# Modulation of Atherothrombotic Factors: Novel Strategies for Plaque Stabilization

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# 8 General Discussion and Perspectives

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## 1. Introduction

Atherothrombosis is the actual cause of death of atherosclerotic disease in the Western world. Occlusion of an artery by a thrombus formed after rupture of an atherosclerotic lesion may lead to the clinical manifestations such as myocardial infarction (acute coronary events) or stroke[1-3]. The risk of plaque rupture and subsequent thrombus formation largely depends on the morphology and composition of the plaque[4]. In particular, the balance between fibrous cap thickness and lipid core size is regarded essential for the stability of the plaque. A disturbed balance will render the plaque more liable to rupture but will also promote, through the highly thrombogenic content of the plaque core, activation of the coagulation cascade and thrombus formation. Conceivably, modulation of the thrombogenicity of the plaque lipid core, the strength of the fibrous cap or the extracellular matrix content can provide a new therapeutic entry to plaque stabilization and the prevention of acute cardiovascular disease.
A major problem in atherothrombosis research is the apparent resistance of plaques of atherosclerosis-prone mice to plaque rupture and subsequent thrombus formation[5,6]. As described in the general introduction, *p53* upregulation in pre-existing carotid artery plaques by means of adenoviral transfer was found to increase the risk of plaque rupture[7]. Furthermore, in the brachiocephalic artery of ApoE$^{-/-}$ mice plaque rupture has been detected after only 8-9 weeks of a high fat and high cholesterol diet[8]. However, in this model no actual thrombosis is observed. In practice, these models for plaque rupture suffer various pitfalls. The brachiocephalic artery is not easily accessible for local therapeutic interventions. The *p53* induced model is very laborious and plaque rupture is based on apoptosis of smooth muscle cells. Plaque rupture in the brachiocephalic artery is the consequence of plaque expansion. When the therapeutic intervention targets a different mechanism of plaque destabilization or atherothrombosis, one might question the validity of both models. In atherosclerosis-prone mice however, intraplaque hemorrhages are more often observed than actual plaque ruptures. Both in advanced carotid artery collar or ligation models and in the brachiocephalic artery, evidence of intraplaque hemorrhages is frequently shown[9-11]. Also in human atherosclerotic lesions, intraplaque hemorrhage is increasingly recognized as an important factor in plaque stability[12,13]. Erythrocyte accumulation in the plaque leads to enhanced intra- and extracellular deposition of erythrocyte derived cholesterol, which will enlarge the necrotic core and increase the risk of plaque rupture[14].
Nonetheless, on basis of several morphological parameters including macrophage content, fibrous cap thickness and necrotic core size, one can have a good estimate of the stability of plaques generated in atherosclerosis-prone mice. This makes the mouse model very usefull for plaque stability research. Throughout this thesis, we have made use of the carotid artery collar model in hypercholesterolemic ApoE$^{-/-}$ or LDLr$^{-/-}$ mice[15], at which plaques are easily accessible for modulation and the initial atherogenic

stimulus, i.e. shear stress and hypercholesterolemia, are essentially similar to the human situation.
In this thesis, we have studied factors, which can shift the atherosclerotic plaque morphology either to a more stable phenotype, such as the protease inhibitors Serp-1 and Serp-2, and the immunosuppressant FK506, or to a more unstable one (e.g. adventitial mast cell activation). Hence, we have divided this thesis in two parts, the first part describing effects of modifiers of extracellular matrix content and cellular homeostasis on plaque stability and thrombogenicity. In the second part, the focus is more on the role of inflammation at different stages of atherosclerotic lesion development. It should however be realized that the extracellular matrix, cellular homeostasis and inflammation are highly interrelated and cannot be judged apart from one another.

## 2. Matrix and Cell Homeostasis

*Protease Inhibitors*
In atherosclerotic plaque development, proteases cover a wide variety of functions, which all may to some extent influence plaque morphology[16]. Several protease families can be discriminated, e.g. metalloproteinases[17,18], serine proteases[19,20] and cysteine proteases[21,22]. Metalloproteinases, and especially the collagenases and the gelatinases, have been associated with plaque destabilization, as these MMPs are capable of extracellular matrix degradation[23]. They have therefore been extensively studied[24,25] and various MMPs were shown to be highly active in advanced and ruptured atherosclerotic plaques. Various cysteine proteases, and in particular cathepsins[21], are key proteins in atherosclerotic lesion development. For instance, cathepsins S and K, both potent elastolytic enzymes, have been thoroughly investigated with respect to matrix and elastin degrading capacity[26].
Serine proteases play, amongst others, an important role in coagulation. Serine proteases have been implicated in atherosclerotic plaque development, e.g. via tissue factor, which activates the serine proteases of the coagulation system[19,20]. Serine proteases can also activate the inflammatory response and tissue repair. Protein fragments produced after cleavage by serine proteases have also been associated with increased cytokine responses, extracellular matrix remodelling and activation of macrophages[27-30]. Inhibition of serine proteases could thus act beneficial on atherosclerotic lesion progression and form an attractive strategy for plaque stabilization.
A particular class of inhibitors, the so-called serpins, are irreversible ‘suicide’ protease inhibitors and very interesting in this regard[31]. The exact role of individual serine protease inhibitors in atherogenesis has not been elucidated yet and findings thus far have not always been convincing. As also described in Chapter 2, Plasminogen Activator Inhibitor-1 (PAI-1) has

been extensively studied[32] and PAI-1 deficiency was demonstrated to leave atherogenesis in both LDLr$^{-/-}$ and ApoE$^{-/-}$ mice unaffected[33]. In other studies, PAI-1 was found to accelerate atherosclerosis or restenosis[34,35], illustrating that the overall effect of these pleiotropic proteins depends on disease stage and model used. In Chapter 2, we have described the effect of infusion with the myxoma virus derived serine protease inhibitor Serp-1 for 4 weeks on *de novo* atherogenesis and on advanced atherosclerosis. Serp-1 treatment was demonstrated to reduce plaque size by three-fold, when applied during plaque development. This reduction in plaque size was accompanied by an increase in collagen content and a striking reduction in macrophage content of the plaque. Likewise, treatment of advanced lesions with Serp-1 resulted in an inhibition of plaque progression and an increased collagen and vSMC content. In both studies, plaque cellularity was increased at the expense of necrotic core size[36]. Although the exact mode of action is still unclear, it may involve the uPA/uPAR dyad as Serp-1 was shown to interact with this system[37]. Additionally, Serp-1 mediates cytokine signaling during myxoma virus infection, which may partly explain the reduced macrophage content of the early lesions after Serp-1 treatment[38]. However, we did not observe a difference in white blood cell content between the control and Serp-1 treated mice. In conclusion, Serp-1 treatment inhibits both early lesion development and plaque progression in carotid arteries of ApoE$^{-/-}$ mice and results in a more stable plaque phenotype. To appreciate its therapeutic potential, it is necessary that side effects on thrombosis and homeostasis are mapped. Relevant in this regard is that while Serp-1 is known to interfere with the plasminogen activator system, we did not observe any effect of Serp-1 on the fibrin content of advanced plaques.

In Chapter 3, we have investigated the capacity of two cross-class protease inhibitors, which inhibit both serine and cysteine proteases, to attenuate plaque development. The proteins in question, CrmA and Serp-2, have been shown to inhibit Interleukin-1β Converting Enzyme (ICE) *in vitro*, CrmA being more potent than Serp-2[39]. ICE catalyzes the conversion of both pro-IL-1β and pro-IL-18 into active IL-1β and IL-18, which are both pro-inflammatory cytokines. In addition, ICE is also known as caspase 1, an activator of the caspase signaling pathway and thus of apoptosis. CrmA and Serp-2 can inhibit Granzyme B activity[40], implying that these protease inhibitors can inhibit both the intrinsic and the extrinsic apoptosis pathway. These two protease inhibitors were evaluated in various models of vasculopathy, notably neointima formation after iliofemoral artery angioplasty and aortic transplant (both in rats) and of atherosclerosis in ApoE$^{-/-}$ mice (i.e. collar-induced carotid artery atherosclerosis and during spontaneous lesion development in the aortic root). In these studies we show effective inhibition of plaque formation in all models by Serp-2 and strikingly, not by CrmA. Also, reactive center loop (RCL) mutants of Serp-2 were ineffective, indicating that the inhibition of lesion formation involves an interaction of the RCL to its target. The underlying mechanism was further delineated by *in vitro* studies in endothelial cell, monocyte and T-lymphocyte cell lines, which revealed that

Serp-2 is able to inhibit T-cell apoptosis and to a lesser extent macrophage apoptosis. This anti-apoptotic activity of Serp-2 was mainly mediated via inhibition of the Granzyme B/perforin pathway. In fact, Granzyme B and perforin are key executioners of the granule exocytosis pathway, which is the primary mechanism through which the immune system targets and kills cells[41,42]. Cytotoxic T-cells release both Granzyme B and perforin, after which Granzyme B will enter the target cells via mannose-6-phosphate receptor mediated endocytosis. Granzyme B will be released from the endocytic vesicles and induce target cell apoptosis.
This study suggests that cytotoxic T-cell mediated induction of apoptosis probably is a critical step in the development of neointimal or atherosclerotic lesions, although involvement of Granzyme B and perforin still has to be established *in vivo*. In previous studies, it has already been shown that Granzyme B and perforin were involved in endothelial cell and vascular smooth muscle cell apoptosis during transplant vasculopathy and in arterial allograft rejection and that in Granzyme B deficient mice luminal narrowing after transplantation was significantly reduced[43,44]. Furthermore a human serpin, i.e. protease inhibitor 9 (PI-9, the human orthologue of SPI-6 in mice) was found to inhibit Granzyme B activity[45] and to regulate the susceptibility to lymphocyte cytotoxicity *in vivo* and *in vitro*[46.] PI-9 and CrmA both were reported to inhibit CTL-mediated apoptosis, but only when both Granzyme B and perforin are present[47]. We have demonstrated here that Serp-2 mediated inhibition of CTL-induced Granzyme B activity is ablated after blocking of perforin.
Although our studies clearly demonstrate that Serp-2 could act anti-atherogenic, it cannot be excluded that part of its effect is mediated by interference with ICE activity. We do observe less mononuclear cell invasion in the rat model of angioplasty upon treatment with Serp-2, which may point to an anti-inflammatory pathway involving ICE. However, the question remains why CrmA does not exert any anti-atherogenic effects.

*Lysophosphatidic Acid in Atherosclerosis*
Lysophosphatidic acid (LPA) is one of the most thrombogenic lipids present in the lipid core of atherosclerotic lesions[48,49]. During lesion initiation, LPA mainly accumulates in the vascular wall by extravasation of LPA enriched modified LDL[50] and subsequent uptake by subendothelial macrophages. During lesion progression, LPA may still be delivered through LDL, however the intraplaque formation of LPA from its precursors will become increasingly important.
In Chapter 4 we have investigated LPA accumulation and the regulation and expression of genes involved in LPA metabolism in the vascular wall, during diet induced lesion formation in $LDLr^{-/-}$ mice. First, we describe that in $LDLr^{-/-}$ mice LPA accumulates in the intima during lesion progression to a similar extent as in advanced human lesions[49]. Accumulation of LPA and other lipids in the plaque may lead to cell death due to necrosis as shown for lipid-laden macrophages (foam cells). It is plausible that progressive build-up of

LPA will enhance the thrombogenicity of the plaque and may help to prime platelets toward coagulation upon rupture of the plaque[51]. This could increase the risk of thrombotic complications following plaque rupture. To determine whether or not the metabolism of LPA in the cellular content of the plaque is disturbed during atherosclerotic lesion progression, we analyzed mRNA expression levels of enzymes involved in LPA conversion.

It is shown that during atherosclerotic lesion development the expression pattern of intracellular enzymes in LPA homeostasis shifted to favor LPA synthesis, as enzymes involved in synthesis were upregulated ($PLD_3$, cPLA2IVA), whereas a key enzyme involved in degradation (LPAATα) was downregulated. LPAATα is the most uniformly expressed LPAAT of the two major isoforms present in mammalian tissue[52]. Interestingly, inhibition of LPAATβ induced cytotoxicity in various tumor cell types, while in most non-tumor cells it affected growth arrest and quiescence[53]. In analogy, downregulation of LPAATα as seen in lesion tissue may therefore result in cytotoxic effects in dedifferentiated or dysregulated cells of the plaque. Further study is awaited to adress this hypothesis.

Fatty acid binding proteins (FABP), which can bind intracellular LPA, have been shown to play a role in atherosclerosis[54,55]. For instance, absence of FABP4 in macrophages attenuated atherogenesis in hypercholesterolemic mice[56,57]. Downregulation of FABP3 during atherogenesis in mice is consistent with previous reports, showing a reduction of FABP3 activity in atherosclerotic rabbit aortas on cholesterol diet, while an age-dependent increase was observed in the normal chow-fed rabbits[58]. The net result of FABP3 downregulation for LPA reactivity and its consequence for plaque size and composition still has to be evaluated.

These data demonstrate that LPA indeed accumulates during atherosclerotic lesion progression. However, the relative contribution of intraplaque LPA synthesis versus LDL mediated delivery still remains to be determined. Apparently, a significant amount of LPA has accumulated in the plaque in the first two weeks after collar placement. At this time point, only fatty streaks have developed and we believe that at this stage the delivery via modified LDL is contributing most to the LPA pool. When the lesion further progresses, local synthesis of LPA may become more important. It should, however, be taken into account that we have only determined mRNA expression levels of enzymes involved in LPA metabolism, which may not necessarily be reflective of protein expression. Additional research will be required to determine also expression of these enzymes at a protein level. In conclusion, LPA accumulates in the plaque, already at the initial stage of atherosclerotic lesion development. The disturbed expression of key enzymes in LPA metabolism favors accumulation during plaque progression. By correction of the expression of one of the key enzymes in LPA metabolism, the LPA content in the plaque might be reduced resulting in a concomitant reduction in plaque thrombogenicity.

## 3. Inflammation and Plaque Stabilization

*Immunosuppression*

Several studies have provided evidence for the impact of immunosuppressive drugs on atherosclerotic plaque development[59-61]. Recently, it was demonstrated that the NFAT signaling pathways regulate the expression of various pro-inflammatory Th1-cytokines, such as IL-2, IL-6 and IFNγ[62]. Thrombin, VEGF and PDGF are all capable of inducing a pro-inflammatory response by activation of NFAT[63,64]. Inhibition of this pro-inflammatory response by the immunosuppressive drugs CsA, FK506 or sirolimus (rapamycin) could reduce atherogenesis, which could be, among others, ascribed to a downregulation of CD40 ligand, Fas ligand and TF expression[65-67]. In addition, CsA and FK506 are able to upregulate TGFβ, which stimulates vSMC proliferation and ECM synthesis, and fibrogenic factors such as collagen and fibronectin[68,69]. The activity profile of the immunosuppressive drugs suggests that they could potentially stabilize advanced atherosclerotic plaques. Previous studies on CsA, sirolimus, FK506 and atherosclerosis have been rather contradictory in that both inhibition and stimulation of atherosclerosis has been reported[61,70-73]. However, different experimental setups with respect to disease models and FK506 dose use could account for the non-consistent outcomes of these efficacy studies.

In Chapter 5, we describe the evaluation of the therapeutic potential of a low dose FK506 immunosuppression on collar-induced atherosclerosis and on spontaneous plaque development in the aortic arch of $ApoE^{-/-}$ mice. Collar-induced plaque development was significantly reduced in mice receiving FK506 and intriguingly, plaque progression was almost completely blocked after treatment with a low dose of FK506 (0.05 mg/kg/day). The FK506 blood concentration of approximately 0.2 ng/mL was sufficient to inhibit NFAT mediated transcription in vSMCs and macrophages, but had no effect the transcription factor NFκB. Interestingly, analysis of the plaque morphology revealed an increased plaque stability as judged from the necrotic core size, collagen content and increased cellularity. In both studies, the ASMA positive vascular smooth muscle cell content tended to be increased, which could partly be responsible for the observed increase in collagen, although it cannot be excluded that FK506 may also directly promote collagen production. Furthermore, macrophages, which are the major producers of collagen degrading enzymes such as MMP9, were slightly diminished in the FK506 treated versus control mice, which also favours a net accumulation of collagen and a reduced necrotic core formation in the plaque. *In vitro* studies revealed that FK506 is able to inhibit vSMC apoptosis, hereby explaining the increased vSMC content of the plaque.

Thus, stabilization of the atherosclerotic plaque after treatment with FK506 may reduce the risk of atherothrombosis. Compared to CsA, FK506 displayed an antithrombotic activity after cardiac transplantation, which is an important step in the development of cardiac allograft vasculopathy[74]. On the

other hand, FK506 treatment of transplantation rejection was in some studies reported to increase the risk of thrombotic microangiopathy, a prothrombotic state due to endothelial damage by the immunosuppressive drugs[75,76]. Nonetheless, in these patients the FK506 serum concentration was approximately 100-fold higher than in our animal studies. The "sub-therapeutic" dose that we have used did not lead to nephrotoxicity, another reported side effect of immunosuppressive drugs[77], although more long-term toxicity studies will be needed to establish the absence of side effects of low dose FK506 treatment.
In conclusion, in Chapter 5 we have shown that FK506 treatment reduces atherosclerotic plaque development and inhibits plaque progression, while improving plaque stability by increasing collagen content and reducing necrotic core formation. These findings led us to conclude that low dose FK506 treatment could serve as a valuable anti-atherosclerotic therapy.

*Adventitial Inflammation*
Inflammation of the adventitia, the perivascular tissue, is recognized to become increasingly important in atherosclerosis research. Recently, the extent of adventitial inflammation was found to correlate with the severity of atherosclerotic plaque progression[78]. Moreover, during atherosclerotic plaque development, microvessels will sprout from the adventitial *vasa vasorum* and penetrate the plaque not only to supply oxygen and nutrients, but also allow the recruitment of inflammatory cells to the core region[79]. In Chapter 6, we have investigated the role of a specific inflammatory cell type, the mast cell, in the adventitia of advanced atherosclerotic plaques on lesion progression. Mast cells are present in human atherosclerotic plaques, especially in the shoulder regions of ruptured lesions[80]. Also, mast cells reside in the adventitia of atherosclerotic arteries and their number was found to correlate with the progression state of the plaques[81]. It is unclear whether the mast cell is a causal factor in plaque rupture, or that they are recruited to the plaque secondary to rupture. In this study, we attracted and activated mast cells in the adventitia of advanced collar-induced atherosclerotic plaques in ApoE deficient mice via a DNP sensitization/challenge protocol. Strikingly, in DNP challenged mice with activated adventitial mast cells, intraplaque hemorrhage was a frequent event. Although intraplaque hemorrhage is clinically not as relevant as plaque rupture, lesions with intraplaque hemorrhage will be classified as Type VI lesions, and thus unstable, according to the AHA classification system[82]. Intraplaque hemorrhage will lead to erythrocyte derived cholesterol deposition and increased necrotic core formation. The increase in incidence of hemorrhages was accompanied by an increased apoptosis of plaque macrophages, which will also increase the necrotic core size. Also, apoptotic macrophage residues (apoptotic bodies) are rich in activated TF, rendering the necrotic core highly thrombogenic[83]. The mast cell constituents histamine, chymase and tryptase appeared to be responsible for the induced macrophage apoptosis. Moreover, proteases released from the mast cells

after degranulation, including chymase, tryptase and several cathepsins, are able to degrade extracellular matrix, thereby further destabilizing the plaque.

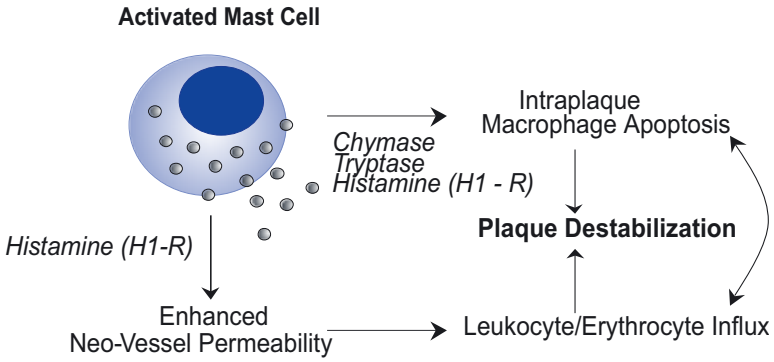


**Figure 1**. Proposed mechanism of activated mast cell in the adventitia of atherosclerotic lesions.

In addition, we showed that microvessels, present in the intima, media and adventitia of the plaques, may become leaky in response to the local high levels of mast cell derived histamine. It is plausible that the increased leakiness contributed to the high incidence of hemorrhage in the plaque. *In vitro* and *in vivo* studies revealed that the histamine $H_1$-receptor is an important factor in mast cell induced plaque destabilization. The $H_1$-receptor antagonist triprolidine was not only able to inhibit the mast cell induced macrophage apoptosis, but also to prevent increased vascular leakage, while $H_2$- and $H_3$- receptor antagonists had no effect. The histamine $H_1$-receptor has frequently been implicated in diseases such as asthma[84]. Interestingly, the $H_1$-receptor has been reported to play a significant role in intimal thickening[85]. Recently, the histamine $H_4$-receptor has been discovered[86] and may have an activity profile similar to the $H_1$-receptor. Specific antagonists are currently being developed and they will allow us to investigate the role of $H_4$-receptors in mast cell induced plaque destabilization in more detail.

Importantly, simultaneous administration of a mast cell stabilizer cromolyn not only prevented the adventitial mast cell activation *in vivo*, but also the associated increase in macrophage apoptosis, vascular leakage and intraplaque hemorrhage. Research on mast cell stabilizers and atherosclerosis has not been substantial, however tranilast, a rather unspecific mast cell stabilizer with anti-inflammatory activity, was shown to inhibit transplant atherosclerosis in two studies[87,88].

The question remains what the actual endogenous trigger is for mast cell activation and degranulation. Oxidized LDL, which could enter the adventitia via the *vasa vasorum* or be produced locally by adventitial macrophages, has been demonstrated to induce mast cell degranulation[89]. Also, mast cells often colocalize with sensory neurons in the adventitia[90,91], especially in

advanced atherosclerotic plaques. These neurons stained positive for the neuropeptides Substance P and calcitonin gene-related peptide, both capable of mast cell activation[91]. Preliminary data from our lab suggest that indeed adventitial activation of mast cells by locally administered Substance P promoted the incidence of intraplaque hemorrhage in advanced atherosclerotic lesions in ApoE$^{-/-}$ mice, albeit to a lesser extent that after local challenge with DNP. Mast cells are also known to express LPA receptors and recently, Gabba *et al*. described that LPA, via LPA receptors, can accelerate mast cell proliferation and differentiation[92]. In addition, phospholipases D, which convert PC into the LPA precursor PA, are known to induce mast cell degranulation[93]. These data may point to a role for LPA, which accumulates in plaques during lesion progression, in mast cell activation.
To conclude, we show in Chapter 6 that activated adventitial mast cell are instrumental in plaque destabilization and that they increase, by promoting macrophage apoptosis, the thrombogenic activity of the plaque. Therefore, we postulate that mast cell stabilization provides a new therapeutic entry in the prevention of plaque destabilization.

## 4. Research models

Animal models are widely used in all areas of biomedical research and the generation of the hyperlipidemic mouse strains such as the ApoE$^{-/-}$ and the LDLr$^{-/-}$ mice was a major breakthrough in atherosclerosis research[94,95]. However, to address the role of genes in atherosclerosis, the creation of knockout mice and subsequent back-crossing to a hyperlipidemic background will be equally necessary. This generally is very time-consuming and even impossible when the deletion of the particular gene leads to embryonic lethality. Research with transgenes can also be difficult when cell specific gene expression is required. This may be obviated in part by bone marrow transplantations, but even this approach requires the generation of knockouts or transgenes.
To speed up the experimental progress, we explored the potential of transplantation of lentivirally transduced bone marrow. Downregulation of genes by means of siRNA or shRNA has proven its usefulness in the last few years *in vitro* as well as *in vivo*[96,97] and several research groups have demonstrated that shRNA constructs can be efficiently delivered to different cell types by lentiviruses[98,99]. In Chapter 7, we have elaborated this strategy further and transduced bone marrow cells with shRNA lentivirus and the subsequently transplantated them to lethally irradiated recipient mice. We used CC-Chemokine Receptor 2 as model gene to establish the “proof of principle”, since the key role of CCR2 in leukocyte migration has already been extensively described[100]. At 7 weeks after transplantation of the recipient mice with bone marrow transduced with either H1.Empty control virus or H1.shCRR2 lentivirus, we indeed observed a 70% downregulation of

CCR2 expression by macrophages isolated from the peritoneal cavity. This downregulation in CCR2 mRNA levels resulted in a complete loss of CCR2 function as judged from the sharply reduced number of isolated macrophages, which was identical to that isolated from mice transplanted with *CCR2*$^{-/-}$ mice. Thus despite the *CCR2*$^{+/+}$ genotype, mice that had been transplanted with H1.shCCR2 lentivirus transduced bone marrow, displayed a *CCR2*$^{-/-}$ phenotype. PCR analysis on the Y-chromosomal *SRY* gene in the recipient bone marrow revealed that the transduced male donor bone marrow was not outcompeted by residual female recipient bone marrow after irradiation for at least 7 weeks after transplantation. Further long-term follow-up of these studies will be necessary to determine the persistence of CCR2 silencing after lentivirally transduced bone marrow transplantation. Moreover, studies are currently underway to validate this approach in disease models of atherosclerosis rather than leukocyte migration per se. It is expected that CCR2 knockdown will lead to a reduced atherosclerotic plaque development, as has been demonstrated by Guo *et al.*[101] for mice deficient in macrophage *CCR2*. Nevertheless, in this study we are the first to show effective delivery of lentiviral shRNA to bone marrow cells and subsequent transplantation into irradiated recipient mice as a strategy to generate hematopoietic knockdowns with silenced CCR2 expression. The speed and efficiency renders this strategy very helpful for addressing the role of other leukocyte genes in inflammatory disorders.
One of the possible target genes is considered to be Stromal cell Derived Factor-1α (SDF-1α), which has been shown to be highly expressed in atherosclerotic plaques and to play a crucial role in neointima formation after wire-injury[102,103]. Lentiviral transduction of a carotid artery of ApoE$^{-/-}$ mice after wire injury of a known functional SDF-1α antagonist, the P2G mutant, led to an over 50% decrease in neointimal area[104]. Another elegant tool in this regard is the so-called SDF-1α-degrakine, which specifically and stably inactivates the corresponding chemokine receptor CXCR4 by redirecting the receptor via a HIV-1 protein, a Vpu-tagged SDF-1α fusion protein, to the host proteasome machinery. This results in a complete loss of CXCR4 protein expression on the cell surface[105]. Transduction of bone marrow with either SDF-1α antagonist lentivirus or lentivirus containing the CXCR4 degrakine construct and subsequent transplantation into lethally irradiated recipient mice could allow us to elucidate the role of CXCR4/SDF-1α dyad in atherosclerotic lesion development.

## 5. Perspectives

This thesis presents an overview of various plaque stabilizing strategies. It was divided into two parts, the first focussing on matrix and cell homeostasis, while the second focussed mainly on inflammation. We have demonstrated that viral protease inhibitors Serp-1 and Serp-2 offer the potential of stabilizing atherosclerotic lesions in different disease and animal models.

Also, we have firmly established that the immunosuppressive drug FK506 displays a marked plaque stabilizing capacity. These studies may have therapeutic implications, although of course the extrapolation from mice to the human situation is difficult and side effects of both strategies still need to be mapped.
In addition, we describe in Chapters 4 and 6 plaque components that contribute to plaque instability and plaque thrombogenicity. The atherosclerotic plaque was found to contain an increasing amount of the highly thrombogenic lipid LPA during lesion progression, which can at least in part be accounted for by increased intraplaque production of LPA. We have identified new protein targets for correction of the LPA homeostasis that could lead to novel strategies for intervention in atherothrombosis. Moreover, the role of activated adventitial mast cells was delineated, revealing that mast cell activation indeed promotes plaque destabilization by increasing macrophage apoptosis, vascular leakage and intraplaque hemorrhage. This study also underlined the relevance of the adventitia for lesion development and CVD. Further study of adventitial inflammation and the cellular composition of the adventitia will give more insight into the role of the adventitia in atherosclerotic plaque development. Also, the identification of the potential trigger of mast cell activation in the adventitia can lead to plaque stabilization. In this thesis, mast cell stabilization already leads to reduced plaque instability, which could be an effective new therapeutic entry in the prevention of acute coronary syndromes or its sequelae.
Finally, a new research model is described, which allows faster and more efficient research with respect to leukocyte genes in atherosclerotic plaque development. Using this new technique, more potential candidates for future therapeutic interventions with respect to plaque stabilization and reduction of plaque thrombogenicity can be discovered.

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