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Monocytes in ischemic heart disease

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Chapter 12

Summary of the thesis, concluding remarks and future directions

Anja M. van der Laan

SUMMARY OF THE THESIS

The link between the immune system and ischemic heart disease has been recognized for years and great improvements have been made in understanding the role of immune cells in the context of infarct healing, atherosclerosis and arteriogenesis, using experimental and *in vitro* models. However, the role of each cell subset is complex, and considerable differences exist between the reparative responses in experimental models and patients. Therefore, the main aim of this thesis was to gain more insight into the role of immune cells, particularly monocytes, in the reparative responses to acute and chronic ischemic heart disease in patients, and hence, to find new clues for treatment. The results presented in this thesis are derived from clinical studies, autopsy studies, experimental studies and *in vitro* studies, and were performed in close collaboration with various research groups. This interdisciplinary effort has resulted in a more integrated understanding of how monocytes are involved in the reparative responses to acute and chronic ischemic heart disease.

Bone marrow cell therapy to augment infarct healing

To date, reperfusion strategies and advances in pharmacological management have resulted in an increasing proportion of survivors of acute myocardial infarction (AMI) at heightened risk of developing left ventricular (LV) remodelling and heart failure. Over the past decades, cell-based therapies have evoked great interest as an adjuvant therapy to augment the reparative response after AMI. However, the mechanisms by which cell therapy confers benefit are still unclear. Experimental studies have shown that bone marrow mononuclear cell (BMMC) therapy enhances neovascularisation and improves functional recovery following AMI.^{1,2} Thus, the predominant mechanisms of BMMCs for improving cardiac function have pointed towards paracrine effects rather than transdifferentiation into cardiomyocytes. However, in patients, evidence for this hypothesis is scarce.³

In **chapter 2**, the effect of BMMC and peripheral blood mononuclear cell (PBMC) therapy on the recovery of the microcirculation was assessed in AMI patients that participated in the HEBE trial, using intracoronary Doppler flow measurements. Paired Doppler flow measurements were available for 23 patients in the BMMC group, 18 in the PBMC group, and 19 in the control group. Coronary flow was assessed with intracoronary Doppler flow measurements at 3 to 8 days after primary percutaneous coronary intervention (PCI) and repeated at 4 months of follow-up. No differences were found in the change of coronary flow velocity reserve and hyperemic microvascular resistance index from baseline to 4-month follow-up between the two treatment groups and the control group. Thus, adjuvant therapy with BMMCs or PBMCs does not improve the recovery of microcirculation in the HEBE trial. This contradicts the hypothesis of enhanced neovascularisation after this mode of cell therapy.

It has been hypothesized that BMMC therapy may exert its beneficial effects on a longer term than 4 months. **Chapter 3** describes the long-term effect of BMMC and PBMC therapy on the recovery of the myocardial function in the HEBE trial. At 2 years of follow-up, there were no significant differences between the two treatment groups and the control group, regarding the percentage of dysfunctional segments

at baseline with improved segmental wall thickening and LV ejection fraction.

Monocytes in infarct healing

Monocytes are important mediators of healing after AMI. Clinical studies have shown that elevated levels of circulating monocytes are associated with adverse LV remodelling after AMI and development of heart failure.^{4,5} It has been speculated that excessive breakdown of extracellular matrix by monocytes/macrophages early after AMI may mechanically weaken tissue, and thus, may lead to LV dilation, aneurysm formation or even rupture. Furthermore, enhanced fibrosis during the proliferative and maturation phase may stiffen the left ventricle and reduce LV compliance. Accordingly, modulation of the monocyte response in patients may serve as a potential therapeutic target for attenuating adverse LV remodelling after AMI. Hence, increased insight into the systemic monocyte response following AMI is essential to find new therapeutic avenues for treatment.

In **chapter 4**, circulating levels of classical and nonclassical monocytes were evaluated in 58 AMI patients that participated in the HEBE trial to investigate their relation with myocardial injury at baseline and functional outcome at 4 months of follow-up. Patients with high levels of classical monocytes had impaired LV ejection fraction, larger infarct size, and often presence of microvascular obstruction at baseline. At 4 months of follow up, high levels of classical monocytes were negatively associated with the regional systolic LV function, independent of the transmural extent of infarction. In contrast, positive associations for the levels of nonclassical monocytes were observed. Overall, a significant association between high levels of classical monocytes, and severe myocardial injury and poor functional outcome following AMI was found.

Monocytes not only consist of distinct subsets, but monocytes can also adapt their functional properties in response to tissue injury. **Chapter 5** describes a search for additional monocytic characteristics that relate to adverse LV remodelling following AMI in patients. Transcriptional profiling and pathway analysis of the circulating monocytes from 51 patients that participated in the HEBE trial revealed that AMI patients with a higher increase in LV end-diastolic volume from baseline to 4 months follow-up showed attenuated type I interferon (IFN) signalling. Furthermore, in patients that died after AMI, local expression of IFN- α protein by monocytes/macrophages that infiltrated the infarcted myocardium was observed. These findings reveal a link between adverse LV remodelling following AMI and attenuated type I IFN signalling in monocytes, suggesting that type I IFNs may beneficially influence the LV remodelling process in patients with AMI.

Chapter 6 describes a detailed histological analysis of clinical autopsy material to gain further insights into the systemic monocyte response following AMI in patients. This chapter shows a unique spatio-temporal pattern of monocyte accumulation in the human myocardium following AMI. Monocytes, recruited 12 h–5 days after AMI, predominantly accumulate in the infarct border zone and are located adjacent and also adherent to cardiomyocytes. In contrast, 5–14 days after AMI, monocytes almost exclusively invade the infarct core, consisting of granulation tissue. Subsets analysis showed abundant presence of the classical subset in the border zone at 12 h–5 days after AMI, whereas comparable proportions of classical and

nonclassical monocytes were present in the infarct core at 5–14 days. Importantly, in the AMI patients, the number of monocytes was significantly decreased by 39% in the bone marrow and by 58% in the spleen, in comparison to control patients, suggesting that the human spleen contains an important reservoir function for monocytes.

So far, it is unclear whether monocytes also play a role in remodelling of the noninfarcted myocardium following AMI. The study in **chapter 7** unequivocally demonstrates the influx of monocytes in the infarcted area as well as the noninfarcted myocardium following AMI in mice, using advanced positron emission tomography/computed tomography and magnetic resonance imaging techniques. This finding was mirrored by the presence of macrophages in the noninfarcted myocardium of patients that died after AMI. Furthermore, an increase of recruiting adhesion molecules, chemokines and matrix metalloproteinase activity was found in the noninfarcted myocardium following AMI, as compared to myocardium from control mice, suggesting that monocyte recruitment to the remote zone may contribute to post-AMI remodelling of the noninfarcted myocardium.

Monocytes and atherosclerosis

It is well established that monocytes are involved in the pathophysiology of atherosclerosis. The recruitment of monocytes into plaques drives progression of the disease. Infiltrated monocytes are known to elicit pathogenic immune responses in the vascular wall, contributing to the local inflammatory environment. The identification of factors that associate with atherosclerosis may reveal new mechanisms that underlie the progression of atherosclerotic disease.

In **chapter 8**, the transcriptome of resting and stimulated monocytes, macrophages, and T-cells is compared between patients with coronary artery disease and control patients to find gene expression profiles that relate to atherosclerotic disease. Cautious patient matching revealed only small differences in transcriptional activity in different mononuclear cell types. Paradoxically, inflammatory genes and surface markers were found to be downregulated in monocytes of atherosclerotic patients. The inflammatory vascular milieu in these patients might in fact be the cause of this suppressed inflammatory gene expression in circulating monocytes.

Since the level of circulating monocytes increases after AMI, it is 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. The study in **chapter 9** shows that *Apoe*^{-/-} mice develop larger atherosclerotic lesions when subjected to AMI. This disease acceleration persisted over many weeks and was associated with increased monocyte recruitment. Upon AMI, liberation of haematopoietic stem and progenitor cells from bone marrow niches was triggered via sympathetic nervous system signalling. These progenitors subsequently seeded the spleen, yielding a sustained boost in monocyte production. These findings provide new mechanistic insight into atherogenesis and provide a novel therapeutic opportunity to mitigate atherosclerotic disease progression.

Monocytes in arteriogenesis

Arteriogenesis is a natural escape mechanism to progressive atherosclerotic disease, and is critically mediated by infiltrating monocytes, supplying locally the necessary growth factors and degrading enzymes. Knowledge on factors involved in human arteriogenesis is scarce. Previously it has been shown that differences in RNA and protein expression of stimulated monocytes are related to the extent of the arteriogenic response, implicating IFN- β signalling as an inhibitor of arteriogenesis in both patients and in an experimental model.⁶ The study in **chapter 10** investigates whether the arteriogenic response can be enhanced by attenuating IFN- β signalling. Inhibition of the IFN- β receptor (IFNAR) increased the proliferation of vascular smooth muscle cells. Furthermore, expression of genes related to apoptosis was attenuated in monocytes from IFNAR^{-/-} mice, indicating reduced monocyte apoptosis. Hindlimb perfusion restoration one week after femoral artery ligation was improved in IFNAR^{-/-} mice compared to wildtype mice. Finally, in stimulated monocytes from patients with chronic total coronary artery occlusion, whole genome expression analysis confirmed increased expression of IFN- β -regulated genes in patients with a low arteriogenic response. These data suggest that blocking IFN- β -signalling may be a novel approach to promote arteriogenesis.

In **chapter 11**, a study was conducted to identify additional targets that relate to the arteriogenic response in patients with a chronic total coronary occlusion. Increased mRNA expression of galectin-2 was found in monocytes of patients with a low arteriogenic response, pointing at an inhibitory role of galectin-2 in arteriogenesis. Additionally, the mRNA expression level of galectin-2 was significantly associated with the rs7291467 polymorphism in *LGALS2* encoding galectin-2. Finally, the study demonstrates that galectin-2 impairs arteriogenesis upon femoral artery occlusion in mice.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Over the last 5 years, during which the studies described in this thesis were performed, interest in the link between the immune system and ischemic heart disease has further increased, and the importance of understanding the role of immune cells in patients is now widely acknowledged. The studies described in this thesis have made significant contributions to our understanding of the role of immune cells, especially monocytes, in the reparative responses to acute and chronic ischemic heart disease, and may pave the way for the next challenge: the development of an immunomodulatory treatment that modulates the immune response, without impairing reparative functions, incapacitating the defence system against pathogens or disrupt homeostatic functions. This will only be successful if the relevant cell subset or characteristics are identified accurately in patients. In the clinical studies described in this thesis, we observed a profound heterogeneity in the natural reparative responses to acute and chronic ischemic heart disease. Using this heterogeneity, we identified several characteristics of the monocyte response that relate to parameters of outcome, and thus, that may serve as a potential therapeutic target for patients with ischemic heart disease. In the following paragraphs, the major

findings of this thesis are discussed, and future research is proposed.

A promising strategy for improving outcome after AMI includes cell therapy to augment repair of the damaged myocardium. In the current thesis, we did not find a beneficial effect of intracoronary delivery of BMSCs following AMI in patients in the HEBE trial, the results of other clinical trials varied considerably.⁷⁻¹⁰ A meta-analysis of these trials showed at least promising results in terms of LV function and LV volumes.¹¹ It has been suggested that these various results are related to differences in study protocols. The use of various protocols along with various clinical endpoints in the clinical trials have left us with many unanswered questions regarding the use of the ideal cell type, the number of cells needed to be delivered for maximal efficacy, optimal isolation and storage techniques, ideal delivery route and optimal time of administration. Furthermore, the mechanisms by which BMSCs exert their putative beneficial effects are still unclear. Additional research on these issues is required to further assess efficacy of this mode of therapy.

So far, clinical studies have primarily focused on the enhancement of cardiac repair using cell-based therapeutic strategies, with only modest success. Very few studies, on the other hand, have dealt with the inflammatory reaction early after AMI, which is critically mediated by monocytes. In this thesis we showed that AMI patients with high levels of circulating classical monocytes have increased myocardial injury and impaired recovery of myocardial function, which is in line with the findings of others.¹² Timely inhibition of classical monocyte recruitment to the ischemic myocardium, and hence, prevention of excessive extracellular matrix degradation seems a worthwhile strategy to maintain structural integrity of the damaged myocardium and to arrest infarct expansion and LV dilation that offset the adverse LV remodelling process. To our best knowledge, direct evidence pointing at a causal role of classical monocytes in the pathophysiology of myocardial injury is still lacking in patients with AMI. Alternatively, monocytes may be recruited because of severe myocardial damage. This “chicken or the egg” causality dilemma has been addressed in experimental studies, showing that inhibition of monocyte recruitment results in enhanced myocardial salvage.¹³ A large clinical study might shed light on the here proposed causative role of classical monocytes in mediating post-infarct myocardial injury. Interestingly, a number of compounds that interfere with classical monocyte recruitment are already at various stages of development. CC chemokine ligand CCL-2 is a potent attractant for monocytes, especially for the classical monocyte subset, as these cells display high membrane expression of its receptor, the CC chemokine receptor (CCR)-2. Experimental studies have shown that targeting CCL-2/CCR-2 can beneficially influence LV remodelling after AMI.¹⁴⁻¹⁶ However, CCR-2 is also expressed by other cells. Thus, additional research is necessary to investigate potential detrimental effects before translation into clinical research can be pursued.

We further demonstrated that monocytes accumulate in distinct regions of the infarcted myocardium at different time points following AMI in patients. Strikingly, classical monocytes accumulated primarily in the infarct border zone (and not the necrotic infarct core) during the inflammatory phase of healing after AMI, adjacent but also adhering to cardiomyocytes, surrounding the necrotic infarct core. Furthermore, classical monocytes also invaded the noninfarcted myocardium (remote from the

infarcted area). It is intriguing to speculate that classical monocytes may contribute to the fate of the cardiomyocytes that survived the primary ischemic period. Future studies are warranted to answer the questions why classical monocytes cells invade the infarct border zone and remote zone, and what their biological role is in these areas.

For a long time, the paradigm dictated that monocytes are recruited from the bone marrow where they originate from hematopoietic stem cells and progenitor cells, and subsequently enter the circulation where they are made available to sites of tissue injury. However, we now provided evidence for the spleen as a major source of monocytes in patients, which parallels the findings from experimental studies.^{17,18} Notably, previous studies have demonstrated that the rapid release of splenic monocytes into the circulation is mediated by angiotensin II, and not by CCR-2, which mediates mobilization of monocytes from bone marrow¹⁷, suggesting that the mobilization of monocytes from the spleen depends on different cues compared to mobilization from the bone marrow.¹⁷ Increased understanding of the mechanisms that regulate storage and release of splenic monocytes in AMI patients may provide new clues for the modulation of the monocyte response.

This thesis showed that the increased availability of classical monocytes not only associates with poor infarct healing, but also detrimentally affects the progression of atherosclerotic disease. Upon AMI, mobilization of haematopoietic stem and progenitor cells from bone marrow to the spleen was triggered via sympathetic nervous system signalling to boost monocyte production in the spleen. Hence, interference with the supply of monocytes after AMI seems an attractive option to curb inflammation in the infarcted area and also mitigate the progression of atherosclerotic disease. Mobilization of hematopoietic stem and progenitor cells is likely mediated through the β -2 and β -3 receptor.¹⁹ A randomized control trial is warranted to investigate the effects of early β -2 and β -3 receptor blockage on parameters of myocardial injury and recovery, and the clinical event rate on the long-term.

Nevertheless, targeting the monocyte response in patients with ischemic heart disease may also have a potential downside. Overlap between the mechanisms that regulate inflammation in the context of infarct healing, atherosclerosis and arteriogenesis has introduced a benefit versus risk phenomenon, as all three processes are critically mediated by monocytes. Thus, therapies aiming at the attenuation of monocyte recruitment in the context of atherosclerosis and infarct healing may impair the arteriogenic response. Vice versa, any pro-arteriogenic compound that enhances monocyte recruitment is likely to have detrimental effects on arteriogenesis. In this thesis we found an intriguing role for type I IFN signalling in both infarct healing and arteriogenesis. Adverse LV remodelling after AMI in patients was associated with attenuated type I IFN signalling, suggesting that type I IFNs, and hence, induction of type I IFN signalling, may beneficially influence post-AMI healing and attenuate adverse LV remodelling. This is in line with the experimental study of Veldhuis *et al.* showing that exogenous administration of the type I IFN inhibits the influx of monocytes into the ischemic area of the brain, and reduces infarct size after stroke in rats.²⁰ On the other hand, this thesis also showed evidence for an inhibitory role of type I IFN signalling in arteriogenesis, indicating that administration

of type I IFNs may impair the arteriogenic response. However, it must be noted that patients with AMI display normal patency of epicardial arteries after successful primary PCI. Therefore, the arteriogenic response is considered to be a process of minor importance, as the trigger for arteriogenesis (i.e. a pressure deficit between a donor artery and the recipient artery), is not present. Furthermore, conflicting data have been reported regarding effects of type I IFN on atherosclerosis in experimental models.^{21,22} Collectively, the administration of type I IFNs following AMI to improve outcome holds promise, but needs careful evaluation.

Finally, in the present thesis, we identified galectin-2 as an inhibitor of arteriogenesis. In addition, galectin-2 expression was associated with a genetic polymorphism (rs7291467). As already mentioned above, future studies testing compounds that block galectin-2 to stimulate arteriogenesis should also evaluate the effects on atherosclerosis and AMI. The association between the polymorphism and susceptibility to AMI has been investigated extensively in clinical studies; however, it was concluded, that a direct association with the rs7291467 polymorphism is unlikely.²³ Nevertheless, these data require further investigation before translation into clinical research can be pursued.

Taken together, a number of promising immunomodulatory strategies mentioned above are worthwhile to pursue, but warrant further studies, of which the results are eagerly awaited prior to their clinical evaluation. In this regard, the development of reliable clinically applicable noninvasive imaging modalities and advanced cell labelling techniques are required, not only to increase knowledge on the systemic monocyte response in patients with ischemic heart disease, but also to provide a template for assessing new immunomodulatory therapies.

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