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

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Chapter 7

General discussion

Cardiovascular disease is still the leading cause of death in the Western countries. Major progress in research has been made in the past decade in the prevention and treatment of cardiovascular disease. Many research has focused on the role of inflammation and the immune system in the pathogenesis of cardiovascular disease. In this thesis, we have studied the immune modulating effects of TLR activation (**chapter 2**), type I IFN signaling (**chapter 3**), p66Shc activation (**chapter 4**), glucocorticosteroids and M-CSF (**chapter 5**), and galectin-2 (**chapter 6**) in the pathogenesis and treatment of ischemic heart disease (IHD), the most common cause of cardiovascular disease, with an emphasis on their role in bone marrow-derived cells. The results described in this thesis show novel immune modulating targets and strategies to prevent and/or treat IHD.

Immune modulators in AMI

Many studies in experimental animals have shown that TLR2 and TLR4 expression and activation have harmful effects on atherosclerosis¹⁻⁴, infarct size⁵ and cardiac function after AMI^{6,7}. In humans, the levels of circulating TLR4-positive monocytes, monocytic TLR4 expression and TLR4 signaling are increased in patients with unstable angina and myocardial infarction^{8,9}. Furthermore, increased monocytic TLR2 expression is reported in patients with AMI¹⁰. However, the clinical consequences of TLR2 and TLR4 expression and activation on circulating leukocytes during AMI in humans are unknown, including its role in long-term outcome of cardiac function. In **chapter 2**, we have studied 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. We have shown TLR pathway activation by increased expression of TLR4 and its downstream genes, including IL-18R1, IL-18R2, IL-8, MMP9, HIF1A, and NFKBIA. In contrast, expression of the classical TLR-induced genes, TNF- α and IL-1B, was reduced during AMI. This increased non-canonical TLR4 response during AMI can be explained by the activation of transcription factor HIF-1 α . Increased TLR4, HIF1A and IL-18R1/2 expression appeared before troponin T release, a marker for myocardial necrosis. Only HIF1A and IL-18R1/2 expression correlated with the ischemic area, suggesting that cardiac ischemia is a sufficient trigger to induce the expression of HIF1A and IL18R in circulating leukocytes. Cardiac ischemia induces the release of endogenous TLR ligands¹¹, explaining this aforementioned TLR response. Additionally, cardiac ischemia induces the expression of the pro-inflammatory cytokine IL-18¹² in vascular endothelial cells, resident myocardial macrophages and cardiomyocytes¹³. Increased circulating levels of IL-18 have been demonstrated in patients with acute coronary syndromes after prolonged periods of ischemia^{14,15} and are independently associated with future clinical events^{16,17}. Active IL-18 binds a heterodimeric receptor consisting of IL-18R α (1) and IL-18R β (2) subunits, which in turn mediates signaling through the canonical IL-1R superfamily signaling cascade that includes myeloid differentiation 88 and nuclear factor-kB (NF-KB)¹⁸. IL-18 may depress myocardial

contractility *in vitro*¹² and high circulating IL18 levels correlate with decreased left ventricular ejection fraction¹⁴. IL-18 induces the production of ECM components and remodeling by cardiac fibroblasts, implicating this cytokine in myocardial fibrosis^{19,20} depressing myocardial contractility. In our study, we could not detect high circulating levels of IL-18 in all patients with AMI, which may be due to the short ischemic period. Moreover, TLR4, HIF1A and IL-18R1/2 expression did not correlate with outcome of cardiac function (salvage index, change in left ventricle end diastolic volume, and change in left ventricle ejection fraction), which may be also ascribed to the short ischemic period. IL-18 may also function as a chemoattractant for IL-18R expressing monocytes²¹ and may activate monocytes to secrete pro-inflammatory cytokines^{22,23}. In conclusion, cardiac ischemia may induce the release of endogenous TLR ligands, leading to HIF-1 α activation and IL-18R expression in circulating monocytes, increasing their responsiveness to local cardiac expressed IL-18. IL-18 may be induced by prolonged periods of cardiac ischemia, allowing monocytes to migrate to the ischemic area, and secrete pro-inflammatory cytokines (Figure 1).

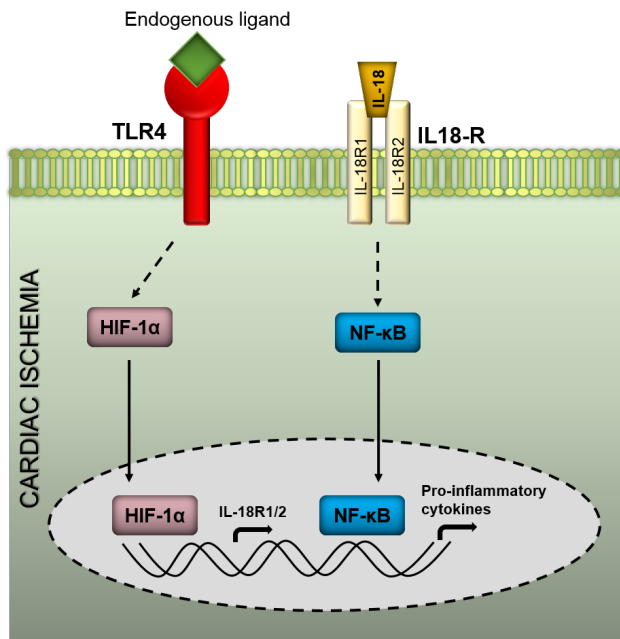


Figure 1. TLR4 and IL-18 signaling in monocytes during AMI. Cardiac ischemia induces the release of endogenous TLR ligands, leading to activation of TLR4 pathway and transcription factor HIF-1 α , which induces IL-18R1 and -2 expression. After prolonged ischemia, pro-inflammatory cytokine IL-18 is released in the circulation, which binds a heterodimeric receptor consisting of IL-18R1 and IL18R2, leading to NF- κ B activation and induction of pro-inflammatory cytokines. IL-18 may also function as a chemoattractant for IL-18R expressing monocytes, allowing their migration to the IL-18 producing, ischemic area at risk. TLR, Toll-like receptor; HIF-1 α , Hypoxia-inducible factor 1-alpha; IL-18R, interleukin-18 receptor; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells.

Immune modulators in neovascularization

TLR4 activation also induces the production of type I IFNs, such as IFN- β . IFN- β plays a pivotal role in atherosclerosis progression²⁴, inhibition of angiogenesis²⁵, and in the inhibition of arteriogenesis in CAD patients with insufficient myocardial perfusion²⁶. Given the potential beneficial effects of IFN- β inhibition on atherogenesis and neovascularization, blocking of IFN- β downstream signaling constitutes a challenging novel approach to relieve the symptoms of IHD. Circulating angiogenic cells (CACs), previously designated as early endothelial progenitor cells (EPCs), are bone marrow-derived blood cells that share features of endothelial cells and monocytes/macrophages after *in vitro* conditioning²⁷⁻³¹. EPCs, which are important effectors of neovascularization^{30,31} are affected by type I IFNs in different disease entities^{32,33}. Although it is clear that IFN- β impairs arteriogenesis in CAD patients with insufficient myocardial perfusion, the pathways by which this cytokine exerts its anti-arteriogenic effects in CACs have not been discovered. In **chapter 3**, we have identified a potential downstream effector molecule of IFN- β in CACs, that might explain the inhibitory role of IFN- β in arteriogenesis in CAD patients with insufficient myocardial perfusion. Therefore, we have investigated the effects of IFN- β and calpain 1, a Ca²⁺-dependent cysteine protease involved cell adhesion, migration and apoptosis^{34,35}, on CAC number *in vitro* and function by an *in vitro* assay of neovascularization. During neovascularization the expression of fibronectin, an ECM protein is highly increased, favoring cell adhesion^{36,37}. We have shown that IFN- β reduced the number of CACs both during differentiation from mononuclear cells (MNCs) and after differentiation on fibronectin *in vitro*. The reduced CAC number by IFN- β was not directly caused by apoptosis, but by impaired adhesion to- and spreading on fibronectin, which was dependent on $\alpha 5\beta 1$ (VLA-5) integrin. IFN- β affected the functional activity of VLA-5 in mature CACs, leading to rounding and detachment of cells from fibronectin, by induction of calpain 1 activity. Cell rounding and detachment was completely reversed by inhibition of calpain 1 activity in mature CACs. Our data suggested a direct involvement of calpain 1 in the adverse effects of IFN- β on CAC-matrix adhesion by cleavage of VLA-5 (Figure 2 upper panel). The involvement of calpain in the cleavage of the cytoplasmic domain of $\beta 1$ integrin has been demonstrated³⁸, resulting in inactivation. The inhibitory role of calpain 1 in angiogenesis has been shown by several studies³⁹⁻⁴¹. During our *in vitro* capillary-like tube formation assay, in the absence of IFN- β , calpain 1 inhibition enhanced endothelial cell tube formation in fibrin matrix, but not the CAC-induced optimal endothelial cell tube formation. The adhesion of endothelial cells to fibrin is mediated by VLA-5 and $\alpha v\beta 3$ integrins, which stimulate capillary-like tube formation *in vitro*⁴². Calpain 1 inactivation in endothelial cells probably leads to enhanced VLA-5 and $\alpha v\beta 3$ activation, enhancing endothelial cell-matrix adhesion and thus capillary-like tube formation. CACs adhere to endothelial cells mainly via $\beta 2$ integrin, which is important for homing and neovascularization capacity of CACs *in vivo*^{43,44}. CACs increased endothelial cell tube formation in our study, which is likely mediated by $\beta 2$

integrin activation. Probably, calpain 1 inactivation in CACs does not lead to enhanced $\beta 2$ integrin activation. The presence of IFN- β in the capillary-like tube formation assay impaired endothelial cell tube formation and CAC-induced endothelial cell tube formation. Calpain 1 inhibition was not sufficient to rescue IFN- β -impaired endothelial cell tube formation and CAC-induced endothelial cell tube formation, indicating that other factors than calpain 1 may be more responsible for reduced neovascularization with IFN- β *in vivo*. IFN- β may strongly affect endothelial cell function. In addition, IFN- β may affect the function of CACs in neovascularization by reducing their secretion of paracrine pro-angiogenic factors (Figure 2 upper panel). The role of paracrine pro-angiogenic factor secretion by CACs in enhancing neovascularization has been described before⁴⁵⁻⁴⁷.

Based on current EPC knowledge, we hypothesized that CAC number and function are deregulated by cardiovascular risk factors such as diabetes mellitus leading to impairments in neovascularization, which results from the deleterious action of high glucose levels by inducing mitochondrial ROS generation via pro-oxidant adaptor protein p66Shc^{48,49}. Besides glucose, also free or nonesterified fatty acids (NEFAs) are elevated in diabetic patients, and contribute to increased ROS generation⁵⁰. However, the role of elevated circulating levels of NEFAs have been poorly studied in this context. In **chapter 4**, we have investigated the effect of high levels of palmitic acid, a saturated free fatty acid on oxidative stress induction in CACs, and the number and function of CACs *in vitro*, associated with a diabetic patient trial. We have also explored the role of p66Shc as a mediator of palmitic acid effects on oxidative status and function of CACs *in vitro*. Furthermore, the beneficial action of resveratrol, a grape- and red wine-derived polyphenol with anti-oxidant potential^{51,52}, on palmitic acid induced effects on CACs was determined. High levels of palmitic acid induced oxidative stress, p66Shc overexpression, but did not affect CAC number. Additionally, high levels of palmitic acid may affect the pro-angiogenic potential of CACs by inducing TNF- α and VEGF-A expression, and reducing SDF-1 α , PECAM-1, CXCR4, VEGFR-2 and Tie2 expression. TNF- α is one of the major deleterious cytokine upregulated in diabetic patients affecting EPC number and migration, and tube formation capacity of EPCs^{53,54}. TNF- α did not induce p66Shc expression, suggesting that p66Shc may be an inducer of TNF- α . Furthermore, TNF- α did not induce VEGF-A expression, also pointing to a direct induction by p66Shc. VEGF-A increases VEGFR-2 internalization and degradation⁵⁵, explaining the reduced VEGFR-2 expression by palmitic acid. VEGF-A may also induce vascular permeability in diabetes⁵⁶. Reduced expression of SDF-1 α ^{57,58}, PECAM-1⁵⁹, CXCR4⁶⁰, VEGFR-2^{61,62} and Tie2⁶³ may have deleterious effects on neovascularization *in vivo* by reducing the mobilization and homing of CACs. P66Shc silencing did not affect palmitic acid-induced oxidative stress, suggesting that p66Shc is an effector of oxidative stress or dominance of other sources of oxidative stress over mitochondrial-generated oxidative stress by p66Shc exist. P66Shc silencing reduced palmitic acid-induced TNF- α and VEGF-A expression. Interestingly, scavenging of ROS by

N-acetyl cysteine (NAC) did not affect palmitic acid-induced p66Shc, TNF- α and VEGF-A expression, indicating that p66Shc is not an effector of oxidative stress. Furthermore, neither ROS scavenging nor p66Shc silencing restored palmitic acid-reduced SDF-1 α and PECAM-1 expression, indicating ROS- and p66Shc-independent regulation of these two genes. CACs from diabetic patients were also not affected in number, but showed increased oxidative stress, which was attenuated by resveratrol. Resveratrol reversed palmitic acid-induced oxidative stress and p66Shc expression, normalized TNF- α , VEGF-A, PECAM-1 and CXCR4, but not SDF-1 α and VEGFR-2 expression levels. Furthermore, resveratrol had a beneficial action on the VEGF-A activation pathway, particularly in improving palmitic acid-affected CAC migration towards VEGF-A, likely by increasing the functional sensitivity of VEGFR-2. Resveratrol improved palmitic acid-affected chemokinesis, a function previously shown to be controlled by NO production in CACs⁶⁴. In our study, we did not observe any change of eNOS expression levels after palmitic acid treatment, suggesting that resveratrol improves eNOS activity and/or NO bioavailability by reducing palmitic acid-induced oxidative stress. A summary of the findings is shown in Figure 2 (lower panel).

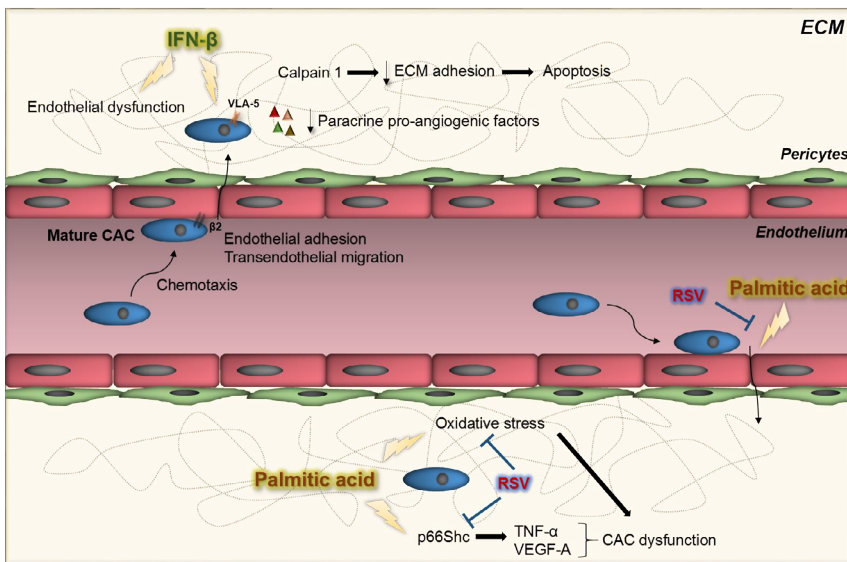


Figure 2. IFN- β and palmitic acid affect CACs, impairing neovascularization. IFN- β affects mature CAC number and function. CAC number is affected by induction of calpain 1 activity, causing impaired adhesion and spreading on ECM by inactivation of VLA-5 integrin which may lead to apoptosis. CAC function may be affected by reduced secretion of paracrine pro-angiogenic factors (Upper left). Palmitic acid affects CAC function by inducing oxidative stress and p66Shc expression leading to TNF- α and VEGF-A expression, which are attenuated by resveratrol (Lower right). IFN, interferon; CAC, circulating angiogenic cell; ECM, extracellular matrix; VLA-5, very late activation antigen 5; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; RSV, resveratrol.

Despite the success of drug therapy and surgical revascularization therapies, prevention of clinical events of atherosclerosis remains a major problem in current-day cardiology, rendering promotion of therapeutic neovascularization by EPCs a potential approach. EPC therapy is effective in animal models of hind limb ischemia^{30,31}, and is safe, feasible and effective for the treatment of cardiovascular diseases as demonstrated in clinical trials^{65,66}. However, some clinical trials show limited efficacy of EPC therapy following AMI⁶⁷⁻⁶⁹, which largely arise as the result of using i.e. ill-defined, heterogeneous bone marrow-MNC preparations and different methods for cell preparation. Thus, there is still a lack of consensus as to the definition of an EPC and the methods used to isolate and culture them. Recently, early EPCs were referred to as CACs which display close similarity to monocytes/macrophages (M2)^{45,70}. CACs hold a great promise as novel therapeutics in vascular repair *in vivo*⁴⁵. However, little is known regarding the pro-angiogenic factors and mechanisms responsible for the therapeutic effect of CACs *in vivo*. This also reignites interest regarding the identification of new strategies to optimize human CACs for therapeutic neovascularization to successfully translate it to cell therapy in clinical trials in the setting of IHD. Since, therapeutic neovascularization requires augmentation of CAC numbers or improvement in their function, new strategies may include making modifications in the culture methods of CACs. In **chapter 5**, we have described 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 have analyzed their relation to monocyte and macrophage subtypes. We have shown that all human monocyte subsets (classical, intermediate and non-classical) differentiate to CACs when cultured in endothelial growth medium (EGM), as judged by their adherence to fibronectin, uptake of Dil-Ac-LDL and binding to Ulex-Lectin. EGM-derived CACs expressed VEGFR-2 and the angiopoietin receptor Tie2 surface proteins, which are important for their pro-angiogenic properties. From the EGM components, we identified the pro-angiogenic factor essential for CAC differentiation. Specifically, hydrocortisone induced Tie2 (and VEGFR-2 only at highest pharmacological concentration), and reduced CD14 surface protein expression, which indicated that CACs have obtained the M2 macrophage phenotype⁷¹. These effects were reproduced by the related glucocorticosteroid, dexamethasone. It has been reported that glucocorticosteroids induce the expression of macrophage colony-stimulating factor (M-CSF) receptor (CSF-1R) in monocyte-derived macrophages⁷², suggesting that glucocorticosteroids may increase the responsiveness of CACs to M-CSF. Treatment with M-CSF augmented the effect of hydrocortisone on VEGFR-2 surface protein expression to levels exceeding the expression levels on M2 macrophages. CACs shared a significant number of M2 differentiation markers, but their pro-angiogenic capacity seems much higher than M2 macrophages as judged by higher gene expression levels of paracrine pro-angiogenic factors such as IL-8, VEGF-B, HGF, PDGF-B, PDGF-C, and CXCL12. However, glucocorticosteroids and M-CSF were of minor importance for paracrine pro-angiogenic

factor production when compared to endothelial basal medium (EBM), particularly IL-8 was reduced by both compounds. Interestingly, this was also reflected in an *in vitro* angiogenesis model. VEGF-driven endothelial cell tube formation was only significantly enhanced by EBM-derived CACs, while hydrocortisone and M-CSF maintained the supportive potential of EBM. The fact that hydrocortisone and M-CSF did not further enhance VEGF-driven angiogenesis by CACs *in vitro* could be ascribed to reduced IL-8 secretion by these molecules. Reduced IL-8 secretion may decrease VEGFR-2 transactivation in CACs⁴⁵, inactivating VEGFR-2 downstream signaling pathway. Surprisingly, M-CSF also did not enhance VEGF-driven angiogenic response by enhanced VEGFR-2 expression. However, M-CSF increased the number of CACs during differentiation from MNCs by inducing proliferation *in vitro*, while hydrocortisone partly prevented cell proliferation. When added in equal numbers, M-CSF-expanded CACs had equal stimulating effects on endothelial cell tube formation compared to CACs grown in the absence of M-CSF. A summary of the findings is shown in Figure 3.

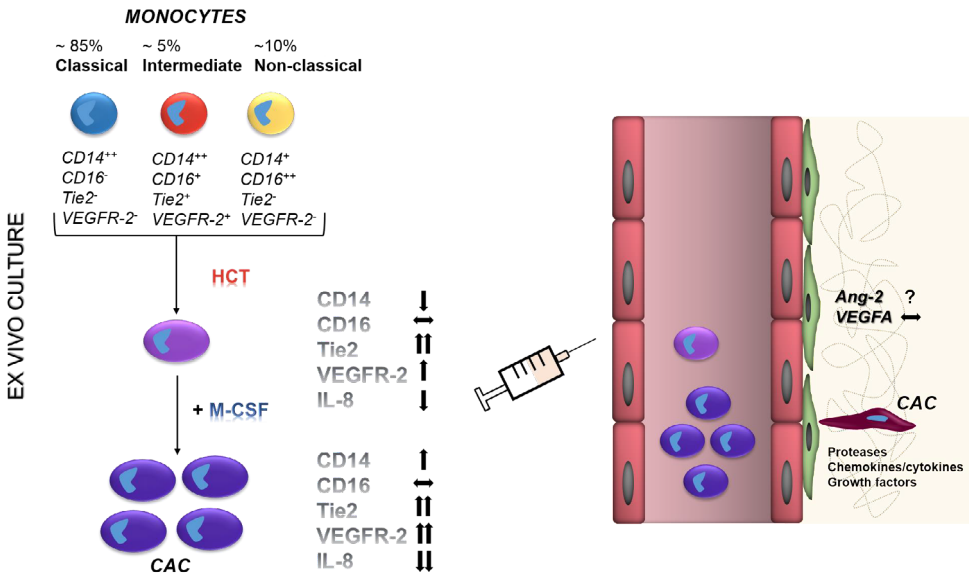


Figure 3. Hydrocortisone and M-CSF enhance neovascularization by CACs. The three human monocyte subsets characterized by different expression levels of CD14, CD16, Tie2 and VEGFR-2 differentiate to CACs by hydrocortisone. Hydrocortisone highly induces Tie2 expression, while VEGFR-2 is slightly induced. CD14 and IL-8 expression are downregulated by hydrocortisone. M-CSF augments the effect of hydrocortisone on VEGFR-2 expression, upregulates CD14, and further downregulates IL-8, while Tie2 expression remains equal. CD16 expression remains the same in both hydrocortisone and M-CSF-derived CACs. M-CSF expands the CAC number by inducing proliferation, while hydrocortisone partly prevents it. Both hydrocortisone- and M-CSF-derived CACs do not enhance VEGF-driven angiogenesis *in vitro* when added in equal numbers, which may be ascribed to reduced IL-8 expression. Angiopoietin-2 may enhance angiogenesis by both hydrocortisone- and M-CSF-derived CACs. CAC, circulating angiogenic cell; VEGFR, vascular endothelial growth factor receptor; IL-8, interleukin-8; HCT, hydrocortisone; M-CSF, macrophage colony-stimulating factor; Ang-2, angiopoietin-2.

Next to CACs, monocytes/macrophages are important effectors of arteriogenesis⁷³. Galectin-2 mRNA expression was increased in monocytes/macrophages of CAD patients with a low arteriogenic response. Additionally, in a murine hind limb model of arteriogenesis, galectin-2 significantly impaired arteriogenesis. Interestingly, galectin-2 treatment reduced the number of perivascular macrophages *in vivo*, suggesting that galectin-2 may inhibit arteriogenesis by modulating monocyte/macrophage responses⁷⁴. In **chapter 6**, we have studied 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*. We have shown that galectin-2 binds monocytes by CD14. Galectin-2 is a carbohydrate-binding lectin, suggesting that galectin-2 binding to CD14 is carbohydrate-dependent. However, the binding of galectin-2 on monocytes was not affected by carbohydrates, indicating a specific protein-protein interaction with CD14. Furthermore, galectin-2 binding was higher in monocyte subsets with CD14^{high} expression (classical and intermediate) compared to CD14^{low} subset (non-classical). Galectin-2 induced pro-inflammatory cytokines in monocytes by CD14/TLR4 in a carbohydrate-independent manner. CD14 is also a co-receptor for TLR2 in rare instances^{75,76}, indicating that signaling through TLR2 may also exist providing an important aspect of future research. Galectin-2 impaired monocyte migration, and expression of pro-arteriogenic factors by monocytes. Furthermore, galectin-2 also bound macrophages and induced a M1-like phenotype in two different macrophage subtypes, characterized by elongated cell shape, expression of filamentous actin, reduced motility, and increased expression of pro-inflammatory cytokines, accompanied by a switch in surface protein expression to CD40-high and CD206-low (M1 phenotype). Galectin-2 partially modified differentiated M2 macrophages to M1 macrophages, since persistent expression of some M2 markers were found in M2 macrophages. This can be due to the differences in CD14 expression levels seen between the several macrophage subtypes, which may have an impact on galectin-2 binding. M2 macrophages express lower amounts of CD14 protein compared to M1 macrophages, which is caused by IL-4^{71,77}. In a murine hind limb model of arteriogenesis, we showed that the anti-arteriogenic effect of galectin-2 is accompanied by increased numbers of pro-inflammatory CD40-positive (M1) and reduced numbers of anti-inflammatory CD206-positive (M2) macrophages around collateral arteries. A summary of the findings is shown in Figure 4.

Concluding remarks and future perspectives

Over the past decade, major progress in research has been made in the prevention and treatment of IHD. Many research has focused on the role of inflammation and the immune system in the pathogenesis of IHD. The studies described in this thesis have led to novel targets and strategies in bone marrow-derived cells such as monocytes/macrophages and CACs to prevent and/or treat IHD, which may limit the extent of heart damage and improve

cardiac function. A number of immune modulating molecules in bone marrow-derived cells are presented in this thesis, which can be used to prevent atherosclerosis progression and to improve their vascular repair properties reducing the risk for cardiovascular events.

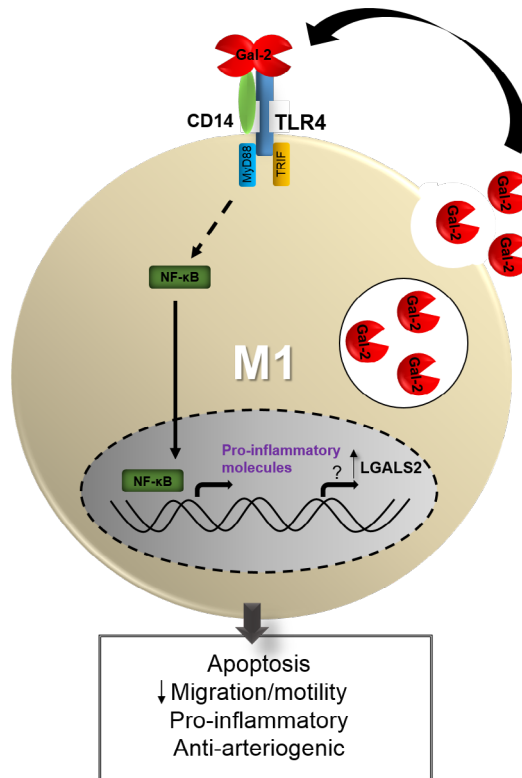


Figure 4. Mechanism by which galectin-2 modulates monocytes/macrophages to inhibit arteriogenesis. Galectin-2 gene (LGALS2) expression is increased in monocytes/macrophages of CAD patients with a low arteriogenic response. The transcription factors that are essential for galectin-2 gene expression are unknown. Galectin-2 protein directly binds CD14/TLR4 after secretion and stimulates the expression of pro-inflammatory molecules, likely by activating the transcription factor NF-κB, inducing a M1 phenotype, monocyte apoptosis, reduced monocyte migration and macrophage motility. CAD, coronary artery disease; Gal-2, galectin-2; TLR, Toll-like receptor; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells.

Firstly, we identified a novel HIF-1 α -dependent non-canonical TLR4 activation pathway in circulating leukocytes of AMI patients leading to enhanced IL-18R expression which correlated with the magnitude of the cardiac ischemic area. This might increase homing of leukocytes to the IL-18 producing, ischemic area at risk. This knowledge may contribute to our mechanistic understanding of the involvement of the innate immune system during AMI and may yield diagnostic and prognostic value for patients with myocardial infarction. A potential target to

improve cardiac function during AMI might be IL-18 by neutralization of the circulating levels using IL-18-binding protein (BP), a naturally occurring specific endogenous IL-18 inhibitor or neutralizing IL-18 antibody. IL-18 participates in the pathogenesis and progression of atherosclerosis. IL-18 has been shown to be highly expressed in human atherosclerotic plaques, mainly in plaque macrophages, and in particular in unstable plaques⁷⁸. Additionally, elevated levels of IL-18 correlate with increased vascular stiffness and intima media thickness in men without evidence of coronary artery disease, indicating that this cytokine may play a role in early stages of atherosclerosis development⁷⁹. Further, IL-18 levels correlate with instability of atherosclerotic plaque and strongly serve as an independent predictor of cardiovascular death in patients with subclinical atherosclerosis⁸⁰. Neutralization of IL-18 by blocking antibody inhibits neointima formation in a rat model of balloon-induced vascular injury⁸¹. Overexpression of IL-18BP prevents the spontaneous development of plaque, limits plaque progression, and promotes plaque stability in ApoE knockout mice⁸². IL-18 also displays anti-angiogenic activities. Treatment with IL-18BP, stimulates ischemia-induced neovascularization in a murine model of hind limb ischemia which is associated with activation of VEGF/Akt signaling⁸³, making IL-18 inhibition a promising strategy for the treatment of IHD. In animal models this strategy has already proven successful. Injection of IL-18BP overexpressing mesenchymal stem cells improves myocardial function, and reduces adverse ventricular remodeling and infarct size in rat models of ischemia and AMI⁸⁴. Furthermore, administration of IL-18 neutralizing antibody reduces infarct size in mice subjected to experimental ischemia⁸⁵. However, the safety and efficacy of recombinant human IL-18BP and of a blocking humanized monoclonal IL-18 antibody is still under investigation in initial phases of clinical trials for psoriasis and rheumatoid arthritis and type 2 diabetes⁸⁶. Furthermore, pre-clinical studies of IL-18 targeted treatments with IL-18BP or IL-18 antibody investigating the safety and efficacy of these drugs during cardiac ischemia or myocardial infarction in humans are lacking, opening a new area of research.

Secondly, this thesis showed that the IFN- β -induced reduction of the numbers of *in vitro* differentiated CACs is based on activation of calpain 1, resulting in a diminished adhesion and spreading to ECM protein fibronectin via VLA-5. *In vivo* this could lead to inhibition of neovascularization due to reduction of the locally recruited CAC numbers and their paracrine pro-angiogenic factors. Calpain 1 inhibition might improve the numbers of differentiated CACs in the neovascularization area *in vivo*, but it will not restore the influence of IFN- β on CAC function. Further studies are needed to understand the *in vivo* effect of calpain 1 activity in differentiated CACs in relation to neovascularization. First, animal experiments should be performed to assess the effect of IFN- β treatment on calpain 1 activity in differentiated CACs from muscle sections in an *in vivo* model of hind limb ischemia. Therefore, it is also important to characterize differentiated CACs *in vivo*, however research shows we still lag on understanding the immunophenotypic characteristics of CACs. Next experiment could

be then assessing the effect of active calpain 1 overexpression in differentiated CACs on neovascularization in an *in vivo* model of hind limb ischemia in which also the specific mechanism by which calpain 1 activity interferes with CAC adhesive capacity needs to be elucidated. Since calpain 1 does not clarify all effects of IFN- β in reducing neovascularization, other factors can also be evaluated. For instance, secretome analysis of IFN- β -regulated pro-angiogenic factors in *in vitro* differentiated human CACs can be performed, whereafter *in vivo* evaluation of muscle sections in an animal model of hind limb ischemia after IFN- β treatment can be explored. Another experiment could be analyzing the effect of IFN- β and eventually calpain 1 on human CAC migration *in vitro* and the effect on pro-angiogenic receptors. Overall, calpain 1 inhibition in CACs holds a chance in IHD patients with insufficient myocardial perfusion, but needs further investigation. Calpain activity is associated with several important human pathological disorders including ischaemia/reperfusion injury, atherosclerosis and myocardial infarction⁸⁷. Pharmacological inhibition of calpain improves cardiac function in animal models of ischemia/reperfusion injury^{88,89}. Furthermore, pharmacological inhibition of calpain 1 specifically attenuates AngII-induced abdominal aortic aneurysm formation and atherosclerosis development in low-density lipoprotein receptor-/- mice⁹⁰. Finally, pharmacological inhibition of calpain reduces infarct size and improves cardiac function in an animal model of myocardial infarction^{91,92}. However, calpain inhibitors may have side effects. The effect of systemic use of calpain inhibitors is broad, affecting vascular inflammation⁹⁰, which is important for neovascularization. Therefore, cell type-specific targeting of calpain is essential to address this issue. Furthermore, isoform-specific calpain inhibitors are currently lacking for clinical use, also providing an important aspect of future research⁸⁷.

Thirdly, p66Shc might be a potential therapeutic target for treatment of cardiovascular complications in diabetes patients. P66Shc-/- mice exhibit less atherosclerosis during a high-fat diet⁹³ and apoE-/- background⁹⁴ and increased p66shc gene expression levels in peripheral blood monocytes is independently associated with CAD⁹⁵. Furthermore, p66Shc mediates diabetic impairment of angiogenesis *in vivo*⁴⁸. In line with the latter study, we have shown that exposure of human CACs to a pro-diabetic condition (high NEFA) *in vitro* increases oxidative stress in parallel to an upregulation of pro-oxidant protein p66Shc, which may affect the mobilization, homing, and pro-angiogenic capacity of CACs by inducing TNF- α and VEGF-A. To more clarify this, it would be interesting to investigate whether migration, pro-vascularizing receptors/factors, and angiogenesis are dysregulated by high TNF- α and VEGF-A levels *in vitro*, which can be established by overexpressing p66Shc and neutralizing high TNF- α and VEGF-A levels in CACs *in vitro*. Another interesting point is that several studies have shown that p66Shc is involved in the production of mitochondrial ROS, while we showed that NEFA induces oxidative stress independently of p66Shc. However, in our experiment ROS was measured in the cytoplasm, and not specifically in the mitochondria,

which will need further investigation before we can conclude whether dominance of other sources of oxidative stress over mitochondrial-generated oxidative stress by p66Shc exist. In this case, it would be interesting to investigate which other sources of oxidative stress are activated by NEFA, and whether and how this NEFA-induced oxidative stress may affect CAC migration (pro-vascularizing receptors) and function (eNOS activity, NO bioavailability, pro-vascularizing factors, angiogenesis) in a p66Shc knocking-down context *in vitro*. Intriguingly, resveratrol pre-treatment may reverse NEFA-induced CAC dysfunction, possibly by modifying the cells towards an anti-oxidant status through attenuation of p66Shc-independent induction of oxidative stress and by attenuating p66Shc-dependent pathway. One interesting experiment could be investigating the mechanism how resveratrol affects p66Shc expression and activation *in vitro* in CACs exposed to high NEFA. Several molecular mechanisms have been described that might regulate p66Shc expression and activation, among which are 1) p53 acetylation^{96,97}, 2) PKC- β II phosphorylation^{98,99}, 3) MEK-ERK activation¹⁰⁰, 4) serine 36 phosphorylation¹⁰¹, and 5) pin1 activation¹⁰². If dominance of other sources of oxidative stress over p66Shc-generated oxidative stress exist in CACs exposed to NEFA, it would be interesting to investigate resveratrol's effects on the p66Shc-independent pathway, inter alia whether and how resveratrol improves NEFA-impaired CAC migration (pro-vascularizing receptors) and function (eNOS activity, NO bioavailability, pro-vascularizing factors, angiogenesis) in a p66Shc knocking-down context *in vitro*. We further demonstrated that CACs from diabetic patients showed increased oxidative stress, even after 7 days of *in vitro* culture, which can be attributed to metabolic memory. A recent study underlined the role of p66Shc in the process of hyperglycemic memory in endothelial cells¹⁰¹. It would be also interesting to investigate whether diabetic CACs display p66Shc protein expression and activation *in vitro*, and the effects of silencing on mitochondrial-generated oxidative stress.

Fourthly, we identified a new *ex vivo* strategy to optimize human CACs for therapeutic neovascularization *in vivo* in the setting of IHD. We have shown that *ex vivo* treatment with hydrocortisone and M-CSF promote a pro-angiogenic phenotype in CACs. Especially, hydrocortisone induces Tie2, while M-CSF induces VEGFR-2 expression and expands CAC numbers. However, VEGFR-2 on CACs may be non-functional during neovascularization *in vivo*, which may be ascribed to reduced IL-8 expression levels. Intriguingly, increased Tie2 expression might have a potential in enhancing neovascularization by CACs *in vivo* (Figure 3). Exposure of Tie2-expressing monocytes/macrophages (TEMs) to Tie2 ligand, angiopoietin-2 (Ang-2) enhances their pro-angiogenic activity in a tumor model using genetically modified mice with Ang-2 overexpression specifically in endothelial cells. This results in tumors with more vascularization and higher numbers of infiltrated TEMs than WT-mice¹⁰³. In plasma, more than 90% of cortisol is bound to plasma proteins (corticosteroid-binding globulin, CBG and albumin). Alterations in the affinity and level of the binding

proteins affect the free cortisol concentration in plasma. Only unbound or free cortisol (~5-10%) is directly responsible for the physiological function of the hormone. In healthy people, free cortisol levels range between 1.4-14 ng/ml, which corresponds to ~0.004-0.04 μM ¹⁰⁴. We tested pharmacological doses between 0.01-5 μM on CACs, which partly overlaps with the normal range. The use of glucocorticosteroids should be restricted to the *in vitro* generation of CACs, because glucocorticosteroids (pharmacological doses) inhibit angiogenesis and arteriogenesis both *in vitro* and *in vivo*¹⁰⁵⁻¹⁰⁸. Furthermore, cortisol is associated with atherosclerosis development^{109,110}, increased myocardial infarction size^{111,112}, and major adverse cardiovascular events in post-AMI patients¹¹³. On the other hand, systemic use of M-CSF has also some disadvantages. While M-CSF accelerates neovascularization of ischemic hind limbs by stimulating the mobilization of EPCs from the bone marrow¹¹⁴, it may contribute to atherosclerotic plaque progression¹¹⁵ and is associated with cardiac dysfunction in patients with AMI¹¹⁶.

Finally, we identified a mechanism by which galectin-2 may impair arteriogenesis in CAD patients. We have shown that human galectin-2 is an endogenous ligand for CD14, inducing a pro-inflammatory phenotype in monocytes and macrophages by activating TLR4 signaling pathway. In contrast, other studies have shown that activation of TLR4 signaling pathway in monocytes/macrophages is favorable for arteriogenesis, irrespective of the type of ligand, by inducing monocyte/macrophage recruitment from the bone marrow to the growing collaterals^{117,118}. This can be explained by the fact that the TLR4-accessory molecule RP105 (CD180) is present on monocytes/macrophages, which specifically controls TLR4-mediated inflammatory response by inhibiting TLR4-ligand binding and thus TLR4 over-activation¹¹⁹. However, in our study galectin-2 possibly reduces RP105 expression or function, which may lead to an uncontrolled TLR4-mediated inflammatory response and thus TLR4 over-activation in monocytes/macrophages leading to their reduced migration to the growing collaterals¹¹⁹. It has been reported that the fungal form of galectin-2 can be secreted via the non-classical secretory pathway¹²⁰, suggesting that secretion of human galectin-2 may occur by a similar molecular mechanism. However, human galectin-2 cannot be detected in supernatants of cultured monocytes, but was detected on the monocytic cell surface⁷⁴, suggesting that human galectin-2 directly adheres to the monocytic cell surface after secretion (Figure 4). Thus, when considering a treatment for CAD patients with a low arteriogenic response, neutralization of human galectin-2 protein by a blocking antibody in blood plasma of these patients might be challenging. One solution might be modulating galectin-2/TLR4-mediated inflammatory response in monocytes/macrophages of CAD patients with a low arteriogenic response by inducing RP105 expression or restoring its function. Van der Laan *et al.* reported a SNP (rs7291467; 3279 C>T in intron 1) of galectin-2 gene, associated with a low arteriogenic response in CAD patients, with CC genotype resulting in higher galectin-2 mRNA expression in monocytes/macrophages⁷⁴. It will be also interesting to interfere with

the high galectin-2 mRNA expression in these cells of CAD patients. To establish this, we also need to know the galectin-2 expression levels in the different monocyte subsets and macrophage subtypes to specifically target the proper cells. A reason why galectin-2 may be a good therapeutic target in CAD patients with a low arteriogenic response is that galectin-2 is also expressed by vascular cells of human atherosclerotic lesions¹²¹, indicating its involvement in atherosclerosis. Thus, inhibition of galectin-2 may improve arteriogenesis and prevent atherosclerosis. In conclusion, suppression of galectin-2 expression or signaling in monocytes/macrophages is a promising future therapeutic strategy for stimulation of arteriogenesis in CAD patients with a low arteriogenic response.

Taken together, we have described a number of novel immune modulating targets and strategies in bone marrow-derived cells which can be used to prevent atherosclerosis progression and to improve neovascularization in patients with IHD. However, further studies are required before these targets and strategies can be applied into the clinic.

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