Innate immune modulation in atherosclerosis and vascular

Wezel, A.

Chapter 9

General summary and perspectives

Summary

The general term "cardiovascular disease" (CVD) comprises diseases of the heart and circulation, including myocardial infarction, stroke and peripheral artery disease. The major underlying cause of CVD is atherosclerosis, in which lesions develop over several decades at predisposed areas in the arterial vasculature, influenced by processes involved in cholesterol metabolism and inflammation. Advances in treatment options, including interventions such as bypass or stent placement, lifestyle changes and lipid-lowering or anti-hypertensive drugs, have contributed to a considerably improved prognosis of CVD. However, despite these developments, CVD are still among the leading causes of death worldwide[1]. Therefore, investigating the underlying mechanisms of atherosclerosis is crucial to identify new therapeutic leads for the treatment of this disease, in order to prevent acute cardiovascular syndromes (ACS).

The formation of early lesions is initiated by endothelial damage and transmigration of monocytes into the vessel wall, which subsequently differentiate into macrophages and scavenge lipids, transforming them into foam cells. Atherogenesis is further aggravated by the influx and inflammatory actions of other immune cells, such as T cells and mast cells. Research has focused on the prevention of destabilization of advanced atherosclerotic plaques, since at this stage most patients present themselves at the clinic. So-called unstable lesions consist of a large lipid core, covered by a thin fibrous cap depleted of smooth muscle cells and collagen. The inflammatory cell count as well as the intraplaque density of microvessels is high, and luminal erosions may occur[2]. Rupture of the plaque results in thrombus formation, which can lead to distal embolization with subsequent tissue ischemia. Therefore, it is promising to direct therapeutic interventions not only towards lesional growth, but also towards stabilization of the atherosclerotic plaque. Furthermore, problems arise after treatment of patients with acute cardiovascular syndromes. A surgical intervention strategy commonly used to restore blood flow to the ischemic tissue is venous bypass grafting. Unfortunately, the patency of these venous conduits is often poor due to failure of the grafts, which may be the result of intimal hyperplasia. Endothelial damage, excessive smooth muscle cell proliferation and inflammation all play a crucial role in vein graft disease (VGD)[3], and more research is necessary to fully elucidate the mechanisms involved in immune responses in this disease.

In this thesis, the involvement and modulation of several components of the innate immune system were investigated in relation to both early atherosclerosis, late stage plaque destabilization and in vein graft disease. In **Chapter 2** the role of mast cell derived mediators in early atherogenesis and advanced atherosclerosis were discussed, as well as ligands capable of mast cell activation in the setting

of atherosclerosis. **Chapter 3** has demonstrated a role for the complement component C5a in the attraction and activation of mast cells in the initiation of vein graft disease. Late stage activation with C5a was seen to result in lesional vein graft disruptions independent of mast cells in **Chapter 4.** In **Chapter 5,** mast cell mediated neutrophil influx in the setting of atherosclerosis was described. Exacerbated vein graft disease accompanied by increased mast cell activation in RP105 deficient mice was observed in **Chapter 6** while the role of the monocyte and RP105 deficiency in early atherogenesis were investigated in **Chapter 7**. Finally, modulation of multiple processes involved in atherosclerosis by use of a microRNA inhibitor was studied in **Chapter 8.**

Mast cells and complement component C5a in atherosclerosis and vein graft disease

Chapter 2 provides a general overview of ligands capable of activating mast cells in the setting of atherosclerosis, as well as the diverse effects that mast cell derived mediators may exert on atherosclerosis. Interestingly, besides an important role for mast cells in atherosclerosis, mast cells were also seen to accumulate in the perivascular tissue of vein grafts during the progression of vein graft disease, as was demonstrated in **Chapter 3.** To establish whether mast cells play a causal role in vein graft disease, venous grafts were placed in mast cell deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice and control C57BL/6 mice. Indeed, a significant decrease in vessel wall thickening was observed in mice lacking mast cells. Accordingly, local activation of perivascular mast cells with a DNP hapten resulted in aggravated lesion formation, accompanied by a decrease in the percentage of lesional smooth muscle cells and endothelial cell coverage. We then aimed to elucidate which ligand is mainly responsible for mast cell activation in the setting of vein graft disease. Previously, mast cells have been found to co-localize with C5aR in atherosclerotic plaques[4]. C5a is one of the end products of the complement system, capable of acting as a chemoattractant as well as inducing a potent inflammatory reaction. Mast cells can be activated via the C5aR and therefore we determined whether C5a may act as a mast cell activator in vein graft disease. First, we provided both mRNA and protein expression patterns of C5 and C5aR during vein graft disease. A distinct peak in C5 expression was visible 6 hours after surgery, while the expression of C5aR was maximally increased 1 day after vein graft placement. Next, we investigated the effects of local treatment with C5a at time of vein graft placement, which significantly increased the vessel wall area. In line with these findings, mice treated with the C5a receptor antagonist PMX-205 displayed reduced lesional area. Interestingly, perivascular mast cell numbers in mice treated with PMX-205 were reduced as well. Finally, to test whether C5a affects vein graft disease specifically via mast cell activation, mice were again locally treated with C5a, this time in combination with systemic injections of the mast cell stabilizer cromolyn or PBS

as a control. Intriguingly, the increase in vessel wall thickening after treatment with C5a could significantly be reduced by cromolyn treatment, indeed indicating a mast cell-dependent mechanism.

In addition to lesion formation, mast cells are known to play a crucial role in lesion destabilization. Previously, Bot *et al.* demonstrated that perivascular mast cell activation with DNP increased the frequency of intraplaque hemorrhages as well as the percentage of lesional apoptotic cells, both indicative of reduced atherosclerotic plaque stability[5]. Therefore, we aimed to determine in **Chapter 4** whether late stage exposure to C5a would increase perivascular mast cell activation with subsequent plaque rupture. Investigating plaque ruptures in murine experimental models is a major challenge, since these events do not usually occur in mice similar to the human situation. Interestingly, signs of disruptions accompanied by fibrin layers and intraplaque hemorrhage have been found in the murine vein graft lesions[6], making this a useful model to study the underlying mechanisms of lesion disruptions. Venous grafts were placed in apoE$^{-/-}$ mice and 24 days after surgery, when advanced plaques were formed, C5a or PBS was focally applied in a pluronic gel at the lesion site. To dissect out potential mast cell mediated effects, C5a treated mice received systemic injections with either cromolyn or PBS. Local application of C5a resulted in a profound increase in the amount of plaque disruptions, with concomitant intraplaque hemorrhage. The observed disruptions were not inhibited by cromolyn treatment, suggesting that at this late stage, C5a does not primarily exert its effects via the mast cell. The contrasting results of C5a activation in early versus advanced lesions can be explained by a difference in the number of cells present in these lesions and the plaque composition. In early lesion formation recruitment of inflammatory cells plays a dominant role, possibly via a range of mast cell derived chemokines such as CXCL2, CCL5 and CCL2. In advanced lesions, the number of immune cells present in the vessel wall is already strongly increased. Therefore, C5a itself may have more direct effects on other cell types involved in plaque stability. Indeed, we observed a dose-dependent increase in cellular apoptosis upon stimulation of both endothelial and smooth muscle cells *in vitro*. This was confirmed by increased lesional cell apoptosis in mice treated with C5a as well, which may contribute to plaque destabilization.

Mast cell mediated leukocyte recruitment

Mast cells excrete a range of mediators upon activation, and for most of these mediators a detrimental role in atherosclerosis has been established. For instance, mast cell specific proteases induce matrix degradation and smooth muscle apoptosis, leading to thinning of the fibrous cap. Basic fibroblast growth factor may increase the frequency of neovessels, while histamine and tryptase contribute to

the leakiness of these vessels. The secreted inflammatory cytokines activate other immune cells and drive the ongoing inflammatory reaction in the vessel wall. However, a definite role for mast cell derived chemokines has not been established yet in atherosclerosis. In **Chapter 5**, we thus investigated whether mast cells can actively recruit inflammatory cells to the plaque by chronic mast cell activation with IgE in µChain mice, which lack endogenous IgE. Interestingly, a striking increase was observed in the number of neutrophils in the intima, and in particular in the adventitia where mast cells reside. To dissect out a direct mast cell mediated effect, mast cell deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice and control mice received intraperitoneal injections with the mast cell activator compound 48/80. No differences were observed in the recruitment of monocyte or lymphocyte populations, however, a profound influx of neutrophils was observed in the control mice, which was absent in mice lacking mast cells. Also, *in vitro* migration of neutrophils was significantly increased after addition of supernatant from activated mast cells to a transwell setup. Thus, these data suggest that mast cell mediated neutrophil influx may be a novel mechanism via which the mast cell can aggravate the ongoing inflammatory process in the setting of atherosclerosis.

Regulatory actions of RP105 in vein graft disease and atherosclerosis

Toll-like receptor 4 is capable of inducing powerful inflammatory signalling; and its detrimental role in atherosclerosis and vein graft disease has previously been established[7,8]. As with many components of the immune system, excessive activation is prevented by the presence of inhibitory molecules to avoid unnecessary tissue damage. In the case of TLR4, inflammatory signalling in macrophages and dendritic cells is inhibited by the accessory molecule RP105 through a direct extracellular interaction. However, RP105 seems to exert a completely different effect on the B cell, leading to its activation rather than inhibition. These dichotomous actions make RP105 a difficult, but even more so an interesting molecule to study. In **Chapter 6** vein grafts were placed in RP105 deficient mice, and we hypothesized that lesion formation would be aggravated due to increased TLR4 signalling. Indeed, vessel wall thickening was markedly increased in mice that lack RP105, moreover, an increased number of plaque disruptions was observed. These findings are in line with a previous study performed by our group, in which damage-induced neointima formation was exacerbated in $RP105^{-/-}$ mice[9]. As vein graft disease is often accompanied by superimposed atherosclerosis, we also investigated how RP105 affects vein graft disease in a hypercholesterolemic setting. In $LDLR^{-/-}$ mice fed a western type diet, RP105 deficiency resulted in a less stable plaque phenotype of the vein grafts. Further *in vitro* investigations demonstrated excessive CCL2 secretion by $RP105^{-/-}$ smooth muscle cells, also, the activation status as well as the proliferative capacity of $RP105^{-/-}$ mast cells was markedly increased. The observed effects on vein graft lesions may therefore

at least partly be explained by aggravated mast cell activation, which is in line with the findings in chapter 3, where we established that perivascular mast cell activation exacerbates vein graft disease.
Previously, we have shown that transfer of RP105$^{-/-}$ bone marrow into control mice results in a reduction of atherosclerotic lesion formation[10]. This was in fact an unexpected finding, taking into consideration the above described effects of RP105 deficiency on vein graft disease and damage-induced neointima formation. The beneficial effects on atherosclerosis were suggested to be caused by alterations in B cells, in particular due to a decrease in activated B2 cells. Since in this study only myeloid cells lacked RP105, we investigated the effect of total body RP105 deficiency on atherosclerosis in **Chapter 7**. Atherosclerotic lesions induced by carotid collars were decreased in RP105 deficient mice compared to control; moreover, lesional macrophage content was significantly reduced. We aimed to further elucidate the mechanisms behind the decrease in macrophages by investigation of the migratory capacity of the monocyte in these mice. Intriguingly, monocyte recruitment to the peritoneum was impaired in RP105$^{-/-}$ mice compared to control mice. *In vitro*, downregulation of the chemokine receptor CCR2 was observed after stimulating monocytes with LPS, which was more profound in monocytes lacking RP105. Therefore, the decreased monocyte recruitment may be caused by exaggerated CCR2 downregulation, possibly contributing to the reduction in lesional macrophages.

Modulating multiple processes involved in atherosclerosis via microRNA inhibition
MicroRNAs (miRs) are short, non-coding RNA strands capable of regulating the expression of multiple genes. It is this specific ability that makes them suitable targets for the treatment of complex diseases involving various processes, such as atherosclerosis. In order to identify miRs that may have a major impact on atherosclerosis, we made use of a unique Reversed Target Prediction in **Chapter 8**. Instead of taking a miR that is differentially expressed in atherosclerosis, we took 164 atherosclerosis-related genes as a starting point. Next, we determined the number of miRs with predicted binding sites for these genes, and counted them manually. We found enrichment of binding sites for multiple miRs from the 14q32 miR gene cluster (chromosome 12Fl in mice). From this cluster, miR-494 was seen to be abundantly expressed in human atherosclerotic plaques, as well as in murine organs involved in the development of atherosclerosis. Therefore, we selected miR-494 for further investigations. To investigate the effect of miR-494 on atherosclerosis, we placed collars in apoE$^{-/-}$ mice fed a western type diet and inhibited miR-494 in these mice by means of gene silencing oligonucleotides (GSOs). Effective uptake of GSOs in the affected areas of the arteries was confirmed using fluorescently labeled GSOs. Interestingly, we observed a marked decrease of lesion formation in mice treated with GSO-494; moreover, lesions

were more stable, as measured by increased collagen content and a reduction of the necrotic core area. Although we were unable to confirm de-repression of some of the target genes, we detected *in vivo* upregulation of a number of the predicted target genes, such as TIMP3, TGFB2, and IL33, in the atherosclerotic plaque. This is in fact a common challenge in miR research; and as van Rooij *et al*[11] has previously postulated, it is often impossible to attribute the effects of a miR to the regulation of a few specific mRNA targets due to the minor increase in the expression of these targets. Therefore, the mode of action via which miRs are suggested to act is more likely caused by the accumulation of all delicate changes induced in the expression of their target genes.

Future perspectives

In this thesis, modulation of different components from the innate immune system in atherosclerosis and vein graft disease has been investigated. Our data provide new mechanistic insights, as well as possible therapeutic targets for the treatment of cardiovascular diseases. We have shown that mast cell activation, mediated by C5a, aggravates vein graft lesion development. Also, we demonstrated that mast cell mediated neutrophil influx may aggravate atherosclerosis. These data add to an increasing amount of evidence towards a detrimental role for mast cells in vascular remodelling. As of yet, limited clinical trials in humans with cardiovascular disease have been performed using mast cell stabilizers. The PRESTO trial investigated the use of the the anti-allergic drug Tranilast in 10.000 patients undergoing percutaneous coronary intervention[12]. A 9-month follow-up period did not show any effect on major adverse cardiovascular events or restenosis. However, since Tranilast exerts effects on fibroblasts and endothelial cells as well, it is difficult to draw any conclusions regarding mast cell specificity. Recently, a patent for the use of mast cell stabilizers in the treatment and prevention of cardiovascular disease has been published (US8445437 B2)[13]. Thus in the near future, research directed at mast cell stabilization in patients with cardiovascular disease may deliver promising results. It should be noted however that mast cells do not only exert harmful effects. Mast cells are of importance in for example wound healing and they play a vital role as sentinels in our bodies, acting as a first line of defense against bacterial and parasite infections. Therefore, care must be taken with complete systemic mast cell inhibition and unwanted side-effects such as infections should be tightly monitored.

Instead of complete mast cell stabilization, a different approach may also be used, for instance via the inhibition of a specific ligand responsible for one route of mast cell activation in the setting of atherosclerosis. One of these ligands which may be advantageous for drug targeting is complement factor C5a. Our

research has demonstrated adverse effects for C5a mediated mast cell activation on vein graft lesion development. Moreover, we have demonstrated that C5a itself has direct effects on late stage lesional disruptions, indeed making C5a a promising therapeutic target. Previous studies have reported increased serum levels of C5a in patients with CVD[14]; also, high C5a serum levels directly before stent placement have been correlated with increased in-stent restenosis[15]. The therapeutic potential in cardiovascular disease of a C5 inhibitor, the monoclonal antibody Pexelizumab, has already been tested in phase III trials. For instance, patients undergoing coronary artery bypass surgery and reperfusion therapy for myocardial infarction have received treatment with Pexelizumab. Interestingly, a systemic meta-analysis pointed out that Pexelizumab was associated with a 26% reduction in risk of death after coronary artery bypass surgery[16]. Taken together, these data indicate that C5a may be a promising therapeutic target in the treatment of cardiovascular disease.

With regard to the accessory protein RP105, we have made some progress in unraveling the mechanisms behind the regulatory effects of RP105 on inflammation and vascular remodelling. We demonstrated that RP105$^{-/-}$ mice develop exacerbated vessel wall thickening and lesion disruptions in an experimental vein graft model. This is in accordance with a previous report by our group, which showed that neointima formation is increased in RP105$^{-/-}$ mice, while overexpression of the RP105-MD1 complex resulted in reduced neointima formation[9]. These data would suggest that RP105 can be used to develop a novel therapeutic strategy to inhibit neointima formation and vein graft disease. However, in this thesis we have also established that deficiency of RP105 decreases atherosclerosis by reducing monocyte influx. It is therefore of high importance to be aware of differences in pathophysiology underlying atherosclerosis versus neointima formation and vein graft disease. In the latter two cases, smooth muscle cell migration and proliferation plays a more predominant role. Smooth muscle cells lacking RP105 display an increased proliferation rate; also, they excrete increased levels of CCL2, thereby aggravating the disease process. In atherosclerosis, monocyte influx is a crucial step in the initiation of the lesion and defects in monocyte recruitment, as observed in RP105$^{-/-}$ mice, may thus attenuate atherosclerosis. In addition, it has also been shown by our group that RP105 deficiency alters B cell activation and proliferation, which may also reduce atherosclerotic lesion formation[10]. These contrasting data on vein graft disease and neointima formation versus atherosclerosis make it difficult to conclude on the therapeutic potential of RP105. After all, venous grafts and stents are most often placed in patients suffering from atherosclerosis; and improving one disease process while aggravating the other would not be a desirable treatment option. In conclusion, additional research

into RP105 is necessary to establish its therapeutic potential. For instance, vein grafts are particularly accessible for local or even *ex-vivo* treatment. Site-specific RP105 overexpression may thus attenuate vein graft disease without affecting atherosclerosis elsewhere in the arterial vasculature.

MiRs have a unique ability to regulate the expression of multiple genes; and in this thesis, we have aimed to utilize this particular characteristic in order to single out a miR that may exert a broad effect on atherosclerosis. The profound effects on lesion formation and stability after miR-494 inhibition in our study indicates that miRs indeed may serve as an interesting drug target. Only one miR drug, a miR-122 inhibitor (SPC3649), has been used in clinical studies yet for the treatment of hepatitis C[17]. Technical challenges with miR inhibition usually involve delivery, stability and trying to avoid eliciting an immune response. For our studies, we made use of GSOs, which have a modified phosphorothioate backbone to improve stability and linked 5'ends in order to prevent TLR-mediated immune activation. However, additional pre-clinical evaluations of GSOs are required to further investigate their exact mode of action, the time-frame of their inhibitory effects and possible off-target effects. This holds true for the miR we inhibited in our study as well: miR-494. Since we aimed to exert broad effects on atherosclerosis, it is important to exclude the possibility of side-effects. Furthermore, to ensure clinical relevance, it would be interesting to investigate if we could induce plaque regression by inhibiting miR-494. Taken together, miR research is a promising, fast-developing field; and additional studies will have to point out whether their inhibition in patients with cardiovascular disease is a feasible treatment option.

In conclusion, this thesis provides novel insight into the inflammatory and regulatory mechanisms of multiple components from the innate immune system. Furthermore, experimental studies have identified potential therapeutic targets in the setting of atherosclerosis and vein graft disease.

References

1. World Health Organization. The leading causes of death in the world 2012. Fact sheet N°310.
2. Shah, P. K. Mechanisms of plaque vulnerability and rupture. *J. Am. Coll. Cardiol.* 2003;41:15S–22S.
3. Kim, F. Y., Marhefka, G., Ruggiero, N. J., Adams, S. & Whellan, D. J. Saphenous vein graft disease: review of pathophysiology, prevention, and treatment. *Cardiol. Rev.* 2013;21:101–109.
4. Oksjoki R, Laine P, Helske S, Vehmaan-Kreula P, Mäyränpää MI, Gasque P, Kovanen PT, Pentikäinen MO. Receptors for the anaphylatoxins C3a and C5a are expressed in human atherosclerotic coronary plaques. Atherosclerosis. 2007;195:90-9.
5. Bot, I. *et al.* Perivascular mast cells promote atherogenesis and induce plaque destabilization in apolipoprotein E-deficient mice. *Circulation* 2007;115:2516–2525.
6. De Vries, M. R. *et al.* Plaque rupture complications in murine atherosclerotic vein grafts can be prevented by TIMP-1 overexpression. *PloS One* 2012;7:e47134.
7. Lu Z, Zhang X, Li Y, Jin J, Huang Y. TLR4 antagonist reduces early-stage atherosclerosis in diabetic apolipoprotein E-deficient mice. *J Endocrinol* 2013;216:61-71

8. Karper JC, de Vries MR, van den Brand BT, et al. Toll-like receptor 4 is involved in human and mouse vein graft remodeling, and local gene silencing reduces vein graft disease in hypercholesterolemic APOE*3Leiden mice. *Arterioscler Thromb Vasc Biol*. 2011;31:1033-40.
9. Karper JC, Ewing MM, de Vries MR, et al. TLR accessory molecule RP105 (CD180) is involved in post-interventional vascular remodeling and soluble RP105 modulates neointima formation. *PloS One*. 2013;8:e67923.
10. Karper JC, de Jager SC, Ewing MM, de Vries MR, Bot I, van Santbrink PJ, Redeker A, Mallat Z, Binder CJ, Arens R, Jukema JW, Kuiper J, Quax PH. An unexpected intriguing effect of Toll-like receptor regulator RP105 (CD180) on atherosclerosis formation with alterations on B-cell activation. *Arterioscler Thromb Vasc Biol* 2013;33:2810-7.
11. van Rooij E, Olson EN. MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles. *Nat Rev Drug Discov*. 2012;11:860-72.
12. Holmes DR Jr, Savage M, LaBlanche JM, Grip L, Serruys PW, Fitzgerald P, Fischman D, Goldberg S, Brinker JA, Zeiher AM, Shapiro LM, Willerson J, Davis BR, Ferguson JJ, Popma J, King SB 3rd, Lincoff AM, Tcheng JE, Chan R, Granett JR, Poland M. Results of Prevention of REStenosis with Tranilast and its Outcomes (PRESTO) trial. *Circulation*. 2002;106:1243-50.
13. Guo-ping, Shi. 2013. Treatment and prevention of cardiovascular disease using mast cell stabilizers. U.S. patent 8445437 B2, filed July 24, 2007, and issued May 21, 2013
14. Speidl WS, Exner M, Amighi J, *et al*. Complement component C5a predicts future cardiovascular events in patients with advanced atherosclerosis. *Eur Heart J*. 2005;26:2294-9
15. Speidl WS, Katsaros KM, Kastl SP, *et al*. Coronary late lumen loss of drug eluting stents is associated with increased serum levels of the complement components C3a and C5a. *Atherosclerosis*. 2010; 208:285-9.
16. Testa L, Van Gaal WJ, Bhindi R, Biondi-Zoccai GG, Abbate A, Agostoni P, Porto I, Andreotti F, Crea F, Banning AP. Pexelizumab in ischemic heart disease: a systematic review and meta-analysis on 15,196 patients. *J Thorac Cardiovasc Surg*. 2008;136:884-93.
17. Nana-Sinkam SP, Croce CM. Clinical applications for microRNAs in cancer. *Clin Pharmacol Ther*. 2013;93:98-104.