Immune modulation of bone marrow-derived cells in Ischemic Heart Disease

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Chapter 1

General introduction

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Interferon-Beta, a Decisive Factor in Angiogenesis and Arteriogenesis
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The emerging role of galectins in cardiovascular disease
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1. INTRODUCTION

Cardiovascular disease (CVD) remains the major cause of death worldwide. Presence of atherosclerosis in the coronary artery is a determining risk factor for the development of CVD. Atherosclerosis might lead to insufficient blood supply to the heart causing ischemia. Despite the success of drug- and surgical revascularization therapies, prevention of clinical events of atherosclerosis remains still a major problem in current-day cardiology, rendering promotion of therapeutic neovascularization a potential approach. Bone marrow-derived cells such as monocytes/macrophages and circulating angiogenic cells (CACs) are important effectors of neovascularization. The research described in this thesis addresses novel targets and strategies using bone marrow-derived cells, which can be used to increase therapeutic neovascularization and to treat ischemic heart disease (IHD), the most common cause of CVD.

2. CARDIOVASCULAR DISEASE

2.1 From atherosclerosis to myocardial infarction

Atherosclerosis is the leading cause of myocardial infarction (MI) and the incidence with its associated morbidity and mortality is increasing globally[1]. The disease begins to develop early in life but the speed of progression varies greatly and is difficult to predict. However, it usually takes decades to develop the advanced lesions responsible for clinical disease[2]. Atherosclerosis is a focal disease process in large and medium sized arteries[2], characterized by arterial lesions containing cholesterol, fibrotic tissue, and inflammatory cell infiltrates[3]. It occurs predominantly at sites of disturbed laminar flow, notably, arterial branch points and bifurcations[4].

Atherosclerosis is recognized as a chronic inflammatory/immunomodulatory response to the presence of oxidized low-density lipoproteins (LDL) within the arterial wall[1]. Risk factors such as family history, high blood pressure, hypercholesterolemia, smoking, diabetes mellitus (DM) or altered changes in arterial blood flow shear stress (low and oscillatory) cause endothelial dysfunction resulting in LDL accumulation and oxidative modification of LDL (oxLDL)[1,2]. This oxLDL activates the endothelium, upregulating the expression of endothelial adhesion molecules such as E- and P-selectin, vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), to facilitate the entry of immune cells into the vessel wall intima[1,3,5,6] (a small area between the endothelium and the underlying smooth muscle cells of the media)[4].

The pathogenesis of atherosclerosis involves many immune cell types with a well-accepted role for monocyte/macrophages[7]. Monocytes are recruited in response to chemoattractive stimuli (CCL2/MCP-1)[5] secreted by activated endothelial cells (ECs), and adhere to the activated endothelium by specific selectins (P-selectin glycoprotein ligand-1/PSGL-1) and

integrins such as very late antigen-4 (VLA-4; α4β1; CD49d/CD29), lymphocyte function-associated antigen 1 (LFA-1; CD18/CD11a) and macrophage-1 antigen (MAC-1; CD18/CD11b)[4,6]. Following adhesion, monocytes are recruited into the intimal layer of the vascular wall by diapedesis, which is primarily dependent on platelet-endothelial-cell adhesion molecule-1 (PECAM-1; CD31) and CD99. Circulating monocytes constitute a heterogeneous population of cells expressing distinctive adhesion molecules and chemokine receptors with different adhesive and migratory properties[7]. It remains unclear whether there is differential recruitment of monocyte subsets into human atherosclerotic plaques[8] *(see paragraph "role of monocyte subsets in CVD").*

In the intima, monocytes differentiate into macrophages under the influence of endothelium-derived growth factors[6]. It is now well recognized that at least two if not three subtypes of macrophages can be observed in atherosclerotic plaques[2] *(see paragraph "role of macrophage subsets in CVD").* Macrophages respond to and phagocytose oxLDL[7]. Pattern recognition receptors, such as scavenger receptors, notably the type A scavenger receptor (SRA) and a member of the type B family, CD36, mediate macrophage uptake of oxLDL leading to the intracellular accumulation of cholesterol[1,4]. Other pattern recognition receptors such as toll-like receptors 2 and 4 (TLR2 and TLR4) are also involved in atherosclerosis[6] (*see paragraph "role of TLR activation in CVD"*). Cholesterol-loaded macrophages are a characteristic feature of plaques and are major players in all stages of plaque development (Figure 1). If engulfed cholesterol cannot exit the cell then macrophages become lipid-laden foam cells. Foam cells within the developing fatty streak produce pro-inflammatory cytokines, chemokines, proteases, growth factors, angiogenic factors, and toxic oxygen and nitrogen radicals that not only direct and amplify the local immune response but also leads to tissue injury. Furthermore, foam cells secrete extracellular matrix (ECM) components that further support lipoprotein retention within the sub-endothelium once lesions becomes established. This also leads to migration of vascular smooth muscle cells (SMCs) from the media to the intima of the artery[7].

In the intima, SMCs proliferate in response to foam cell secreted mediators, and produce ECM molecules, including interstitial collagen and elastin, contributing to a fibrous cap that covers the plaque[7,9]. This cap typically overlies a collection of cholesterol-loaded macrophages, some of which die by apoptosis, leaving behind a soft and destabilizing lipid-rich cavity containing cholesterol crystals that accumulate extracellularly[2,9]. Macrophage apoptosis coupled with defective phagocytic clearance of the apoptotic cells (efferocytosis)[2,4] promotes the accumulation of cellular debris and extracellular lipids, forming a lipid-rich pool called the necrotic core of the plaque[9]. The formation of a fibrous cap maintains plaque integrity and avoids contact of the thrombogenic necrotic core with flowing blood, creating a mature fibroatheroma[1,2].

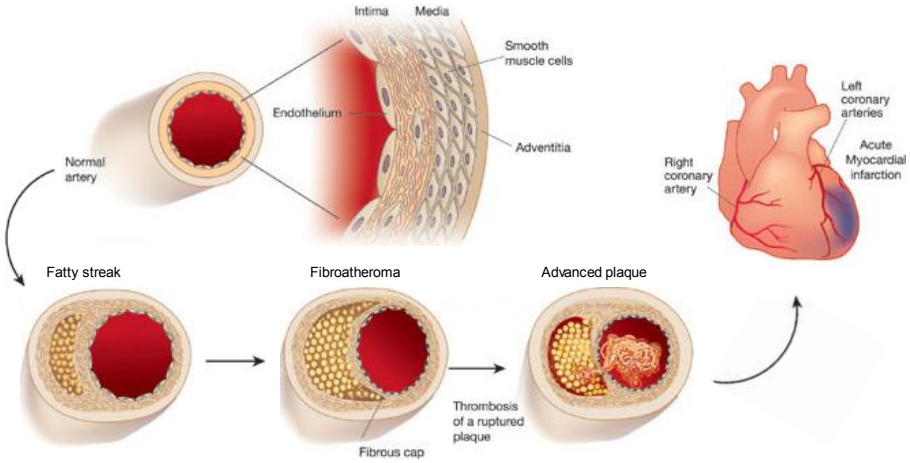

Figure 1. Progression of atherosclerosis. Atherosclerotic lesions are characterized by cholesterol, inflammatory cell infiltrates, and fibrosis in the intimal layer of an artery. Cholesterol-loaded macrophages, also called foam cells are major players during all stages of atherosclerotic plaque development. Foam cells within the developing fatty streak produce pro-inflammatory cytokines, chemokines, proteases, growth factors, angiogenic factors, toxic oxygen and nitrogen radicals, and ECM components that direct and amplify the local immune response, leads to tissue injury, support lipoprotein retention, and leads to SMC migration and proliferation. SMCs produce ECM components, including interstitial collagen and elastin, contributing to a fibrous cap that covers the plaque. This cap overlies a collection of cholesterol-loaded macrophages, some of which die by apoptosis, leaving behind a soft and destabilizing lipid-rich cavity containing cholesterol crystals that accumulate extracellularly. Macrophage apoptosis coupled with defective phagocytic clearance of the apoptotic cells (efferocytosis) promotes the accumulation of cellular debris and extracellular lipids, forming a lipid-rich pool called the necrotic core of the plaque. The formation of a fibrous cap maintains plaque integrity and avoids contact of the thrombogenic necrotic core with flowing blood, creating a mature fibroatheroma. Over time active plaque inflammation leads to advanced lesions with an increased risk of clinical instability eliciting plaque rupture and thrombus formation. Acute thrombosis may lead to acute myocardial infarction. ECM; extracellular matrix; SMC, smooth muscle cell. *Adapted by permission from Macmillan Publishers Ltd: Nature 420: 868-74, copyright 2002.*

Over time active plaque inflammation leads to advanced lesions with an increased risk of clinical instability eliciting plaque rupture (Figure 1). Atherosclerotic plaque rupture is caused by a combination of plaque biomechanical forces that are dependent on the necrotic core thickness, fibrous cap thickness, and the extent of positive coronary arterial remodeling (outward vessel wall expansion by preserving lumen)[1]. High-risk atherosclerotic lesions such as those with positive remodeling, large lipid-rich necrotic core (>30% of plaque) covered by a thin-fibrous cap (<65 µm) highly inflamed by pro-inflammatory macrophages and less by SMCs, intra-arterial neovascularization (angiogenesis), adventitial inflammation, and spotty pattern of calcifications may rupture[2], allowing the pro-coagulant lesion to come in contact with the coagulation proteins and platelets in the flowing blood, leading to thrombus formation[4]. The macrophages within the cap cause plaque destabilization, and ultimate cap rupture and thrombosis by secreting proteolytic enzymes such as matrix metalloproteinases

(MMPs) that degrade matrix components[2], coagulation initiator tissue factor that renders the lipid core thrombogenic, and by generating mediators that provoke the death of SMCs[9]. Macrophages may also reduce collagen synthesis in SMCs without actually killing the cells[4]. Neovascularization inside the arterial wall and atherosclerotic plaques plays a critical role in pathogenesis of heart attacks. The two known mechanisms resulting in the formation of new vessels within the plaque are local ischemia, and inflammation[10]. As the plaque progresses, small capillaries in the adventitia (vasa vasorum) start to proliferate crossing into the intima from the media which leads to neovascularization within the plaque[2,10]. Neovascularization contributes to atherosclerotic lesion progression in two ways. It leads to the focal accumulation of inflammatory cells (i.e. monocytes/macrophages) inducing a pathological circle[10]. In addition, these neovessels lack supporting cells and are fragile and leaky, which favor plaque haemorrhage[2,11]. Angiogenesis-derived plaque haemorrhage is defined as the presence of plasma proteins and red blood cells (erythrocytes) within a plaque. Erythrocytes are important source of free cholesterol and cholesterol esters, which may induce plaque expansion.

Plaque haemorrhage not only occurs from neovascularization (angiogenesis), but may also occur from plaque rupture. During intraplaque haemorrhage, plaque ruptures and an intraintimal thrombus forms containing not just erythrocytes but mainly fibrin and platelets[2]. Acute thrombosis may lead to sudden cardiac death, acute MI ("heart attack"), unstable angina (accelerating chest pain due to ongoing heart muscle ischemia) or ischemic stroke[2,4].

2.2 Role of monocyte subsets

Monocytes are mononuclear phagocytes of myeloid origin that account for about 6% of total peripheral blood leukocytes in adults[12]. Monocytes are the main component of the innate immune system that is responsible for counteracting exogenous bacterial, viral, and fungal infections mainly by phagocytosis. However, they are also involved in endogenous inflammatory processes[13]. Monocytes constitute a heterogenous cell population, containing subsets with distinct biological functions. In humans and mice at least two monocyte subsets have been described[14].

In humans they are usually classified according to the expression levels of the cell-surface lipopolysaccharide (LPS) receptor CD14 and the FcγIII receptor CD16[12,14]. Human monocytes have been divided into three populations: the classical $CD14^{++}CD16^{-}$ monocyte subset which constitute ~85% of total monocytes in the peripheral blood, an intermediate $CD14^{++}CD16^{+}$ subset (~5% of total monocytes), and a non-classical $CD14^{+}CD16^{++}$ subset (~10% of total monocytes). These three subsets differ significantly in phenotype and function[12]. The classical $CD14^{++}CD16^{-}$ subset highly express CCR2, FcγRI (CD64), CD62L (L-selectin), scavenger receptor class A (ScR-A) and VEGFR-1. The non-classical $CD14^{+}CD16^{++}$ cells display higher levels of HLA-DR, CD11c, CX3CR1 and CCR5, but do not express CCR2. The intermediate $CD14^{++}CD16^{+}$

subset can be clearly discriminated from the non-classical $CD14^{+}CD16^{++}$ subset by the expression of CCR2. Nonetheless, this subset has some unique features which separates it from the other two subsets. For instance, $CD14^{++}CD16^{+}$ monocytes have higher expression of CXCR4, Tie2, KDR (VEGFR-2), endoglin (CD105), MHC class II and HLA-DR compared to the other two subsets[12,13]. In mice, two main monocyte subsets can be distinguished based on their expression of Ly-6C/Gr-1. Ly-6Chigh/Gr1+ monocytes are CCR2high CX3CR1low CD62L+ PSGL-1high (inflammatory), whereas Ly-6Clow/Gr1- monocytes are CCR2low CX3CR1high CD62L- PSGL-1low (resident/patrolling)[14]. The Gr1- monocytes also intensively express CCR5[12]. The $CD14^{++}CD16^{-}$ and $CD14^{++}CD16^{+}$ monocytes resemble the murine Ly-6Chigh/Gr1+ monocytes, whereas $CD14^{+}CD16^{++}$ monocytes resemble Ly-6Clow/Gr1- monocytes[14,15]. During steady state conditions, inflammatory and resident monocytes exist approximately at a 1:1 ratio in the blood of mice[16].

The involvement of different monocyte subsets during primary atherosclerosis as well as their contribution to angiogenesis and tissue regeneration secondary to plaque rupture have recently emerged[12]. Human monocyte subsets do not reflect the same behavior as murine monocyte subsets in the setting of CVD. Clinical and experimental studies point to a prominent role of CD16-positive monocytes i.e. intermediate and/or non-classical monocytes in human atherosclerosis, while classical Ly-6Chigh/Gr1+ monocytes are considered as central drivers of murine atherosclerosis[6,14]. A number of studies have also explored how monocyte subsets change during acute cardiovascular events. Most studies report an association of intermediate monocytes with active CVD, whereas some suggest that classical monocytes are a negative prognostic indicator[14]. One might also speculate that the intermediate monocyte subset might play a role in myocardial healing post-MI[13] since these monocytes phenotypically resemble pro-angiogenic monocytes[10]. However, the cause of such associations remains unclear, and the exact function of the intermediate monocytes is still under investigation[10]. A mouse model of MI demonstrated that Ly-6Chigh monocytes dominate the early stages exhibiting phagocytic, proteolytic, and inflammatory functions while Ly-6Clow monocytes promote healing and angiogenesis in the later stages[17]. A balanced and coordinated subset recruitment is likely important because atherosclerotic mice with Ly-6Chigh monocytosis have impaired healing of the infarcted myocardium[18]. By contrast, depletion of Ly-6Chigh monocytes early post-infarct led to increased areas of debris and necrotic tissue with impaired ventricular healing[17].

2.3 Role of macrophage subsets

Monocytes can give rise to phenotypically and functionally distinct macrophages. It is not known whether a transition from a distinct monocyte subpopulation to a specific macrophage type exists or whether macrophage phenotypes within plaques are influenced by the heterogeneity of circulating monocytes[7]. Macrophages are known to be plastic cells

that retain their ability to respond to their microenvironment, and change their phenotype, once differentiated. Therefore changes in the plaque microenvironment are likely to profoundly alter the function of macrophages within it. Changes in macrophage phenotype and function within the atherosclerotic plaque have profound consequences for plaque biology, including rupture and thrombosis leading to clinical events such as MI[8].

The most common macrophage phenotype in both murine and human atherosclerotic plaques are the classically activated M1 macrophages, which dominate with plaque progression. M1 macrophages are induced by IFN-γ or other T helper 1 (Th1) cytokines, and secrete pro-inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and -12 (IL-12), and reactive nitrogen and oxygen intermediates. This promotes continued recruitment of immune cells, the activation of ECs by upregulation of adhesion molecules, additional oxidation and accumulation of LDL-derived cholesterol within the arterial wall, vascular SMCs migration from the media to the intima of affected arteries, and cell apoptosis[1,2,5,7-9]. Within the atherosclerotic plaque, these M1 macrophages are foamy in nature (high lipid intake) and show MHC class II expression required for antigen presentation and activation of T cells[2].

However, depending on the microenvironment, macrophages can also develop functions that facilitate tissue repair, remodeling and restoration of normal tissue homeostasis[7]. The alternative M2 macrophages are activated by a different pathway through T helper 2 (Th2) cytokines, i.e. IL-4 and IL-13[2]. M2 macrophages may inhibit the atherosclerotic disease progression by dampening the inflammatory response through secretion of anti-inflammatory cytokines such as IL-10 and transforming growth factor β (TGF-β)[1]. These macrophages express mannose receptor, phagocytose cytotoxic lipoproteins, clear apoptotic bodies, induce ECM synthesis and SMC proliferation that stabilize vulnerable plaques[7]. A third type of anti-inflammatory macrophages existing only in the human atherosclerotic plaque occurs at the sites of haemorrhage or angiogenesis[2]. These haemoglobin stimulated macrophages express both CD163 and mannose receptors, and lack scavenger receptors. Instead they have high expression of ATP-binding cassette transporters inducing efflux of excess cholesterol, mediating reverse cholesterol transport and plaque regression. They produce IL-10, but do not secrete pro-inflammatory cytokines such as TNF-α and have reduced production of inducible nitric oxide synthase (iNOS) compared to foam cells[19,20].

Moreover, besides inflammatory signals of leukocyte origin, also microbial products drive different forms of macrophage activation and polarization[5] (*see paragraph "role of TLR activation in CVD"*).

2.4 Role of TLR activation

Pattern recognition receptors, of which the TLRs are a major family, serve to initiate inflammatory signaling in response to the detection of conserved microbial pathogen-associated molecular patterns (PAMPs). PAMPs are highly conserved molecules that are present in bacteria, yeast or viruses but not in mammalian cells[21]. Ten functional TLRs (TLRs 1-10) have been identified in humans. TLRs 1, 2, 5 and 6 are expressed on the cell surface of mammalian cells, whereas TLR 3, 7, 8 and 9 are expressed in intracellular compartments, primarily endosomes and the endoplasmic reticulum. TLR4 can signal both at the plasma membrane and at endosomes. TLR10 is the most recent member of the human TLR receptor family discovered with unknown function[22,23]. TLRs are type 1 membrane-spanning receptors that contain a leucine-rich repeat extracellular motif required to recognize PAMPs and an intracellular signaling motif that is similar to those of interleukin (IL)-1 and IL-18 receptors[22,24]. This common intracellular signaling motif is termed the 'Toll-IL-1 receptor' (TIR) homology domain[24]. TLRs signal by forming homo- or heterodimers[22]. Each TLR activates a number of signaling pathways, some of which are common to all TLRs and some of which are specific to particular TLR types[24]. The TLR signaling pathway is dependent on whether the cytoplasmic adaptor protein myeloid differentiation factor 88 (MyD88) or TIR-domain-containing adaptor inducing interferon-β (TRIF) is recruited to the TIR domain. All TLRs, except TLR3, use the MyD88 dependent pathway to initiate downstream signaling, although both TLR3 and TLR4 use the interferon regulatory factor-3 (IRF-3) dependent pathway via recruitment of adaptor molecule TRIF. IRF3 mediates the production of type I IFNs and subsequently IFN-induced genes. TLR2 and TLR4 have been shown to be involved in triggering a cascade ultimately resulting in nuclear factor-kappaB (NF-KB) activation and pro-inflammatory cytokine release[16] (Figure 2). TLRs are expressed on all cell types associated with atherosclerotic plaques, such as monocytes/macrophages, ECs, vascular SMCs and platelets[25].

Recent genetic studies in atherosclerosis-prone apolipoprotein E (apoE) or low-density lipoprotein receptor (LDLR)-deficient mice have revealed a central role for TLR-signaling in the development of atherosclerosis[21]. TLR2 and TLR4 have been extensively studied in relation to inflammation in CVD. TLR2 and TLR4 expression[26-31] and their activation by exogenous ligands have been demonstrated to be implicated in atherosclerosis. TLR4 agonist LPS[32-34], or TLR2 agonists peptidoglycan (PGN)[35], and Pam3CSK4[29,36], were shown to accelerate atherogenesis in experimental- and patient studies. PAMPs such as PGN[37] and LPS[38] have been identified as constituents of human atheroma. PAMPs can promote EC activation, monocyte adhesion and foam cell formation, key processes of atherosclerosis[21]. It has been proposed that in addition to exogenous molecules i.e. PAMPs, endogenous molecules i.e. products of host tissue damage, so-called damage associated molecular patterns (DAMPs) may also stimulate TLR-signaling[39-42]. Several endogenous TLR activators are described in relation to atherosclerotic disease. Such endogenous TLR activators include heat-shock

protein (HSP)-60 and -70[43-45], myeloid-related protein (MRP)-8 and -14 also known as S100A8 and S100A9[46-48], high mobility group box-1 (HMGB-1)[49], oxLDL[50] and minimally modified LDL (mmLDL)[51-53] or fibronectin extra domain A (EDA)[54,55].

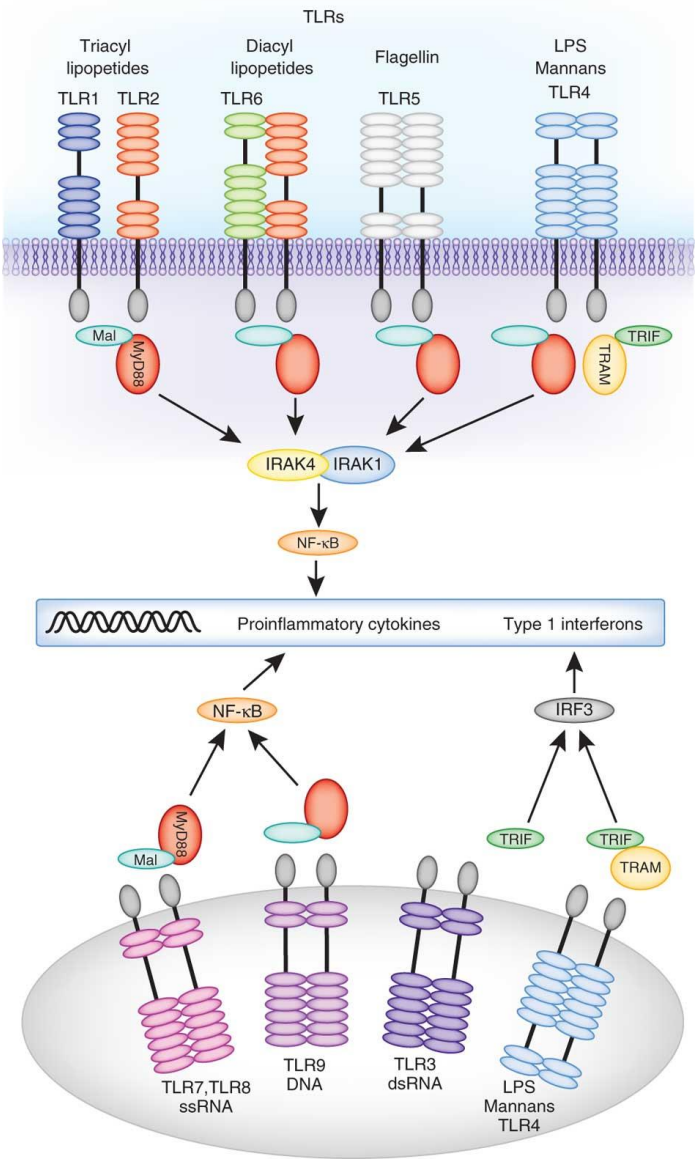

Figure 2. TLRs, their main ligands and some of the intracellular pathways that lead to the induction of pro-inflammatory cytokine and type I IFN release. TLR, Toll-like receptor; IFN, interferon. *Reprinted by permission from Macmillan Publishers Ltd: Nature Immunology. 13: 535-42, copyright 2012.*

In addition to promoting atherosclerosis, studies have suggested that TLR-induced inflammation may influence atherosclerotic plaque stability, and hence may contribute to the development of acute coronary syndrome (ACS) in patients with coronary artery disease (CAD)[56]. In humans, levels of circulating TLR4-positive monocytes, monocytic TLR4 expression and TLR4 signaling are increased in patients with unstable angina and MI[57-59]. Furthermore, increased TLR2 expression is found in circulating monocytes of patients with acute MI[60]. Experimental data are in line with these clinical findings. Mice with a deficiency of TLR4[61] have reduced infarct sizes when compared to wild-type controls. Moreover, mortality and left ventricular remodeling are reduced in mice with deficiency of TLR4[62] or TLR2[63]. TLRs could be pivotal in the polarization of macrophages during atherosclerosis. TLR4 has been implicated in M1 polarisation[64], and their genetic deletion reduces atherosclerosis development[28].

2.5 Role of diabetes and oxidative stress

In DM, accelerated atherosclerosis and reduced angiogenic response to myocardial ischemia has been observed[65]. Cardiac morbidity and mortality of patients with DM is greatly increased[66]. Hyperglycemia is one of the major risk factors for vascular complications in DM[67]. In patients with DM, the production of reactive oxygen species (ROS) is increased, which could contribute to the onset and development of vascular complications[68]. ROS can be generated from numerous sources within the cell, including mitochondria, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase, xanthine oxidase, and endothelial nitric oxide synthase (eNOS) uncoupling[69]. Mitochondrial ROS production has been recognized as an important mediator of hyperglycemic vascular damage[70].

2.6 Treatment

Cardiovascular medicine faces a need for effective prevention of MI, which constitutes the clinical manifestations of atherosclerosis[71]. Medical management of atherosclerosis and its manifestation aims at retardation of progression of plaque formation, prevention of plaque rupture, and subsequent events and treatment of symptoms. In addition to a healthy lifestyle, drug therapy aims at retardation of atherosclerosis progression, or even its regression, and at prevention of cardiovascular events such as death or MI. Preventive drug treatments includes aspirin (anti-thrombotic), statins, angiotensin-converting-enzyme (ACE)-inhibitors, angiotensin receptor blockers, and β blockers in patients after MI. In addition, anti-thrombotic agents such as clopidogrel, prasugrel, and ticagrelor reduce recurrent events after an ACS. Revascularization by either percutaneous coronary intervention (PCI) or coronary artery bypass surgery (CABG) is performed as treatment of flow-limiting coronary stenosis to reduce myocardial ischemia and its manifestations. In high-risk patients with ACS, a routine invasive strategy with revascularization in most patients provides the best outcome with a significant reduction in MI and death compared with an initial conservative non-invasive strategy i.e. drug therapy[72].

Despite the success of drug therapy and invasive revascularization therapies, prevention of clinical events of atherosclerosis remains a major problem in current-day cardiology[71]. Many patients with CAD suffer from disabling symptoms despite intense pharmacotherapy and are not eligible for invasive revascularization by PCI or surgery[73]. The above approaches may not always be feasible for patients with severe diffuse occlusive arterial disease and poor target vessels for grafting. Furthermore, the risks associated with current revascularization approaches include acute thrombotic occlusion or gradual restenosis of stents, and occlusion of bypass grafts[74]. Thus, better therapeutic opportunities are needed to limit the extent of heart damage starting once acute ischemia has occurred, rendering promotion of therapeutic neovascularization a potential approach.

3. BONE MARROW-DERIVED CELLS IN NEOVASCULARIZATION

Therapeutic neovascularization offers promise as a novel treatment for IHD, particularly for patients who are not candidates for current methods of revascularization[73]. After birth two processes of neovascularization can be distinguished, namely angiogenesis and arteriogenesis. Angiogenesis and arteriogenesis represent the final targets of therapeutic neovascularization aimed at providing an alternative treatment strategy for patients with CAD[75]. Angiogenesis and arteriogenesis are critical processes involved in the response of the organism to ischemic injury, and contribute to tissue revascularization and organ preservation[76]. Angiogenesis describes the growth of new capillaries by sprouting from pre-existing vessels in an ischemic area through the migration and proliferation of mature ECs[77]. Capillaries are cellular tubes comprising only of ECs and lacking other typical wall cells like SMCs or fibroblasts[78]. Arteriogenesis, i.e. the growth and outward remodeling of pre-existing collaterals into functional conductance arteries after proximal arterial obstruction, also relies on the proliferation of ECs and on SMCs, as well as fibroblasts, and strongly depends on leukocyte accumulation in the perivascular space[77,79]. Both processes are driven by distinct, but partially overlapping, cellular and molecular pathways[75]. For example, hypoxia (lack of oxygen) is the major driving force for angiogenesis in the setting of ischemia and involves the activation of transcription factor hypoxia-inducible factor 1-alpha (HIF-1α), whereas fluid shear stress (FSS) might be the most important trigger for initiation of collateral growth which proceeds independently of hypoxia[75,78]. Besides these specific initial triggers, angiogenesis and arteriogenesis share cellular and molecular mediators including inflammatory cells and pro-inflammatory cytokines, growth factors, chemokines, proteases, which play different roles in promoting and refining these processes[75].

Optimization of pro-angiogenic therapies might include stimulation of both angiogenesis and vessel maturation by arteriogenesis[80]. Although angiogenesis alone stimulates the formation of immature, leaky, and unstable blood vessels leading to poor tissue perfusion,

arteriogenesis is indispensible producing mature high-conductance vessels[80,81]. Thus, arteriogenesis is the main blood vessel growth process which can compensate for blood flow deficits caused by arterial occlusions. It has been known that patients with ischemic vascular diseases spontaneously develop natural collateral arteries bypassing the occlusion side[78]. A good collateral circulation has a significant benefit and lowers mortality in patients with CAD[82].

Arteriogenesis is associated with an increase in blood flow and ultimately FSS over pre-existing collaterals occurring after arterial occlusion. FSS induces EC activation, with a subsequent upregulation of cell adhesion molecules (ICAM-1 and -2, and VCAM-1) and chemokines (i.e. monocyte chemoattractant protein-1, MCP-1) and consequent enhanced recruitment of leukocytes i.e. monocytes to the perivascular space[75,78,79,83]. Once in the perivascular region, monocytes differentiate into macrophages and create an inflammatory environment[83]. There is convincing evidence that monocytes and macrophages promote arteriogenesis through paracrine effects[74]. The potential importance of monocyte subsets and macrophage skewing in arteriogenesis will be discussed later in the paragraphs *"role of monocyte subsets in neovascularization"* and *"role of macrophage subsets in neovascularization"*. Monocytes/macrophages produce a pool of factors that sustain inflammation such as TNF-α, thus increasing leukocyte recruitment, participate in ECM remodeling through the secretion of MMPs, and stimulate the proliferation of fibroblasts, ECs and SMCs by secreting fibroblast growth factor (FGF)-2 or bFGF, platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF)-A. The stimuli that trigger the production of these cytokines, growth factors, and enzymes by monocytes and macrophages at sites of collateral growth are largely unknown[79]. Monocytes/macrophages also express iNOS. When induced, macrophages release abundant amounts of nitric oxide (NO), which might contribute to arteriogenesis[74]. Recently, the importance of the expression of receptors for PAMPs such as TLR2 and TLR4 by leukocytes infiltrating sites of collateral artery growth has been discovered[79] (*See paragraph "role of TLR activation in neovascularization"*).

New revascularization strategies have been employed with the goal of relieving the symptoms of CAD and improving cardiac function by restoring blood flow to the ischemic tissue limiting tissue necrosis. Such strategies to improve neovascularization focused on the growth of new blood vessels in the myocardium using various potential angiogenic growth factors with limited success such as VEGF-A or bFGF[73,78,80,81,83]. VEGF-A selectively stimulates ECs to proliferate, while FGF-2 not only stimulates ECs more potently, but additionally stimulates SMCs and fibroblasts to proliferate[81]. Since the growth process includes complex interactions between factors to mediate cell proliferation and migration as well as digestion and reassembly of wall structures, the application of single factors most likely did not reach far enough to encompass all steps of the neovascularization process[78].

Another complicating factor has been the fact that many of these compounds that are able to stimulate vascular growth also have potential adverse effects including hypotension, local edema, anemia, thrombocytopenia, and renal toxicity, and accelerate atherosclerosis and plaque destabilization by inducing plaque angiogenesis, precluding their use in a clinical setting[73]. Apart from VEGF-A and FGF-2, also MCP-1 treatment stimulated atherosclerosis besides arteriogenesis stimulation[84]. Aside from growth factor administration, the principal alternative approach currently under investigation is cell-based therapy. Endothelial progenitor cells (EPCs) are the cell types most widely-researched for their ability to induce cardiac neovascularization[81].

3.1 Role of endothelial progenitor cells

Hitherto, EPCs represent the most widely studied adult human progenitor cell subpopulation[85]. EPCs are involved in the maintenance of endothelial homeostasis and in the process of neovascularization of ischemic tissue[3]. EPCs, first isolated by Asahara *et al.* in 1997 from adult peripheral blood, play an important role in the recovery and repair of injured endothelium. Hence, these cells have been termed putative EPCs and were described to express the hematopoietic stem cell marker CD34 and endothelial cell marker vascular endothelial growth factor receptor (VEGFR-2, also known as kinase domain insert receptor, KDR)[86]. Accordingly, another primitive marker that has been used to identify putative EPCs is AC133 or CD133[87]. EPCs are precursor cells that express some cell surface markers characteristic of mature endothelium and some from hematopoietic stem cells. EPCs are mobilized from the bone marrow (BM) into the peripheral blood in response to a chemical or mechanical injury of the endothelium caused by tissue ischemia or trauma[3]. Other sources of EPCs include the spleen, adipose tissue, the vascular wall and the adventitia[87]. One of the most essential triggers for mobilization of EPCs is the CXC chemokine stromal cell-derived factor-1α (SDF-1α/CXCL12), which specifically binds to the CXC chemokine receptor-4 (CXCR4). In addition, hypoxia and some other cytokines (e.g. VEGF-A; erythropoietin; macrophage colony-stimulating factor, M-CSF; granulocyte colony- stimulating factor, G-CSF; and granulocyte monocyte colony-stimulating factor, GM-CSF) have been shown to increase the number of EPCs[85,88,89]. The mobilized EPCs migrate to sites of injured endothelium where they differentiate into mature ECs, incorporate into sites of neovascularization and contribute to re-endothelialization of injured arteries by replacing dysfunctional ECs[3]. EPCs also display paracrine activity within the vascular system by delivering angiogenic growth factors on mature ECs, and proteases inducing neovascularization[3,88,90].

Currently, two types of EPCs, namely early and late outgrowth EPCs, can be derived and identified from peripheral blood with differing properties and origin, and the potential for therapeutic use in neovascularization. Although they have different roles in neovascularization, most of the previous studies mainly focused on early rather than late

EPCs[91,92]. Currently, the definition of EPCs and their functional characteristics are under debate[88]. EPCs correspond to a heterogeneous population of multiple origins and phenotype. Recent data have additionally described some $CD14^{+}$/low myeloid subsets as functional angiogenic cells with contributions to endothelial repair and ischemic neovascularization. EPCs are cultured by plating of isolated peripheral blood-derived mononuclear cells (PBMCs) on fibronectin- or collagen-coated plastic in endothelial-specific growth medium (EGM). This *in vitro* approach results in two major types of EPCs which have been classified according to the time at which they appear in culture, early EPCs and late outgrowth EPCs.

First, early EPCs develop after short-term culture of 4-7 days described as adherent spindle-shaped cells, also referred as circulating angiogenic cells (CACs). Notably, these "early" endothelial-like cells share common phenotypical properties of monocytes (e.g. CD14, pan-leukocyte marker CD45) and ECs (CD31; KDR; eNOS) and contribute indirectly to neovascularization mainly by secreting angiogenic cytokines and proteases in a paracrine manner. These cells have lower capacity for EC marker expression than late outgrowth EPCs[85,87,88,90,93]. Furthermore, early EPCs exhibit low proliferative capacity and do not incorporate into vascular structures[94]. EPC-colony-forming units (CFU) is another culture method used by Hill *et al.*[95] for isolating and enumerating early EPCs. EPC-CFU are derived from re-plated non-adherent cells of seeded PBMCs after 48 h onto fibronectin-coated plates in a special differentiation medium. These cells form tight clusters of T-lymphocytes surrounded by monocytic cells and promote *in vitro* angiogenesis indirectly via paracrine mechanisms[88,93,94,96]. Early EPCs are consistent with the original cells described by Asahara *et al.*[86], containing $CD34^{+}KDR^{+}$ cells[88]. Second, after long-term culture of 2-3 weeks a "late" proliferative outgrowth EPC (endothelial colony forming cells, ECFCs) with characteristics of mature ECs and cobblestone morphology arises and these cells directly enhance neovascularization by developing into mature ECs incorporating into vascular structures[85,87,88]. It has been suggested that late outgrowth EPCs are derived from cells in the $CD34^{+}$ fraction of PBMCs that do not express the pan-leukocyte marker CD45, nor CD14 and CD133[94]. These findings suggest that late outgrowth EPCs and not early EPCs are 'true' progenitors of ECs[87]. Late outgrowth EPCs can also be derived from umbilical cord blood[97]. Dil-Ac-LDL uptake and lectin binding are typical functional properties of ECs and both early and late outgrowth EPCs are $acLDL^{+}Lectin^{+}$ [85,88].

Numerous experimental studies have reported the therapeutic potential of exogenously administered EPCs in neovascularization. Both early and late outgrowth EPCs show comparable *in vivo* neovascularization capacity in an animal model of hind limb ischemia[91,92]. Furthermore, mixed transplantation of early and late EPCs has synergistic effects on neovascularization in an animal model of hind limb ischemia[92] (Figure 3). A large number of preclinical and clinical studies have examined the use of EPC therapy post-MI to improve healing and restore cardiac function. Transplantation of *ex vivo* expanded EPCs

improves neovascularization and left ventricular function after MI in a rat model[98]. It has been demonstrated in initial pilot studies that the use of EPCs was safe and feasible for the treatment of CVD[99,100]. However, recent randomized clinical trials showed mixed results of EPC therapy following AMI[101-104], which largely arise as the result of using i.e. ill-defined, heterogeneous bone marrow mononuclear cell preparations and different methods for cell preparation.

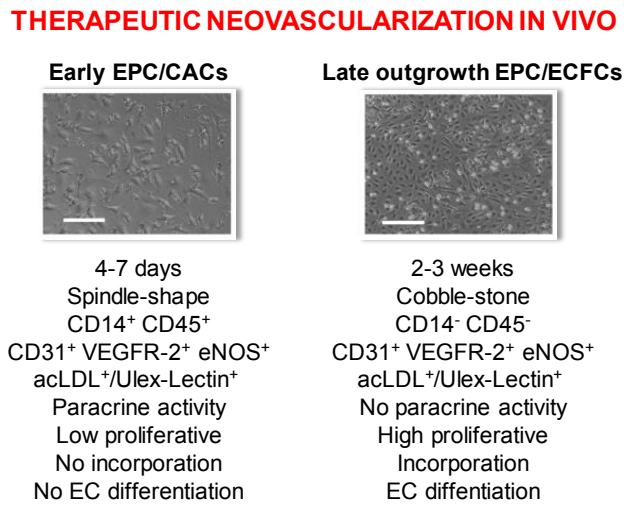

Figure 3. Morphological, immunophenotypical and functional characterization of early EPC/CACs and late outgrowth EPC/ECFCs. Early EPC/CACs are derived after 4-7 days, and late outgrowth EPC/ECFCs after 2-3 weeks of in vitro culture of bone marrow- and/or peripheral blood-derived mononuclear cells in EGM. Both EPC types promote therapeutic neovascularization in vivo by different mechanisms. EPC, endothelial progenitor cell; CAC, circulating angiogenic cell; ECFC; endothelial colony forming cell; EGM, endothelial growth medium; EC, endothelial cell. *This figure was published in Experimental Cell Research. Vol 314, Mukai, N et al. A comparison of the tube forming potentials of early and late endothelial progenitor cells, pp 430-40, Copyright Elsevier (2008).*

Numerous clinical studies have supported the notion that EPCs are also deregulated by cardiovascular risk factors or in CVD. Several clinical conditions characterized by both increased inflammation and oxidative stress such as DM, hypertension, and hyperlipidemia are significantly associated with reduced numbers and impaired functionality of EPCs. In addition, experimental and clinical studies have shown that atherosclerosis is associated with reduced numbers and dysfunction of EPCs[3,105]. For example, in DM, hyperglycemia is a key risk factor for the development of vascular complications such as impaired arteriogenesis[66,106-108]. Recently, hyperglycemia was shown to reduce the number and function of circulating blood–derived progenitor cells, mainly early EPCs, both *in vitro*[109,110] and *in vivo*[111-113]. The reduced number and function of early EPCs were also found to associate with the pathogenesis of

vascular complications in either type 1[112,114] or type 2[111,113,115] DM. Deficiencies in generation of eNOS-derived NO[116] and an increased level of oxidative stress[117,118] have been proposed as mechanisms responsible for EPC dysfunction in DM. Besides glucose, also free or non-esterified fatty acids (NEFAs) are elevated in DM patients, and contribute to increased oxidative stress generation[119].

3.2 Role of monocyte subsets

Post-ischemic neovascularization is highly dependent on monocyte levels in the bloodstream[76]. As discussed previously, Ly-6Chigh and Ly-6Clow monocytes infiltrate the ischemic heart sequentially, Ly-6Chigh monocytes being predominant in the early phase of myocardial healing, while Ly-6Clow monocyte infiltration peaks at later time points. The specific role of these mouse subsets in post-MI neovascularization, as well as that of their human counterparts remains unclear.

Although Nahrendorf *et al.* proposed that Ly-6Clow monocytes promoted angiogenesis through VEGF secretion in the ischemic myocardium, depletion of either subset in mice hampers neovascularization after MI[17]. Moreover, several line of evidence suggest that Ly-6Chigh, but not Ly-6Clow monocytes promote angiogenesis in hind limb ischemia models[76,120], presumably in a MMP-9 dependent manner[76]. On the other hand, both subsets enhanced arteriogenesis[76]. Few studies have so far addressed the role of human monocyte subsets during neovascularization. Gene expression profile analysis in monocyte subsets recently linked human $CD14^{++}CD16^{+}$ monocytes to angiogenesis by their expression of the pro-angiogenic receptor Tie2[121]. Moreover, the angiogenic potential of Tie2-expressing monocytes, a cell type in some aspects resembling $CD14^{++}CD16^{+}$ monocytes was demonstrated *in vivo*[122]. In a previous study, transfusion of freshly isolated $CD14^{+}$ monocytes did not improve neovascularization, whereas injection of cultivated $CD14^{+}$ cells did enhance blood flow recovery after murine hind limb ischemia[123]. In contrast, Awad *et al.* found that also unstimulated $CD14^{+}$ PBMCs were able to increase vascular healing and growth[124]. In another study, *in vitro* angiogenesis by ECs was enhanced by freshly isolated unfractionated monocytes and, to a lesser extent, by $CD16^{-}$ monocytes in a paracrine manner. In the mouse hind limb ischemia model, increased perfusion was observed in mice treated with unfractionated monocytes, irrespective of their subtype. Interestingly, mice that received $CD16^{+}$ monocytes had an increased arteriole size (arteriogenesis), whereas the number of capillaries (angiogenesis) did not significantly differ between unfractionated monocytes, $CD16^{-}$ and $CD16^{+}$ monocytes. The above findings indicate that most probably both $CD16^{-}$ and $CD16^{+}$ monocyte subsets contribute to neovascularization in a synergistic, paracrine manner[77].

3.3 Role of macrophage subsets

It is known that different subsets of macrophages coexist *in vivo* and exert different functions depending on their environmental stimulus. Classically activated M1 macrophages are induced by LPS, IFN-γ and/or TNF-α and are pro-inflammatory. On the other hand, M2 macrophages can be induced by IL-4/IL-13 (M2a) or IL10/glucocorticosteroids (M2c) and are considered anti-inflammatory[125]. Several inducers and functions of different polarized macrophage populations are shown in Figure 4.

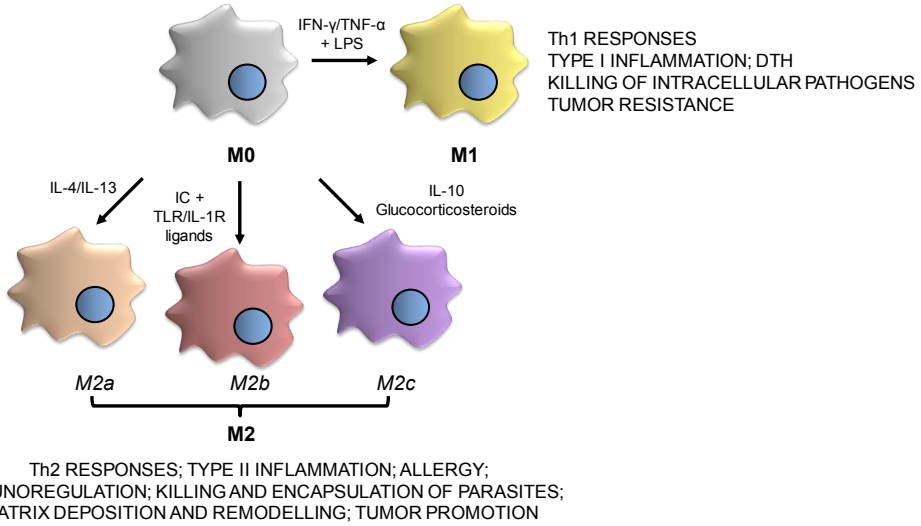

Figure 4. Inducers and functions of different polarized macrophage populations. M0 macrophages can polarize to M1 macrophages after exposure to IFN-γ or TNF-α and LPS and exert cytotoxic and antitumoral functions. Additionally, M0 macrophages can polarize to M2 macrophages, which have immunoregulatory and protumoral activities. M2 macrophages can be subdivided in three different macrophage subtypes, in particular M2a (after exposure to IL-4 or IL-13), M2b (after combined exposure to immune complexes and TLR or IL-1R agonists), and M2c (after exposure to IL-10 or glucocorticosteroids). M2a and M2b exert immunoregulatory functions and drive type II responses, whereas M2c macrophages are more related to suppression of immune responses and tissue remodeling. IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; IC, immune complexes; IL-1R, interleukin-1 receptor; TLR, Toll-like receptor; DTH, delayed-type hypersensitivity. *Adapted from Trends Immunology, Vol 25, Mantovani, A et al. The chemokine system in diverse forms of macrophage activation and polarization, pp 677-86, Copyright (2004), with permission from Elsevier.*

The effect of macrophage heterogeneity on arteriogenesis was investigated in a study performed by Takeda *et al*. They have recently shown that haplodeficiency of the prolyl hydroxylase domain-containing protein 2 (PHD2), an oxygen sensor involved in the ubiquitin mediated proteasomal degradation of HIF, determines macrophage skewing toward the M2 phenotype and protects against ischemia by inducing arteriogenesis in mice.

Improved arteriogenesis in PHD2 heterozygous mice was due to an expansion of M2-like macrophages and increased production of pro-arteriogenic factors, leading to enhanced SMC recruitment and growth[126]. Another study showed that local injection of exogenous polarized murine macrophages, M1 (IFN-γ), M2a (IL-4) or M2c (IL-10) into mice after femoral artery ligation improves reperfusion recovery regardless of the subset. Furthermore, preventing endogenous M2c polarization did not affect reperfusion recovery suggesting that M2c's are not required for arteriogenesis, but are sufficient to induce arteriogenesis upon exogenous administration[127]. Polarization of macrophages therefore is important for arteriogenesis. Although the effect seems to be independent of the direction of polarization, the mode of action of macrophage subtypes on arteriogenesis may differ. This was confirmed by a recent study by Troidl *et al*.[128] in a rat model with chronically elevated FSS, where they showed that M1 and M2 macrophages both contribute to arteriogenesis and show distinct temporal and spatial distribution. Both populations increased over time, but the M2 macrophages dominated at the site of collateral growth. Whereas M1 macrophages were detected adjacent to the media, M2 macrophages were localized in the outer perivascular region of the growing collateral vessel. They speculated that during arteriogenesis these M1 macrophages increase the recruitment of circulating monocytes and that M2 macrophages display vascular remodeling function. Furthermore, the activation state of macrophages was systemically modulated in mice with femoral artery ligation by injections of either glucocorticosteroids or the interleukins IL-10, IL-4/IL-13. Suppressing the global inflammatory response with glucocorticosteroids led to impaired perfusion recovery, whereas increasing M2 macrophage differentiation by IL10 or IL4/IL13 administration significantly increased perfusion recovery[128].

3.4 Role of TLR activation

TLR activation has also been associated with neovascularization. Blocking TLR2 signaling promotes angiogenesis by ECs *in vitro*. In a murine model of hind limb ischemia, using TLR2-deficient or anti-TLR2 antibody-treated wild-type mice resulted in increased new capillary formation and enhanced reperfusion[129]. In contrast, many studies showed that TLRs may activate neovascularization processes. Pollet *et al.* showed that LPS stimulates EC sprouting *in vitro* and angiogenesis *in vivo* by signaling via TLR4[130]. Endogenous ligands such as HMGB1 released by ischemic endothelial and skeletal muscle cells, mediates angiogenic behavior of ECs, and increases perfusion in murine ischemic hind limbs[131]. In another study it was shown that TLR2 activation promotes EC migration and permeability, and lymphocyte invasion through endothelial cells *in vitro*. *In vivo*, TLR2 activation promotes limb perfusion, which may be associated with the high serum levels of TNF-α and IL-6, and lymphocyte invasion[132]. Infusion of the TLR4 ligand LPS has shown to result in stimulation of angiogenesis and arteriogenesis after hind limb ischemia by recruiting and activating monocytes[133]. Another

study showed that TLR2-deficient and TLR4-defective mice have a significant reduction of collateral perfusion. Attenuated perfusion restoration is likely due to decreased extravasation of leukocytes. Indeed, both strains showed significantly reduced monocyte/macrophage accumulation in the perivascular tissue of growing collateral arteries compared to their wild-types. Furthermore, transplantation of wild-type bone marrow into TLR2 deficient and TLR4 defective mice rescued the impaired arteriogenesis, while injection of TLR2 deficient and TLR4 defective bone marrow into wild-type mice significantly reduced arteriogenesis to levels of TLR deficient/defective mice. Interestingly, two endogenous TLR ligands fibronectin EDA and Hsp60 were shown to be significantly increased during collateral artery growth. This study also implies TLR activation in monocytes/macrophages during collateral artery growth by endogenous ligands[134].

4. IMMUNE MODULATING EFFECTOR MOLECULES

4.1 Interferons

Interferons (IFNs) are pleiotropic cytokines with antiviral, antitumor and immunoregulatory functions. The IFN family comprises type I IFN, type II IFN and type III IFN, which can be further divided in several subtypes. Type I IFNs include the subtypes IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω. Type II IFN consists only of IFN-γ and type III IFNs can be divided into IFN-λ_1, IFN-λ_2, and IFN-λ_3[135,136]. Type I IFNs are produced during infection by immune cells as part of an effective host defense against pathogens. In this setting, PAMPs are the major stimuli for the production of type I IFNs, which are sensed by cell surface or endosomal TLRs. TLRs 2, 3, 4, 7/8 and 9 have been linked to the induction of type I IFNs. Type I IFNs have been successfully used for the treatment of several diseases e.g. chronic viral infections, multiple sclerosis (MS), rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and cancer[135].

All type I IFNs bind a common cell-surface receptor, which is known as the type I IFN receptor consisting of two transmembrane subunits, IFNAR1 and IFNAR2. Hence, the janus activated kinase (JAK)/signal transducers and activators of transcription (STAT) signaling pathway is activated, involving the janus family kinases tyrosine kinase 2 (Tyk2) and Jak1 and the transcription factors STAT1 and STAT2. This pathway leads to the transcription of several IFN-responsive genes[136] (Figure 5).

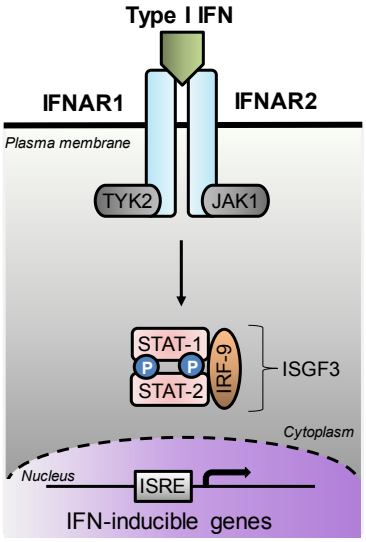

Figure 5. Type I IFN signaling pathway. Type I IFNs bind IFNAR, which is composed of two subunits, IFNAR1 and IFNAR2. These receptor subunits are associated with the JAKs: Tyk2 and Jak1. Activation of both JAKs induces STAT-1 and -2 phosphorylation, activating STATs, which form a complex with IRF-9 to form the transcription factor ISGF-3. This transcription factor complex translocates to the nucleus and binds ISREs in genes to induce their expression. IFN, interferon; TYK2, tyrosine kinase 2; JAK1, janus activated kinase 1; signal transducer and activator of transcription, STAT; IRF-9, IFN-regulatory factor 9; ISGF3, IFN-stimulated gene factor 3; ISRE; IFN-stimulated response elements.

4.1.1 Role of type I interferons in angiogenesis

The relation between type I IFNs and angiogenesis have been extensively studied in the field of cancer. McCarty *et al.* showed that endogenous IFN-α/IFN-β is involved in the inhibition of tumor growth and angiogenesis using IFN-α/IFN-β receptor deficient mice, which lack a functional type I IFN receptor. In this study, tumor growth and angiogenesis were found to be significantly increased in IFN-α/βR-/- mice[137]. Endogenously produced type I IFNs suppress the generation of tumor-associated macrophages (TAMs), which may in turn account for inhibition of tumor growth and angiogenesis[138]. In another study, an adeno-associated virus (AAV) vector with the human IFN-β transgene was used to continuously deliver IFN-β in murine tumor models, which led to inhibition of tumor angiogenesis and growth[139]. In a study by Cao *et al.* tumors in nude mice were injected with adenoviral vector encoding for the murine IFN-β gene (AdIFN-β). IFN-β therapy led to the suppression of tumor growth in a dose-dependent manner. At a high dose of AdIFN-β, these tumors showed increased expression of iNOS, reduced levels of bFGF and TGF-β1, increased EC apoptosis and downregulation of angiogenesis[140]. Intriguingly, transduction of EPCs with the lentiviral vector encoding human IFN-β gene counteracted the tumor-progressive function of EPCs *in vivo* leading to reduced tumor growth and neovascularization, which was due to the reduced secretion of VEGF[141].

4.1.2 Role of type I interferons in arteriogenesis

Arteriogenesis involves the recruitment of monocytes/macrophages, and proliferation of ECs and SMCs. Schirmer *et al.* showed that CAD patients with a low arteriogenic response have increased gene expression levels of IFN-β and several IFN-responsive genes (e.g. chemokine (C-X-C motif) ligand CXCL9, CXCL10, CXCL11), and apoptosis-related proteins in their monocytes after LPS stimulation. Monocytes were activated with LPS to more closely resemble the phenotype of monocytes/macrophages during arteriogenesis. These findings were corroborated in a murine hind limb model of femoral artery ligation, where exogenous application of IFN-β was associated with attenuated arteriogenesis[142]. Conversely, arteriogenesis was enhanced in IFNAR1-/- mice compared to wild-type mice[143]. In line with these results, another study showed that increased plasma chemokine levels of CXCL9, CXCL10 and CXCL11 are associated with lower extent of coronary collaterals in patients with chronic IHD[144].

The mechanisms of IFN-β-induced inhibition of arteriogenesis are only partly known. IFN-β dose-dependently inhibited vascular SMC proliferation *in vitro*[142,143]. Furthermore, IFN-β treated monocytes showed enhanced apoptosis *in vitro*, while IFN-β signaling and apoptosis-related gene expression were attenuated in monocytes from IFNAR1-/- mice[143]. These results demonstrate that IFN-β may inhibit arteriogenesis through inhibition of vascular SMC proliferation and induction of monocyte apoptosis[143].

4.1.3 Role of type I interferons in atherosclerosis

The role of type I IFNs in atherosclerosis has also been established. Low dose IFN-α has been shown to accelerate atherosclerosis in an LDLR-deficient mouse model[145]. Patients with systemic lupus erythematosus (SLE), an autoimmune disorder, develop accelerated atherosclerosis due to reduced number and function of EPCs, which is triggered by elevated levels of type I IFNs[146,147]. IFN-α priming promotes lipid uptake and macrophage-derived foam cell formation by upregulating SR-A expression[148]. Type I IFNs induce endothelial dysfunction, impaired neoangiogenesis by reduced EPC number and function, inflammatory cell infiltration by macrophages and T cells, plaque progression and thrombosis in murine models of SLE and atherosclerosis[149]. Another finding showed that type I IFNs enhance atherosclerosis in two different mouse models of atherosclerosis by stimulating macrophage recruitment to lesions[150]. Concomitantly, absence of endogenous type I IFN signaling in myeloid cells reduces atherosclerosis development, protects against lesional accumulation of macrophages, and reduces necrotic core formation. Finally, type I IFN signaling is upregulated in ruptured human atherosclerotic lesions[150].

4.2 Galectins

Galectins, also known as carbohydrate-binding proteins, are β-galactoside-specific lectins that have an intracellular and extracellular function in vertebrates. Extracellular galectins may mediate cell-cell or cell-matrix adhesion by recognizing specific oligosaccharides, ligand glycoproteins and glycolipids on cell surface or in the ECM[151]. Intracellularly, galectins bind to cytoplasmic and nuclear proteins in a carbohydrate-independent manner, thereby regulating signal transduction and biological responses[152]. Hitherto, 15 different galectins have been identified. All galectins contain a conserved carbohydrate-recognition domain (CRD) of about 130 amino acids, that ensures the carbohydrate-binding. Based on structure, formed by different numbers of the CRD domain, three different types of galectins can be distinguished; the prototypical, chimeric and the tandem-repeat. The proto-type, which consists of galectin-1, -2, -5, -7, -10, -11, -13, -14 and -15, contains only one CRD. The chimera-type which has unusual tandem repeats fused onto one CRD, consists only of galectin-3. The last group, consisting of galectin-4, -6, -8, -9 and -12 are of the tandem repeat-type and contain two distinct CRDs[153] (Figure 6). Some galectins are relatively similar to each other. For example, human galectin-2 has a greater similarity to human galectin-1 than to any other galectin, sharing 43% amino-acid sequence identity[154]. Exogenous galectins bind to monocytes and macrophages, which can lead to cell activation, cytokine secretion, apoptosis, and inhibition of migration[155]. Furthermore, galectins play a crucial role in linking the innate- and adaptive immune system[156]. Galectins have been shown to play a role in cancer, IBD, wound healing[157,158], and CVD[159].

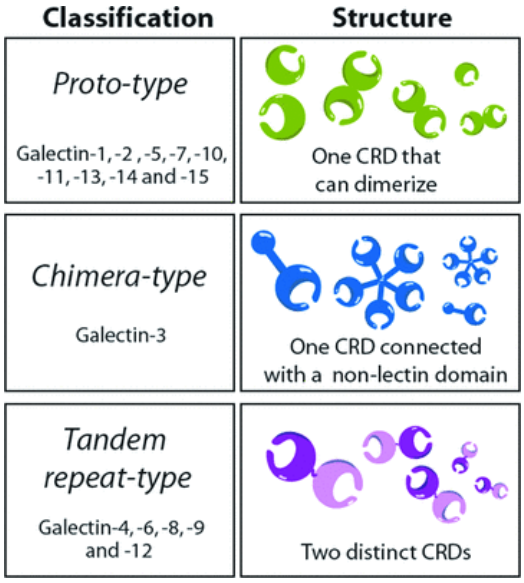

Figure 6. Members of the galectin family. The galectin family has been classified into three types according to their structure. The proto-type consists of galectin-1, -2, -5, -7, -10, -11, -13, -14 and -15, containing one CRD which can form homodimers. The chimera-type consists of galectin-3, containing unusual tandem repeats fused onto one CRD which can form a pentamer. The tandem repeat-type consists of galectin-4, -6, -8, -9 and -12, containing two distinct CRDs connected by a linker peptide have two carbohydrate-binding sites. CRD, carbohydrate-recognition domain. *This figure was published in Annals of the New York Academy of Sciences. Vol 1183, Liu, FT et al. Galectins: regulators of acute and chronic inflammation, pp 158-82, Copyright John Wiley and Sons (2010).*

4.2.1 Role of galectins in angiogenesis

The role of galectin-1 and -3 have been extensively studied in relation to angiogenesis, particularly in cancer using knockout models or blocking antibodies.

Increased expression of galectin-1 has been observed in tumor cells[160], and activated- and tumor ECs[161,162]. Galectin-1 is secreted from tumor cells, taken up and used by tumor-associated ECs to stimulate EC proliferation and migration *in vitro*[160]. *In vivo*, galectin-1 expression is associated with tumor growth and angiogenesis[160,161]. Galectin-1 also induces angiogenesis in an *in vivo* chorioallantoic membrane assay. Knocking-down galectin-1 expression during zebrafish development results in abnormal and irregular vessel growth[161]. Two separate mechanisms have been proposed for galectin-1-mediated angiogenesis stimulation. Galectin-1 binds to neuropilin-1 (NRP-1) on vascular ECs, which is a co-receptor for VEGFR-2. Hsieh *et al.* showed that the binding of galectin-1 to NRP-1 activates VEGFR-2 signaling, and induces proliferation, adhesion and migration of vascular ECs[163]. Jouve *et al.* showed that galectin-1 also binds to CD146, another co-receptor for VEGFR-2 on vascular ECs, protecting against apoptosis induced by galectin-1 *in vitro*[164], which might enhance angiogenesis.

Similar to galectin-1, galectin-3 is also expressed and secreted by tumor cells. Galectin-3 secreted from tumor cells enhances the infiltration of M2 macrophages, tumor growth and angiogenesis *in vivo*[165]. Galectin-3 is also a substrate for cleavage by MMP-2 and -9 to yield a more angiogenic variant[166], which induces tumor cell migration, invasion and adhesion to vascular endothelium, and EC migration, resulting in angiogenesis *in vitro* and *in vivo*[166,167]. Markowska *et al.* showed that galectin-3 mediates VEGF and bFGF-mediated angiogenesis *in vitro* and *in vivo* using a mouse cornea model. This effect was mediated by interaction of galectin-3 with αvβ3 integrin on vascular ECs, which leads to integrin clustering and activation of signaling pathways that promote angiogenesis[168]. Same group showed that galectin-3 induces VEGF-mediated angiogenesis *in vitro* and *in vivo* by also interacting with VEGFR-2 on vascular ECs, promoting VEGFR-2 clustering, cell surface retention and activation of VEGFR-2 signaling[169]. Wan *et al.* investigated the effect of galectin-3 on ECs differentiated from rat bone marrow mesenchymal stem cells. Galectin-3 enhanced EC proliferation and angiogenesis *in vitro*[170]. Furthermore, Hu *et al.* found that overexpression of galectin-3 reduces tumor cell apoptosis induced by epidermal growth factor (EGF), which indicates another means for galectin-3 promoting tumor angiogenesis[171].

4.2.2 Role of galectin-2 in arteriogenesis

For the role of galectins in arteriogenesis, data is only available for galectin-2, described by van der Laan *et al*[172]. In this report it was shown that galectin-2 inhibits arteriogenesis. Increased mRNA expression of galectin-2 was found in patients with CAD with a less well developed coronary collateral circulation. This increased galectin-2 expression was found in

both resting monocytes, LPS-stimulated monocytes, and in cultured macrophages derived from patients with an low arteriogenic response. This expression turned out to be linked to a common single nucleotide polymorphism (SNP) for galectin-2, the so-called rs7291467 SNP (3279 C>T in intron 1 of LGALS2 gene). CAD patients with rs7291467 CC genotype displayed the highest galectin-2 expression, which was associated with a low arteriogenic response, while patients with rs7291467 TT genotype showed the lowest galectin-2 expression, associated with a high arteriogenic response. To validate the clinical observation, a murine hind limb model was used to test the effect of galectin-2 on arteriogenesis *in vivo*, showing that galectin-2 hampered arteriogenesis[172].

The mechanism by which galectin-2 affects arteriogenesis remains unclear, but a recent study showed hampered trafficking and increased apoptosis of monocytes *in vitro* in the presence of galectin-2[155]. Interestingly, van der Laan *et al.* also observed reduced number of perivascular macrophages after galectin-2 treatment *in vivo*[172], suggesting that galectin-2 may inhibit arteriogenesis by modulating monocyte/macrophage responses.

4.2.3 Role of galectins in atherosclerosis

Galectin-1 and -3 have been widely studied for their association with atherosclerosis. Vascular SMC proliferation and migration play a significant role in the pathogenesis of atherosclerosis. Galectin-1 is highly upregulated in human atherosclerotic lesions. Galectin-1 induces vascular SMC adhesion to ECM proteins and proliferation[173]. Galectin-1 also causes accumulation of lipoprotein(a) [Lp(a); a modified LDL molecule] in atherosclerotic lesions by binding Lp(a)[174]. Increased expression of galectin-3 is also found in human atherosclerotic lesions[175], in which galectin-3 is predominantly expressed and secreted by foam cells and macrophages[175,176]. Galectin-3 deficiency reduces the progression of atherosclerosis in ApoE-/- mice[176,177], which is associated with a lower number of perivascular inflammatory infiltrates and mast cells[176]. The effect of galectin-3 on atherosclerosis could be related to its function in stimulation of monocyte/macrophage chemotaxis[178], lipid uptake[179], and LPS binding[180]. Mackinnon *et al.* also observed reduced M2 activation of plaque macrophages in ApoE/Gal3-deficient mice, while the function of these M2 plaque macrophages is unknown[177]. Lee *et al.* showed that galectin-3 is the predominant galectin within atherosclerotic plaques of apoE-/- mice, while galectin-2 was not detected[181]. Furthermore, galectin-3 has been postulated as a biomarker for ACS[182]. Increased expression of galectin-2 was observed in SMCs and macrophages of human atherosclerotic lesions. Endogenous galectin-2 binds to- and increases lymphotoxin-α (LTA) protein secretion, which mediates inflammation during atherosclerosis[183].

Different studies examined which polymorphisms of the galectins are associated with atherosclerosis. Knowledge of these genetic factors contributing to the pathogenesis of atherosclerosis and even MI will lead to improved diagnosis, treatment and prevention for

the patients. The most studied SNP in association with atherosclerosis is of the LGAL2 gene, encoding galectin-2. Genome-wide association study identified a functional rs7291467 SNP (3279 C>T) for galectin-2 was associated with reduced risk for MI, at least in Japanese population. Carriage of the "C" allele variant of this SNP is associated with increased LGALS2 transcript levels, while "T" allele variant is associated with reduced LGALS2 transcript levels *in vitro*[183]. Asselbergs *et al.* found that this SNP of the LGALS2 gene is associated with a decreased risk of coronary heart disease in women[184]. In contrast, a Japanese-Korean study investigated the association between the LGALS2 polymorphism, MI and severity of CAD in Japanese and Korean populations. They showed that this polymorphism was not associated with severity of coronary atherosclerosis and MI in both populations[185]. Also among British patients no significant association was found between the LGALS2 polymorphism and risk of MI[186]. In addition, Sedlacek *et al.* also found no association between the LGALS2 polymorphism and MI in two different German populations with MI[187].

4.3 Adaptor molecule p66(Shc)

Mitochondrial ROS production has been recognized as an important mediator of hyperglycemic vascular damage[70]. The adaptor protein p66Shc is implicated in mitochondrial ROS generation and translation of oxidative signals into apoptosis in response to high glucose. Indeed, p66(Shc-/-) mice show reduced production of intracellular oxidants and increased resistance to oxidative stress-induced apoptosis, interestingly resulting in prolonged lifespan[188,189]. In diabetic wild-type mice, the expression of p66Shc protein is upregulated in aortas, thus underlining a causal relationship between high glucose and p66Shc. Genetic deletion of p66Shc prevents hyperglycemia-induced endothelial dysfunction and oxidative stress in diabetic mice, by upregulating protein expression and activity of eNOS and the anti-oxidant enzyme heme oxygenase-1 (HO-1)[190], preserving NO bioavailability. P66Shc remains upregulated even after normoglycemia restoration in human ECs exposed to high glucose and in aortas of diabetic mice. The persistent p66Shc upregulation and its mitochondrial translocation from cytoplasm were associated with continued ROS production, reduced NO bioavailability, and apoptosis. *In vivo* gene silencing of p66Shc blunts ROS production and endothelial dysfunction in diabetic mice[191]. P66Shc gene expression is increased in peripheral blood monocytes from patients with DM and positively correlates with plasma isoprostanes, an *in vivo* marker of oxidative stress[192]. Moreover, the reduced production of ROS provided by silencing the p66Shc gene is associated with resistance to apoptosis induced by a variety of different mediators, including hydrogen peroxide (H_2O_2), growth factor deprivation, ultraviolet radiation, and calcium ionophore[189,193,194]. P66Shc was shown to participate in mitochondrial ROS production by serving as a redox-sensitive enzyme that oxidizes cytochrome *c*, thus generating proapoptotic ROS in response to specific stress signals through a PKC-β dependent pathway[190,193,195-198]. Many studies demonstrated that ROS

production in diabetic EPCs is also significantly higher than normal EPCs. This supports the notion that increased ROS generation contributes to diabetic EPC dysfunction[105]. BM-derived EPCs cultured in the presence of high glucose upregulated p66Shc protein expression and high glucose exposure markedly decreased the number of BM-derived EPCs[199]. P66Shc knockout in BM-derived EPCs resulted in resistance to high glucose inhibition of EPC number, by reducing apoptosis and oxidative stress, and increasing NO bioavailability. Furthermore, p66Shc knockout prevented diabetic impairment of angiogenesis *in vivo*[199].
In conclusion, these studies indicate the involvement of p66Shc in the production of mitochondrial oxidative stress, which may impair cell number and function during neovascularization.

5. THESIS OUTLINE

Research into the inflammatory nature of CVD has led to improved mechanistic understanding of its pathogenesis which can be used to identify novel therapeutic targets and strategies. In this thesis a number of studies are presented aiming at improving the vascular repair properties of bone marrow-derived cells and preventing atherosclerosis progression through thorough mechanistic understanding.
In ***chapter 2***, we investigate the expression of TLRs and their downstream effectors in circulating leukocytes during AMI in a specific patient trial, and how they relate to the outcome of cardiac function.
In ***chapter 3***, we identify a potential downstream effector molecule of IFN-β in CACs using *in vitro* studies, that might explain the inhibitory role of IFN-β in arteriogenesis in CAD patients with insufficient myocardial perfusion.
In ***chapter 4***, we investigate the effect of high levels of NEFA, palmitic acid, on oxidative stress induction in CACs, and the number and function of CACs *in vitro*, associated with a diabetic patient trial. We also explore the role of p66Shc as a mediator of palmitic acid effects on CACs oxidative status and function *in vitro*. Furthermore, the beneficial action of resveratrol, a grape- and red wine-derived polyphenol with anti-oxidant potential in EPCs[200], on palmitic acid induced effects on CACs is determined.
In ***chapter 5***, we describe the potential of several factors to increase the neovascularization capacity of human CACs by investigating the effects on number and function of CACs *in vitro*, and we analyze their relation to monocyte and macrophage subtypes.
In ***chapter 6***, we study the mechanism through which human galectin-2 exerts its anti-arteriogenic effect in both monocytes and macrophages by examining the changes in their phenotype and physiology *in vitro* and *in vivo*.
Finally, in ***chapter 7***, we summarize the findings from the studies described in this thesis and discuss the relevance of these new findings in the context of recent advances in CVD. Additionally, we use these findings to define new areas for future research.

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