/db* mice still have an average wound area of $42.9\% \pm 19.1$ at this time point.

Figure 4. The effect of macrophage subsets on wound healing in diabetic mice (n=8). **A**, Representative pictures of *db/db* mice treated with RPMI medium, non-polarized M0, M2a or M2c macrophages. **B**, Wound area in percentage of the original wound area. The data represent the mean $\pm$ SEM and statistical significance was determined by two-way ANOVA. * $p < 0.05$ compared to M0, # $p < 0.01$ compared to control.

The course of wound healing in *db/db* mice was not differently influenced by the different treatment options until post-wounding day 5 (Figure 4A,B). However, from day 7 onwards we observed a decreased healing in wounds treated with M2a or M2c with an average wound area of $42.2\% \pm 36.9$ and $29.7\% \pm 18.0$ respectively compared to treatment with M0 ($15.0\% \pm 15.6$) macrophages and control ($20.1\% \pm 29.2$) on day 13.

Inflammation is sustained in wounds of *db/db* mice treated with M2 macrophages

In order to further characterize wound healing after treatment with M2 macrophages we performed immunohistological stainings for endothelial cells (CD31, as a marker for angiogenesis), macrophages (F4/80) and neutrophils (NIMP-1) (Figure 5A-D).

Figure 5. Histological analysis of *db/db* wounds (n=8). **A**, Representative pictures of CD31, F4/80 and NIMP-1 staining (100x magnification). **B**, Percentage CD31 per high power field was measured and a trend towards increased angiogenesis under influence of M2 macrophages could be observed. **C**, Macrophage number is unchanged after treatment with macrophage subsets. **D**, Neutrophil (NIMP-1) number per HPF was significantly increased by M2 macrophage treatment (**p<0.01).

After 15 days we observed a significant increase in the number of neutrophils in wounds treated with either M2a or M2c macrophages ($p < 0.01$), which is indicative for a persistent and unresolved inflammatory reaction. Especially in the area underneath the scab accumulation of neutrophils was striking, as was the accompanied absence of macrophages in this region which were located in the surrounding granulation tissue. Neither the number of macrophages nor the number of endothelial cells was different between experimental groups, although endothelial cells tended to an increase.

Discussion

Macrophages are a very dynamic cell population involved in many processes ranging from inflammation to tissue repair. We investigated the wound healing capacity of M2 macrophages *in vivo* and administered exogenously polarized macrophages into cutaneous wounds of both healthy and diabetic mice. In the healthy situation, treatment with M2 macrophages did not show an additional effect on the course of wound healing since wound closure completed very rapidly which is confirmed by other researchers¹⁶. Probably the young age of the mice allowed such a fast healing as treatment with exogenous macrophages was reported to be efficacious in aged mice¹⁷. Wound healing of diabetic mice was significantly delayed compared to normoglycemic animals. Surprisingly, treatment of diabetic wounds with M2 macrophages did not improve, but further aggravated wound healing. Additionally, we detected highly increased neutrophil numbers in M2 treated wounds, which points towards sustained inflammation possibly contributing to the aggravated wound phenotype.

To stimulate wound healing we treated wounds with exogenously polarized M2 macrophages, with the intention of creating an anti-inflammatory pro-wound healing environment. Supporting wound healing by administration of exogenous macrophages was previously investigated as well by Drey Mueller *et al.*¹⁸. They studied the wound healing capacity of M2-like embryonic stem cell-derived macrophages (ESDM) and M1-like bone-marrow derived macrophages (BMDM) in C57BL/6 mice. For this purpose, a skin flap on the tail base was removed and macrophages were applied immediately after wounding on the wound area, which was sealed with a fibrin gel. Treatment

with ESDM as well as BMDM prolonged the healing of dermal wounds of C57BL/6 mice compared to cell-free treatment. In line with our findings, M2-like ESDM treatment resulted in higher cell densities in the wound, indicative for prolonged inflammation status, and impaired wound closure even to a higher extent compared to BMDM treatment. In our study, we used IL-4 to phenotypically skew macrophages towards an M2a status addressing the arginine pathway to stimulate collagen production and promoting wound closure.

The major fraction of the inflammatory cells recruited into the wound site consists of macrophages and neutrophils¹⁹. The role of macrophages in wound healing is twofold as they control tissue injury by protecting the host from invading pathogens and stimulate tissue repair. Leibovich and Ross were the first to describe the essence of the inflammation phase and the role of macrophages for normal wound healing²⁰. By temporarily depleting macrophages during selective phases of wound healing it was discovered that the lack of macrophages in the inflammatory phase hampered the healing process the most, leading to impaired epithelialization and reduced formation of vascularized granulation tissue⁶.

Additionally, the number of alternatively activated infiltrated macrophages in a later stage was significantly reduced by the absence of macrophages during the early phase of repair. Evidently, macrophages are key players in the inflammatory phase delineating the wound phenotype and influencing the course of wound healing. Neutrophils conduct a more specific task as they are specialized pro-inflammatory cells efficiently eliminating infection within the first three days after wounding^{19, 21}. However, persistence of neutrophils in wound areas is associated with impaired healing as cellular products such as pro-inflammatory cytokines, reactive oxygen species (ROS) and proteases directly delay the healing process by damaging the newly formed tissue²². On the other hand, when neutrophils are absent wound fluids contain high amounts of pro-inflammatory cytokines (increased TNF α and IL-6, but decreased TGF β concentrations)¹⁹, produced by wound macrophages. Herein, neutrophils have a modulating anti-inflammatory function to suppress the inflammatory phenotype of wound macrophages. We found increased neutrophil numbers in diabetic mice in combination with the treatment with M2 macrophages, which probably added up to a very sustained

anti-inflammatory wound character and could partly explain the significant delay in the wound healing process.

Diabetic wounds fail to heal efficiently and frequently remain in a chronic inflammatory state accompanied by persistent macrophage and neutrophil accumulation and elevated levels of pro-inflammatory cytokines²³⁻²⁵. Expression levels of pro-inflammatory markers (IL1 β , MMP-9 and iNOS) persist throughout post-wounding day 20 in combination with suppressed expression levels of mannose receptor (CD206) and scavenger receptor CD36 in diabetic mice, which suggests a delayed switch towards an anti-inflammatory phenotype of diabetic macrophages²⁶. Furthermore, wounds of diabetic mice have lower levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the early phase of wound healing. Treatment with recombinant GM-CSF enhanced wound healing and elevated pro-inflammatory cytokine levels of IL-6 and monocyte chemoattractant protein-1 (MCP-1) which correlated with increased neovascularization and infiltration of neutrophils and macrophages²⁷. One of the underlying causes of impaired wound healing in diabetics is macrophage dysfunction^{23, 28}. Sun *et al.* investigated the effect of streptozotocin-induced diabetes on the phenotypical changes of macrophages²⁹. Expression of pro-inflammatory factors such as TNF α , IL-6 and NO was significantly reduced when peritoneal macrophages of these mice were stimulated with LPS and IFN γ , whereas arginase activity was increased upon stimulation with IL-4. These findings were confirmed by *in vitro* experiments and could be reversed by insulin treatment. As a result of this hyperglycemic state, the phagocytic function of macrophages is directly suppressed, which leads to increased apoptotic cell burden in the wound³⁰. As a consequence, protease activity in diabetic wounds is increased leading to rapid ECM degradation further hampering wound closure³¹. Thus, insulin deficiency accompanied by high glucose levels in the blood strongly dampens wound macrophages in their inflammatory response and thus in their host defense functions.

Conclusively, our study shows that wound healing associated macrophages do not improve wound healing in normoglycemic animals and even are detrimental to wound healing in diabetic mice. Bacterial burden, macrophage dysfunction and an inherent prolonged inflammatory state are definable parameters for the progression of diseased wound phenotype.

Further research is needed to investigate the possible beneficial effect of M2 macrophages at a later time point in diabetic wound healing.

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Chapter 6

General Discussion

Hypothesis and findings

The formation of the vascular system is the foundation of every form of vertebrate life and essential for the development and growth of an individual. In the adult life however, vascular endothelium is quiescent and rarely sprouts or forms new branches. Nevertheless, the activation of the endothelium and formation of new blood vessels occurs in pathologies such as cancer and atherosclerosis and is negatively correlated with progression of the disease. On the other hand, neovascularization in the adult life also has beneficial effects and is, for instance, essential for the course of wound healing. Since inflammation is suggested to be one of the driving factors for neovascularization in disease, many studies have concentrated on the effect of macrophages on neovascularization, yet the fact that macrophages are a heterogeneous cell population and can exert different functions depending on their phenotype is not taken into consideration. The goal of this thesis was to elucidate the role of different macrophage subsets in neovascularization.

We hypothesized that the anti-inflammatory subset or M2 macrophages are pro-angiogenic and promote not only angiogenesis, but also stimulate collateral remodeling and improve reperfusion recovery and wound healing. To test these hypotheses we performed different *in vitro* and *in vivo* experiments and aimed to gain better insight into the mechanism behind M2-induced angiogenesis. Furthermore, we investigated the option of using macrophage subsets as alternative for treating ischemia by promoting reperfusion recovery and for stimulating wound healing in diabetes.

The main findings of this thesis are that:

1. Macrophages of the M2 subset promote angiogenesis *in vitro* and *in vivo* (chapter 2).
2. Arteriogenesis can be positively influenced by exogenous administration of pro-inflammatory M1 as well as anti-inflammatory M2 macrophages (chapter 3).
3. MIF treatment impairs reperfusion recovery and even attenuates collateral growth but does not function as inducer of macrophage polarization (chapter 4).
4. Diabetic wound healing is deteriorated by treatment with pro-angiogenic, anti-inflammatory M2 macrophages (chapter 5).

Macrophages and neovascularization: a double-edged sword

A common characteristic of pathological neovascularization is that it is induced and driven by inflammation as the accumulation of leukocytes is correlated with angiogenesis in e.g. tumor development ¹ or atherosclerosis and is recognized to stimulate collateral remodeling in arteriogenesis ^{2, 3}. From extensive studies it is evident that the accumulation of inflammatory cells in tumors contributes to tumor growth and metastasis formation ⁴⁻⁶. The attraction of leukocytes by tumor-derived angiogenic and chemotactic factors fuels the production of angiogenic and arteriogenic factors such as VEGF, FGF-2, PDGF, MCP-1 and TNF α by the leukocytes, which in turn stimulates proliferation and migration of endothelial cells, attracts smooth muscle cells and even more leukocytes ⁷. Though inflammation induced neovascularization strongly correlates with a poor prognosis in tumors, this phenomenon can be both beneficial as well as detrimental in cardiovascular disease. Here, inflammation is an inevitable reaction to damaged tissue, yet, chronic inflammation is unwanted for the recovery process. In case of myocardial infarction, inflammatory cells are recruited to clear the necrotic tissue, however, along with this leukocyte invasion comes the secretion of pro-inflammatory cytokines which stresses and damages the vessel wall, limits angiogenesis and arteriogenesis and thereby the remodeling of the myocardium ⁸. Accumulation of inflammatory cells also drives plaque progression and although the formation of plaque microvessels is usually a response to hypoxia, this also correlates with the number of macrophages ^{9, 10}. On the one hand these microvessels prevent cell death inside the lesion by providing oxygen and nutrients, but on the other hand they contribute to plaque growth by permitting the accumulation of lipids and infiltration of inflammatory cells. This ultimately destabilizes the lesion and results in cap degradation and plaque rupture leading to e.g. coronary thrombosis and myocardial infarction. The complexity of inflammation and its relation to disease outcome was investigated in a study where temporal deletion of macrophages was induced ¹¹. Wound healing was heavily impaired when macrophages were deleted in the early (inflammatory) phase, leading to hampered contraction and granulation tissue formation. Subsequently, by deleting macrophages in the proliferation phase, healing was deteriorated by destabilization of the vascular bed leading to hemorrhages. Thus, similar

disease outcome is in this case determined by inflammatory cells, though differently regulated. As it is recognized that macrophages vary in phenotype and function *in vivo* ¹²⁻¹⁴, their heterogeneity could elucidate the complex mechanism behind inflammation induced neovascularization.

Macrophage heterogeneity and neovascularization

The presence of different macrophage subsets has been demonstrated in tissues with neovascularization, e.g. tumors and atherosclerotic lesions. Although we currently distinguish M1 and M2 macrophages with a further subdivision of the M2 phenotype in M2a and M2c macrophages, most studies linking macrophages with neovascularization have been performed in tumors where alternatively activated macrophage subsets have been differently annotated. Tumor associated macrophages (TAM) make up a major proportion of the myeloid cells found in tumors. In progressing tumors, TAMs represent an alternatively activated M2-like phenotype and produce a wide array of pro-angiogenic factors and cellular mediators that stimulate angiogenesis and promote immune suppression to enhance tumor cell survival ^{15, 16}. Another macrophage phenotype associated with tumor angiogenesis is the Tie2-expressing monocytes/macrophages (TEM) ¹⁷. TEMs are a distinct lineage of pro-angiogenic cells defined by their specificity for the Tie2-ligand angiopoietin-2 (Ang2) secreted by activated endothelium ¹⁸ and are also considered to be M2-like. The identification of other myeloid subsets that drive tumor angiogenesis is challenging, since markers are shared by many cell types and combinations of these markers change in response to local signals in the tumor microenvironment ¹⁹.

In cardiovascular tissues, macrophages are important during the healing phase of a myocardial infarct, in atherosclerotic plaque formation and in collateral formation. In a myocardial infarct, macrophages basically have the same functions as in any form of wound healing (see above), with a likely specific temporal pattern of M1 macrophage predominance followed by a growing number of M2 macrophages. In human atherosclerotic plaques macrophage heterogeneity is demonstrated since both M1 and M2 macrophages are simultaneously present throughout atherogenesis. Here, a distinct pattern of subset localization is observed as M1 macrophages are highly represented in the shoulder region, whereas M2 macrophages localize

in the vascular adventitia ²⁰. However, how this distribution of subsets is related to plaque neovascularization has not been clarified.

In arteriogenesis, macrophage heterogeneity is observed by means of histological analysis at a given time point. Both M1 as M2 macrophages could be localized around remodeled collateral vessels in the hind limb ²¹, suggesting an equal contribution of different macrophage subsets to arteriogenesis. However, this suggestion is speculative and until now it could not be confirmed by other studies in this field.

In this thesis we examined the role of macrophage subsets in both angiogenesis and arteriogenesis, two out of three mechanisms of neovascularization. Since the cellular environment determines the phenotype of the macrophage ²²⁻²⁴, the question rises whether different triggers for neovascularization can affect endogenous macrophage polarization. Whereas angiogenesis is largely dependent on hypoxia and characterized by sprout formation of endothelial cells leading to new vasculature, arteriogenesis is regulated by hemodynamic changes and characterized by the outward remodeling of existing vasculature driven by SMC activity.

Hypoxia, being the driving force for tumor development and plaque neovascularization, is associated with macrophage accumulation ^{9, 25}. It is debated if HIFs, transcription factors mediating the effects of hypoxia, contribute to macrophage polarization. Both HIF-1 α and -2 α are factors that accumulate in macrophages in response to hypoxia, upregulating HIF target genes such as the proangiogenic growth factor VEGF ²⁶. For example, human TAMs express abundant levels of HIF-1 in response to hypoxia both *in vivo* and *in vitro*, suggesting a leading role for HIF-1 in macrophages and its potential for gene therapy ²⁷. Werno *et al.* showed *in vitro* that HIF-1 α -/- tumor associated macrophages (TAMs) infiltrate tumor spheroids and were alternatively activated, but express TAM markers CD206 (MR) and stabilin-1 to a much higher extent than WT TAMs ²⁸. Furthermore, the cytotoxic activity of HIF-1 α -/- TAMs was diminished and these TAMs were found to be less responsive towards tumor cells. Together, these findings suggest that HIF-1 α is required for maintenance of an M1 phenotype. Additionally, WT TAMs infiltrated in tumor spheroids enhanced CD31 expression, being an indicator for angiogenesis, yet, this expression was attenuated by infiltration of HIF-1 α -/- TAMs. This observation confirms the polarizing capacity of hypoxia regulated

by HIF-1 α in TAMs (towards a more M1 phenotype) and its key role in angiogenesis.

Hemodynamic changes trigger the outward remodeling of collateral arteries, yet, the effect on the polarization status of resident and circulatory macrophages remains to be elucidated. The observation of Arras *et al.* illustrates the presence of two different macrophage subsets in the perivascular space of the hind limb muscle after ligation ²¹. By means of immunohistochemical analysis TNF α and FGF-2 expressing macrophages could be discriminated around collateral arteries. Based on our gene expression data in chapter 2 we can speculate that both macrophages of the M1 (TNF α) as well as the M2 (FGF-2) subset accumulate in the perivascular space and stimulate collateral formation. Whether hypoxia and hemodynamic changes affect (re)polarization of macrophages remains to be determined.

Mechanisms of pro-angiogenic and pro-arteriogenic effect of macrophage subsets

We evaluated the effect of different exogenously polarized macrophage subsets on angiogenesis and arteriogenesis (Table 1). From our studies, we could conclude that by exogenous administration of M2 macrophages both angiogenesis and arteriogenesis are promoted. However, the effect of M1 macrophages is not so straight forward, as angiogenesis is not influenced by this subset in contrast to the stimulatory effect on arteriogenesis. What determines the discrepancy in effects of different macrophage subsets on neovascularization?

Angiogenesis and arteriogenesis are often considered to be similar processes, judging from the fact that pro-angiogenic growth factors function also in favor of arteriogenesis ²⁹. As described above however, the triggers, cell types involved and specific mechanisms of angiogenesis and arteriogenesis are quite different. Therefore, the role of distinct macrophage subsets in these processes may vary.

Table 1. Schematic overview of study results

Neovascularization	Model	Trigger	Effector cell; Process	Effect M1	Effect M2
Arteriogenesis	Ischemic disease	Hemodynamic changes	EC/SMC; remodeling vessel wall	↑	↑
	Ischemic disease	Hypoxia	EC; sprouting	n.d	n.d
Angiogenesis	Matrigel plug	Inflammation	EC; sprouting	↓	↑
	Wound healing*	Inflammation, tissue damage	EC/SMC/ fibroblasts; remodeling wound	n.d	↓

* in diabetic mice

Not only mechanistically we see differences between angiogenesis and arteriogenesis, but these processes determine also disease outcome to a large extent. Overall, angiogenesis is associated with excessive tumor growth, diabetic retinopathy and plaque destabilization and rupture, whereas arteriogenesis functions as a compensatory mechanism protecting ischemia-induced cell death ³⁰. The leaky phenotype of newly formed microvessels contributes to excessive growth and development of tumors and also deteriorates lesion phenotype as plaque microvessel formation ultimately leads to destabilization and rupture. Supporting cells such as pericytes and SMCs protect the endothelial layer of leakage and damage resulting in a mature vasculature as is seen during arterial remodeling.

In normal wound healing, inflammation is essential for the course of wound healing, but must be tightly regulated ³¹. Initially, (M1) macrophages and neutrophils clear apoptotic cells and bacterial debris. Subsequently, M2 macrophages promote wound healing and stimulate angiogenesis thereby indirectly contributing to the healing process. Thus, macrophages fulfill a double function in wound healing, which is related to the phenotypic switch (from pro-inflammatory M1 to anti-inflammatory M2) they undergo during the healing process ³². In diabetes, this switch does not take place which leads to chronic inflammation and severe impaired wound healing, which is irresolvable and leads to infections if left untreated. Chronic venous leg ulcers fail to progress through the normal pattern of wound healing not only driven by

chronic inflammation³³, but also by impaired arteriogenesis³⁴⁻³⁶. In order to prevent the development of chronic inflammation, we treated diabetic *db/db* mice with M2 macrophages one day after wounding (chapter 5). Unexpectedly, this treatment strategy tended to deteriorate the healing process even more (Table 1). From this observation we may conclude that if the pro-inflammatory phase is cut short (by injecting the anti-inflammatory subset too early), wound healing is impaired and results in the type of wounds comparable to those observed in patients who suffer from chronic inflammation. Indeed, Lucas *et al.* showed in their study that macrophages have other functions in diverse phases of wound healing¹¹. Thus, further research needs to elucidate if treatment at a later time point can skew the wound environment towards a wound healing phenotype and resolve chronic inflammation.

Paracrine mechanisms of macrophages

To initiate neovascularization, a tightly regulated communication amongst endothelial cells is required. In addition, the interaction of macrophages with endothelial cells, but also with other cell types such as SMC, platelets and pericytes defines the success of neovascularization. In other words, there is a tight cross-talk between inflammation and neovascularization³⁷. Figure 1 indicates several mechanisms by which macrophage subsets may influence neovascularization. The communication between macrophages and other cell types involved in neovascularization may go through a paracrine fashion, as macrophages produce and secrete a large variety of mediators. Chemokines produced by inflammatory cells can act both as leukocyte attractants and angiogenic inducers³⁸. Additionally, pro-inflammatory cytokines induce angiogenesis directly by interaction with endothelial cells and indirectly by stimulating leukocytes and endothelial cells to produce angiogenic mediators. Furthermore, macrophages produce angiogenic factors (e.g. VEGF and Ang-1) that elicit pro-inflammatory responses in endothelial cells^{39,40} and directly and indirectly stimulate vessel growth. In chapter 2 the pro-angiogenic effect of M2 macrophages was demonstrated, which could be attributed to the production of FGF and PlGF. However, it is not known what the exact trigger is for this production of growth factors by macrophages *in vivo* as it depends on a concerted reaction of micro-environmental changes such as (de-)activation by

endothelial cells, structural alterations in ECM and release of mediators by other cells ⁴¹.

Preparatory function

Next to paracrine release of growth factors, chemokines and cytokines, macrophages also release molecules with 'preparatory functions', i.e. degradation of the extracellular matrix surrounding endothelial cells, which keeps them in place and supports the vascular structure. Consequently, growth factors that are stored in the matrix are released and stimulate endothelial cell sprouting. In addition to this, macrophages create tunnels supporting migration of vascular cells for the formation of future blood vessels. Indeed, Anghelina and coworkers showed that macrophages eat their way through the matrix facilitating the ingrowth of new vessels as endothelial cells pave the tunnel that was created ⁴². A range of MMPs that is produced by macrophages facilitate this process ⁴³. Variations in MMP-secretome between macrophage subsets likely contribute to the differential effects observed on angiogenic and arteriogenic functions. In chapter 2, we describe gene expression profiles of three macrophage subsets and observed an increased gene expression of MMP-9 in the M2a subset (data not shown). The role for MMP-9 in vascular remodeling is described by Hazan and coworkers ⁴⁴. MMP-9 expressing macrophages were observed in close association with blood vessels during the early phase of remodeling. Thus, macrophages support sprout formation directly by the release of growth factors and indirectly by matrix degradation.

Cell-cell contact mechanisms

To induce sprouting and microvessel formation there must be a tight regulation and communication between endothelial cells and supporter cells, such as macrophages. Above we described the paracrine communication and preparatory function of macrophages, yet other mechanisms are also involved. It was demonstrated that Notch signaling in endothelial cells is important for macrophage recruitment and localization ⁴⁵. Macrophages cluster at vascular branch points and between tip cells, expressing the Notch ligand Delta-like-4 (DII4). Furthermore, in mice with myeloid specific loss of Notch1 neighboring endothelial sprouts failed to fuse with one another, suggesting the requirement for Notch signaling in the interaction between endothelial cells

and macrophages. Additionally, Notch signaling is involved in MCP-1 regulated macrophage accumulation as was shown by Fukuda *et al.*⁴⁶. DII4 induces MCP-1 expression in arteries and adipose tissue, leading to accumulation of macrophages, which are an additional source of MCP-1 and may therefore be part of a self-reinforcing mechanism.

Notch activation not only modulates macrophage activation^{47, 48} or accumulation, it also influences macrophage phenotype as was shown by Wang and co-workers⁴⁹. Activation of Notch signaling resulted in a distinct M1 expression profile (increased IL-12), whereas Notch signaling deficient macrophages showed a more variable phenotype resembling M2 macrophages. Hence, Notch signaling in macrophages may modulate angiogenic and arteriogenic processes by influencing macrophage phenotypes. Whether distinct macrophage subsets also differentially affect Notch signaling in endothelial cells remains to be determined.

Fantin and coworkers provided by their study a mechanistic insight into the role of tissue resident macrophages in the development of new vasculature⁵⁰. They found that macrophages are associated with endothelial tip cells during retinal angiogenesis in the mouse and in vessel fusion in zebrafish. Macrophages seem to function as cellular chaperones, connecting filopodia from endothelial tip cells reaching out for each other to form tube-like structures. They have a great affinity for tip cell filopodia and by their flexibility and mobility they are the ideal partner cells to establish contact and vessel fusion. Interestingly, we demonstrated in chapter 2 that macrophages incorporate in the tubular structures formed by endothelial cells. Furthermore, several studies suggest contact requirement between endothelial cells and macrophages for vascular network formation via the Tie2/Ang2 pathway⁵¹⁻⁵³. Altogether, this shows that, aside from the paracrine communication between macrophages and endothelial cells, cell-cell contact is also of essence in completing new vasculature (Figure 1).

Figure 1. Proposed pro-angiogenic mechanisms of macrophages. Macrophages stimulate neovascularization in a paracrine fashion by **1.** excretion of pro-angiogenic growth factors and **2.** production of MMPs that degrade matrix and make tunnels. **3.** Subsequently, cell-cell interaction of macrophages with endothelial cells induces sprouting, regulated via Dll4/Notch or Tie2/Ang2 signaling. **4.** Additionally, macrophages can behave as chaperone cells to connect neighboring tip cells.

Clinical implications

Several studies suggest stimulation or inhibition of neovascularization as a therapeutic target for the treatment of different cardiovascular diseases^{54, 55}. Current strategies to inhibit neovascularization in order to restrict tumor development are mainly based on targeting the VEGF pathway^{54, 56, 57}. Prevention of the binding of VEGF to its receptors (Bevacuzimab), the use of

soluble receptors to trap VEGF (Aflibercept) and small molecule tyrosine kinase inhibitors (Sunitinib) are successful strategies, which are used in combination with standard chemotherapy. Additionally, the VEGF pathway is also targeted to inhibit plaque angiogenesis and reduce plaque destabilization⁵⁸. On the other hand, therapies to stimulate neovascularization (therapeutic neovascularization) and improve reperfusion recovery gained attention over the past decades. In the context of therapeutic neovascularization, we proposed in chapter 4 the potential of macrophage migration inhibitory factor (MIF) to stimulate vascular remodeling. Since MIF was found to have pro-angiogenic potential, we hypothesized that treatment with recombinant mouse-MIF (rmMIF) antibody would stimulate arteriogenesis. However, MIF treatment impaired collateral remodeling whereas treatment with anti-MIF antibody improved perfusion recovery. Next to this, we investigated *in vitro* the polarizing capacity of MIF in order to determine the use of it as *in vivo* polarizing agent. Macrophages were exposed to different concentrations of rmMIF and screened for M1 and M2 markers, yet, polarizing markers were not upregulated by rmMIF treatment. Although in our hands MIF failed to stimulate arteriogenesis and *in vitro* macrophage polarization, several studies have been conducted to determine the success of other cytokines for these purposes. However, there is a large discrepancy in the translation of pre-clinical studies in animals into clinical treatments for humans. Pre-clinical trials that were conducted in animals put forward many options (e.g. MCP-1, GM-CSF, FGF, VEGF), which however could not be translated to a successful treatment in humans⁵⁹.

In search of the best therapeutic approach to alleviate the consequences of peripheral artery disease, pre-clinical studies have been performed using growth factors such as VEGF and FGF-2 to stimulate arteriogenesis⁶⁰⁻⁶². At first, the focus was on monotherapy, delivering one angiogenic protein or cytokine to ischemic tissues. These showed short term beneficial effects, but failed to prolong their stimulatory effects on the long run. Of general concern is the unstable and fragile phenotype of the newly formed vasculature. Therefore, the effectiveness of combination therapy, combining pro-angiogenic and pro-arteriogenic factors, was investigated. Cao *et al.* showed in both rat and rabbit hind limb ischemia models that the combination of FGF-2 and PDGF-BB results in the formation of robust and long-

term stable vessels and significantly improved vascularization^{63, 64}. In addition, this specific combination of proteins was found to be successful for reestablishing the stability of the vessels and improve myocardial perfusion and function in pigs⁶⁵. Based on their broad secretion pallet of growth factors, macrophage subsets offer perspectives as therapeutic strategy to stimulate the formation of new and stable vasculature.

Additionally, current therapies show adverse effects on other pathologies, which can lead to life threatening situations. An example of such a drug is IFN β , which is used to treat patients suffering from multiple sclerosis. In humans, it was shown that treatment with IFN β results in impaired arteriogenesis and reperfusion recovery⁶⁶. Additionally, Goossens *et al.* showed in different mouse models that IFN β treatment accelerated plaque formation and increased macrophage accumulation into the plaque⁶⁷. Thus, the challenge of developing new drug therapies lies within maximizing their effectiveness and minimizing their side effects.

Different studies focused already on the potential of cell based therapy for therapeutic angiogenesis⁶⁸⁻⁷². We discussed in chapters 2, 3 and 5 the use of different macrophage subsets to induce angiogenesis, arteriogenesis and stimulate wound healing. A great advantage of such a treatment compared to the current treatments is the local application, reducing the adverse effects on other non-target organs. Furthermore, cytokines have short half-lives and are broken down very fast. Thus, to reach the intended effect, one should administer every day injections or a sustained release formulation, such as cytokines in a biomaterial that slowly degrades and releases the growth factor. We showed in chapter 3 that macrophages injected in muscle tissue stay resident for at least 6 days, which could serve as a local source of growth factors and mediators for a longer period of time.

Future perspectives and concluding remarks

We acknowledge the potential of macrophage subsets for the use as therapeutic strategies, especially for therapeutic neovascularization. Another option to change the local environment and to steer resident macrophages into a certain direction is to polarize these cells *in vivo*. Takeda *et al.* have investigated the possibility of *in vivo* polarization of macrophages by the deletion of the *Phd2* gene on a single allele⁷³. Arteriogenesis was improved by

an increased resident macrophage M2 population, which stimulated SMC recruitment by the release of arteriogenic factors. Several studies suggested already the key role of tissue resident macrophages for the maintenance of the tissue and stimulation of neovascularization ⁷⁴. Jenkins and coworkers concluded that local proliferation of macrophages is essential in inflammatory responses driven by Th2-cytokines such as IL-4 ⁷⁵ in order to increase population density of the local macrophage pool that has an effect on neovascularization. Recently it was found that local macrophage proliferation inside the atherosclerotic plaque contributes to a large extent to the mass representation of lesional macrophages, rather than recruited monocytes from the blood ⁷⁶. Altogether, this might give new insights in the *in situ* polarization of tissue resident macrophages. Also, timing of the treatment determines the success of therapeutic neovascularization as we concluded from our wound healing study in diabetic mice (chapter 5).

In this thesis we established the role of different macrophage subsets in neovascularization, in particular angiogenesis and arteriogenesis. The potential of macrophage subsets to induce therapeutic neovascularization was shown in our studies. Local delivery of exogenous macrophages resulted in stimulation of angiogenesis and arteriogenesis, such that M2 macrophages stimulated angiogenesis and both M1 and M2 enhanced arteriogenesis. Conclusions on the role of exogenous macrophage treatment in diabetic wound healing require more investigation, especially focusing on the timing of the treatment. Furthermore, the *in vivo* polarization of resident tissue macrophages towards a certain phenotype to locally treat pathologies, minimizing the side effects to other non-target tissues should gain more attention in the future. We expected MIF to be effective in this respect, but our results did not support this. The translation of using macrophages as a therapeutic strategy needs further and more thorough research regarding the amount of cells, the timing of treatment and the long term effects.

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Summary

Cardiovascular diseases are the leading cause of death worldwide, mostly manifested as stroke and myocardial infarction often caused by ruptured fat accumulations in the vessel wall (atherosclerotic plaque). Progression of plaque phenotype is not only driven by environmental risk factors such as smoking, stress, increased fat intake, but also by accumulation of macrophages and the formation of plaque microvessels.

The formation of new vasculature, or neovascularization, is essential for embryonic development (angiogenesis) and adult homeostasis (arteriogenesis). Vascular endothelium is quiescent in adult life, yet can be activated again by a disturbed balance of pro-and anti-angiogenic factors leading to the 'angiogenic switch'. Angiogenesis describes the formation of new microvasculature out of pre-existing blood vessels by capillary sprouting and is associated with development of cardiovascular disease. Plaque infiltrating microvessels enable a constant supply of oxygen and nutrients further aggravating plaque phenotype. Whereas angiogenesis is associated with disease progression, arteriogenesis functions as a natural compensation mechanism. Narrowing or occlusion of the vasculature by plaque formation results in a lack of oxygen in the underlying tissue and in an increased blood pressure in the collaterals. In response to this increased shear stress, outward remodeling of small capillaries into larger arterioles restores blood flow and protects the tissue from necrosis.

Macrophages are key for tissue homeostasis and host defense, but also contribute to the progression of diseases. For several years it is recognized that macrophages can be divided into different subsets characterized by their function, which is induced by micro-environmental triggers. By the expression pattern of cytokines and chemokines we can roughly distinguish between pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages, of which the latter further can be subdivided into M2a (IL-4/IL-13), M2b (IC/LPS) and M2c macrophages (IL-10). Recent studies have observed an association between tumor angiogenesis and the accumulation of M2-like macrophages, however, a causal effect was not clearly investigated. In this thesis, we investigated the effect of different macrophage subsets on neovascularization. We hypothesized that the M2 subset promotes neovascularization.

In chapter 2, the effect of macrophage subsets on angiogenesis was studied *in vivo* by means of injection of Matrigel mixed with different subsets

of macrophages. Under the influence of M2a and M2c macrophages significantly more microvessels were observed. Using a co-culture of endothelial cells and macrophages in an in vitro a tube formation assay we observed significantly more tubes in the presence of M2a and M2c macrophages, whereas tube formation was inhibited by the presence of the M1 subset. Gene expression analysis showed that M2 macrophages highly express pro-angiogenic growth factor genes, amongst others FGF2 and PlGF. Neutralizing the FGF and PlGF signaling in vitro reduced the stimulatory effect of both M2a and M2c macrophages, suggesting that these macrophages stimulate angiogenesis in a paracrine fashion.

In chapter 3, the effect of macrophage subsets on arteriogenesis during reperfusion recovery was investigated in a hind limb ischemia model. Mice underwent femoral artery ligation and were treated with different macrophage subsets. Local intramuscular injection of both M1 and M2 macrophages, improved the reperfusion recovery significantly. Temporary depletion of circulating monocytes using clodronate liposomes did not influence the stimulatory effect of locally injected macrophages. To differentiate between the effect of the exogenously injected macrophages and endogenously M2c polarized macrophages, we induced hind limb ischemia in mice with a macrophage specific deficiency of the IL10 receptor. Reperfusion recovery was not influenced by this deletion, however, the improvement on reperfusion recovery was still present after local treatment with M2c macrophages suggesting that the stimulatory effect is caused only by the exogenous administration of macrophages.

In chapter 4, the effect of MIF was investigated as potential stimulator of arteriogenesis. Mice underwent hind limb ischemia and were treated with PBS, recombinant MIF or anti-MIF antibody. Treatment with recombinant MIF inhibited reperfusion recovery, whereas under influence of the antibody reperfusion recovery was improved. Furthermore, treatment with recombinant MIF reduced the accumulation of MAC2⁺ macrophages, the number of circulating CD11b⁺ monocytes as well as the transmigrating capacity of macrophages in vitro. However, MIF did not affect macrophage polarization. Thus, blocking MIF signaling enhanced arteriogenesis likely by affecting the circulating monocyte population.

In chapter 5, the therapeutic effect of M2 macrophages on wound healing was investigated. Full-thickness skin wounds were inflicted on the back of wildtype C57Bl6 mice and different subsets of macrophages were injected at 3 sites around the wound area. An additional effect of macrophage treatment on wound healing was not detected, as in all groups the wound area was reduced by ~70% after 3 days, meaning that the healing response occurred very fast. Since diabetics suffer from a delayed wound healing response, we used diabetic db/db mice and performed the same wounding procedure and treatment regimen. Interestingly, compared to treatment with PBS or M0 macrophages, wound healing was even further delayed after treatment with M2 macrophages accompanied by significantly increased numbers of neutrophils. Future experiments should show whether timing of treatment is of essence and could suppress sustained inflammation in diabetic wounds.

In chapter 6, the results described in this thesis were discussed and put in perspective. Implications of the study were made and new ideas for therapeutic options for treatment of cardiovascular disease with macrophage subsets were suggested. In conclusion, this thesis provides new insight in the relation between macrophage subsets and neovascularization.

Samenvatting

Hart- en vaatziekten zijn de grootste doodsoorzaak wereldwijd, die zich manifesteren als een beroerte of hartinfarct vaak veroorzaakt door een losgeschoten vetophoping in de vaatwand (atherosclerotische plaque). Progressie van plaque fenotype wordt niet alleen bevorderd door milieufactorisicofactoren zoals roken, stress en vet eten, maar ook door ophoping van macrofagen en het binnendringen van microvaatjes in de plaque.

Het ontstaan van nieuwe bloedvaten, ofwel neovascularisatie, is essentieel voor de embryonale ontwikkeling (angiogenese) en homeostase in het volwassen stadium (arteriogenese). Normaal gezien is vasculair endotheel in het volwassen stadium in rust, maar kan opnieuw worden geactiveerd door verstoring van het evenwicht van pro- en anti-angiogene factoren, wat resulteert in de zogenaamde 'angiogenic switch'. Angiogenese is het ontstaan van nieuwe capillairen uit reeds bestaande bloedvaten en wordt geassocieerd met de ontwikkeling van hart- en vaatziekten. Microvaatjes die de plaque zijn binnen gedrongen zorgen voor constante toevoer van zuurstof en voedingsstoffen en verslechteren op deze manier de toestand van de plaque. Waar angiogenese geassocieerd wordt met progressie van de ziekte, fungeert arteriogenese als een natuurlijk compensatie mechanisme. Vernauwing of verstopping van een bloedvat door plaquevorming resulteert in een zuurstofgebrek in het onderliggende weefsel en een verhoogde bloeddruk in de omliggende collateralen. In reactie op deze verhoogde bloeddruk, vormen kleinere collateralen zich om tot functionele arteriolen om zo de bloedstroom te herstellen en het weefsel te beschermen tegen necrose.

Macrofagen zijn essentieel in de afweer tegen ziekteverwekkers en voor het onderhoud van weefsel, maar dragen ook bij aan de progressie van ziekten. Sinds enkele jaren is erkend dat er verschillende subtypen macrofagen bestaan, gekarakteriseerd op basis van hun functie die bepaald wordt door factoren uit hun directe omgeving. Op basis van het expressiepatroon van cytokines en chemokines kunnen we ruwweg onderscheid maken tussen pro-inflammatoire M1 macrofagen en anti-inflammatoire M2 macrofagen, welke laatste verder onderverdeeld worden in M2a (IL-4/IL-13), M2b (IC/LPS) en M2c macrofagen (IL-10). Recente studies hebben een correlatie tussen tumor angiogenese en de accumulatie van M2-achtige macrofagen waargenomen, maar een causaal effect werd tot nu toe nog niet duidelijk onderzocht. In dit proefschrift hebben we het effect van verschillende macrofaag subtypes op

neovascularisatie nader onderzocht met de hypothese dat M2 macrofagen neovascularisatie stimuleren.

In hoofdstuk 2 is het effect van verschillende macrofaag subtypen op angiogenese in vivo onderzocht door muizen te injecteren met Matrigel gemengd met verschillende subtypen macrofagen. Onder invloed van M2a en M2c macrofagen konden significant meer microvaatjes waargenomen worden. Een co-cultuur van endotheelcellen en macrofagen in een in vitro tube formation assay toonde aanzienlijk meer tubes in de aanwezigheid van M2a en M2c macrofagen, terwijl de vorming van tubes werd geremd door de aanwezigheid van M1 macrofagen. Met behulp van genexpressie vonden we verhoogde expressie van pro-angiogene groeifactor genen zoals FGF2 en PIGF in M2 macrofagen. Door FGF en PIGF signalering in vitro te neutraliseren werd het stimulerende effect van zowel M2a en M2c macrofagen gereduceerd, wat suggereert dat deze macrofagen angiogenese paracrien stimuleren door het uitscheiden van groeifactoren.

In hoofdstuk 3 is het effect van macrofaag subtypen op arteriogenese onderzocht in een muis model waarbij ischemie in de achterpoot werd geïnduceerd. Muizen ondergingen ligatie van de femorale arterie en werden behandeld met verschillende subtypen macrofagen. Lokale intramusculaire injectie van zowel M1 en M2 macrofagen, verbeterde het reperfusie herstel aanzienlijk. Tijdelijke depletie van circulerende monocytten onder invloed van clodronaat liposomen had geen invloed op het stimulerende effect van lokaal geïnjecteerde macrofagen. Om onderscheid te maken tussen het effect van de exogeen geïnjecteerde macrofagen en endogeen M2c gepolariseerde macrofagen hebben we ischemie geïnduceerd in muizen met een macrofaag specifiek deficiëntie van IL10 receptor. Reperfusie herstel werd niet beïnvloed door deze deletie, echter, de verbetering van reperfusie herstel was nog aanwezig na lokale behandeling met M2c macrofagen, wat erop wijst dat het stimulerende effect veroorzaakt wordt door de exogene toediening van macrofagen.

In hoofdstuk 4 hebben we het effect van MIF als potentiële stimulator van arteriogenese onderzocht. Muizen ondergingen een operatie om ischemie in de achterpoot te creëren en werden behandeld met PBS, recombinant MIF of een anti-MIF antilichaam. Reperfusie herstel werd geremd na behandeling met recombinant MIF, terwijl onder invloed van anti-MIF antilichaam

reperfusie hertel werd verbeterd. Daarnaast verminderde de accumulatie van MAC2+ macrofagen, het aantal circulerende CD11b+ monocyt en de transmigration capaciteit van macrofagen in vitro onder invloed van recombinant MIF. Echter, MIF had geen invloed op macrofaag polarisatie. Het blokkeren van MIF signalering bevordert arteriogenese waarschijnlijk door de populatie circulerende monocyt te beïnvloeden.

In hoofdstuk 5 werd het therapeutische effect van M2 macrofagen op wondgenezing onderzocht. Huidwonden werden aangebracht op de rug van C57Bl6 wildtype muizen en verschillende subtypen macrofagen werden geïnjecteerd op 3 locaties in het wondgebied. Een positief effect van de behandeling met macrofagen kon niet worden waargenomen, omdat al na 3 dagen het wondoppervlak verminderd was met ~70% in alle groepen. Omdat diabetici lijden aan een verstoorde wondgenezing, hebben we het experiment herhaald in diabetische db/db muizen. Opvallend genoeg werd door de behandeling met M2 macrofagen de wondgenezing verder vertraagd in vergelijking met de behandeling met PBS of M0 macrofagen. Dit ging daarnaast gepaard met significant verhoogde aantallen neutrofielen, wat wijst op een aanhoudende ontsteking. Toekomstige experimenten moeten uitwijzen of de timing van de behandeling met M2 macrofagen essentieel is en aanhoudende ontsteking bij diabetische wonden alsnog kan onderdrukken.

In hoofdstuk 6 zijn de resultaten van dit proefschrift besproken en in perspectief gezet. Implicaties van de studie werden bediscussieerd en nieuwe ideeën voor therapeutische inzet van macrofaag subtypen voor de behandeling van hart- en vaatziekten werden voorgesteld. Dit proefschrift geeft nieuwe inzichten in de relatie tussen macrofaag subtypen en neovascularisatie.