Chemokines in atherosclerotic lesion development and stability : from mice to man

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1 Introduction

Contents

1. Atherosclerosis

Cardiovascular diseases are the major cause of morbidity and mortality in western societies[1]. The most common clinical manifestations are stroke and acute myocardial infarction and in both ailments atherosclerosis is the underlying culprit. In general, atherosclerosis is regarded a progressive, multi-factorial disease, already initiated during early adolescence[2, 3]. Initially lesion progression remains at a subclinical stage due to arterial plasticity or in other words the capacity of vessels remodelling to compensate for luminal loss[4]. Depending on the site, plaque composition and affected vessel, advanced atherosclerotic lesions are prone to rupture[5-7]. Upon rupture, the highly thrombogenic content of the plaque will be exposed to the circulation, triggering blood coagulation and thrombus formation[8, 9]. The ensuing (total) arterial occlusion can induce acute complications like cerebral ischemia (stroke), angina pectoris, peripheral arterial occlusive disease and myocardial infarction and might eventually lead to death.

Several locations in the vasculature appear to be pre-disposed for atherosclerosis, especially vascular segments with curves and branches, like the left anterior descending artery (LAD) of the coronary arteries, the common carotid arteries at the bifurcation and all main branching points of the aorta[10, 11]. These site-specific effects are attributable to hemodynamic factors, such as low shear stress, oscillatory flow and turbulent flow[12]. Apart from genetic and spatial predisposition to atherogenesis, several behavioural features can affect disease progression, such as smoking[13], high fat diet[14], stress[15, 16] and physical inactivity[17, 18]. Also hypertension[13, 19], hyperhomocysteinemia[20, 21], diabetes[22-25] and obesity[26, 27] generate an increased risk for cardiovascluar disease. Surgical intervention by e.g. bypass surgery, percutaneous transluminal coronary angioplasty (PTCA), stenting or atherectomy is frequently required to restore obstructed blood flow, however the success rate of these interventions is often impaired by re-stenosis[28].

2. Atherosclerotic Plaque Development

2.1. Plaque Initiation

Atherosclerosis mostly occurs in the medium and large sized arteries[29, 30]. Under normal conditions the artery consists of an endothelial layer covering the media of smooth muscle cells that is framed by the internal and external elastic lamina. On the perivascular site, the artery is surrounded by adventitial tissue. As already discussed above, atherosclerosis is initiated, at predisposed sites (e.g. arterial branches or bi-furcations), by endothelial dysfunction caused by low turbulent or oscillatory shear stress in combination with the presence of atherogenic factors like high lipoproteins (VLDL, LDL) levels and hypertension: the so-called 'response to injury' theory[31, 32]. The first crucial step in atherogenesis is the interaction of atherogenic lipoproteins with freely exposed proteoglycans just underneath the endothelial layer[33], consequently resulting in lipid accumulation. Simultaneously, endothelial cells activated by flow disturban-ces increase the expression of cellular adhesion molecules such as E- and P-selectin[34, 35] on their cell surface, which mediates the rolling of monocytes to the endothelial layer (*Figure 1*). Subsequently circulating leukocytes are arrested to the vascular endothelium during rolling by cellular activation via several members of the chemokine family[36, 37] or by interaction with cellular integrins[34]. Firm adhesion of leukocytes is induced by integrin clustering or structural rearrangement[38, 39,] which in turn will initiate intracellular signalling pathways thereby further strengthening cell adhesion. The final step in the process of leukocyte emigration into the subendothelial intimal area in atherosclerosis is transmigration or diapedesis. Transmigration is usually preceeded by a process currently known as crawling[40, 41] as leukocytes scan for the most convenient site for transmigration. During extravasation, leukocytes will have to penetrate the endothelial cell barrier, the endothelial cell basement membrane and finally the pericytes. Probably the unique combination of adhesion molecules, chemokines and integrins presented in the

context of atherosclerosis results in recruitment of specific leukocyte subsets to 'coded areas' of the atherosclerotic lesion.

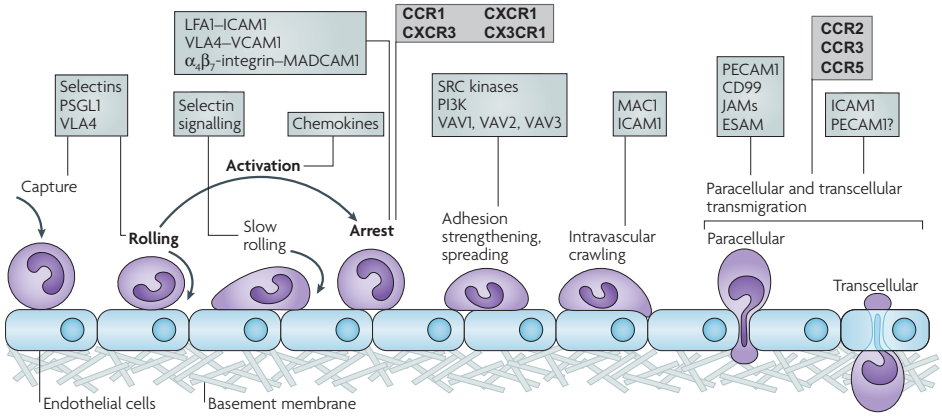

Figure 1: *Leukocyte rolling, arrest, adherence and diapedesis through the vascular wall are mediated by adhesion molecules and chemokines. Adapted from Ley et al.*[214]

As a result of locally produced pro-inflammatory signals, such as Macrophage Colony Stimulating Factor (M-CSF), Tumor Necrosis Factor α (TNFα), Interferon γ (IFNγ), Interleukin-1 and growth factors (like Placental Derived Growth Factor (PDGF)) the migrated leukocytes will differentiate into tissue macrophages[42-44]. The maintenance crew of the immune system: the intimal macrophages, will ingest accumulated cholesterol and modified lipoprotein particles, thereby converting into foam cells[45] (*Figure 2*). This initial, quiescent plaque is classified as a type I lesion according to the classification criteria of the American Heart Association (AHA) system[46-49]. Type I lesions will further accumulate lipid-laden macrophages, attract T-lymphocytes and develop into type II fatty streaks[50]. Under the influence of several growth factors, as Fibroblast Growth Factor (FGF) and Tumor Growth Factor β (TGFβ), medial vascular smooth muscle cells (vSMC) will start to migrate toward the endothelial lining and consequently the lesion progresses into a type III lesion.

2.2. Plaque Progression

Type III intermediate lesions contain small extracellular lipid deposits under a layer of migrated vSMCs. This class of plaques can be regarded as the transition stage between the fatty streak and an advanced atherosclerotic lesion and are also referred to as pre-atheroma plaques[48, 49]. In type IV lesions the intimal lipid deposits have expanded into large a-cellular lipid pools containing a substantial amount of cholesterol crystals, due to either apoptosis or necrosis of intimal foam cells or to accumulation of infiltrated lipoprotein particles. The type IV atheroma is the first stage of an advanced atherosclerotic lesion and is distinguished by a large lesion core and by intimal capillaries that most likely originate from the vasa vasorum. Type IV atheromas can induce clinical symptoms known as angina pectoris. During further progression, even more fibroblasts and vSMCs accumulate subendothelially to produce, via interaction with extracellular matrix material like collagen and proteoglycans, a fibrous cap covering the lipid core. However due to local death signals apoptosis can occur in fibrous cap cells resulting in rupture-prone vulnerable areas in the atherosclerotic lining. The type V lesion is known as the fibro-atheroma[51] and rupture preferentially occurs in this lesion type, as these lesions are biomechanically vulnerable and are constantly exposed to high blood flow forces[5]. In fact, type V lesions are subdivided into 3 subcategories, of which the first (type Va) is described above, type Vb that is highly calcified and type Vc lesion, is relatively lipid poor. In practice, type IV and V lesion are difficult to discriminate, and nowa-

days frequently termed as 'thick' and 'thin' fibrous cap atheroma, respectively[5].

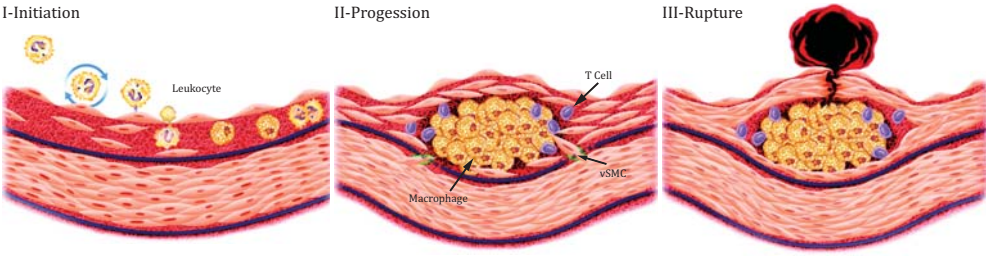

Figure 2: *Leukocyte adherence to the endothelial layer during atherosclerotic lesion initiation (panel I). During lesion progression transmigrated monocytes differentiate into macrophages, which release pro-inflammatory cytokines resulting in accumulation of T cells in the plaque (panel II). Thinning of the fibrous cap can eventually lead to plaque rupture (panel III). Adapted from Packard et al.[215]*

Ruptured lesions with an intramural or luminal thrombus or lesions containing intra-plaque haemorrhage are classified type VI atherosclerotic lesions (*Figure 2*). Type VI lesions without noticeable cap breaks are referred to as eroded[52, 53]. The various subclasses can be distinguished on the basis of three different criteria: The 'fibrous cap atheroma with erosion', which has a thick fibrous cap and a luminal thrombus but without physical lumen-plaque core interaction. Next is the 'thin fibrous cap with plaque rupture', where a luminal thrombus is in direct contact with the lipid core of the lesion. The third subtype describes the 'calcified nodule with erosion', with an eruptive nodular calcification with overlying luminal thrombus.

3. The Unstable Plaque

All plaques that have progressed beyond type IV are considered 'unstable' and are accountable for the majority of clinical manifestations as stroke and myocardial infarction. In the pathobiology of atherosclerosis there is a delicate balance between necro-tic core size and fibrous cap rigidity. Disturbance of this delicate balance may lead to fibrous cap rupture. Due to rupture the highly thrombogenic lipid core will come in immediate contact with the circulation leading to coagulation, thrombus formation and finally results in acute cardiovascular syndromes or stroke. Several mechanisms have been implicated in the induction or acceleration of plaque destabilization. In particular, extra cellular matrix degradation[54-56], vascular wall cell apoptosis, intimal macrophage apoptosis[54, 57-59] and platelets adherence[60, 61] are regarded to be key regulators of sta-bility. In turn many of these processes are influenced by the local inflammatory status. Atherosclerotic plaques and in particular the unstable plaque, contains many leukocyte subsets that can induce various pro-inflammatory interleukins, cytokines, chemokines[62-64], which in turn regulate leukocyte homeostasis.

4. Leukocyte Homeostasis in Atherosclerosis

4.1. Monocytes/Macrophages

At present time atherosclerosis is broadly accepted as a lipid driven process with features of a chronic inflammatory disease. The first notion for this was provided by pathology studies revealing the presence of inflammatory infiltrates in atherosclerotic lesions[65-67]. One of the major effector cells in the initiation and progression of atherosclerosis is the macrophage. Macrophages are part of the innate immune system and normally function in immediate host defence against pathogens. Plaque macrophages engulf lipid particles, such as oxidized LDL (oxLDL), via several scavenger receptors (e.g. SR-A, SR-B1, CD36, CD68 and CXCL16)[68-73]. Uptake of oxLDL results in cellular activation and differentiation and as lipid particles further accumulate macrophages gradu-

ally transform into foam cells (Figure 3A).

Once activated intimal macrophages produce and release a broad panel of pro-inflammatory cytokines and growth factors. These soluble mediators can either influence the endothelial lining of the vascular wall or they can further stimulate foam cell formation, macrophage activation or T cell stimulation (Figure 3B). For instance IL1α, TNFα and IFNγ[74-76] have been implicated in the induction of adhesion molecules and chemokines, particularly MCP-1 (CCL2), IL-8 (CXCL8) and Fractalkine (CX3CL1)[77-80], on the vascular wall thereby promoting further cellular infiltration in the plaque. Furthermore some cytokines can influence foam cell formation either by attenuation or augmentation of lipid uptake. For instance IFNγ can inhibit scavenger receptor expression[81, 82] thereby influencing cholesterol uptake, while on the other hand it also attenuates cholesterol efflux[83]. Conversely TGFβ was seen to inhibit scavenger receptor activity in human macrophages[84]. Although macrophages are not specialized in antigen presentation they are capable of presenting antigen on their cell surface. Oxidized LDL particles are processed by macrophages leading to oxLDL peptide presentation on MHC-II molecules and subsequent T cel activation[85]. Indeed blockade of MHC-II molecules resulted in decreased activation and proliferation of oxLDL specific T cells[86].

4.2. Lymphocytes

Already a few decades ago Jonasson *et al.* demonstrated the precensce of T cells in human atherosclerotic plaques[87]. The majority of these T cells are of the $CD4^+$ subtype[88]. Ablation of $CD4^+$ T cells[89-91] attenuated lesion formation in $LDLr^{-/-}$ mice, while adoptive transfer of $CD4^+$ T cells to immune deprived B and T cell deficient $RAG^{-/-}$ mice accelerated atherogenesis[89]. Furthermore it has become clear that T cell activation predominantly occurs during progression of atherosclerosis, but it is virtually absent during initiation[92]. Cytotoxic $CD8^+$ T cells have also been identified in atherosclerosis, but their role in the disease progress is not unambiguous[93-95]. Classically $CD4^+$ helper T cells are categorized into two subclasses based on their cytokine profile. The T helper 1 (Th1) subset is classified as an inducer of cellular immunity, while the T helper 2 (Th2) subset induce a humoral response[96, 97], with regards to atherosclerosis these subsets are considered pro- and anti-inflammatory respectively. Under normal conditions the balance between these two T cells subsets is static, while during episodes of inflammation this balance is polarized towards Th1 or Th2. Atherosclerosis has been identified as a pro-inflammatory Th1 driven disease[98] (Figure 3C). Conceivably modulation of the immune response towards Th2 might favourably influence atherogenesis or plaque stability. In keeping with this conception, immunization of mice against MDA-LDL or oxLDL attenuated atherosclerotic lesion formation or neo-intima formation respectively[99, 100]. Interestingly recent studies revealed that protein vaccination might prove a useful strategy to prevent atherosclerosis. For instance protein vaccination against interleukin-12 and VEGF-RII both inhibit atherogenesis[101, 102]. Moreover tolerance induction to oxidized LDL was shown to emeliorate atherogenesis, mainly due to increased levels of regulatory T cells[103].

Already in the late seventies an immune suppressive T cell was identified[104], however scientific attention was quenched during the 90s due to lack of suitable markers. Recently this cell type recurred in science as the regulatory T cell. Regulatory T cells (Treg) represent a novel subset of $CD4^+$ T cells with the capacity to regulate local immune responses[105, 106]. Currently 3 different subsets of Treg cells have been identified. First naturally occurring Tregs, which derive from thymic selection and are distinguished as $CD4^+$, $CD25^{high}$, FoxP3 expressing and TGFβ responsive cells[107-109]. Second antigen specific inducible Tregs have been described, the so-called Tr-1 cells, whose major effector protein is interleukin 10[110, 111]. The third identified subset of Tregs, Th3 cells are antigen non-specific and exert their regulatory potential as bystander inhibitors, mainly via TGFβ[110, 111]. Also within the field of vascular biology regulatory T cells play an important role. Induction of ovalbumin specific Tr1 cells was shown to attenu-

ate atherosclerosis development in apolipoprotein E (ApoE) deficient mice. Adoptive transfer of these Tr1 cells resulted in decreased T cell and macrophage numbers and increased interleukin-10 expression within the atherosclerotic lesion[112]. Moreover, combined hematopoietic deficiency of co-stimulatory molecules CD80 and CD86 resulted in decreased $CD4^+$ $CD25^{high}$ naturally occurring Treg and subsequent acceleration of atherogenesis. Additionally, transfer of Treg depleted splenocytes induced atherosclerotic lesion in T cell deficient mice, whereas adoptive transfer of naturally occurring Tregs attenuated atherogenesis[113].

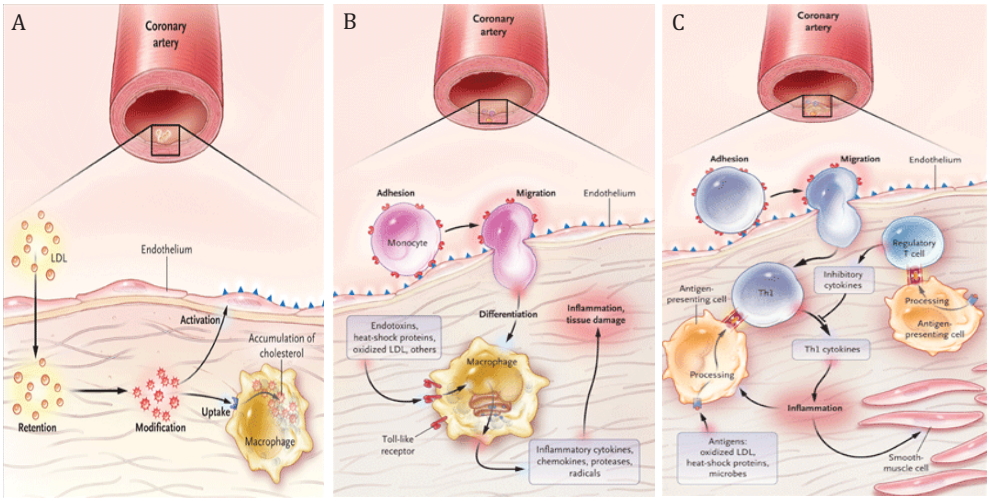

Figure 3: *Modification of intimal LDL cholesterol and subsequent uptake by macrophages (A). Monocyte differentiation and release of pro-inflammaroty cytokines from macrophages (B) results in inflammtory cell accumulation (C). Adapted from Hansson[63]*

4.3. Mast Cells

Mast cells (MC) 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 actively release their granules into the surrounding tissue. Already in 1878, MCs were identified by Paul Ehrlich who believed that this curious cell type contained nutrients for its neighbouring cells[114, 115]. He therefore termed these 'fertilizing' cells Mastzellen, which resulted in the english term Mast cells. MCs originate from $CD34^+$ progenitors in the bone marrow under control of interleukin-3 and Stem Cell Factor and are released into the circulation in an immature form[116-118]. These immature MCs migrate into different tissues were they fully maturate into either mucosal or connective tissue mast cells depending on their surroundings[119, 120]. MCs express the high affinity IgE receptor, FcεRI, via which they usually are activated during episodes of allergies or asthma[121-124]. Binding of multiple IgE molecules leads to cross linking of the FcεRI and subsequent cellular activation resulting in the release of MC granules[125-128]. Next to this classical activation of MCs it was recently shown that activation can also occur by binding of immunoglobulin Light Chain (IgLC) to a currently unkown receptor on the MCs[129]. Neurogenic stimulation by for instance substance P can also lead to activation and degranulation of MCs, while several inflammatory stimuli (e.g. TNF and IL-1) and complement factors (e.g. C3a and C5a) act similarly. MC granules contain a plethora of proteases (histamine, chymase, tryptase), cytokines (TNFa, IFNg, IL-2, IL13, IL15), chemokines (CCL2, CCL3, CCL4, CCL5, CXCL10) but also growth factors (SCF, VEGF, TGFβ)[130-135].

Relevant to atherosclerosis MCs have been identified in the shoulder region of human plaques and they were associated with plaque rupture[136, 137]. Interestingly, acti-

vated mast cells were found to be abundantly present also in the adventitia of atherosclerotic lesions and their number were seen to correlate with the stage of atherosclerotic plaque development and the incidence of plaque rupture[138, 139]. *In vitro* studies have revealed that the release of heparin proteoglycans from MCs can induce the uptake of lipids by macrophages[140, 141], suggesting that MCs have the potential to modulate atherogenesis. More so the MC protease chymase can effectively proteolyse Apo-A1 containing lipoproteins, thereby reducing cholesterol efflux[142]. Next to the effects on foam cell formation, heparin proteoglycans have the potential to inhibit SMC proliferation, while MC derived chymase can provoke SMC apoptosis and induce matrix degradation[143-146]. Moreover MC can release angiogenic factors (e.g. VEGF and bFGF) and therefore have been implied in plaque neovascularisation[147, 148]. Only very recent direct experimental evidence for MC involvement in atherosclerosis was provided by use of MC deficient mice. The absence of MC diminished aortic plaque progression by 50%. The percentage of macrophages and T cells was significantly reduced, while collagen deposition was enhanced. Reconstitution of these mice with *ex vivo* cultured mast cells normalized plaque growth to that of of control MC^{+} mice. Moreover adoptive transfer of TNFα, IL6 and IFNγ deficient MC revealed a significant role for both MC derived IL6 and IFNγ in lesion progression, while MC derived TNFα does not influence lesion development[149]. Clearly these findings provide evidence for a key role of the MCs in atherogenesis (*Figure 4*).

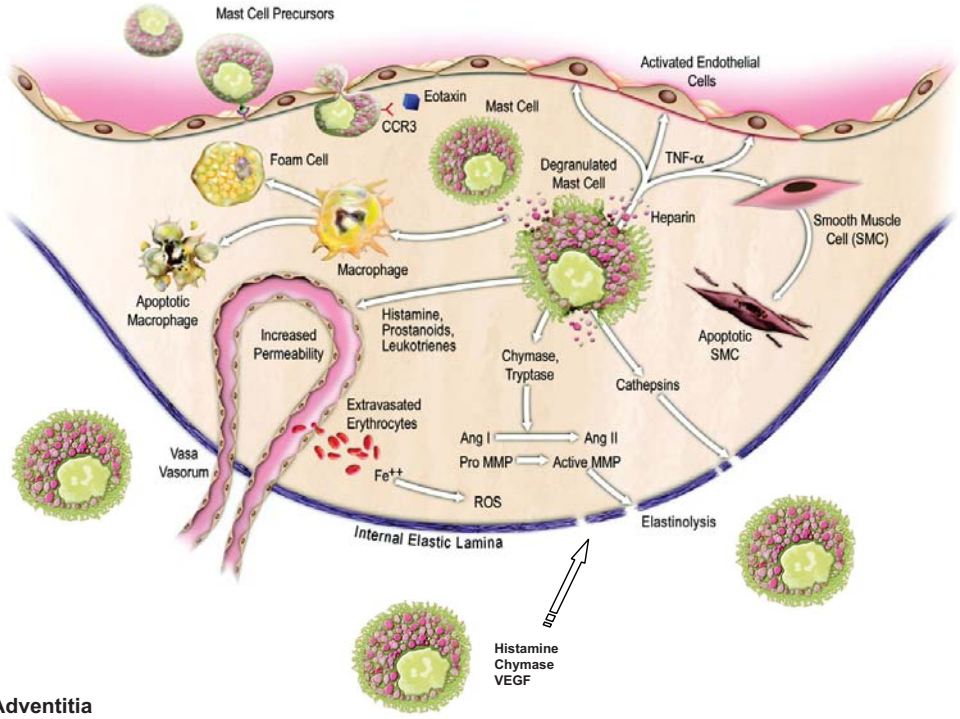

Figure 4: *Activation of adventitial and intimal MCs results in plaque destabilization as a result of increased intimal apoptosis, matrix degradation and erythrocyte extravasation. Adapted from Libby et al.*[216]

4.4. Chemokines

Chemokines are members of the cytokine family, with strong chemotactic capacity[150, 151]. Chemokines and their receptors have conventionally been divided into four families on the basis of the structural arrangement of the N-terminal conserved cystein residues (CXC, CC, C and CX3C). Next to their structural classification, chemokines can also be

functionally classified as being either homeostatic or inflammatory chemokines. Homeostatic chemokines are constitutively expressed and regulate leukocyte navigation during immune surveillance. However, the vast majority of chemokines are inducible and regulate cellular recruitment especially to sites of inflammation[152]. Chemokines are soluble proteins that can be released from many inflammatory cell types including, endothelial cells, platelets, MCs, macrophages and lymphocytes[134, 153-155]. Chemokines contain one to three disulfide bonds, with the exception of CX3CL1 and CXCL16, which contain a membrane-anchored mucin stalk[156, 157]. Chemokines characteristically fold to a structure that consists of an N-terminal domain, a three-stranded β-sheet and a C-terminal helix. Chemokines bind to dedicated receptors of the G Protein Coupled Receptor (GPCR) family of 7 transmembrane receptors coupled to heterotrimeric G-proteins and ligand binding generally induces Gi mediated calcium release and subsequent activation of downstream signalling cascades [158, 159]. A detailed overview of chemokine participation in the pathology of cardiovascular disease and possible treatment strategies is provided in chapter 2.

4.5. G Protein Coupled Receptor Kinases

The activity of most GPCRs is regulated not only at the level of receptor expression but also at a functional level. An important mechanism for controlling receptor activity involves receptor desensitization, which dampens the response to prolonged or repeated stimuli[160, 161]. Desensitization occurs within seconds after receptor stimulation and is primarily mediated by uncoupling of the GPCR from associated G-proteins[162, 163]. Dedicated GPCR kinases (GRKs) can induce receptor desensitization by phosphorylation of the ligand occupied receptor, which subsequently enhances its affinity for cytosolic inhibitor proteins, so-called arrestin family members. Binding of arrestins to the phosphorylated receptor results in uncoupling and internalization of the receptor [164, 165]. Currently the GRK family consist of 7 ubiquitously expressed serine/threonine kinase members[163]. The GRK family has been categorized into three subfamilies based on functional and structural similarities: (1) rhodopsin kinases (GRK1 and GRK-7) (2) β-adrenergic receptor kinases (GRK2 and GRK3) and (3) GRK4 like kinases (GRK4, GRK5 and GRK6)[162].

While several of the GRK family members have been implicated in human pathology, GRK2 has been most frequently related to cardiovascular diseases. GRK2 contributes to chronic heart failure by desensitization of the β1-adrenergic receptor, resulting in loss of cardiac contractility[166, 167]. Furthermore GRK2 was shown to influence vascular resistance and induce hypertension by inhibiting β-adrenergic agonist stimulation[168-170]. Transgenic mice with vSMC specific overexpression of GRK2 have an attenuated vasodilatory response to beta adrenergic stimuli[171]. Moreover GRK2 can affect hypertension by regulation of epithelial Na^+ channels activity[172] and by impairment of endothelial cell nitric oxide synthase (eNOS) activity[173]. GRKs also regulate inflammatory responses that may be relevant to atherosclerosis. Indeed patients suffering from rheumatoid arthritis, an inflammatory disease which shares many features with atherosclerosis, were shown to have decreased GRK2 levels. Rheumatoid arthritis specific cytokines (IFNγ, interleukin-6) are able to decrease GRK2 synthesis[174, 175]. In a rat model of experimental arthritis GRK activity was significantly down regulated during disease manifestation, where it was most evident in B- and $CD4^+$ T cells[176]. Moreover GRK2 protein levels were demonstrated to be reduced in patients with both active and secondary progressive multiple sclerosis (MS) and interestingly the decrease in protein level was similar during remission. Initiation of experimental MS was similar in both wild-type and $GRK2^{+/-}$ mice. However $GRK2^{+/-}$ mice only developed an acute phase of the disease, which was accompanied by massive influx of both T cells and macrophages, without any episodes of relapse[177]. Collectively these findings indicate that *in vivo* inflammation induces tissue- and immune cell-specific downregulation of various GRKs. Conceivably, downregulation of GRKs might result in excessive cellular migration towards inflammatory sites like the atherosclerotic plaque, possibly influencing plaque

progression or stability, hence rendering GRK modulation an intriguing target for the treatment of atherosclerosis.

5. Adventitial Inflammation

The adventitia, or perivasuclar tissue is increasingly recognized as an important substrate in atherosclerosis research. The adventitia consists of extracellular matrix material, a network of capillary blood vessels (vasa vasorum), sensory nerves, tertiary lymphoid structures, fibroblasts, progenitor cells and also inflammatory cells[139, 178-180]. During progression of atherosclerotic lesion development the adventitia expands and adventitial inflammation is gradually enhanced[181-183]. Moreover expression of cytokines in aortic adventitia was shown to be associated with advanced atherosclerosis[184]. The adventitia of ruptured lesions was shown to contain significantly more inflammatory cells, such as monocytes, T-lymphocytes and MCs[138, 182, 185, 186], than that of non-ruptured lesions. Illustratively, expression of the chemokine MCP-1 and its receptor CCR2 are reported to be abundantly present on adventitial macrophages during early atherogenesis. Interestingly while MCP-1 was also expressed in intimal macrophages, the expression of CCR2 appeared specific for adventitial macrophages[187]. In culprit lesions, significantly more $CD4^+$ and $CD8^+$ lymphocytes were observed at the adventitial rim, accompanied by an increased amount of capillaries, compared to stable athero-sclerotic lesions[188]. Furthermore the adventitia, rather than the media, was suggested to be the major source of myofibroblast proliferation after balloon angioplasty and thereby implicated in re-stenosis as well[189]. Adventitial fibroblast were also shown to be activated during atherogenesis in $ApoE^{-/-}$ mice before the formation of intimal lesions[190, 191]. Post mortem examination of human atherosclerotic lesions provided evidence that ruptured lesions displayed enhanced adventitial inflammation, accompanied by increased elastic lamina breaks[182]. Finally adventitial innervation has been proposed as the link between diabetes, smoking, exercise or aging and atherosclerosis, all as a result of dysfunctional autonomic adventitial innervation[192]

The vasa vasorum, a network of adventitial capillaries, is increasingly recognized as an important factor in atherosclerotic lesion development, as it is a major source of intimal neovessels[193-196]. Although luminal infiltration of neovessels may occur as well, this particularly occurs at earlier stages of lesion formation. The exact mechanism of neovessel formation from the vasa vasorum into the plaque is only poorly understood[197]. Possibly, intimal hypoxia and ischemia may induce the expression of Hypoxia-Inducible Factor (HIF-1)[198], which in turn upregulates the expression of Vascular Endothelial Growth Factor (VEGF) and other angiogenic factors by inflammatory cells of the vasa vasorum. Additionally, activated macrophages, particularly in the inner core of the atheroma, stimulate the angiogenic system by inducing endothelial cell secretion of basic Fibroblast Growth Factor (bFGF) and VEGF[197], which further induce endothelial cell proliferation. Moreover adventitial MCs can release a whole set of angiogenic factors such as Histamine, IL-8 and VEGF upon receptor mediated activati on, thereby possibly regulating angiogenesis from the adventitia toward the plaque intima[199-201]. *In vivo* models have revealed that vasa vasorum neovascularisation is correlated to aortic plaque progression in both ApoE and LDL receptor knock out mice[202, 203.] The vasa vasorum also represents an alternative entry point for inflammatory cells and plasma constituents into the plaque, which is critical in plaque progression[204]. In post mortem studies, hyperplasia of the vasa vasorum and the consequential macrophage infiltration were found to be associated with plaque rupture[205]. Currently, a high density of vasa vasorum is considered as one of the determinants of a "vulnerable plaque"[194].

Angiogenesis and ensuing adventitial vasa vasorum neovascularization of the intima may predispose to intraplaque hemorrhage (IPH), which has been associated with plaque instability[196]. Kolodgie and colleagues[206] have recently provided compelling evidence that intraplaque hemorrhage often colocalizes with leaky microvessels and may significantly contribute to the expansion of the lipid core. Extravasated erythro-

cytes form a rich source of free cholesterol, which will be deposited in the core, thereby unbalancing the equilibrium between lipid core size and cap thickness. Moreover excess cholesterol that is taken up by macrophages may induce apoptosis of these cells[207]. Finally, IPH will increase macrophage infiltration, platelet deposition and foam cell formation, all factors that destabilize plaques. Macrophage apoptosis will be accompanied by enhanced TF activity in the plaque which in turn increases VEGF expression and angiogenesis, thus creating a self-perpetuating circuit. In patients with peripheral artery disease, both VEGF and TF levels were significantly increased and the expression of both factors appeared to be interrelated, suggesting a direct link between thrombosis and angiogenesis[195]. Focal inhibition of angiogenesis could results in reduced vasa vasorum development and decreased plaque formation[208].

6. Study Aims

The most common clinical manifestations of cardiovascular disorders, stroke and acute myocardial infarction, are a result of atherosclerotic plaque rupture and subsequent thrombosis. As chemokines are generally considered key regulators of leukocyte transmigration into the vessel wall, we anticipated that specific chemokines might have a distinctive role in leukocyte homeostasis at specific stages of atherosclerotic disease progression and during ischemia-reperfusion injury. We also suggest that patient specific local regulation of leukocyte homeostasis by means of modulated chemokine-directed leukocyte migration might therapeutically modulate atherosclerotic plaque progression and stability and additionally could improve tissue recovery after ischemic injury. In this thesis, the first aim was to identify cardiovascular disease specific chemokine or chemokine pattern regulation in humans by use of multiplex chemokine analysis. Secondly we aimed to mechanistically validate the chemokine markers obtained from the human profiling studies and attenuate lesion progression by modulation of several other chemokine targets *in vivo*. Finally, we aimed to integrate human and mouse studies in order to identify new chemokine targets or chemokine patterns for future therapeutic intervention.

7. Thesis Outline

Prevention of clinical complications as myocardial infarction or stroke due to plaque rupture in unstable angina pectoris patients is likely to result in a decreased cardiovascular death rate in the Western Society. Currently angina pectoris treatment is mainly focused on anti-coagulants and cholesterol lowering, often followed by invasive treatments such as primary percutaneous intervention (PCI). To prevent patient hospitalization and invasive surgery, strategies to attenuate plaque progression and even so improve plaque stability of an atherosclerotic lesion could offer a suitable therapeutic alternative.

In this thesis, it was aimed to improve plaque stability by modulation of the local and/or systemic leukocyte homeostasis. **Chapter 2** provides an overview on the current status of chemokine research in several cardiovascular disorders. Furthermore possible treatment strategies are suggested. In **Chapters 3 and 4**, patient material from two different cohorts comprised of acute myocardial infarction patients (MISSION!) and angina pectoris patients (APRAIS) was analyzed for chemokine distribution patterns by use of a multiplex immuno assay. The chemokines CCL3 (MIP1α), CCL5 (RANTES) and CCL18 (PARC) emerged as the most promising therapeutic targets. In **Chapter 5** one of the targets obtained from the human profiling, CCL3 was studied for its contribution to atherogenesis. CCL3 is an inducible inflammatory chemokine also known as Macrophage Inflammatory Protein-1α (MIP-1 α), which is higly expressed by macrophages, lymphocytes and MCs. CCL3 binds to three different chemokine receptors, CCR1, CCR3 and CCR5 and it can form dimers with CCL4. In this study we pursued a bone marrow transplant (BMT) approach where wild type bone marrow was replaced by CCL3 defi-

cient bone marrow in LDLr$^{-/-}$ recipients, thereby inducing leukocyte specific CCL3 knock out. After a full engraftment of the bone marrow the animals received a high fat, high cholesterol diet after which lesion formation and composition in the aortic leaflet area was determined. In **Chapter 6** a CXCR3 antagonist (NBI-74330) was used to establish the role of CXCR3 expressing leukocytes in atherosclerosis. LDLr$^{-/-}$ mice were treated daily with NBI-74330 during the entire experiment and the effect of CXCR3 blockage on both collar induced and 'natural' atherosclerosis was evaluated. In **Chapter 7** the TGFβ family member Macrophage Inhibitory Cytokine-1 (MIC-1) was studied for its role in early atherogenesis and plaque stability. MIC-1 was first implicated in atherosclerosis in patient studies, where it was shown to be an independent risk factor for acute coronary syndromes[209, 210]. Furthermore MIC-1 was shown to co-localize with intimal macrophages and is a potent inducer of p53 mediated apoptosis[211, 212], thereby rendering this growth factor a detrimental player in atherosclerosis. For this study we used the BMT strategy to study the effects of leukocyte specific MIC-1 deficiency on atherogenesis. In **Chapter 8** we studied the effect of chemokine receptor desensitization on atherogenesis and plaque stability. Receptor desensitization, by for instance GRKs, is an important mechanism for controlling receptor activity, which dampens the response to prolonged or repeated stimuli[12, 13]. GRK2 has been frequently related to cardiovascular diseases. For instance, GRK2 contributes to chronic heart failure by desensitization of the β1-adrenergic receptor, resulting in loss of cardiac contractility[18, 19]. Furthermore GRK2 was shown to influence vascular resistance and induce hypertension by inhibiting β-adrenergic agonist stimulation[20-22]. For this study we used the BMT strategy to study the effects of leukocyte specific partial GRK2 deficiency on atherogenesis and plaque morphology. In **Chapter 9** we have studied an inflammatory cell type, the mast cell, which, in human plaque rupture, has been shown to be abundantly present in the adventitia of affected arteries. Although the MC content of the adventitia was linked to the severity of disease, it remained unclear whether adventitial mast cells causally contribute to or are recruited in response to plaque rupture. In this study, we have persued a novel adapted delayed type hypersensitivity approach to attract MCs to the adventitia of carotid artery lesions in ApoE$^{-/-}$ mice and evaluated its impact on plaque morphology. **Chapter 10** represents a detour to the field of immuno haematology as we have by serendipity found that CCR7 may be critical to the development of Graft versus Host Disease (GvHD). Lethally irradiated animals were transplanted with either WT or CCR7$^{-/-}$ bone marrow. After approximately 5 weeks post transplantation the CCR7$^{-/-}$ recipients started to show signs of chronic GvHD. In an additional experiment we have attempted to rescue CCR7$^{-/-}$ recipients from chronic GvHD by dilution of CCR7 deficiency. Finally, **Chapter 11** provides a discussion of the most relevant findings of this thesis and offers an overview of future perspectives the therapeutic implications.

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