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Chemokines and Atherosclerotic Plaque Progression: Towards Therapeutic Targeting?

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Abstract:
Atherosclerosis is currently viewed as an inflammatory disease in which the initiation and progression of the atherosclerotic plaque towards a rupture prone, unstable plaque is driven by leukocyte recruitment mediated by various inflammatory mediators. Recently, interest in chemotactic cytokines or chemokines with regard to atherosclersis has been growing as chemokines mediate the influx of leukocytes that is typical of atherothrombosis. The activity of the majority of chemokines is overlapping and chemokines are not only produced by the various cellular constituents of the atherosclerotic plaque but also by activated platelets. Consequently, the direct influence of individual chemokines on plaque destabilisation and rupture is widespread and rather unclear. Experimental research has already established the role of a number of chemokines in advanced atherosclerosis. Nevertheless, given the complexity and size of the chemokine family, further screening of cardiovascular disease for chemokine level and genetic polymorphisms for chemokines will be warranted as the search for viable biomarkers of plaque destabilization as well as novel therapeutic targets for specific atheroregressive therapeutic compounds is ongoing. With regard to the latter, clinical trials with specific chemokine inhibitory strategies, like chemokine receptor antagonists, are already underway in other inflammatory disorders. Summarizing, chemokine inhibition likely constitutes an important therapeutic option next to already established drugs in the management of cardiovascular disease.

Introduction

Cardiovascular disease (CVD) is the largest contributor to morbidity and mortality in the industrialized world. In 2002, the prevalence of CVD in the American population was 34.2%, accounting for estimated direct and indirect health care costs of 393.5 billion dollars in 2005 and claiming 2600 deaths each day[1]. The largest contributor to CVD, among others, is the slumbering process of atherosclerosis and the culprit for its clinical consequences, atherothrombosis.

Over the past 20 years, our knowledge on the pathophysiology of atherosclerosis and, to a lesser extent atherothrombosis, has increased tremendously. Various in-depth reviews summarize a very compelling body of evidence indicating that atherosclerosis is a chronic inflammatory process[2, 3], as first postulated by Ross[4]. While current paradigm views atherosclerosis as an inflammatory, progressive disease of complex aetiology, hyperlipidaemia, among other risk factors, is intimately linked to vascular wall inflammation and intimal stenosis. Interestingly, clinical events were found to be associated with the composition and vulnerability of the plaque rather than with the degree of stenosis[5]. Together with the observation on atherosclerotic plaques obtained at autopsy that thin capped fibroatheroma are mechanically weaker than those with thicker caps, these finding have prompted the introduction of the vulnerable plaque concept[6-8]. In brief, atherosclerotic lesions are formed in the intima of the vessel wall and what begins with an asymptomatic intimal fatty streak gradually becomes a more advanced lesion through the recruitment and influx of various inflammatory cell types, migration of smooth muscle cells (SMCs) and the formation of a lipid-rich necrotic core with an overlying fibrous cap. Eventually, the plaque will advance to an unstable vulnerable plaque phenotype or thin cap fibroatheroma[9, 10]. The progression of an advanced to a vulnerable plaque is suggested to result from a local dysbalance in cellular as well as extracellular composition of the plaque leading to progressive erosion of the fibrous cap and concomitant expansion and disintegration of the necrotic core. Ultimately, upon rupture of the latter plaque its highly thrombogenic content will be extruded to cause thrombus formation, arterial occlusion and ischemic symptoms or infarction. Thus, a thorough understanding of the molecular pathways underlying leukocyte influx in advanced and vulnerable plaques is from a clinical point of view of utmost importance.

The majority of studies have centred on addressing leukocyte influx in plaque initiation, establishing a prime role for selectin family members (i.e. E-selectin, L-selectin and P-selectin) in the capture, tethering and rolling of circulating monocytes onto the inflamed endothelium and for endothelial adhesion molecules (e.g. ICAM-1, VCAM-1), which mediate leukocyte arrest by interacting with integrins on activated monocytes[11, 12]. Moreover these studies have revealed a key role of a specific subclass of small chemotactic cytokines with strong leukocyte homing capacity, the chemokines, in this process. Chemokines were seen to contribute to the recruitment of leukocytes in initial stages of atherosclerosis by triggering chemotaxis and by assisting in arrest and diapedesis[13-15]. Given the clinical relevance of plaque rupture, leukocyte influx in advanced plaques has been subject of surprisingly few studies. Nevertheless it is conceivable that leukocyte influx in advanced plaques will differ in many aspects from that to inflamed endothelium or initial plaques. The altered composition of advanced plaques likely translates in a changed panel of secretory mediators and chemokine receptors[16]. Moreover, the reported presence of vasa vasorum in close proximity to and neovessels within advanced lesions will certainly impact the pattern of leukocyte recruitment[17], as these vessels will serve as an alternative entry site for circulating leukocytes.

While adequate (mouse) models for plaque initiation and progression are available, there still is considerable debate on the relevance of models for studying (thrombotic) plaque rupture. Although plaque rupture have incidentally been observed in aged atherosclerotic mice, at specific locations (i.e. the brachiocephalic artery) and after me-

chanical or genetic manipulation[18, 19], compelling evidence of thrombus formation at the site of presumed rupture is lacking, possibly due to the high fibrinolytic propensity of mice[20]. Thus, until now a golden standard for (thrombotic) plaque rupture is still not available and by necessity we will have to live with surrogate rather than end-point markers of plaque rupture such as collagen content, cap thickness, lipid core content, buried caps, and intraplaque hemorrhage. Hence any data on effects of chemokines on plaque stability should be handled with considerable caution and can not be directly extrapolated to human situation. In this review, we will present our current knowledge on the role of chemokines in atherothrombosis and plaque stability with special emphasis on emerging therapeutic opportunities for stabilizing the vulnerable plaque.

Chemokines

Chemokines are organised into 2 major (CC and CXC) and 2 minor (C and CX3C) families based on the N-terminal cysteine residues[21]. So far, approximately 50 chemokines have been described, as well as more than 20 of their seven transmembrane G-protein coupled receptors (GPCRs), while it has been shown that CC and CXC chemokine receptors can have multiple high affinity ligands. Conversely, chemokines can bind various receptors as well, which makes chemokine activity very redundant and overlapping. Chemokines are key actors in leukocyte chemotaxis, leukocyte degranulation and mediator release, integrin activation during leukocyte-endothelial interaction and the induction of angiogenesis. Therefore, chemokines have a profound influence on various components of immunology and, as atherosclerosis is regarded as a chronic inflammatory process, an increasing body of research is performed that underscore the influence of chemokines in atherothrombosis. From basic animal research as well as clinical research, 20 of these chemokines were reported to affect atherosclerosis (Tables 1, 2 and 3)[22]. Endothelial cells, vSMCs and macrophages express chemokine receptors, which make chemokines important players in plaque formation and destabilization by orchestrating selectin and integrin mediated monocyte recruitment, as well as supporting intimal migration of SMCs. As the relative contribution of these cell types changes during lesion progression, the contribution of involved chemokines will likely change as well. Illustratively, Lutgens *et al.*, using gene profiling techniques in $ApoE^{-/-}$ mice, showed a time dependent up-regulation of monocyte chemotactic protein-1 (MCP-1), monocyte chemotactic protein-5 (MCP-5), macrophage inflammatory protein-1α (MIP-1α), MIP-1α and fractalkine with plaque progression, mainly in plaque macrophages. This upregulation was accompanied by an increase in MCP-1 and MCP-5 serum levels.

A recent advance in the understanding of chemokine function and atherothrombosis is their synergy with platelets. While platelets not only respond to and are activated by membrane-bound chemokines, they also release chemokines, such as PF4 (CXCL4) and RANTES (CCL5), and mediate deposition of these and other chemokines on the endothelium as recently reviewed[14]. Moreover, PF-4 even has an influence on lipoprotein metabolism modulating lipoprotein uptake by macrophages, illustrative of the pleiotropic effects of chemokines in atherogenesis. Interestingly, increased serum levels and genetic polymorphisms of various chemokines have been reported in atherosclerosis and its clinical sequelae (Tables 3 and 4). This suggests that chemokines might not only play a prominent role in plaque initiation, progression and destabilization, but also in plaque rupture. Below, we will discuss the current insights on chemokine function in atherothrombosis from an experimental as well as a clinical point of view and we shall review the relative contribution of each chemokine to atherothrombosis based on available experimental and clinical data.

MCP-1 / CCL2

The CC chemokines are the largest family of chemokines, encompassing 28 different proteins. One of the CC chemokines, monocyte chemotactic protein-1 (MCP-1/CCL2), has been extensively studied with regard to atherosclerosis in animal models as well as in clinical studies. MCP-1 is expressed in macrophage-rich regions in human atheroscle-

rotic plaques and contributes to the recruitment of monocytes to the vessel wall[23, 24].

	Synonyms	Receptor	Source	Actions
CCL2	Small inducible cytokine A2, Monocyte Chemoattractant Protein-1 (MCP-1), SCYA2	CCR1 CCR2	MΦ/TL	Mc, MΦ recruitment
CCL3	Small inducible cytokine A3, Macrophage Inflammatory Protein 1 alpha (MIP1α), SCYA3, G0S19-1	CCR1 CCR4 CCR5	MΦ/TL/ NK/P	MΦ , Eo, N, BL recruitment
CCL4	Small inducible cytokine A4, Macrophage Inflammatory Protein 1 beta (MIP1β), SCYA4	CCR1 CCR5 CCR8	TL/NK	Mc, MΦ , Eo, TL recruitment
CCL5	Small inducible cytokine A5, Regulated on Activation Normal T-cell Expressed and Secreted (RANTES), SCYA5	CCR1 CCR3 CCR4 CCR5	TL/P	Mc, TL, Eo recruitment and adhesion
CCL11	Small inducible cytokine A11, Eotaxin, Eosinophil chemotactic protein, SCYA11	CCR2 CCR3 CCR5	EC	Mc, E, SMC recruitment, B activation
CCL13	Small inducible cytokine A13, Monocyte chemoattractant protein 4, SCYA4	CCR1 CCR2 CCR3 CCR5	EC	Eo, TL (Th2), Mc recruitment Eo, B activation
CCL17	Small inducible cytokine A17, Thymus and activation-regulated chemokine (TARC), SCYA17	CCR4 CCR8	TL/EC/ P/BL	TL (Th2), Eo recruitment
CCL18	Small inducible cytokine A18, Dendritic cell chemokine 1, Macrophage inflammatory protein 4, Pulmonary and activation-regulated chemokine (PARC), SCYA18	CCR3	DC/Mc/ MΦ/Eo,	TL, BL recruitment Mc/ MΦ activation
CCL19	Small inducible cytokine A19, Macrophage inflammatory protein 3 beta (MIP-3β), EBI1-ligand chemokine (ELC), SCYA19	CCR7	TL/SMC/ DC	TL(naïve, memory), BL, DC recruitment
CCL22	Small inducible cytokine A22, Macrophage-derived chemokine (MDC), Stimulated T cell chemotactic protein-1(STCP-1), SCYA22	CCR4	TL/MΦ/ EC/DC/ BL	TL (Th2), DC, Eo, NK recruitment, Eo activation

Table 1 : CC Chemokines. *BL = B Lymphocyte; B = Basophile; DC = Dendritic Cell; EC = Endothelial Cell; Eo = Eosinophile; MΦ = Macrphage; MC = Mast Cell; Mc = Monocyte; NK = Natural Killer Cell; N = Neutrophile; P = Platelet; SMC = Smooth Muscle Cell; TL = T Lymphocyte*

As summarized in recent reviews[22, 25], MCP-1 and its receptor CCR2 are most prominently involved in endothelial monocyte recruitment to sites of endothelial injury[26]. Also, increased levels of recombinant C-reactive protein (CRP), an inflammatory marker shown to be up-regulated in atherosclerosis, was seen to promote monocyte influx by inducing CCR2 surface expression on circulating monocytes[27]. This finding however should be addressed with care, while recently it has been suggested that this response is more likely due to proinflammatory bacterial products which accompany recombinant CRP[28]. Arterial MCP-1 expression levels were markedly elevated in response to hypercholesterolemia in primates and, in agreement, statin treatment directly inhibited MCP-1 mediated human monocyte recruitment in vitro[23, 29]. Several reports show an involvement of MCP-1 in vivo on the development of neo-intimal formation after vascular injury, neo-angiogenesis and also thrombosis. With regard to the latter, activated platelets were shown to augment monocyte recruitment by inducing MCP-1 synthesis

by endothelial cells[30]. Moreover, MCP-1 and CCR2 deficient mice as well as mice with a deficiency in leukocyte CCR2 all showed decreased lesion formation but no differences in collagen content[31-33], and blockage of MCP-1 activity by transfection of 7ND, an N-terminal deletion mutant of human MCP-1 gene, inhibited lesion initiation considerably[34]. Also, 7ND reduced monocyte infiltration and caused an increase in plaque smooth cell and collagen content thereby promoting a stable plaque phenotype[35]. A similar effect was seen after administration of 11k2, a blocking antibody for MCP-1 and MCP-5, which reduced plaque area and concomitantly increased plaque collagen content[36]. Therefore, its relevance in plaque initiation seems undisputed, while its role in plaque progression and rupture is less clear. Viewed in that light it is very interesting to note that clinical studies could confirm elevated MCP-1 serum levels in atherosclerotic complications such as acute myocardial infarction, unstable angina pectoris and ischemic stroke[37, 38]. Unfortunately, a causal influence of elevated MCP-1 serum levels in these conditions could not be shown. Also, a haplotype defined by the MCP1-2578G allele was associated with prevalent MI, providing genetic evidence on a role of MCP-1 in atherosclerosis[39, 40]. However, other studies did not detect any difference in MCP-1 serum levels between subjects with sub-clinical atherosclerosis and those without[41,42]. It is also shown that MCP-1 plays an important role in post infarction myocardial healing[43]. The increase in MCP-1 levels during myocardial infarction could therefore be more indicative of a MCP-1 driven myocardial healing response than a reflection of plaque instability.

To conclude, MCP-1 is a very pleiotropic chemokine. While evidence for its role in plaque initiation seems compelling, its influence on plaque destabilisation is less evident. With regard to the latter, a designated study on plaque stability on advanced lesions would be desirable.

Platelet derived chemokines: RANTES and PF-4

Not only do platelets play an essential role in hemostasis and thrombus formation, they also release various pro-inflammatory substances upon activation, including chemokines. These chemokines are stored in α-granules and are mainly synthesized in the megakaryocytes but in part also endocytosed in situ by circulating platelets. The α-granules contain an abundant range of chemokines which includes platelet factor 4 (PF-4), a member of the CXC chemokine family (CXCL4), and Regulated-on-activation,-normal-T-cell-expressed-andsecreted (RANTES, CCL5)[44, 45]. The latter, originally purified as a product of activated T-cells and a ligand for CCR1 and CCR5, is a chemoattractant of T-lymphocytes, eosinophils and monocytes with potent endothelial arrest properties[46-48]. PF-4 acts chemotactic for circulating monocytes and Th lymfocytes via its receptor CXCR3[49]. It mediates E-selectin expression by endothelial cells, a process critically dependent on NF-κB activation[50], and was seen to enhance the binding of oxidized low density lipoprotein (oxLDL) to endothelial cells and SMCs[51], thereby participating in the onset of atheroma formation as well plaque progression. Upon activation, platelets secrete RANTES and PF-4, triggering monocyte arrest after deposition of RANTES on the monocyte cell surface and on the activated endothelium[52]. Recently, Baltus *et al*. demonstrated that RANTES and PF-4 dose-dependently and synergistically induced endothelial monocyte arrest under conditions of flow which could be inhibited by Met-RANTES, a CCR1 and CCR5 antagonist, but also by a PF-4 blocking antibody[48]. The recent observation that injection of $ApoE^{-/-}$ mice with activated platelets induced monocyte arrest and aggravated atherosclerotic lesion size may thus be partially attributable to platelet CCR5 and PF-4[13, 53]. This was further substantiated by Veillard *et al.* showing that treatment of hyperlipidemic mice with Met-RANTES considerably inhibited atherogenesis resulting in reduced monocyte infiltration and an increase in plaque collagen content, conferring a more stable plaque phenotype[54]. Interestingly however, mice deficient in CCR5 were not protected against early plaque formation[55]. Recently, our lab showed that TAK-779, a HIV entry inhibitor which blocks CCR5 and CXCR3, impaired atherogenesis by blocking the recruitment of T cells ($CD3^+$) to the plaque[56], illustrative of the potential of chemokine receptor antagonists in plaque stabilizing strategies. In keeping

with the pro-atherothrombotic activity in mice models, PF-4 serum levels are elevated in peripheral vascular disease and CAD, possibly reflecting enhanced platelet activity[57]. Pitsilos et al. observed a correlation between PF-4 expression and the lesion progression stage from carotid artery plaques, linking chronic platelet activation and lesion development. They could detect PF-4 deposits on endothelium, macrophages and neovessels of human carotid arteries, even in early lesions, suggesting that PF-4 may play a major role throughout plaque development[58]. Studies in PF-4 knock-out models are eagerly awaited to verify this hypothesis. RANTES was not only shown to be expressed in human atherosclerotic plaques[59], preliminary data show increased serum levels of RANTES in AMI and ACS patient groups as well, independent of other risk factors and inflammatory markers[60]. However, serum levels were decreased in patients with stable CAD compared to controls[41]. Although the outcome of genetic association studies should be interpreted with care[61], the RANTES G-403A polymorphism was independently associated with CAD and this tendency was even more pronounced for subgroups of ACS and smokers, populations with increased platelet activation and inflammation[62].Although RANTES, and to a lesser extent PF-4, have a share in the onset of atherogenesis, their contribution to activity in plaque destabilization is less clear. The elevated serum levels of both chemokines in cardiovascular diseases may be due to platelet activation, yet their effect on plaque stability still has to be determined.

Eotaxin / CCL11

Eotaxin (CCL11) was also shown to be present in human atherosclerotic plaques and its expression localized on SMCs[22, 63]. Originally described as a potent chemotractant and activator of eosinophils, a cell type which is only rarely observed in plaques, it acts by activating the CCR3 receptor on Th2 lymphocytes, dendritic cells, mast cells and other haematopoietic subsets. Moreover, CCR3 is expressed in macrophage-rich regions of atherosclerotic plaques[63]. Another interesting feature is the reported association of increased inflammation, microvascular density and blood clotting in eotaxin-secreting tumors[64]. Recently, Emanuele *et al.* observed that increased plasma eotaxin levels correlated with the severity of CAD, which contrasts however with studies by Mosedale *et al.* and Rothenbacher *et al*., who did not find any difference in serum eotaxin levels in CAD versus controls[41, 42, 65]. An A23T substitution in the CCL11 gene imparted an increased risk of myocardial infarction in a large prospective cohort of healthy American men[66]. Conversely, the –1328A/A promoter variant of the eotaxin gene appeared to confer protection against restenosis[67]. Given these conflicting data, the lack of solid data on eotaxin serum levels during acute ischemic events, and the lack of experimental data with genetically modified mice, a potential influence of eotaxin on plaque destabilization remains to be proven.

MIP-1α / CCL3 AND MIP-1β / CCL4

Macrophage inflammatory protein-1α (MIP-1α/CCL3) and MIP-1β (CCL4) expression have been observed in human plaques, especially in the shoulder zone[59, 68]. They are secreted by a variety of cells. Amongst others, MIP-1α is released by activated platelets on the endothelium[14]. Whether or not MIP-1α levels are changed in atherothrombosis is unknown to date. Elevated levels of platelet derived IL-7 in stable and unstable angina pectoris were seen to be mediated by MIP-1α in a dose dependent manner and preliminary data from our lab indicate that MIP-1α levels are increased during acute myocardial infarction compared to a healthy control group (de Jager *et al.*; personal communication)[69]. These data show that MIP1α could play a role in plaque destabilization, a hypothesis further underscored by the fact that two MIP-1α receptors, i.e. CCR1 and 5, are both expressed on monocytes and endothelial cells, and the presence of the latter was observed in human vascular smooth muscle cells as well[70]. However, it is still unclear if elevated levels of MIP-1α are indicative of a causal relationship with myocardial infarction. For MIP-1β, aortic expression increased with lesion progression in mice[36]. Although no data on MIP-1β serum levels in human CVD are available, MIP-1β expression in peripheral mononuclear cells was increased in CAD patients compared to

controls and atorvastatin could reduce its expression[71]. Even though data on the influence of MIP-1α and MIP-1β in atherosclerosis are sparse, a role in plaque destabilization seems likely. Further experimental data are needed to verify this hypothesis.

IL-8 / CXCL8 AND GRO-α / CXCL1: CXC Chemokines

Interleukin 8 (IL-8, CXCL8) and growth related oncogene alpha (Gro-α, CXCL1, KC in mice) are related chemokines which both activate chemokine receptors CXCR1 and CXCR2 and contribute considerably to monocyte recruitment. Although IL-8 has initially been linked to neutrophil activation and migration, it also appeared to be a potent mediator of firm adhesion of monocytes to the vascular endothelium under flow conditions and thus is deemed to be a key determinant in the initiation of atherogenesis[72]. Likewise, Gro-α plays a significant role in the arrest of monocytes to the endothelium through the activation of the β-2 integrin VLA-4[73]. Circulating human monocytes express CXCR1 and CXCR2 while IL-8 was abundantly present in macrophage-rich human plaques[72]. Leukocyte deficiency of mIL8RH, the murine homologue of CXCR2, attenuated atherosclerosis in hyperlipidemic mice[74]. Another interesting feature of IL-8 is its ability to induce migration and proliferation of endothelial cells and vSMCs in vitro, pointing to a potential role in neovascularization[75, 76]. An elegant study of Simonini *et al.* confirmed that IL-8 is angiogenic, via analysis of human coronary atherectomy tissue and the subsequent application of tissue derived IL-8 in a corneal sprouting assay, which may in the context of the plaque translate in expansive plaque growth and even plaque vulnerability[75]. In agreement, several lines of evidence further support an involvement of IL-8 in atherothrombosis. Not only were serum IL-8 levels recently found to be raised in stable coronary heart disease[41], elevated levels were also noted in patients with acute myocardial infarction versus controls[77, 78]. A causal relationship of IL-8 and myocardial infarction seems unlikely, while IL-8 levels could be enhanced upon activation by coagulation factor Xa in these patients[77]. Holm *et al.* provided mechanistic evidence showing that platelet-rich plasma, stimulated with oxLDL, enhanced IL-8 release by mononuclear cells in particular in CAD patients[79]. Also, peripheral blood mononuclear cells (PBMCs) derived from these patients and stimulated with oxLDL were seen to release more IL-8 and Gro-α than PBMCs from control patients. However, increased IL-8 levels could also be secondary to increased CRP levels, which is believed to cause a downstream upregulation of IL-8[80], but as mentioned before, this effect could be biased by bacterial side products of recombinant CRP[28]. These data are suggestive of a role of IL-8 in plaque destabilization but additional clinical trials as well as experimental research will have to shed further light on this matter. Conversely, no clinical data exist on Gro-α involvement during episodes of (acute) cardiovascular syndromes. Experimental data in ApoE*3 Leiden mice show that KC serum levels are raised on a high cholesterol diet compared to nontransgenic littermates, however no KC expression was seen in aortic lesions[81]. Therefore, Gro-α only seems to play a role in early lesion formation in response to elevated plasma cholesterol levels, but a role in plaque destabilization seems less likely.

IP-10 / CXCL10

IFNγ-inducible protein 10kDa (IP-10/CXCL10) expression has been detected in all three major atheroma-associated cell types in the plaque[82]. Although IP-10 has not yet been correlated with monocyte recruitment, it has been shown to participate in T-cell arrest via its receptor CXCR3 on activated endothelium. As already referred to by its name, IP-10 augments IFN-γ production by Th1 cells and may thus foster the inflammatory response within the atherosclerotic vessel wall. Interestingly, Veillard *et al.* reported that initial lesion formation was inhibited in CCR2 ApoE double knockout mice[83]. However, IP-10 is a potent inhibitor of tumour neo-angiogenesis as well and in agreement, neo-angiogenesis after myocardial infarction was found to be impaired after IP-10 administration[84, 85]. Therefore, IP- 10 in atherosclerotic plaques could act via attenuated angiogenesis, thereby hampering plaque progression and favouring plaque stability. Additional research has to be performed to exclude that IP-10 directly inhibits or promotes plaque progression in vivo, although recently, Cheng *et al.* showed that the de-

velopment of unstable lesions was characterized by upregulation of IP-10 during lesion initiation. While IP-10 serum levels were seen to be significantly increased in a patient group with stable coronary heart disease (CHD) after adjustment for established risk factors[41], a smaller study reported increased IP-10 expression in circulating T-cells of patients with stable versus unstable angina pectoris[86]. Although no conclusive data on IP-10 levels in acute ischemic syndromes are available, and as IP-10 mainly mediates T-cell function, a prominent role for IP-10 in plaque destabilization seems not unlikely. Currently, studies are underway to investigate this matter in patients with AMI.

	Synonyms	Receptor	Source	Action
CXCL1	Small inducible cytokine B1, Growth regulated protein alpha, GRO-α, SCYB1	CXCR2	MΦ/P	N recruitment and activation
CXCL4	Small inducible cytokine B4, Platelet factor 4, Oncostatin A, SCYB4	CXCR3	P	L recruitment, EC, SMC activation
CXCL8	Small inducible cytokine B8, Interleukin 8, Monocyte-derived neutro-phil chemotactic factor, SCYB8	CXCR1 CXCR2	EC/Eo/ MΦ/ SMC	N, Eo, TL , BL, B activation
CXCL10	Small inducible cytokine B10, 10 kDA interferon-gamma inducible protein (IP-10), SCYB10	CXCR3	TL/ SMC	Mc, TL, SMC recruitment and activation Angiostatic factor
CXCL11	Small inducible cytokine B11, Interferon-inducible T-cell alpha chemoattractant (ITAC), Interferon-gamma-inducible protein-9 (IP-9), SCYB11	CXCR3	EC/ Mc/N	TL (Th1), NK recruitment
CXCL12	Small inducible cytokine B12, Stromal cell-derived factor 1 (SDF-1), SCYB12	CXCR4	B/SMC EC	$CD34^+$ Cell, Mc, TL, BL, MC recruitment
CXCL16	Small inducible cytokine B16, Scavenger receptor for phosphatidylserine and oxidized low density lipoprotein, Bonzo ligand, SCYB16	CXCR6	MΦ/TL/ SMC/ DC	TL recruitment foam cell formation
CX3CL1	Small inducible cytokine D1 , Fractalkine (FKN), neurotactin, SCYD1	CX3CR1	EC/N	Mc, SMC, N, MC recruitment, NK, L, P activation and adhesion, SMC proliferation
MIF	Macrophage inhibitory factor	CD74	L/EC	MΦ, SMC activation

Table 2 : CXC, CX3C chemokines and other factors. *BL = B Lymphocyte; B = Basophile; DC = Dendritic Cell; EC = Endothelial Cell; Eo = Eosinophile; MΦ= Macrophage; MC = Mast Cell; Mc = monocyte; NK = Natural Killer cell; N = Neutrophile; P = Platelet; SMC = Smooth Muscle Cell; TL = T Lymphocyte*

SDF-1α / CXCL12

Initially regarded as prime factor in the recruitment of progenitor cells from stroma, stromal cell-derived factor-1α (SDF-1α, CXCL12) appeared to be a rather pleiotropic chemokine, expressed by vSMCs, endothelial cells and macrophages of human atheroma but not in the normal vessel wall[52]. Moreover, it is one of the few chemokines able to activate platelets, by interacting with CXCR4. SDF-1α is expressed constitutively and is a highly efficacious and potent mononuclear cell attractant in vivo[87]. Interestingly, SDF-1α was shown to display anti-inflammatory activity by inhibiting leukocyte entry into inflamed tissue. Furthermore, high concentrations of SDF-1α could have a potentially plaque stabilizing effect by in vitro inhibition of matrix metalloproteinase-9 (MMP-9) expression and enhancement of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) activity in peripheral blood mononuclear cells derived from patients with unstable angina pectoris (UAP)[88]. This was further underscored by the observation that SDF-1α levels were lower in patients with UAP vs. controls or stable angina pectoris patients[88, 89]. Recent publications mainly by Weber and co-workers focussed on the stimulatory

role of SDF-1α in neointima formation after injury, regulating neointimal vSMC influx by recruitment of circulating progenitor cells[90], which could be inhibited in vivo with an anti-SDF-1α antibody[91]. While progress on SDF-1α is limited by lethality of CXCR4 deficiency, other methods of gene manipulation are currently applied to unravel the precise role of SDF-1α in atherothrombosis. For now, it seems that SDF-1α has a beneficial effect on plaque stability, next to its more established stimulatory effect on mediating wound healing.

Chemokine	Protein/RNA Expression
CCL2	Macrophage rich regions atherosclerotic plaques Elevated serum levels in AMI, UAP and ischemic stroke
CCL5	Increased serum levels in AMI and ACS Decreased serum levels in stable CAD
CCL11	Expression in atheroma Controversy on enhanced serum levels
CXCL4	Increased serum levels peripheral vascular disease and CAD
CXCL8	Expressed in macrophage rich plaques Enhanced serum levels in stable CAD and AMI
CXCL10	Enhanced serum levels in stable CAD
CXCL12	Decreased serum levels in UAP
CXCL16	Expression in atheroma
CX3CL1	Time dependent expression in plaques Enhanced serum levels in ACS
MIF	Expressed and active in advanced lesions

Table 3 : Expression of chemokines in human plaques and serum levels in CVD.

CXCL16

CXCL16, also known as SR-PSOX, is an atypical chemokine. It is expressed in human atheroma, where it is localized on macrophages, endothelial cells and SMCs, but not in the unaffected vessel wall[92-94]. It exists as a membrane bound chemokine, at which the chemokine domain is attached to a transmembrane mucin stalk and in a soluble form after cleavage by ADAM-10 (a disintegrin and metalloprotease), its counter receptor is CXCR6[95]. Soluble CXCL16 was reported to mediate migration of activated Tcells towards the intima as well as SMC proliferation. The transmembrane form serves as a scavenger receptor for oxLDL on macrophages and its expression is upregulated by IFN-γ, mediating foam cell formation[96, 97]. Recently, CXCL16 was shown to affect aortic SMC function in an IL-18 and IFN-γ dependent manner, providing additional mechanistic support for the role of IL-18 in plaque destabilisation[98, 99]. Moreover, Kita and co-workers have demonstrated that CXCL16 is a potent angiogenic factor[100], fuelling the notion that CXCL16 could be involved in plaque neovascularisation and destabilization, although conclusive histopathological and in vivo data are still lacking. While evidence is accumulating that CXCL16 mediates T-cell influx, a role in monocyte arrest and influx remains to be established, though macrophages express CXCL16. Nevertheless, CXCL16 may reduce plaque stability by promoting foam-cell formation and subsequent release of proteases, as well as by mediating T-lymphocyte influx and to a lesser extent engage in expansive plaque growth. Lundberg *et al.* recently showed that the A181V polymorphism of CXCL16 gene enhanced coronary artery stenosis[96]. This observation, although tempting, is not directly indicative of a role of CXCL16 in plaque vulnerability, since myocardial infarction is related to plaque instability rather than progressive stenosis. The fact that Sheikine *et al.* recently stated that CXCL16 might have an atheroprotective effect based on reduced plasma levels in UAP patients compared to controls, clarifies that more experiments on CXCL16 on atherogenesis, plaque stability and scavenging capacities are needed in

order to draw definitive conclusions[101].

Chemokine	Polymorphism	Risk
CCL2	MCP-1-2578G	↑ AMI
CCL5	RANTES-G-403A	↑ CAD
CCL11	CCL11-A23T	↑ AMI
CXCL16	CXCL16-A181V	↑ Coronary Artery Stenosis
CX3CL1	CX3CL1-V249I CX3CL1-T280M	↑ CAD ↓ CVD

Table 4 : Genetic polymorphisms**.**

Fractalkine/CX3CL1

The CX3C family only contains a single member, Fractalkine or CX3CL1. While only recently identified[102], a vast body of evidence is already available on its influence in atherothrombosis as recently reviewed[103]. Fractalkine binds to its unique seven transmembrane domain G-coupled protein receptor CX3CR1[104]. Unlike other chemokines, but similar to CXCL16, it is expressed as a membrane-bound protein, consisting of a 76-amino acid chemokine domain linked to a mucin-like stalk. It is highly expressed on the inflamed endothelium, where it can serve as an adhesion molecule mediating selectin- and integrin-independent arrest of CX3CR1 expressing cells, like T-cells, specific monocytes subsets and NK-cells[103]. Furthermore, tumour necrosis factor alpha converting enzyme (TACE or ADAM-17) activity will release soluble fractalkine, which in turn acts as a chemo-attractant of the aforementioned cell types[105]. Membrane bound CX3CL augments the chemotactic activity of other chemokines. Once migrated, $CX3CR1^{+}$ cells, among others, secrete IFN-γ, which enhances endothelial fractalkine expression, thereby forming a paracrine amplification loop[103]. Hence, both forms support crucial steps in atherogenesis, and it is not surprising that fractalkine deeply impacts the pathobiology of atherosclerosis. Indeed, $CX3CR1^{-/-}$ mice on a hyperlipidemic background developed less atherosclerosis at the aortic root and displayed a more stable plaque phenotype[106, 107]. Yet, fractalkine knock-out mice on an $ApoE^{-/-}$ as well as $LDLr^{-/-}$ background showed reduction in brachiocephalic artery plaque formation but not in that of the aortic root[108]. Greaves *et al.* showed that fractalkine is expressed in human macrophages of advanced atherosclerotic lesions, in particular at neovascularized sites[109]. Lucas *et al*. extended this observation by stating that fractalkine expression in the plaque colocalizes with $CX3CR1^{+}$ cells, most notably vSMCs. This observation was confirmed *in vitro* by CX3CR1-mediated vSMC chemotaxis, which pleads for a plaque stabilizing effect of fractalkine[110]. Genetic polymorphism screening of different cardiovascular disease (CVD) populations identified CX3CR1 haplotype polymorphisms V249I and T280M. Intriguingly, the T280M mutation, which translated in impaired fractalkine activity, was independently found to confer protection for CVD in the Offspring Cohort of the Framingham Heart Study[111], whereas the V249I polymorphism associated with an increased risk of CAD[112, 113]. In agreement, Niessner *et al.* showed that patients with CAD carrying the V249I but not the T280M allele were at elevated risk for acute coronary syndromes (ACS)[114]. During ACS, plasma fractalkine levels were higher in V249I carriers as well, an observation which was recently confirmed by Damas *et al.* showing increased fractalkine plasma levels in unstable CAD patients[115]. These data strongly suggest that the CX3CL1/CX3CR1 axis has important implications for plaque development and destabilization. A further sign of multifaceted activity is the fact that fractalkine can induce platelet activation and adhesion. Given its upregulation in atherosclerosis, fractalkine likely plays a dual role in atherothrombosis and subsequent ischemic symptoms[116].

MIF

Macrophage inhibitory factor (MIF) is a very pleiotropic inflammatory cytokine with a wide expression pattern[117]. While it shows strong structural resemblance with chemokines and also was found to display chemotactic activity this cytokine is categorized as a chemokine like factor[118]. Its presence and activity was shown in the onset of atherogenesis as well as in advanced lesions of hypercholesterolemic rabbits and humans[119, 120]. It is expressed abundantly on the endothelium, where it induces ICAM-1 expression and on SMCs and macrophages, where it is also up-regulated after stimulation with oxLDL[121]. Pan *et al.* showed that MIF deficiency considerably reduced atherogenesis in $LDLr^{-/-}$ mice[122]. Although no data are available on MIF serum levels in CVD, Schmeisser recently showed that advanced plaques express MIF, colocalizing mainly in areas of enhanced instability, such as increased mononuclear cell infiltrates and neovessels[123], Kong *et al.* suggested that this might be due to MIF regulated MMP-1 expression by vSMCs[124]. Burger-Kentischer *et al.* observed a decrease in i.e. ICAM-1, MMP-2 and CD40L expression in mice after treatment with an anti-MIF monoclonal antibody as well as a reduced intimal macrophage content, which however was only accompanied by a small reduction in plaque volume[125]. In contrast, Schober *et al.* did not find any significant differences in plaque area after MIF blockade in a restenosis model, however MIF inhibition rendered a more stable intimal phenotype with reduced foam cell and increased SMC content[126]. Although this result, obtained in a wire injury model, should be extrapolated with caution, a significant influence of the chemokine-like factor MIF in plaque destabilization appears plausible.

Other Chemokines

While there are around 50 chemokines, only a few additional chemokines have been implicated in atherothrombosis, however their exact role in plaque rupture and its sequelae still has to be determined. A brief description of recent findings is that MIP-3β (CCL19) and MCP4 (CCL13) were shown to be expressed in human plaques[127, 128]. Furthermore, pulmonary and activation regulated chemokine (PARC, CCL18) could be detected in human plaques, colocalizing with $CD68^{+}$ macrophages[127]. The related thymus-and activation-regulated chemokine (TARC, CCL17) and macrophage derived chemokine (MDC, CCL22) are also associated with macrophages in advanced plaques, especially at sites with increased neovascularization suggesting that these chemokines could be involved in monocyte recruitment or macrophage activity as well[109] . Also, TARC and MDC are able to induce platelet aggregation via their shared receptor CCR4, which could be inhibited by a monoclonal antibody against CCR4[14, 129]. However in patient cohorts, correlative data of PARC, TARC or MDC serum levels or genetic polymorphisms in CVD are still lacking, which renders their local or systemic role in atherothrombosis and plaque rupture unknown. Finally, one of the more exotic ligands of CXCR3 seems to be involved in atherogenesis as increased IFN-inducible T cell alpha chemoattractant (ITAC,CXCL11) serum levels have been demonstrated to correlate with the development of transplant coronary artery disease[130]. Histological and expression studies on chemokines in atheroma are of great importance. Accordingly, the contribution of a majority of functional chemokines in atherogenesis is still not fully understood, thereby warranting chemokine profiling studies with specific patient groups as well as experimental research in representative mouse models for atherosclerosis or coronary ischemia after pharmacological or genetic intervention in chemokine function. These studies will reveal whether and to what extent the described chemokines are involved in atherogenesis or injury repair after myocardial infarction, whether they are associated as a biomarker or as a specific culprit for plaque rupture and, if so, whether these chemokines are instrumental in disease progression and constitute relevant therapeutic targets for intervention.

Therapeutic options for plaque stabilization; current advances

Studies in mice genetically deficient for a single chemokine or receptor have not only

increased our understanding of their role in atherosclerosis, plaque stability and restenosis but also helped to identify new targets for future intervention. While some chemokines, such as MCP-1, Fractalkine and RANTES appear to aggravate the process, others are believed to have an atheroprotective effect. To complicate matters, the activity of some chemokines was shown to be stage dependent. Given their dedicated role in inflammation and high selectivity, chemokines are very interesting therapeutic targets for intervention in atherothrombosis, while their GPCRs are generally favourable drug targets. This notion prompted pharmaceutical companies to put considerable effort in the design of synthetic chemokine receptor antagonists for other inflammatory diseases like multiple sclerosis, HIV infection and rheumatoid arthritis. Various compounds already have entered clinical phase II and in fact CCR1 and CCR5 receptor antagonists already advanced to phase III trials for HIV/AIDS and rheumatoid arthritis[131]. Despite the apparent promise, to our knowledge, trials to validate the efficacy of chemokine receptor antagonists in atherothrombosis or related disorders of various chemokine candidates have not been initiated to date.

Illustratively, injection of Met-RANTES, a combined CCR1 and CCR5 antagonist, halted the progression of atherosclerosis in animal models[54], while our group has recently demonstrated attenuated lesion formation in vivo after treatment with TAK-779, originally designed as an HIV entry inhibitor and a CXCR3 and CCR5 antagonist for Th1 cells[56]. However, a plaque stabilising effect of these compounds in already existent atherosclerosis remains to be determined. Another technical drawback is that chemokine antagonists quite often lack species cross-reactivity mandatory for extrapolation from an experimental to a clinical setting[132]. Apart from synthetic antagonists, which may lack receptor specificity or display an unfavourable activity profile, another effective approach involves the use of viral chemokine antagonists and chemokine mutants. Its efficacy was recently demonstrated by Bursill and co-workers showing dramatically reduced lesion area, macrophage content and cell proliferation in a diet induced atherosclerosis model as well as in a vein graft atherosclerosis model after in vivo administration of the broad spectrum CC chemokine binding protein 35K[133, 134]. On the other hand, no differences in smooth muscle cell or collagen content were seen. Pyo *et al.* showed a reduction in wire induced neo-intimal hyperplasia in mice expressing M3, a herpesvirus protein that binds and inhibits all 4 chemokine subgroups[135]. However, M3 was tested in a mouse model of restenosis for its effect on intimal hyperplasia, a process which, unlike atherosclerosis, is critically dependent on vSMC proliferation. Both 35K and M3 act on a broad range of chemokines, thereby precluding the opportunity of selective chemokine inhibition and enhancing the chance of adverse side effects in humans.

Recently, our lab was able to reduce atherogenesis and induce a more stable plaque phenotype using a novel vaccination strategy against IL-12[136]. Although no such attempts have been undertaken for the blockade of specific chemokines, it may be very well suited for targeted inhibition of the chemokine system. In addition, focal delivery is very interesting, as chemokines are at the very heart of immuno-mobilisation and recruitment. Receptor antagonists are suited for site directed topical application at the aimed lesion site via for instance drug-eluting coronary stents, local viral transfer or gene silencing using siRNA[137]. Plausibly, systemic intervention in chemokine function, although desirable in the context of wide-spread atherosclerosis, could render considerable side effects. A second strategy to confer specificity is co-therapy: while most chemokines act in concert as heterodimers interacting with functionally coupled receptors, anti-dimerizing agents could yield another viable therapeutic possibility[138].

Interestingly, apart from their expected therapeutic targets, current cardiovascular drugs appear to modulate chemokine activity as well. For instance, it has become increasingly clear that inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase or statins exert antiinflammatory properties, independent of their lipid lowering effect. They are shown to decrease chemokine secretion by inhibition of the

geranyl geranylation of GTP-binding intracellular signalling proteins, including Rho, Ras and Rac[139]. By illustration, Pitavastatin (NK-104) was seen to suppress IL-8 production in human endothelial cells [80]. Still, although statin use has proven to promote a stable plaque phenotype, both in animal models and humans, the contribution of chemokine modulation by statins to this effect remains to be established[140]. However, statins are very pleiotropic and various mechanisms may underlie their plaque stabilizing effects, obscuring to the contribution of statin mediated chemokine inhibition to plaque stabilization. Other chemokine modulating drugs include aspirin, which was demonstrated to inhibit TNF-α induced MCP-1 and IL-8 expression and protein levels in endothelial cell as well as monocyte adhesion and transmigration in vitro[141]. Finally, the angiotensin AT-1 receptor antagonist irbesartan, apart of its anti-hypertensive effects, reduced lesion progression in part by inhibiting MCP-1 and Gro-α expression[142], fuelling the notion that angiotensin II is a mediator of vascular wall inflammation[143].

The main drawback for clinical trials on chemokine intervention in cardiovascular disorders is the lack of a reasonable endpoint. As for now, advanced imaging techniques seem most eligible to evaluate the effect of chronic chemokine inhibition in plaque stability. For the evaluation of chemokine therapy in ACS, while it could serve to stabilize inflammation, reduce platelet activity and induce plaque stability, a clear read-out in serum is favoured. Therefore, regardless of the potential of chemokine therapy, the search for a biomarker specifically correlating with lesion severity or plaque destabilization which could very well involve a certain chemokine, is ongoing and of utmost importance. Such a biomarker is applicable for the stratification of high risk patients as well as for the tailoring of athero-protective and regressive therapy.

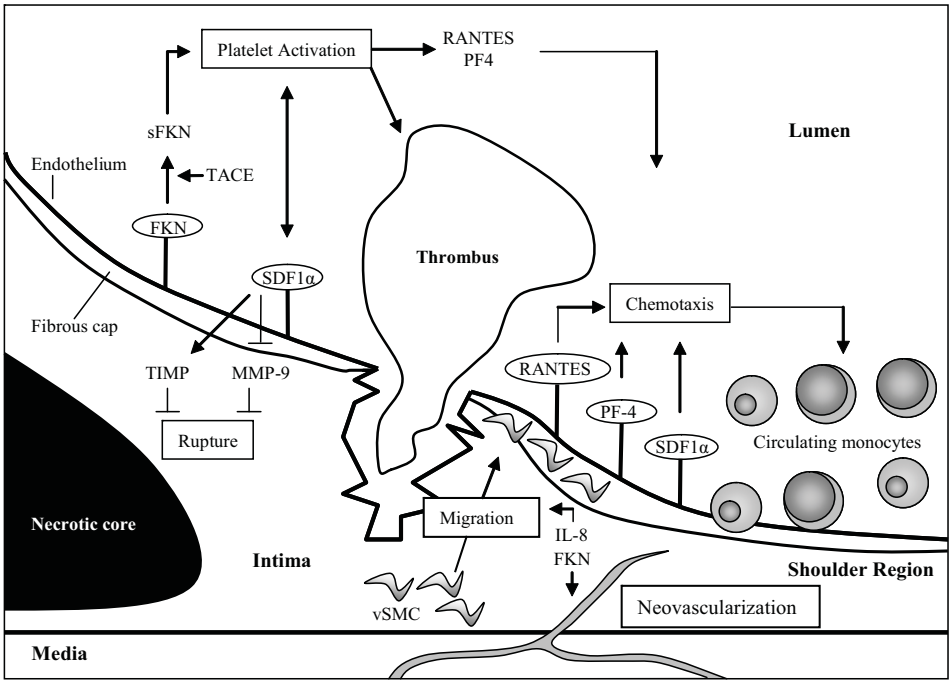

Figure 1: *Schematic overview of mechanisms in atherothrombosis in which chemokines are seen to participate. (s)FKN = (soluble) fractalkine*

Conclusion and future directions/perspectives

Over the last years, our understanding of the role of various chemokines to the patho-

genesis of atherosclerosis and plaque progression has increased tremendously. While the contribution of the whole chemokine system in immunology and atherosclerosis is very complex and overlapping, it is most likely that chemokines, either in concert of after heteromerization, may have their own distinctive role at specific stages of disease progression. This specificity therefore puts the chemokines and their receptors in the therapeutic spotlight. Experimental studies in animal models have already helped to identify a number of interesting targets for intervention in atherosclerotic plaque growth or thrombotic plaque rupture, and it is expected that this selection will rapidly expand in the near future. The fact that chemokine receptors are G-coupled protein receptors render them eligible for drug design, and indeed for many chemokine receptors potent and rather selective antagonists have been developed. Still, validation of their potential in anti-atherothrombotic therapy is hampered by the lack of a good surrogate marker for this disease and one of the reasons why short term clinical trials in humans currently remain cumbersome. Nevertheless, knockout models continue to be developed and dozens of chemokine receptor antagonists have already been tested in other immune disorders. In addition, taking the numerous ways of drug administration and delivery into account, science awaits an exciting increase in our understanding of this complex disease, enabling the development of specific chemokine-targeted atheroregressive therapies besides current lipid lowering strategies.

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