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General Discussion and Perspectives
Chemokines are key regulators of leukocyte transmigration into the vessel wall during lesion formation and progression\(^1,2\). Over the last years, the role of various chemokines in the pathogenesis of atherosclerosis and plaque progression has been extensively studied. The contribution of the chemokine repertoire to atherosclerosis and other inflammatory diseases is very complex due to the extensive redundancy of chemokines and their receptors. It is likely that chemokines, either alone or as heterodimers, have a distinctive role in leukocyte homeostasis at specific stages of disease progression. Conceivably, patient specific, targeted modulation of leukocyte homeostasis could halt atherosclerotic plaque progression and/or improve stability and in this manner prevent patient hospitalization and invasive surgery. In this thesis, initially changes in chemokine profiles during acute cardiovascular disease (AMI+UAP) in humans were analyzed. Subsequently, in mice the contribution of identified candidate chemokines to atherosclerotic lesion formation was assessed and finally the effect of dysregulated leukocyte homeostasis on plaque progression and stability was determined.

**Human Studies**

**Stable/Refractory Unstable Angina Pectoris**

In order to elucidate potential chemokine targets for diagnosis and treatment of high risk patients we set out to determine plasma chemokine patterns in two distinct patient cohorts. Plasma samples from UAP and AMI patients were analyzed for chemokine patterns by use of a tailor-made, highly specific multiplex immunoassay\(^3,4\). In chapter 3 plasma samples of stabilized and refractory UAP patients from the APRAIS cohort were analyzed for chemokine levels at baseline and 180 days follow up. While plasma levels of the vast majority of chemokines were not altered, two chemokines, CCL5 and CCL18, were significantly and independently elevated in refractory patients. Unlike CCL5\(^5-9\), CCL18 has not yet been associated with cardiovascular disorders in patient cohorts, although CCL18 expression was shown in the atherosclerotic plaque and in particular at sites of instability\(^10,11\).

CCL5 and CCL18 quartile distribution revealed a clearcut correlation with the occurrence of refractory symptoms, future cardiovascular events and the need of future revascularisation procedures, whereas that of sCD40L and CRP, two established biomarkers of disease did not. Increased chemokine levels in refractory UAP may either reflect more pronounced thrombosis or be a direct consequence of ischemia/reperfusion injury in these patients. Which of these two mechanisms is most likely involved in refractory disease progression still remains to be clarified. Despite the potential of CCL5 and CCL18 as independent prospective biomarkers for disease, the correlations observed between these chemokines and clinical severity of disease are currently not sufficiently solid to warrant clinical use in diagnosis of high risk patients. Therefore, verification of our findings on plasma CCL5 and CCL18 levels in large scaled studies is eagerly awaited and if affirmative could add further prognostic value to the diagnosis of high risk patients.

**Cardiac Ischemia**

In chapter 4 we aimed to map cardiac ischemia related chemokines in two independent patient cohorts of AMI (MISSION) and UAP (APRAIS), respectively using a multiplex immunoassay and subsequent ELISA verification strategy. Three chemokines (CCL3, CCL5 and CXCL8) were identified that were significantly up-regulated and one chemokine (CXCL10), whose plasma levels were lowered in AMI patients. Interestingly the increased levels of CCL3 were confirmed in the APRAIS cohort of UAP patients, in that CCL3 was transiently raised during ischemia and even showed high prognostic power for future events.

Increased levels of CCL3 during Acute Coronary Syndromes (ACS) and their rapid decline to baseline in the follow-up period further illustrate the profoundly
altered chemokine homeostasis during ACS. Whether increased CCL3 levels represent a risk factor for the development of cardiac ischemia or for a direct response to cardiac ischemia still needs to be elucidated. Interestingly CCL3 release was seen to coincide with cardiac ischemia-repair injury and within two days after ischemia CCL3 levels in UAP returned to baseline levels. This phenomenon has already been alluded to by Parissis et al., reporting CCL3 levels 24 hours post infarct to correlate not only with creatine kinase levels, but also, inversely, with left ventricular ejection fraction. Clearly, this points to a key role of this chemokine in injury and/or repair responses.

Even though it still remains unclear how CCL3 contributes to future cardiovascular events, our study shows that its prognostic power is sufficiently promising to warrant examination in larger scaled trials. To address a potential direct contribution of coronary ischemia itself to plasma CCL3 levels without the underlying substrate of atherosclerosis, we performed coronary ligation studies in normolipidemic, non-atherosclerotic C57Bl/6 mice. Although the outcome of mouse studies cannot be directly extrapolated to the human situation and this experimental set-up has its limitations, our data are supportive of an ischemic rather than atherogenic origin of the CCL3 chemokine response.

Taken together, CCL3 appears transiently secreted during cardiac ischemia, where it potentially functions in the ischemia/repair mechanism by recruitment of CCR5+ T cells, which has already been suggested recently. Furthermore CCL3 appears to be a predictor of future cardiovascular events and therefore might prove useful as biomarker in identifying high-risk patients.

Deducted from the two patient cohorts it can be appreciated that CCL18 and CCL3 represent novel and independent markers of acute coronary syndromes and merits further evaluation of these chemokine markers in larger patient populations.

**Mouse Studies**

**CCL3/MIP1α**

In the second part of this thesis, several chemokines or chemokine like proteins were evaluated for their role in the pathogenesis of atherosclerosis. In chapter 5 one of the identified targets from the human populations, CCL3 was studied for its mechanistic involvement in atherosclerosis. In vitro experiments established that CCL3 is likely to originate from activated macrophages and subsequently can induce macrophage proliferation, which is in concurrence with earlier data. Temporal expression profile of atherosclerotic lesion development revealed that CCL3 is mainly regulated during early lesion progression and not during initiation, clearly suggesting that CCL3 is mainly involved in plaque inflammation.

Atherosclerosis in CCL3−/− mice was significantly attenuated. No effects on macrophage or T cell content were seen, while interestingly the plaque neutrophil content was significantly attenuated. Studies using knock out strategies for both CCL3 receptors, CCR1 and CCR5 showed opposing results. Deficiency in CCR1 accelerates atherosclerosis, while CCR5 deficiency impairs atherosclerosis, suggesting that in concert with our data CCL3 signalling in atherosclerosis mainly occurs via CCR5. Neutrophils were, until recently, not appreciated as major players in atherosclerosis. Studies by Naruko et al. showed that neutrophil infiltration is associated with acute coronary events. Experimental support came from van Leeuwen and coworkers showing the abundant presence of neutrophils in advanced mouse plaques. Furthermore an absence of CXCR4, as apparent during acute cardiovascular events, aggravates atherosclerosis and results in increased neutrophil content in plaques. In this study plaque neutrophils were associated, although not causally linked, with increased
intimal apoptosis and a pro-inflammatory plaque phenotype. Moreover, it was shown that the pro-inflammatory cytokine TNFα, a key component in atherosclerosis, can induce neutrophil chemotaxis. Interestingly, this response can be augmented by CCL3 and mediated via CCR5. In agreement with these findings, we show that in the absence of CCL3, neutrophil migration to and diapedesis into the plaque is significantly attenuated.

Taken together, our data clearly establish a causal role for neutrophils in the development of atherosclerosis. Furthermore, we hypothesize that under conditions of inflammation, leukocyte-derived CCL3 can alter neutrophil homeostasis and enhance neutrophil chemotaxis towards the atherosclerotic plaque to accelerate lesion formation by inducing a focal respiratory burst.

**CXCR3 antagonist**

The chemokine receptor CXCR3 is implicated in migration of leukocytes to sites of inflammation. Antagonizing CXCR3 could therefore be a potential strategy to inhibit inflammation-induced leukocyte migration and subsequently reduce atherosclerotic lesion formation. In chapter 6, we used the selective CXCR3 antagonist NBI-74330 to block CXCR3-mediated signalling in thioglycollate-induced peritonitis and diet-induced atherosclerosis. NBI-74330 is a small molecular weight, high-affinity CXCR3 antagonist with a Ki in the low nanomolar range (~8nM). Leukocytes migration to the peritoneal cavity in response to a single intraperitoneal thioglycollate challenge was significantly attenuated after NBI-74330 treatment. This effect was mainly exerted on the recruitment of CD4+ T cells and macrophages subsets. Lesion formation in the aortic valve leaflet area was significantly inhibited in mice treated with NBI-74330, while macrophage content and collagen deposition in the atherosclerotic lesion were similar. Next to lesion formation in the aortic valve leaflets, NBI-74330 treatment resulted in a 53% reduction in the percentage of lesion area in the aorta, which is in concurrence with earlier studies using CXCR3 knock out mice. Furthermore, administration of a CXCR3 antagonist reduces the leukocyte migration to lymph nodes draining to the site of inflammation. Interestingly, the T-cell population of NBI-74330-treated mice was skewed towards a more regulatory phenotype, which is accompanied by increased plaque expression of TGFβ. This indicates that regulatory T cells are the likely source of the observed increase in TGFβ production within the atherosclerotic plaque and thus inhibit local effector T cell responses.

In general, we hypothesize that the observed reduction in lesion formation and the accompanying induction in regulatory T cell phenotype is a result of a reduction in migration of effector cells from the circulation to the atherosclerotic plaque. In conclusion, selective blockade of CXCR3 migration in vivo using NBI-74330 provides an attractive way to beneficially balance the immune response in an auto-inflammatory situation such as atherosclerosis.

**Macrophage Inhibitory Cytokine 1**

Macrophage Inhibitory Cytokine-1 is a distant member of the TGFβ superfamily, which is notorious for its pleiotropic mode of action. Also, its contribution to cardiovascular disorders such as atherosclerosis is complex and poorly understood. MIC-1 was recently shown to be cardio-protective in mouse models for myocardial infarction and heart failure, and circulating MIC-1 protected the heart from ischemic injury. In chapter 7, we are the first to show that hematopoietic deficiency of MIC-1 attenuates lesion initiation and improves atherosclerotic plaque stability in more advanced stages of disease development. We show that this is attributable to a modulation of the inflammatory status and to CCR2-dependent monocyte influx into the plaque. The beneficial effect of MIC-1 deficiency contrasts with that of other TGFβ family members such as activin-A and TGFβ-1, where neutralization resulted in accelerated atherosclerosis and plaque destabilization with reduced collagen deposition and a...
more pro-inflammatory phenotype. Our studies show that despite the partially contrasting effects of TGFβ and MIC-1, the latter appeared to be a specific trigger of CCR2 signalling, the effects of which can be largely prevented by blocking TGFβRII but not TGFβRI. MIC-1 was shown to co-localize with oxidized LDL in the atherosclerotic plaque and contributes to the local oxidative stress and ensuing apoptosis. In keeping with these findings we now demonstrate that plaques from MIC-1-/- chimeras contain less inflammatory cells and in particular less macrophages. Although pro-apoptotic activity of MIC-1 has been demonstrated in many studies, we were unable to establish any effects of MIC-1 on macrophage apoptosis. In fact, MIC-1 in macrophages is mainly involved in cell cycle regulation rather than apoptosis. Plausibly, lack of MIC-1 will then lead to cell cycle arrest and subsequent loss of cellular mobility.

Collectively, MIC-1 appears to exert its pro-atherogenic effects mainly by TGFβ signaling, suggesting that MIC-1 may in fact act as an acute phase modifier of TGFβ activity. Given this rather exclusive expression of MIC-1 during acute phase responses and inflammatory conditions, our data suggest that focal inhibition of MIC-1 could be a particularly attractive approach to improve plaque stability by simultaneously quenching CCR2 activity, intimal apoptosis and inducing collagen deposition. Furthermore MIC-1 could be an appealing target for the treatment of restenosis as inhibition of MIC-1, an early response gene in vascular injury, simultaneously prevents inflammatory cell recruitment and apoptosis, which both are regarded key culprits in this vasculopathy.

G Protein Coupled Receptor Kinase 2

Rapid chemokine receptor desensitization likely is a key process in accurate tuning of chemotaxis and is regulated by dedicated G-Protein Coupled Receptor Kinases. G Protein Coupled Receptor Kinase-2 is a member of the β-adrenergic receptor kinase family and is known to desensitize CCR5, CCR1 and CCR2, all essential receptors in atherogenesis. In chapter 8 we studied the effects of partial hematopoietic deletion of GRK2 in a murine atherosclerosis model. Contrary to our expectation atherogenesis was attenuated in GRK2+/- chimeras. Interestingly plaque composition was also altered in these mice. In concordance with the stage of atherosclerosis GRK2 +/- plaques contained significantly more macrophages. The amount of intimal necrosis however was, independent of the lesion progression stage, robustly attenuated in these mice, suggesting that GRK2 inhibition directly affects macrophage apoptosis or the clearance of apoptotic remnants. Indeed, next to its classical role in phosphorylation-dependent receptor desensitization, GRK2 can bind to several proteins involved in receptors signalling and trafficking, such as PI3 kinase, Akt, caveolin, MEK1/2 and p38 MAPK. GRK2 was even shown to phosphorylate and inactivate p38-MAPK. The effects of macrophage apoptosis on atherosclerosis are currently under debate and might be dependent on the progression stage. Several studies have shown that increased macrophage apoptosis is beneficial when occurring during lesion initiation and progression, while other have suggested detrimental effects. Macrophage death in advanced atherosclerosis may even promote necrosis and plaque destabilization. Recently it was shown that p38 MAPK signalling is necessary for induction of macrophage apoptosis, which leads us to the intriguing notion that partial deletion of GRK2 may increase p38 mediated macrophage apoptosis to impair atherogenesis, as shown in our study. Furthermore, enhanced chemokine expression and signalling as manifest in GRK2 heterozygosity, may improve the clearance of apoptotic remnants which leads to further reduction in necrotic core formation.

Collectively our data point to a complex and unexpected role of GRK2 in macrophage function during atherogenesis. GRK2 may be specifically relevant for macrophage apoptosis and remnant clearance as necrotic core size is, independent of lesion stage, markedly decreased in GRK2+/- lesions. The fact that even a partial knockdown of GRK2 has such impact on atherogenesis renders this kinase particularly interesting as therapeutic target. Indeed, synthetic antagonists of GRK2 might
prove worthwhile in the treatment of early atherosclerosis, while GRK2 agonists might positively influence advanced atherosclerotic lesions by impairment of local inflammatory stimuli and attenuation of macrophage apoptosis.

Mast Cells
Mast cells are part of the innate immune system and are notorious for their role in allergy and asthma. MCs are large granular cells with the unique ability to release granules into the surrounding tissue upon activation. MC granules contain a plethora of mediators including various chemokines (CCL2, CCL3, CCL4, CCL5, CXCL10). Activated mast cells have been reported to accumulate in the arterial adventitia during plaque progression and are abundantly present in the adventitia of vulnerable and ruptured lesions. To date it remains to be clarified whether these adventitial mast cells contribute to plaque progression and whether these cells are instrumental in plaque rupture. In chapter 9 we recruited and activated mast cells in the adventitia of atherosclerotic lesions in ApoE-/- mice. Systemic mast cell activation was seen to aggravate spontaneous plaque progression in the brachiocephalic artery of ApoE-/- mice, an effect that was not observed after prior mast cell stabilization with cromolyn. In keeping with these data MC deficiency was shown to attenuate lesion formation.

More importantly, as MCs are amply present in type V/VI unstable human atherosclerotic lesions, we have addressed the effect of focal MC activation on pre-existing carotid artery plaques. Local MC activation led to a striking and acute increase in the incidence of intraplaque hemorrhage within three days after challenge. Lesions with intraplaque hemorrhage tended to contain relatively more adventitial MCs than those lacking hemorrhages, which is in line with the observation of Laine et al. Interestingly, it has been reported that intraplaque hemorrhage is a potent pro-atherogenic stimulus and risk factor in plaque destabilization, as it is accompanied by deposition of erythrocyte associated cholesterol, ceroid production and subsequent enlargement of the necrotic core. Treatment of mice with cromolyn during DNP challenge normalized the extent of mast cell degranulation in the adventitia, while preventing intraplaque hemorrhage. Furthermore MC activation increased intimal apoptosis, which was mainly localized in the central atheroma, implying that the majority of apoptotic cells are of macrophage rather than of vSMC origin. In vitro studies disclosed that MC derived histamine is a potent inducer of macrophage apoptosis. It appears that, adventitial MC activation increased leukocyte and in particular monocyte adhesion to the proximal area of atherosclerotic plaques in a CXCR2- and VCAM-1-dependent manner.

In conclusion, MCs contribute significantly to atherosclerotic plaque progression and plaque destabilization. Plaque destabilization is induced by promoting macrophage apoptosis, increased leukocyte influx and enhanced microvascular leakage in pre-existing atherosclerotic plaques, resulting in a sharply increased risk of intraplaque hemorrhage. We propose that mast cell stabilization can be an effective new therapeutic entry in the prevention of acute coronary syndromes.

Perspectives
This thesis encompasses a number of human and experimental mouse studies on the role of chemokine or chemotactic proteins in cardiovascular diseases and most notably on atherosclerosis. From these studies, it is evident that inhibition of chemokine signaling and subsequent leukocyte migration is a very effective measure to attenuate atherosclerosis and stabilize plaques. To date the chemokine family comprises 46 ligands and 18 receptors. As most chemokine ligands have pleiotropic binding capacities to several chemokine receptors, and both ligand and receptor can engage in heterodimerization, the functional effect of chemokine-chemokine receptor interaction is very complex and dependent on the cellular and environmental context. Therefore we have compared specific chemokine patterns rather than individual chemokines in
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acute myocardial infarction and unstable angina pectoris patients and controls by use of multiplex immunoassays. However due to the limited cohort size we were unable to deduce clear patterns from the obtained data that are characteristic for AMI or UAP. We were however able to identify two novel chemokine targets, CCL18 and CCL3, which are currently being examined in a large longitudinal patient cohort. Nevertheless we feel confident that this strategy may yield patterns that are predictive of the patient’s clinical prospect and that chemokine pattern correction may be a particularly effective way to restore leukocyte homeostasis in patients and thus intervene in disease progression.

Surprisingly given the pleiotropic action of most chemokines, examination of the role of various chemokines in the pathobiology of atherosclerosis revealed that even inhibition of individual chemokines, such as CCL3 and CXCR3 or chemotactic proteins (e.g. MIC-1) attenuated atherosclerosis and/or improved plaque stability. For instance absence of CCL3 diminished lesion formation possibly by reducing neutrophil accumulation, while CXCR3 blockade also inhibited lesion formation but in this case by modulating local T cell responses. Leukocyte specific deficiency of the TGFβ family member MIC-1 inhibited plaque initiation, and enhanced plaque stability at later stages of atherosclerosis. These data show that inhibition of chemokine signaling is an effective strategy to intervene in plaque formation. In a chemokine desensitizer knockout we did to our surprise not observe an augmentation, but a robust reduction in atherosclerosis. The observed effect may be due to enhanced macrophage apoptosis, albeit effects on chemotaxis can not be excluded at this stage. In early atherogenesis increased cell death may result in decreased lesion formation, while at later stages of atherosclerosis a same increase may cause destabilization of the plaque. Therefore GRK2 activation may be beneficial to stabilize rupture prone lesion in UAP patients but may at the same time be detrimental when targeting early lesion progression. As most cardiovascular patients enter the emergency care unit with signs of angina pectoris, plaque stabilization is of course the preferred treatment strategy for these patients. We propose that plaque stability can be increased by therapies that effect a reduction of intimal apoptosis in both macrophages and cap smooth muscle cells, and GRK2 may be a good candidate in that regard.

It should be noted that chemokines play a paradoxical role in plaque stability as inhibition of some chemokines (like CCL19 and CCL21) can attenuate the influx of leukocytes into the unstable plaque, while they possibly also can mediate the efflux of the same subset from that plaque. Furthermore due to their pleiotropic properties different leukocyte subsets with a divergent activity profile can be recruited. First, it should be determined which cell type we wish to attract to the lesion and which we want to eliminate. For instance, combinational therapy with a CXCR3 and MIC-1 antagonists will augment regulatory T-cell activity, increased collagen production by vSMCs and removal of macrophages from the intimal area. When adding a third very potent drug, the mast cell stabilizer cromolyn, macrophage apoptosis will be attenuated as will be the formation of leaky neovessels, collectively resulting in improved plaque stability, but of course with the introduction of combinational therapy more side effects can be expected.

Collectively, this thesis provides evidence that leukocyte recruitment to the plaque is delicately balanced. From data obtained from all individual targets studied in this thesis we may infer that combinational therapy for improvement of plaque stability provides the particularly promising therapeutic option. However, when interfering with leukocyte trafficking and subsequent immune balance caution should be taken. Systemic chemokine intervention might lead to disturbed susceptibility to other inflammatory diseases. For instance the use of SDF-1/CXCR4 antagonists to prevent tumour metastasis and growth, may as a side effect contribute to perturbed hematopoiesis as CXCR4 is critical for stromal release and myeloid differentiation. Furthermore systemic stabilization of mast cells could hamper innate immune responses to parasites.
and other pathogens. Preferably intervention should be targeted only during acute phase responses and discontinued as soon as possible to minimize any side effects. As with any therapy, the usefulness of this type of systemic intervention depends on the balance between beneficial effects on the disease outcome and adverse effects on normal physiology.

A clear example of unexpected systemic side effects was seen in a bone marrow transplantation setup (chapter 10) where we intended to study the role of CCR7 on atherogenesis. CC chemokine receptor 7 (CCR7) is one of the key receptors in T cell biology linked to homing of T cells and central and peripheral tolerance. CCR7 is required for migration of naïve T cells to as well as from the cortex to the medulla of the thymus, but has also recently been implicated in the induction of macrophage efflux from advanced atherosclerotic lesions. As a direct result of transplantation of CCR7 deficient bone marrow, T cell selection was attenuated and the thymus in these chimeras severely atrophied. This lack of T cell selection resulted in an uncontrolled proliferation of effector memory T cells, which subsequently led to the induction of chronic Graft versus Host Disease. The massively induced tissue damage in this pathology eventually resulted in enhanced mortality in the CCR7 deficient recipients. These observations clearly show that systemic inhibition of CCR7 signalling results in a disturbed adaptive immune system and might impair accurate immune responses for novel encountered antigens. Systemic inhibition of MIC-1 might be very beneficial for unstable angina pectoris patients, but may adversely induce hypertrophic responses in the heart, possibly progressing into severe cardiomyopathy. Inhibition of chemokines, like CCL3 or CXCR3 ligands, during episodes of ischemia could limit injury severity. However sufficient repair might also be affected as chemokine signalling mediates several other mechanisms besides leukocyte chemotaxis, like regulating angiogenesis and fibrous tissue deposition.

Conceivably the best treatment site would be the affected vessel. Pharmacological substances can either be applied locally, for instance by use of a drug eluting gel applied perivascularly. Unfortunately with current techniques available, this approach would still require surgical intervention, and therefore will be generally disqualified. A more elegant and less invasive option would be cell specific targeting of pharmacological compounds, which has been applied in the field of cancer research.

Deducted from the observed effects of modulation of the leukocyte homeostasis described in this thesis, treatment of UAP patients with a mast cell stabilizer (cromoglycate, ketotifen) or a standard anti-histamine is currently the most promising option as these drugs have been extensively used in the clinic for treatment of mastocytosis, atopic dermatitis and allergies with relatively limited side effects.

In conclusion, the research described in this thesis provided novel candidates that might be of value for the early prediction of high risk patients. Moreover, the identified candidates may also represent valuable targets for tailormade modulation of leukocyte homeostasis in the plaque that could improve atherosclerotic plaque progression and stability and thus prevent cardiovascular disorder related morbidity and mortality. However, the complexity of chemokine receptor interactions and signaling cascades together with their general key role in immune maintenance and inflammation, demands extensive pre-clinical characterization of these potential new drug targets.
References


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