Monocytes in Ischemic heart disease
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General introduction and outline of the thesis

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**Bone marrow cell therapy after acute myocardial infarction: the HEBE trial in perspective, first results**
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**Healing and adverse remodelling after acute myocardial infarction: the role of the cellular immune response**
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**Targeting angiogenesis to restore the microcirculation after reperfused MI**
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**Collateral artery growth in man, from assessment to stimulation**
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GENERAL INTRODUCTION

Ischemic heart disease is the most important cause of heart failure and cardiac death, and a leading health problem in Western countries. This disease is characterized by an impaired coronary blood supply, commonly due to atherosclerosis, leading to myocardial ischemic injury. In many patients, the reparative responses fail to prevent deterioration of cardiac function after myocardial ischemia, resulting in an increased risk for progression to post-infarct congestive heart failure. Therefore, the augmentation of cardiac repair is an appealing concept to improve clinical outcome.

The studies described in this thesis were performed to gain more insight into the role of immune cells in the reparative responses to acute ischemic heart disease, and hence, to find new clues for treatment. We initially studied the effect of adjuvant therapy with intracoronary infusion of autologous bone marrow mononuclear cells (BMMC) and peripheral blood mononuclear cells (PBMC) after acute myocardial infarction (AMI) in the large multicenter, randomized HEBE trial. A sub analysis of the HEBE trial suggested that another type of immune cells, i.e. monocytes, intriguingly regulate healing after AMI. Using clinical and experimental approaches, we studied the source, recruitment and functions of monocytes in the post-AMI healing process. Furthermore, we explored their dual role in atherosclerosis and arteriogenesis, two processes that are known to influence the development of ischemic heart disease.

Healing and adverse remodelling following AMI

AMI is usually caused by a disruption of an atherosclerotic plaque in one of the coronary arteries with superimposition of coronary thrombosis. Sudden obstruction of a coronary artery results in myocardial ischemia and, if such an obstruction persists for longer than 20-30 minutes, infarction of the myocardial tissue.

Immediately after AMI, an intense inflammatory reaction is triggered. In the inflammatory phase of healing, neutrophils and monocytes are attracted to the infarcted area by cytokines and other mediators\(^1,2\) to remove dead cells and to promote extracellular matrix degradation. Thereafter, monocytes/macrophages produce cytokines and growth factors and regulate the formation of granulation tissue during the proliferative phase. In this phase, new blood vessels are formed and fibroblasts produce new extracellular matrix. Finally, in the maturation phase, extracellular matrix is remodelled, fibroblasts and vascular cells undergo apoptosis and a mature collagen-based scar is formed (Figure 1).\(^3,4\)

Patients with AMI exhibit a marked variable response in LV recovery (Figure 2). In a substantial proportion of AMI patients, the repair mechanisms induce profound structural and functional changes, not only in the infarct zone, but also in the noninfarcted area. These patients show myocardial thinning and expansion of the infarct zone in the early phase after AMI, and pathologic cardiomyocyte hypertrophy, apoptosis and extracellular matrix remodelling in the remote zone, a process that may continue for months. These underlying mechanisms induce alterations of the left ventricular (LV) shape, mass, volume, and function, also referred to as adverse LV remodelling (Figure 3). Although some of these changes may be adaptive and physiological as short-term compensation for the sudden loss of contractile function in the infarcted area it may lead over time to heart failure and cardiac death.\(^5\)
Therefore, adverse LV remodelling is considered to be a maladaptive process.

The high incidence of adverse LV remodelling following AMI and its negative prognostic implications justify the search for therapeutic strategies that attenuate this process. Important determinants of adverse LV remodelling after AMI include the extent of infarction and LV loading conditions. So far, most efforts have been focussed on reducing infarct size by timely restoration of myocardial perfusion using mechanical and/or pharmacological interventions, and by unloading of the left ventricle using drugs that target the renin-angiotensin-aldosterone system and the sympathetic nervous system. However, the healing process following AMI is complex and also involves the immune system as a critical modulator of the post-AMI adverse remodelling process. Excessive breakdown of extracellular matrix by neutrophils and monocytes/macrophages early after AMI may mechanically weaken tissue, and thus, may lead to LV dilation, aneurysm formation or even rupture. Furthermore, poor healing and enhanced fibrosis in the proliferative and maturation phase may stiffen the left ventricle and reduce LV compliance. Accordingly, modulation of the immune response may serve as a potential therapeutic strategy for attenuating adverse LV remodelling after AMI.

**Figure 1** Immune cells are centrally involved in post-AMI healing. Images of haematoxylin and eosin staining of normal human myocardium and different phases of infarcted human myocardium at 200x magnification (left panels) and schematic representations (right panels). In the inflammatory phase, neutrophils and classical monocytes transmigrate into the infarcted myocardium, attracted by complement factors, CC chemokine ligand (CCL)-2 (also known as MCP-1) and other mediators, to remove dead cells and debris. Subsequently, non-classical monocytes are recruited to the infarcted area in the proliferative phase. In this phase, new blood vessels are formed and fibroblasts produce extracellular matrix. Finally, fibroblasts and vascular cells undergo apoptosis in the maturation phase and a mature scar is formed (for further details see text). Courtesy of Dr. H.W.M. Niessen, Department of Pathology and Cardiac Surgery, ICar-VU, VU University Medical Centre, Amsterdam, the Netherlands (left panels). CCL-2, CC chemokine ligand 2; CF, complement factor; CM, cardiomyocyte; cM, classical monocyte; FB, fibroblast; IL-10, interleukin 10; L, lumen; Mac, macrophage; MMP, matrix metalloproteinase; N, neutrophil; ncM, nonclassical monocyte; TGFβ, transforming growth factor β.
Bone marrow cell therapy to augment infarct healing

Progenitor cells and stem cells circulate in the peripheral blood in low numbers. However, the blood levels of these cells transiently increase in patients with AMI\(^\text{12,13}\). Bone marrow contains several populations of progenitor cells and stem cells, including endothelial progenitor cells, hematopoietic stem cells and mesenchymal stem cells, which are known for their capability of generating and sustaining fully differentiated cells.

In 2001, Orlic \textit{et al.} showed that intramyocardial injections of bone marrow cells resulted in partial restoration of the infarcted myocardial tissue in a murine model of nonreperfused AMI\(^\text{14}\). Additional evidence for involvement of circulating cells in the preservation of myocardial tissue came from a post-mortem study of Quaini \textit{et al.}, demonstrating migration of precursor cells from the recipient to the donor sex-mismatched transplanted hearts\(^\text{15}\). Since then, circulating progenitor cells and stem cells derived from the bone marrow were thought to contribute to infarct healing, attenuating adverse LV remodelling, either through effects on apoptosis, angiogenesis and fibrosis, or regeneration of myocardium\(^\text{16}\). The capability of bone marrow cells to regenerate infarcted myocardial tissue is still under debate, as other laboratories were unable to reproduce the findings by Orlic \textit{et al.}. The potential beneficial effects of bone marrow cells may be more likely induced by paracrine effects such as enhanced arteriogenesis and angiogenesis following the release of angiogenic and arteriogenic cytokines\(^\text{17}\). This hypothesis was strengthened by the group of Kamihata \textit{et al.}, showing an increased number of arterioles in response to PBMC and BMMC injection in the ischemic myocardium of a pig model of AMI\(^\text{18}\).

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure2.png}
\caption{Recovery of the global LV function following AMI in patients. The lines represent the change in LV ejection fraction from baseline to 4 months follow-up, observed in individual patients randomized to the control group of the HEBE trial (n=60). The LV ejection fraction significantly increased from 42.4±8.3\% to 46.4±9.2\% (P<0.001), as measured by CMR (modified from Hirsch \textit{et al.}\(^\text{26}\)).}
\end{figure}
After the first clinical trials of intracoronary infusion of BMMCs following AMI showed the method to be safe and feasible\(^\text{19,20}\), randomised trials were initiated to further evaluate the therapeutic effect of BMMC therapy after AMI. In 2006, the large randomized Reinfusion of Enriched Progenitor Cells and Infarct Remodelling in Acute Myocardial Infarction (REPAIR-AMI), in which 204 patients were included, reported a significant improvement in LV function following intracoronary BMMC therapy.\(^\text{21}\) However, the results of subsequent trials varied considerably.\(^\text{22-24}\)

To address this issue, the HEBE trial was designed in the Netherlands: a large, multicentre, randomised trial, evaluating the effect of intracoronary infusion of autologous BMMCs and the effect of intracoronary infusion of PBMCs after primary percutaneous coronary intervention (PCI).\(^\text{25}\) As the beneficial effects of the BMMCs may be attributed to the paracrine effects of all mononuclear cell subsets, rather than the progenitor subpopulation, a patient group receiving intracoronary PBMCs was included in the trial. Between 2005 and 2008, a total of 200 patients with AMI successfully treated by primary PCI, were included in the trial. All patients underwent cardiovascular magnetic resonance (CMR) imaging, followed by randomization to treatment with BMMCs (\(n=69\)), PBMCs (\(n=66\)), or no cell therapy (\(n=65\)). After 4 months, CMR was repeated. The trial did not show significant differences between the two treatment groups and the control group with regard to the primary endpoint, the percentage of dysfunctional segments at baseline with improved segmental wall thickening at 4 months, and the secondary endpoints of change in LV ejection fraction LV volumes, LV mass, and infarct size.\(^\text{26}\) A meta-analysis of randomized trials showed at least a modest improvement in LV ejection fraction over conventional therapy at 6 and 12 months after primary PCI.\(^\text{27}\)

**Monocytes in infarct healing**

Although BMMC therapy did not beneficially influence post-AMI recovery in the HEBE trial, data from this trial indicated that another type of immune cells, i.e. monocytes, play an important role in this process. Monocytes originate in the bone marrow, circulate in the blood for several days and travel to peripheral tissues where they differentiate into macrophages or dendritic cells,\(^\text{28,29}\) and contribute to host defence, tissue remodelling and repair. Upon AMI, monocytes are involved in various parts of the healing process. In the inflammatory phase after AMI, monocyte/macrophages clear the infarcted myocardium from dead cells and debris, and promote matrix breakdown. In the proliferative phase, monocytes/macrophages secrete a wide variety of growth factors and cytokines that repress inflammation and stimulate fibroblast growth and angiogenesis. Moreover, monocytes/macrophages participate in the regulation of extracellular matrix remodelling trough the production of matrix metalloproteinase (MMP) and their inhibitors.\(^\text{4}\) Although monocytes are generally referred to as macrophages after infiltration into the infarcted tissue, results from mice studies indicate that monocytes may have distinct fates. While pursuing their functions, infiltrated monocytes may eventually differentiate into macrophages, emigrate from the site of injury, or die by apoptosis or necrosis and are rapidly replenished by newly recruited monocytes.\(^\text{30}\) It has been recently shown after coronary ligation in mice, that a large proportion of these newly recruited monocytes is provided by the spleen, indicating that the spleen contains an important extramedullary reservoir for
In blood, monocytes are a heterogeneous pool of cells, containing classical, intermediate, and nonclassical monocytes, which have distinct phenotypes and functions. Nahrendorf et al. have shown in a murine model of nonreperfused AMI that classical monocytes accumulate primarily in the inflammatory phase and promote inflammation and removal of dead cells and debris by producing tumour necrosis factor (TNF)-α and MMPs. In contrast, nonclassical monocytes produce interleukin (IL)-10 and the profibrotic factor transforming growth factor (TGF)-β and are recruited in the proliferation phase to attenuate inflammation and to promote tissue repair.
There is no doubt that recruitment of monocytes is a prerequisite for proper infarct healing. Monocytes and macrophages are necessary to remove necrotic tissue and to stimulate granulation tissue and scar formation. However, excessive accumulation of activated monocytes, may deleteriously affect the healing process, and thereby evoke adverse LV remodelling. Several clinical studies have measured the levels of circulating monocytes following AMI in patients and reported an association between post-AMI monocytosis and adverse LV remodelling.

So far, experimental models of AMI have been crucial in providing insights into the source, recruitment and roles of monocytes in infarct healing. However, considerable differences exist between the immune systems in experimental models and patients, not to mention the effects of aging and co-morbidities (e.g. dyslipidemia and diabetes mellitus) which are complex to implement in experimental models. Therefore, a better understanding of the monocyte response in patients with AMI is needed. By studying results obtained from AMI patients in experimental models, and vice versa, many mechanisms that regulate the monocyte response following AMI in patients may be uncovered and new options for treatment may be found.

**Monocytes and atherosclerosis**

Atherosclerosis is the major cause of AMI. Atherosclerosis has been recognized as a progressive inflammatory disease which is hallmarked by the accumulation of lipids in the arterial wall and chronic inflammation, resulting in the formation and build-up of atherosclerotic plaques and narrowing of the vessels (stenosis). Studies have shown evidence that monocytes are active mediators of atherosclerosis, rather than innocent bystanders. Monocytes/macrophages are abundantly present in atherosclerotic plaques, and on prolonged residence acquire the phenotype of foam cells. Local activity of these cells leads to the production of proteases, coagulation factors, radicals and other inflammatory mediators that can influence the stability of the plaque and hence, the risk of plaque disruption, arterial occlusion and infarction.

The finding that circulating monocytes consist of several subsets has raised several questions about the role of each monocyte subset in the initiation and progression of atherosclerosis. Studies investigating the dynamics of monocyte subset recruitment to atherosclerotic plaques have shown that classical monocytes continuously enter the growing atherosclerotic plaque, whereas nonclassical monocytes enter the plaque less frequently. Since then, the progression of atherosclerosis in murine models is considered to be driven in large part by the classical monocyte subset. In contrast, higher blood levels of the nonclassical monocyte subset were found in patients with various stages of coronary artery disease in comparison to control patients. Currently, the exact role of monocyte subsets in atherosclerosis in patients is not completely understood.

Despite the contrasting findings, both experimental studies as well as clinical studies have unequivocally demonstrated that the level of circulating monocytes increases after AMI, and that the increased availability of activated monocytes after AMI is associated with LV dilation and a higher propensity to develop heart failure. It is therefore important to clarify whether the increased levels of circulating monocytes after AMI also influences the progression of atherosclerosis and thus the risk for reinfarction, creating a vicious circle.
Monocytes in arteriogenesis

Paradoxically, monocytes contribute to atherosclerosis, but also regulate arteriogenesis, a natural escape mechanism from progressive atherosclerosis. Arteriogenesis refers to the remodelling of pre-existing arteriolar anastomoses into large collateral arteries that divert blood flow around obstructive lesions (Figure 4A-D). This process is triggered by a pressure difference between the vascular territory of the obstructed artery (low pressure) and the territories of one of the other arteries (high pressure), resulting in an increased blood flow and shear stress in the interconnecting arterioles. Approximately 12 hours after activation of the endothelium by shear stress, monocytes start to accumulate into the perivascular space of the growing vessels, peaking between one and three days. After differentiation into macrophages, monocyte/macrophages promote inflammation and secrete MMPs,

Figure 4 Mechanisms of arteriogenesis. (A) Without significant stenosis, there is no significant pressure gradient over pre-existing collateral anastomoses, which are small and barely carry blood. (B) Development of a significant arterial obstruction leads to a drop in pressure and oxygen saturation distal in the vascular bed (purple-blue colour), while proximal pressure and oxygen saturation proximal remain normal (red colour). The pressure gradient over the collateral circulation increases fluid shear stress in these arterioles. (C) Close-up on the cellular level. The endothelium senses increased fluid shear stress via its cytoskeleton, transmembrane proteins (integrins, ion channels) and the glycocalyx. In an activated state, the endothelium expresses adhesion molecules, such as intercellular adhesion molecule (ICAM)-1, to which circulating monocytes bind via their macrophage (Mac)-1 receptor. Monocytes transmigrate into perivascular tissue, differentiate to macrophages and secrete growth factors and cytokines that attract further monocytes and stimulate proliferation of smooth muscle cells and endothelial cells. (D) Adequately developed collateral arteries restore distal perfusion and provide sufficiently oxygenated blood to distal tissues. bFGF, basic fibroblast growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICAM, intercellular adhesion molecule; MCP, monocyte chemoattractant protein; MMPs, matrix metalloproteinases; TGFβ, transforming growth factor β (Reproduced from Schirmer et al., with permission from BMJ Publishing Group Ltd).
creating space for the vessels to expand. Furthermore, these cells produce cytokines and growth factors that promote proliferation of smooth muscle cells and endothelial cells, and remodelling of the arterioles (Figure 4C). Finally, large collateral arteries are formed with the ability to compensate blood flow deficits caused by arterial occlusions.45

In patients with chronic coronary artery disease, many studies have shown an association between collateral flow and improved clinical outcome, i.e. signs of ischemia, preservation of the myocardial function, risk of AMI and survival.23,46,47 Although the severity of stenosis is one of the most important determinants of the extent of the arteriogenic response,48,49 the group of Seiler have demonstrated that even for a given stenosis, there is a large heterogeneity in the arteriogenic response.49 It has been estimated that 20 percent of the patients with coronary artery disease cannot be revascularized by PCI or coronary artery bypass graft (CABG) surgery.50 Hence, the stimulation of collateral artery growth, especially in this patient group, may be a valuable therapy.

Experimental studies have shown that depletion of monocytes using cytostatic agents like 5-fluorouracil or liposomes, containing cytotoxic bisphosphonates, severely impairs the arteriogenic response.44,51 Accordingly, factors such as monocyte chemotactic protein (MCP)-1 and granulocyte macrophage colony-stimulating factor (GM-CSF) that stimulate the recruitment of monocytes were tested for their effect on arteriogenesis.52,53 Although both MCP-1 and GM-CSF were shown to enhance the arteriogenic response in experimental studies, increasing the availability of monocytes may also has a potential downside, as MCP-1 was shown to worsen atherosclerotic disease in a hypercholesterolaemic murine model.52 The mechanisms underlying collateral artery growth closely resemble those of atherosclerosis: both are inflammatory processes involving enhanced monocyte recruitment into the vessel wall, resulting in a trade-off of potential pro-arteriogenic effects and pro-atherogenic effects. Therefore, the challenge is to develop a therapeutic strategy that augments arteriogenesis without aggravating atherosclerosis.

Catheter based hemodynamic measurements at the cardiac catheterization laboratory have opened new avenues to investigate the dynamic behaviour of the coronary collateral circulation in patients with coronary artery disease. Since large heterogeneity exists in the collateral response amongst patients with coronary artery disease, comparative studies of patients with either a reduced collateral flow or enhanced collateral flow may provide important insights into the mechanisms involved in this process, leading to new therapeutic options.54
OUTLINE OF THE THESIS

In the multicentre, randomized HEBE trial, we previously studied the effects of intracoronary infusion of autologous BMMCs and PBMCs after first AMI. Experimental studies suggested that bone marrow cell therapy improves post-AMI functional outcome by enhancing neovascularisation. Therefore, in chapter 2, we examine the effect of bone marrow cell therapy on the recovery of the microcirculation in the HEBE trial using intracoronary Doppler flow measurements. In the same trial, we investigate the long-term effects of bone marrow cell therapy on functional outcome following AMI which are presented in chapter 3.

Next, we move our focus from bone marrow cells towards monocytes. In patients with AMI, monocytosis is a predictor of poor functional outcome. In chapter 4, we present the relation of the level of circulating classical and nonclassical monocytes following AMI in patients with myocardial injury and functional outcome using CMR imaging techniques. In chapter 5, we investigate the transcriptome of these circulating monocytes following AMI to identify additional characteristics of the monocyte response that relate to post-AMI adverse remodelling. In chapter 6 the influx of monocytes into the infarcted myocardium is studied in patients that died at different time points after AMI. Also, we explore the potential source of these monocytes by studying monocytes in the bone marrow and the spleen. So far, it is unclear whether monocytes also play a role in post-AMI adverse remodelling of the noninfarcted zone. Therefore, chapter 7 focuses on the influx of monocytes in both the myocardial infarct zone, as well as the noninfarcted remote zone in a murine model of AMI, using positron emission tomography/computed tomography (PET/CT), CMR imaging, flow cytometric and immunohistochemical techniques. Furthermore, we evaluate the numbers of macrophages in the remote zone in post-mortem tissue specimens of patients that died after AMI.

Monocytes play an important role in atherosclerosis as well as arteriogenesis. In chapter 8, we investigate the gene expression profiles of circulating monocytes from patients with coronary artery disease and control patients to find monocyte characteristics that relate to coronary artery disease. So far, it is unknown whether post-AMI monocytosis influences the progression of atherosclerosis. Therefore, we investigate the effect of post-AMI monocytosis on the progression of atherosclerosis in ApoE−/− mice in chapter 9. We also explore the background mechanisms by which stem cells and progenitor cells contribute to the supply of these monocytes.

Arteriogenesis has previously been reported to be inhibited by interferon-β signalling in monocytes. We explore the underlying mechanism by which interferon-β signalling in monocytes influences the arteriogenic response, and results are presented in chapter 10. In search of additional targets that regulate arteriogenesis, we investigate the gene expression profiles of monocytes from patients with a chronic total coronary occlusion, and relate these transcriptome profiles to the capacity of the collateral circulation in chapter 11.

In the final chapter 12, we summarize our data, implement our findings in the context of recent developments in cardiovascular research, and propose what research is needed to increase understanding of the monocyte response in AMI patients and to ultimately find options to improve recovery following AMI in patients.
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Introduction and outline


