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Regulation of Atherogenesis by Chemokines and Chemokine Receptors

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Abstract

Atherosclerosis is a chronic inflammatory and metabolic disorder affecting large and medium-sized arteries, and the leading cause of mortality worldwide. The pathogenesis of atherosclerosis involves accumulation of lipids and leukocytes in the intima of blood vessel walls creating plaque. How leukocytes accumulate in plaque remains poorly understood, however chemokines acting at specific G protein-coupled receptors appear to be important. Studies using knockout mice suggest that chemokine receptor signaling may either promote or inhibit atherogenesis, depending on the receptor. These proof of concept studies have spurred efforts to develop drugs targeting the chemokine system in atherosclerosis, and several have shown beneficial effects in animal models. This article will review key discoveries in basic and translational research in this area.

Keywords

Atherosclerosis; Cardiology; Immunology; Inflammation; Antagonist

Introduction

Atherosclerosis is the pathologic process underlying most strokes and heart attacks, which together are now the leading cause of death worldwide (Roger et al. 2012). Risk factors for atherosclerosis include age, gender, a high ratio of low-density lipoprotein (LDL) to high-density lipoprotein (HDL) in the blood, hypertension, diabetes, obesity, smoking and inheritance (Berger et al. 2010). It is generally accepted that atherosclerosis is a chronic metabolic and inflammatory disease (Hansson GK 2005). The pathologic hallmark is the atherosclerotic plaque, composed of lipids, collagen, platelets, fibroblasts, smooth muscle cells (SMCs) and leukocytes. Rupture of unstable plaques may result in thrombosis and ischemia in surrounding tissues (Weber et al. 2008; Weber and Noels 2011).

Both innate and adaptive immunity appear to be involved in the development of plaque (Packard et al 2009). Innate immune cells, including macrophages, neutrophils, mast cells and platelets, accumulate early and express reactive oxygen species (ROS), proteinases,

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lipid mediators and various cytokines, leading to smooth muscle cell proliferation, angiogenesis and additional inflammatory cell activation. Adaptive immune cells, including T and B lymphocytes, accumulate later and may accelerate disease progression through complex and poorly understood mechanisms (Weber et al. 2008). In particular, in mouse models the B1 subset of B cells is atheroprotective, whereas the B2 subset is pro-atherogenic; Th1 cells are pro-atherogenic, and regulatory T cells (Tregs) are atheroprotective; whereas the roles of Th2 and Th17 cells are still controversial (Butcher and Galkina 2011; Kyaw et al. 2011; Robertson and Hansson 2006).

The classic mechanism of immune cell recruitment from the blood into tissues, including the blood vessel wall, is thought to involve sequential interactions between leukocytes and endothelial cells (ECs). These include selectin-mediated leukocyte rolling on endothelium, followed by leukocyte activation by chemokines, then integrin-mediated firm arrest of leukocytes to endothelium, culminating in leukocyte transendothelial migration (Ley et al. 2007). Chemokines are a large protein family of small (8–10 kDa) leukocyte chemoattractants that can be divided into four classes--C, CC, CXC and CX3C--according to the number and spacing of conserved cysteines in the *N*-terminal domain of the molecule (Murphy et al. 2000). In human, approximately 46 chemokines and 20 chemokine receptors have been identified (Murphy 2002). Since the discovery in 1998 that the chemokine Ccl2 and its receptor Ccr2 are positive regulators of both the apolipoprotein E-deficient ($ApoE^{-/-}$) and LDL receptor deficient ($Ldlr^{-/-}$) mouse models of atherosclerosis (Boring et al. 1998; Dawson et al. 1999; Gu et al. 1998), roles for many other chemokines and chemokine receptors have been identified using genetic and in some cases pharmacological criteria (Koenen and Weber 2011). In this review, we will discuss current concepts of the chemokine system as an immunoregulator and potential therapeutic target in atherosclerotic cardiovascular disease.

The Chemokine System as an Immunoregulator in Atherogenesis

Chemokines and chemokine receptors are able to regulate leukocyte trafficking in both homeostasis and inflammation (Murphy et al. 2000). Chemokine receptors identified in atherogenesis include inflammatory receptors (e.g. CCR2, CCR5, CXCR2 and CX3CR1), classical homeostatic receptors (e.g. CCR7) as well as receptors that have both inflammatory and homeostatic functions (e.g. CCR6) (Koenen and Weber 2011). Here we will discuss the chemokines and their receptors in three different categories: atherogenic, atheroprotective and controversial (Table 1).

1. Atherogenic chemokines/chemokine receptors

CCL2-CCR2 was the first chemokine/chemokine receptor pair to be implicated in the pathogenesis of atherosclerosis, and the one studied in the greatest detail. CCL2 (also known as Monocyte Chemotactic Protein [MCP]-1) is mainly produced by monocytes, macrophages, ECs and smooth muscle cells (SMCs), and has been identified in both mouse and human atherosclerotic lesions (Rayner et al. 2000; Nelken et al. 1991). In human, single nucleotide polymorphisms (SNP) in the promoter of CCL2, *CCL2-2518G*, and in the open reading frame of CCR2, *CCR2-V64I*, have been associated with increased risk of myocardial infarction (McDermott et al. 2005; Ortlepp et al. 2003; Szalai et al. 2001). *CCL2-2518G*

appears to be functional since individuals with this allele have significantly higher serum CCL2 levels (McDermott et al. 2005). The biochemical effect of *CCR2-V64I* has not been clearly defined. In mice, genetic deletion of either Ccl2 or Ccr2 in both the *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ models of atherosclerosis significantly reduced the size of lesions in the aorta (Boring et al. 1998; Dawson et al. 1999; Gu et al. 1998). In a third model, Ccl2 deficiency decreased atherosclerotic plaque burden in mice that overexpress human apolipoprotein B (Gosling et al. 1999). A pro-atherogenic role for Ccl2/Ccr2 was also demonstrated by blocking Ccr2 with 7ND, an *N*-terminally truncated mutant form of Ccl2, which attenuated the initiation and progression of atherosclerosis in *ApoE*$^{-/-}$ mice (Inoue et al. 2002; Ni W et al. 2001). Study of radiation chimeric mice has attributed the pro-atherogenic effect of Ccr2 to expression on hematopoietic cells. In particular, *Ccr2*$^{-/-}$ but not *Ccr2*$^{+/+}$ bone marrow transplantation into ApoE3-leiden mice reduced the size of atherosclerotic lesions; moreover, overexpression of Ccl2 on hematopoietic cells in *ApoE*$^{-/-}$ mice increased atherosclerotic lesion size (Aiello et al. 1999; Guo et al. 2003). However, blockade of Ccr2 by either 7ND treatment or *Ccr2*$^{-/-}$ bone marrow transplantation did not affect the progression of established atherosclerotic lesion development (de Waard V et al. 2010; Guo et al. 2005). Further studies in mice showed that Ccr2 deficiency abolished the egress of monocytes from the bone marrow and markedly reduced Ccl2-induced recruitment of monocytes from the blood into inflammatory sites (Boring et al 1997; Tacke et al. 2007; Tsou et al. 2007). Considering that monocytes from the bone marrow and the spleen are critical for both the initiation and progression of atherosclerosis (Robbins et al. 2012; Swirski et al. 2009; Weber et al. 2008) (Figure 1), this may partially explain why Ccl2 and Ccr2 deficiency inhibit the development of atherosclerosis.

CCL5-CCR5—The inflammatory chemokine CCL5 (also known as RANTES [Regulated upon Activation, Normal T cell Expressed and Secreted]) is expressed by monocytes, macrophages, T cells and SMCs in both mouse and human atherosclerotic lesions, and acts at the chemokine receptors CCR1, CCR3 and CCR5 (Krohn et al. 2007; Pattison et al. 1996). In human, the gain-of-function polymorphism *CCL5-G403A* affects basal CCL5 expression levels and is associated with a higher risk of coronary artery disease (Boger et al. 2005; Simeoni et al. 2004), and loss-of-function polymorphism *CCR5Δ32* may be associated with a lower risk of myocardial infarction (Gonzalez et al 2001; Simeoni et al. 2004). Although genetic deletion of *Ccr5* in *ApoE*$^{-/-}$ mice was not shown to reduce early spontaneous atherosclerosis in the initial study (Kuziel et al. 2003), later investigations suggested that it reduced both early and late-stage atherosclerosis development in *ApoE*$^{-/-}$ mice fed either a chow or high-fat diet (Braunersreuther et al. 2007; Quinones et al. 2007). Also, *Ldlr*$^{-/-}$ mice reconstituted with *Ccr5*$^{-/-}$ bone marrow showed reduced macrophage accumulation in atherosclerotic lesions and improved plaque stability (Potteaux et al. 2006). The mechanism of CCL5/CCR5 modulation of atherosclerosis has not been clearly defined. Possibilities include regulation of leukocyte trafficking to lesions and modulation of adaptive immunity. As indirect supportive evidence, CCR5 is important for the spreading and trans-endothelial migration of monocytes, neutrophils and Th1 cells, and the atheroprotective effects seen in *Ccr5*$^{-/-}$ mice may be associated with up-regulation of IL-10 and reduction of Th1-type immune responses (Braunersreuther et al. 2007; Drechsler et al. 2010; Potteaux et al. 2006; Weber et al. 2001) (Figure 1).

CCR6—Both CCR6 and its sole chemokine ligand CCL20 (also known as Macrophage Inflammatory Protein [MIP] 3α) are expressed at increased levels in human atherosclerotic plaques, and the circulating level of CCL20 is significantly increased in hypercholesterolemic subjects (Calvayrac et al. 2011; Yilmaz et al. 2007). In mice, Ccr6 and Ccl20 are both present constitutively in healthy aortas and in atherosclerotic plaques, and Ccr6 deletion in *ApoE*$^{-/-}$ mice fed a Western diet significantly reduced the atherosclerotic lesion size in both the whole aorta and the aortic root, accompanied by a reduction of macrophage content in the plaques (Wan et al. 2011a). Bone marrow transplantation suggested that the lesion reduction seen in *Ccr6*$^{-/-}$*ApoE*$^{-/-}$ mice is caused by Ccr6 expression on hematopoietic cells. CCL20 is produced by epithelial cells, ECs and SMCs, whereas CCR6 is mainly expressed by immature dendritic cells, B cells, T cells, neutrophils and monocytes (Schutyser et al. 2003; Wan et al. 2011a). Interestingly, Ccr6-deficient mice on the *ApoE* knockout background were relatively monocytopenic, whereas bone marrow monocyte content was slightly increased. Thus, modulation of atherosclerosis could be due to Ccr6 function on monocytes, mediating their egress from the bone marrow and their recruitment from the blood into the vessel wall (Koenen and Weber 2011; Wan et al. 2011a) (Figure 1). However, the effect of Ccr6 in atherogenesis may be cell type-specific since a second study suggested that Ccr6 might be involved in B cell-mediated atheroprotection (Doran et al. 2012). Because mice globally deficient in Ccr6 have reduced atherosclerosis, the effect of Ccr6 deficiency on non-B cells appears to be dominant over the effect of B cell Ccr6 deficiency (Wan et al. 2011b). Additional work will be necessary to further clarify the underlying mechanism.

CCL17—CCL17 (also known as Thymus and Activation-Regulated Chemokine [TARC]) is a dendritic cell-derived chemokine that induces chemotaxis of T cells through its receptor CCR4 (Imai et al. 1997a). CCL17 has been detected in both advanced mouse and human atherosclerotic lesions, and its expression is upregulated in human atherosclerotic plaques compared with healthy arteries (Greaves et al. 2004; Weber et al. 2011). Genetic targeting of Ccl17 in *ApoE*$^{-/-}$ mice resulted in reduced atherosclerotic lesion size, along with a decrease of macrophages and T cells in the plaques, which was confirmed by *Ccl17*$^{-/-}$ bone marrow transplantation and Cc17 specific antibody treatment (Weber et al. 2011). The beneficial effect may be dependent on Tregs that accumulate in the lymph nodes and aortas in the double knockout mice (Figure 1). Moreover, co-culture of CD4^{+} T cells with *Ccl17*$^{-/-}$ DCs resulted in less apoptosis and enhanced expansion of Tregs, suggesting that Ccl17 is a central regulator of Treg homeostasis (Weber et al. 2011). Taken together, this suggests that CCL17 expressed by dendritic cells may drive atherosclerosis development through inhibiting expansion of Tregs.

CX3CL1-CX3CR1—CX3CL1 (Fractalkine), the sole ligand for CX3CR1, is a unique chemokine that exists in both membrane-tethered and soluble shed forms, thus mediating both cell adhesion and chemotaxis (Bazan et al. 1997). CX3CL1 is primarily produced by ECs and CX3CR1 is expressed on monocytes, lymphocytes, platelets, and DCs (Barlic and Murphy 2007a; Imai et al. 1997b). In addition, both molecules have been identified on foam cells and SMCs in mouse and human atherosclerotic lesions, and CX3CR1 is upregulated on monocytes from coronary artery disease (CAD) patients (Apostolakis et al. 2007; Lucas et

al. 2003). In several retrospective cohort studies and the population-based prospective Framingham Heart Study Offspring Cohort, two SNPs of CX3CR1, *V249I* and *T280M*, were associated with a markedly reduced risk of CAD (Ghilardi et al. 2004; McDermott et al. 2001; McDermott et al. 2003; Moatti et al. 2001; Norata et al. 2006). However, two other studies showed that these polymorphisms were not associated with peripheral arterial disease (522 patients) and were associated with increased risk of restenosis after coronary stenting (365 patients) (Gugl et al. 2003; Niessner et al. 2005). The biochemical effects of these polymorphisms on CX3CR1 function are also in dispute, with two groups reporting that they affect function but one finding a gain of function and the other a loss of function (Daoudi et al. 2004; McDermott et al. 2003). In atherosclerosis mouse models ($ApoE^{-/-}$ mice and $Ldlr^{-/-}$ mice), genetic deletion of Cx3cl1 and Cx3cr1 significantly reduced the size of atherosclerotic lesions and inhibited the recruitment of macrophages/DCs into the vessel wall (Combadiere et al. 2003; Lesnik et al. 2003; Liu et al. 2008; Teupser et al. 2004). The role of Cx3cl1 and Cx3cr1 in atherogenesis appears to be independent from Ccl2, Ccr2 and Ccr5 since additive protection was observed in the *ApoE* knockout model in both $Cx3cl1^{-/-}$Ccr2$^{-/-}$ mice and $Ccl2^{-/-}Cx3cr1^{-/-}$ mice treated with Met-RANTES, a pharmacological inhibitor of Ccr5 (Combadiere et al. 2008; Saederup et al. 2008). The proposed atherogenic mechanisms include Cx3cr1-dependent migration/retention of macrophages in the vessel wall (Barlic and Murphy 2007b; Tacke et al. 2007); Cx3cr1-conferred monocyte/macrophage survival in atherosclerotic plaques (Landsman et al. 2009) and Cx3cr1-supported formation of platelet-monocyte complexes in hyperlipidemic mice (Postea et al. 2012) (Figure 1).

CXCL1-CXCR2—Both CXCR2 and its major ligands, CXCL1 (also known as Growth-related Oncogene [GRO] α) and CXCL8 (also known as Interleukin [IL]-8), have been identified in human atheromata, and mouse Cxcr2 and Cxcl1 have been found in plaque in mouse models of atherosclerosis (Apostolopoulos et al. 1996; Boisvert et al. 2000; Wang et al. 1996). Consistent with this, the plasma level of CXCL1 is significantly increased in patients with coronary artery disease (CAD) (Breland et al. 2008), and expression of CXCL8 and CXCR2 are strongly upregulated on ECs and monocytes after oxidized LDL (oxLDL) stimulation (Lei et al. 2002; Yeh et al. 2001). A pro-atherogenic role for Cxcr2 was suggested by studies in which $Cxcr2^{-/-}$ bone marrow was transplanted into irradiated $Ldlr^{-/-}$ mice, resulting in much smaller lesions with fewer lesional macrophages compared to controls (Boisvert et al. 1998). Also, genetic deletion of *Cxcl1* significantly reduced atherosclerotic lesion size in both the whole aorta and the aortic root of $Ldlr^{-/-}$ mice (Boisvert et al. 2006). CXCL1 is mainly produced by macrophages, neutrophils and epithelial cells, whereas CXCR2 is found most prominently on neutrophils, with some expression on monocytes and mast cells (Murphy et al. 2000). The pro-atherogenic effect of the CXCL1-CXCR2 axis may be due to CXCL1-triggered monocyte arrest on early atherosclerotic endothelium and CXCR2-mediated macrophage accumulation in established lesions (Boisvert et al. 2006; Huo et al. 2001; Papadopoulou et al. 2008). In humans, CXCL8 levels were significantly increased in the setting of acute myocardial infarction (Neumann et al. 1995). Recent studies have suggested that neutrophils are also pro-atherogenic, and thus CXCR2 might be involved in the recruitment of neutrophils to the

vessel wall during the initiation of atherosclerotic plaque formation (Drechsler et al. 2011) (Figure 1).

CXCL10-CXCR3—CXCR3 and its ligand CXCL10 (also known as IFNγ-induced Protein of 10 kDa or IP-10) are highly expressed in lesional T cells (Th1 cells) as well as ECs, SMCs and macrophages in human atheromata (Mach et al. 1999). The percentage of $CXCR3^+$ lymphocytes has been reported to be significantly increased in the blood of CAD patients (Fernandes et al. 2004) and the plasma concentration of CXCL10 was also much higher in CAD patients compared to controls (Kawamura et al. 2003). In $ApoE^{-/-}$ mice, both Cxcr3 and Cxcl10 deficiency resulted in a reduction of atherosclerotic lesion formation, accompanied by a decrease of $CD4^+$ T cell accumulation in the plaques (Heller et al. 2006; Veillard et al. 2005). This was associated with an elevated number of regulatory T cells and increased expression of the anti-inflammatory cytokine IL-10 within lesions, suggesting that the CXCL10-CXCR3 axis may promote atherogenesis by regulating the recruitment and balance of effector T cells and Tregs (Heller et al. 2006) (Figure 1). The role of Cxcr3 in atherogenesis is not redundant since $Cxcr3^{-/-}Ccr2^{-/-}ApoE^{-/-}$ triple knockout mice show a further decrease of lesion formation compared with deletion of either Cxcr3 or Ccr2 alone in $ApoE^{-/-}$ mice (Veillard et al. 2005). Cxcl10 deficiency may also be harmful to the cardiovascular system in the $ApoE^{-/-}$ mouse model, since in addition to reduced atherosclerosis, increased aneurysm formation was identified in these mice (King et al. 2009).

CXCR6—CXCR6 is expressed on monocytes, macrophages, T cells and SMCs in both mouse and human atherosclerotic lesions (Wuttge et al. 2004). Genetic deletion of Cxcr6 in $ApoE^{-/-}$ mice significantly decreased the size of atherosclerotic plaques. This was accompanied by a reduced percentage of macrophages and $Cxcr6^+$ T cells within the aortas (Galkina et al. 2007) (Figure 1). The production of IFNγ within the aortas of $Cxcr6^{-/-}ApoE^{-/-}$ mice was also diminished, consistent with the finding that $Cxcr6^+$ T cells express high amounts of IFNγ upon activation (Calabresi et al. 2002). These results suggested that Cxcr6 might accelerate the progress of atherosclerosis by regulating the migration of T cells into the aortic wall thus influencing the accumulation of macrophages indirectly. The ligand for CXCR6 is CXCL16 and its effect in atherogenesis will be discussed in the following section since an opposite phenotype has been suggested (Aslanian and Charo 2006).

2. Atheroprotective chemokines and chemokine receptors

CCR1—CCR1 is a chemokine receptor for many pro-inflammatory CC chemokines, including CCL3 and CCL5, both of which have been identified in atherosclerotic plaques; it is mainly expressed on monocytes, macrophages and T cells in humans, but important functional roles have been identified for Ccr1 on mouse neutrophils (Murphy et al. 2000; Wilcox et al. 1994). In contrast to the proatherogenic role of Ccl5, $Ccr1^{-/-}$ bone marrow reconstitution of $Ldlr^{-/-}$ mice resulted in markedly increased atherosclerotic lesion size in both the thoracic aorta and the aortic root (Potteaux et al. 2005). Similarly, $Ccr1^{-/-}ApoE^{-/-}$ mice showed increased atherosclerotic lesion formation compared with control mice and there is a significant increase of $CD3^+$ T cells and IFNγ production in the plaques, but the

lesional macrophage content was not affected (Braunersreuther et al. 2007). Also, it has been reported that although both CCR1 and CCR5 support trans-endothelial chemotaxis toward CCL5, only CCR1 mediates CCL5-induced arrest of monocytes and Th1 cells on activated endothelium (Weber et al. 2001) (Figure 1). These results suggest that CCR1 plays a protective role in atherogenesis, possibly by regulating T cell activation and the trafficking of monocytes and T cells into the vessel wall.

CXCR4—CXCR4 is a receptor only for the chemokine CXCL12 (also known as Stromal cell-Derived Factor [SDF]-1α), which has been identified in ECs and SMCs of human atherosclerotic plaques (Abi-Younes et al. 2000). It is well known that the CXCL12-CXCR4 axis plays an important role in hematopoietic stem cell mobilization, organ development and angiogenesis, and recent data suggest that it may also be involved in atherosclerosis (Zernecke and Weber 2010). In particular, plasma levels of CXCL12 in CAD patients were significantly reduced compared to those in healthy controls (Damas et al. 2002) and aged *ApoE*$^{-/-}$ mice had much lower serum and bone marrow levels of Cxcl12 (Xu et al. 2011), indicating that the CXCL12-CXCR4 axis may exert a protective effect in atherogenesis. Consistent with this, chimeric *ApoE*$^{-/-}$ mice transplanted with *Cxcr4*$^{-/-}$ bone marrow showed a marked increase of atherosclerotic lesions in both the whole aorta and the aortic root (Zernecke et al. 2008). This effect was confirmed by either blocking Cxcr4 through long-term administration of a specific pharmacologic antagonist, AMD3465, in *ApoE*$^{-/-}$ mice or by repopulating *Ldlr*$^{-/-}$ mice with bone marrow that had been transduced by a recombinant lentivirus encoding a Cxcr4-specific ‘degrakine’, which traps Cxcr4 in the endoplasmic reticulum (Zernecke et al. 2008). Blocking Cxcr4 with AMD3465 in *ApoE*$^{-/-}$ mice caused a pronounced leukocytosis and an expansion of circulating neutrophils, and correspondingly the recruitment of neutrophils into the plaques was significantly increased. There was also a significant reduction of SMCs and $CD3^+$ T cells in aortic root plaques, implying that the plaques become more vulnerable (Zernecke et al. 2008). Taken together, these results suggest that the CXCL12-CXCR4 axis may play a protective role in atherogenesis possibly by controlling homeostasis of neutrophils and their recruitment to atherosclerotic lesions (Figure 1).

3. Controversial chemokines/chemokine receptors in atherogenesis

CXCL16—CXCL16 (originally named SR-PSOX [Scavenger Receptor for Phosphatidylserine and Oxidized lipoprotein]) is the only other chemokine besides CX3CL1 that exists in both membrane-tethered and soluble shed forms, and it is expressed by macrophages, SMCs and T cells in mouse, rabbit and human atherosclerotic plaques (Hofnagel et al. 2011; Minami et al. 2001a; Minami et al. 2001b; Wuttge et al. 2004). The role of CXCL16 in human atherosclerosis is controversial. One study showed that patients with stable angina pectoris and myocardial infarction have significantly lower plasma levels of CXCL16 (Sheikine et al. 2006), whereas several other groups found that patients with angina, stroke or acute coronary syndromes have elevated plasma CXCL16 levels compared with healthy controls (Lehrke et al. 2007; Smith et al. 2008; Sun et al. 2008; Ueland et al. 2012;Wang et al. 2010; Yi and Zeng, 2008). In candidate gene analysis, two SNPs within the *CXCL16* gene, rs3744700 and *CXCL16-A181V*, were reported to be independently associated with CAD development and the severity of coronary stenosis (Huang et al. 2010;

Lundberg et al. 2005). In *Ldlr*$^{-/-}$ mice, genetic deletion of Cxcl16 significantly aggravated atherosclerosis and enhanced macrophage recruitment into the plaques (Aslanian and Charo 2006). However, this is in contrast to the deletion of its receptor Cxcr6 in *ApoE*$^{-/-}$ mice, which resulted in reduced atherosclerosis (Galkina et al. 2007). Conceptually, an atheroprotective role is plausible since CXCL16 is known to function not just as a chemokine but also as a scavenger receptor for the pro-atherogenic factors phosphatidylserine and oxLDL (Fukumoto et al. 2004; Minami et al. 2001a) (Figure 1). Consistent with this, CXCL16 promoted the internalization of oxLDL in human macrophages and *Cxcl16*$^{-/-}$ mouse macrophages have a significant reduction in the capacity to bind and internalize OxLDL (Aslanian and Charo 2006; Barlic et al. 2009). However, a recent study showed that the plasma level of Cxcl16 was much higher in *ApoE*$^{-/-}$ mice receiving a high-fat diet (Yi et al. 2011). Moreover, overexpression of Cxcl16 in *ApoE*$^{-/-}$ mice did not affect the size of existing atherosclerotic lesions but instead promoted their evolution to vulnerable plaques, suggesting that CXCL16 may be an atherogenic marker in plasma (Yi et al. 2011). Considering the limited studies in atherosclerosis mouse models and the contradictory findings in human CAD patients, more work will be needed to clarify the role of CXCL16 in atherogenesis.

CCR7—CCR7 and its ligands CCL19, CCL21 have been identified in both mouse and human atherosclerotic lesions, especially in macrophages and T-cell rich areas (Damas et al. 2007). In humans, the plasma levels of CCL19 and CCL21 were found to be increased in patients with stable and unstable angina (Damas et al. 2007). In *Ldlr*$^{-/-}$ mice, genetic deletion of Ccr7 attenuated the size of atherosclerotic plaques and reduced the macrophage accumulation in those plaques (Luchtefeld et al. 2010). In contrast, Ccr7 deficiency did not affect the size of atherosclerotic plaques or the plaque macrophage content in *ApoE*$^{-/-}$ mice (Feig et al. 2010; Potteaux et al. 2011). In an atherosclerosis regression mouse model in which a segment of atherosclerotic *ApoE*$^{-/-}$ mouse aortic arch was transplanted into wild type mice, it was found that Ccr7 was upregulated in foam cells during the regression of atherosclerotic lesions, accompanied by a significant reduction of macrophage content in the lesions (Trogan et al. 2006). Plaque regression and foam cell content reduction were both greatly inhibited by the blockade of Ccl19 and Ccl21 with specific antibodies, suggesting that Ccr7 may drive the egress of macrophages from the lesions and have a protective role in atherosclerosis development (Trogan et al. 2006) (Figure 1). Consistent with this, atorvastatin and rosuvastatin treatment significantly increased the expression of Ccr7 in lesional macrophages and enhanced the emigration of macrophages from the plaques in *ApoE*$^{-/-}$ mice, while macrophages from *Ccr7*$^{-/-}$*ApoE*$^{-/-}$ mice failed to emigrate upon statin treatment (Feig et al. 2011a). Also, it was found that liver-X-receptor (LXR) agonist- and HDL-induced atherosclerotic lesion regression in different atherogenic mouse models is associated with increased expression of Ccr7 on plaque macrophages (Feig et al. 2010; Feig et al. 2011b; Verschuren et al. 2009). However, in a non-surgical model it was found that Ccr7 had no effect on atherosclerotic plaque regression in *ApoE*$^{-/-}$ mice, and the loss of plaque macrophages during disease regression was attributed to suppressed monocyte recruitment (Potteaux et al. 2011). In fact, although expression of CCR7 is increased in atherosclerotic plaques, its expression on circulating T cells was significantly decreased in angina patients, indicating that CCR7 may also be involved in infiltration and egress of T

cells from the atherosclerotic vessel wall (Damas et al. 2007) (Figure 1). Clearly, more studies are necessary to dissect the exact role of CCR7 in the development of atherosclerosis.

Chemokine/Chemokine Receptor Antagonists in Atherogenesis

Considering the above evidence from animal models that chemokines and chemokine receptors may modulate atherogenesis (Figure 1), it is reasonable to consider them as potential drug targets for the treatment of cardiovascular disease. In the United States more than 30% of drugs on the market target G protein-coupled receptors and numerous antagonists of chemokine receptors have been developed (Horuk 2009). Here we will focus on the antagonists that have been tested in atherosclerosis (Table 2). The difficulty in demonstrating efficacy for these agents is the endpoint that must be considered: clinical events or inflammation of the blood vessel wall. The former is difficult to assess in a short trial, and the latter is difficult to quantitate with precision.

Drugs targeting CCR2 and its ligand CCL2 have been the most extensively evaluated in preclinical and clinical studies. In a phase II clinical trial of patients at risk for atherosclerotic cardiovascular disease, **MLN1202** (Millennium), a specific humanized monoclonal antibody directed against CCR2, was found to significantly reduce the serum levels of C-reactive protein, a surrogate marker of inflammation in cardiovascular disease (Gilbert et al. 2011). **CCX140** (Chemocentryx) is a small molecule inhibitor of CCR2 that has successfully completed a phase II clinical trial in type 2 diabetics, but has not yet been tested in cardiovascular disease (Koenen and Weber 2011). The CCR2 small molecule antagonists **INCB-3344** and **GSK1344386B** (Glaxo-Smith-Kline) did not affect the size of atherosclerotic lesions in *ApoE*$^{-/-}$ mice, even though they markedly reduced the number of circulating inflammatory monocytes and macrophage content in plaques (Aiello et al. 2010; Olzinski et al. 2010). As for CCL2 antagonists, **11K2** (an inhibitory antibody against CCL2 and CCL12) (Biogen Idec Inc) treatment of *ApoE*$^{-/-}$ mice significantly reduced both atherosclerotic plaque formation and lesional macrophage content (Lutgens et al. 2005). Treatment of *ApoE*$^{-/-}$ mice with **PA508**, a modified CCL2 form with increased glycosaminoglycan binding activity but reduced affinity for CCR2, markedly reduced neointimal plaque formation after arterial injury, due to reduced inflammatory monocyte recruitment to the lesions (Liehn et al. 2010). A humanized monoclonal antibody against CCL2, **ABN-912 (Novartis)**, has been tested in patients with rheumatoid arthritis, but the treatment did not show any benefit and whether it may affect atherosclerosis still remains unknown (Haringman et al. 2006).

CCR5 and its ligand CCL5 are another chemokine/chemokine receptor pair that has been targeted in atherogenesis. **TAK-779** is a small molecule antagonist for both CCR5 and CXCR3 that was originally developed as an HIV entry inhibitor (Baba et al. 1999; Gao et al. 2003). TAK-779 treatment of *Ldlr*$^{-/-}$ mice significantly reduced atherosclerotic plaque formation and Th1 cell infiltration into the lesions (van Wanrooij et al. 2005). **Maraviroc (Pfizer)** is a small molecule CCR5 antagonist approved by the FDA for HIV treatment and **HGS004/HGS101** (antibodies raised against CCR5) (Human Genome Sciences) has also been used to treat HIV in clinical trials (Latinovic et al. 2011), but whether they can affect

atherosclerosis is still unclear. Treatment of *Ldlr*$^{-/-}$ mice with Met-RANTES, an N-methionylated variant of CCL5 with potent CCR5 antagonist activity (Proudfoot et al. 1996), reduced the size of atherosclerotic lesions in both the whole aorta and the aortic root, accompanied by increased plaque stability (Veillard et al. 2004). Met-RANTES treatment of *ApoE*$^{-/-}$*Ccl2*$^{-/-}$*Cx3cr1*$^{-/-}$ triple knockout mice induced a further decrease of atherosclerotic plaque size, suggesting that it might also be considered alone or in combination with other chemokine receptor antagonists to treat atherosclerosis (Combadiere et al. 2008). Another antagonist for CCL5 is [**^{44}AANA^{47}]-CCL5**, a CCL5 variant with specific mutations in the principal RANTES/GAG binding site, that was shown to prevent the progression of established atherosclerotic lesions in *Ldlr*$^{-/-}$ mice (Braunersreuther et al. 2008). **MKEY** (Carolus Therapeutics Inc) is a synthetic peptide designed to disrupt the heteromerization of CCL5 and CXCL4, and administration of MKEY into *ApoE*$^{-/-}$ mice markedly reduced the formation of atherosclerotic lesions as well as macrophage accumulation in plaque (Koenen et al. 2009).

Antagonists against CXCR2, CXCR3 and CXCR4 have also been developed and tested in atherosclerosis models. **SB-517785-M** (Glaxo-Smith-Kline) is an antagonist that selectively inhibits CXCR2. This agent significantly reduced angiotensin II-induced infiltration of mononuclear cells and neutrophils into rat arterioles, suggesting that it may inhibit atherogenesis by controlling recruitment of neutrophils into the vessel wall (Nabah et al. 2007; Drechsler et al. 2011). Treatment of *Ldlr*$^{-/-}$ mice with **NBI-74330**, a CXCR3 antagonist, markedly increased the content of Tregs in atherosclerotic plaques but reduced effector T cells in lymph nodes draining the aortic arch, thus attenuating atherosclerotic lesion formation in these mice (van Wanrooij et al. 2008). As discussed in the previous section, CXCR4 blockade by its antagonist **AMD3465** led to aggravated atherosclerotic lesion formation in *ApoE*$^{-/-}$ mice, probably due to increased neutrophil expansion in the blood and their recruitment into plaque (Zernecke et al. 2008). **AMD3100** (Plerixafor) (Genzyme) is a CXCR4 antagonist that has been approved by the FDA for stem cell mobilization for autologous transplantation in the setting of ablative chemotherapy for multiple myeloma and non-Hodgkin's lymphoma, but its role in atherogenesis is still unknown (Keating 2011).

Human CX3CL1 has been modified at the N-terminus to produce a CX3CR1 antagonist named **F1** (Dorgham et al. 2009). F1 did not induce signaling through CX3CR1 but potently inhibited CX3CL1-induced chemotaxis, calcium flux and cell adhesion. In a peritonitis mouse model F1 was found to strongly inhibit macrophage accumulation, indicating that it has anti-inflammatory activity (Dorgham et al. 2009). However, it is still not clear whether this antagonist may affect the progress of atherosclerosis.

There are also some naturally occurring chemokine blocking agents that target more than one chemokine. For example, Evasins are a group of multipotential chemokine binding proteins found in tick saliva. Evasin-1 binds to CCL3, CCL4, and CCL18; Evasin-3 binds to CXCL1 and CXCL8; and Evasin-4 binds to CCL5 and CCL11 (Deruaz et al. 2008). A single administration of **Evasin-3** significantly reduced post-ischemic infarct size during myocardial ischemia in C57BL/6 mice, associated with reduced infiltration of neutrophils into the site of injury and decreased production of reactive oxygen species (Montecucco et

al. 2010). These results suggest that Evasins have anti-inflammatory functions that may be useful for the treatment of atherosclerosis. **M-T7** is a 37-kDa glycoprotein that also binds to a broad range of C, CC and CXC chemokines through the conserved C-terminal GAG binding domain of the chemokines (Lalani et al. 1997). Administration of purified M-T7 into rats caused a significant reduction of intimal hyperplasia after angioplasty injury (Liu et al. 2000) and inhibited aortic allograft vasculopathy associated with reduced inflammatory cell invasion (Liu et al. 2004; Dai et al. 2010), indicating that it may prevent recurrent atherosclerotic plaque growth. **NR58-3.14.3** is a broad-spectrum chemokine-blocking peptide that effectively inhibits the activities of CCL2, CCL3, CXCL8 and CXCL12, and treatment of *ApoE*$^{-/-}$ mice with this peptide significantly reduced macrophage accumulation in vascular lesions and increased the content of collagen and smooth muscle cells, although the vascular lipid lesion area was not changed (Reckless et al. 2005). Also, tail-vein injection of a recombinant adenovirus encoding soluble protein **35K**, a broad-spectrum CC-chemokine blocking agent encoded by Vaccinia virus, into *ApoE*$^{-/-}$ mice markedly reduced atherosclerotic plaque formation and atherosclerosis in carotid-caval vein grafts, accompanied by decreased macrophage recruitment into the lesions (Ali et al. 2005; Bursill et al. 2004). These results have suggested that broad-spectrum chemokine-blocking peptides might be useful to inhibit the progress of atherosclerosis and promote the stabilization of atherosclerotic plaques.

Recent studies suggest that statins (HMG-CoA reductase inhibitors), a class of drugs used for the primary and secondary prevention of coronary artery disease, may also affect the expression of chemokines and chemokine receptors during atherosclerosis (Feig et al. 2011a; Wahre et al. 2003). Statins are known to lower cholesterol levels in hypercholesterolemic patients and it has been shown that normalization of plasma lipid levels may cause atherosclerosis regression in both humans and atherosclerosis-prone mouse models (Nissen et al. 2006; Trogan et al. 2006). The regression of atherosclerosis is accompanied by reduced plaque inflammatory cell content (e.g. macrophages), which may be due to increased efflux of leukocytes from plauqes (Trogan et al. 2006) and decreased influx of inflammatory cells into plaques (Potteaux et al. 2011). The increased efflux and reduced influx of leukocytes are associated with increased expression of Ccr7 and reduced expression of Ccl2, respectively (Lieu et al. 2003; Trogan et al. 2006). In support of this, statin treatment significantly increased the expression of Ccr7 in lesional macrophages and enhanced their emigration from the plaques of *ApoE*$^{-/-}$ mice (Feig et al. 2011a). *In vitro*, statin treatment significantly inhibited the expression of CCL2 in PBMC, ECs and monocytes/macrophages (Jougasaki et al. 2010; Morikawa et al. 2002; Romano et al. 2000; Veillard et al. 2006). *In vivo*, simvastatin treatment reduced expression of CCL2 and CCR2 on circulating monocytes in both hypercholesterolemic patients and healthy people (Han et al. 2005; Rezaie-Majd A et al. 2002); both atorvastatin and simvastatin significantly inhibited the expression of CCR2 and CX3CR1 on PBMCs in CAD patients (Damas et al. 2005; Waehre et al. 2003). In addition, statin treatment has been reported to down-regulate the expression of CCR1, CCL3, CCL4, CCL19, CCL21, CXCL8, CXCL16 and CX3CL1 in different studies (Damas et al. 2005; Damas et al. 2007; Smith et al. 2008; Waehre et al. 2003), suggesting that statins may have a broad effect on the expression of atherogenic chemokines during the reversal of hypercholesterolemia in cardiovascular patients.

However, not all patients can benefit from using statins and as many as 20% of the patients who use statins develop adverse effects such as muscle fatigue and weakness and impaired cognition (Maningat et al. 2011). New drugs are needed to treat those statin-intolerant patients and chemokine and chemokine receptor antagonists are worth considering in this setting, either as prevention or in combination with statins in treatment of atherosclerosis.

Conclusions

Atherosclerosis is the leading cause of mortality worldwide. Although many drugs in several classes are approved for the treatment of cardiovascular disease, so far only statins target atherosclerosis by lowering the cholesterol level. However, not all patients benefit from statins, and new therapeutics are needed. In the past decade, numerous studies in mice and humans have suggested that chemokines and their corresponding G protein-coupled chemokine receptors play important roles in the pathophysiology of atherosclerosis development. For example, CCL2-CCR2, CCL5-CCR5 and CX3CL1-CX3CR1 are found to be pathogenic whereas CCR1 and CXCR4 are protective, indicating that antagonists for CCR2, CCR5, CX3CR1 and agonists for CCR1 and CXCR4 may be beneficial for therapeutic intervention. However, although more than 30% of the drugs on the U.S. market are targeted against G protein-coupled receptors and many chemokine receptor antagonists have been tested in different mouse models and clinical trials, only two antagonists against chemokine receptors have been approved by the FDA: the CCR5 antagonist Maraviroc for HIV treatment and the CXCR4 antagonist AMD3100 (Plerixafor) for multiple myeloma and non-Hodgkin’s lymphoma. Despite substantial progress in identifying chemokine system targets and developing lead compounds, much more research will be necessary to define the cellular mechanisms by which they operate as well as to evaluate current drug candidates and identify new ones. Also, it would be necessary to evaluate the effect of these new drugs on the immune system and antimicrobial host defense during long-term use.

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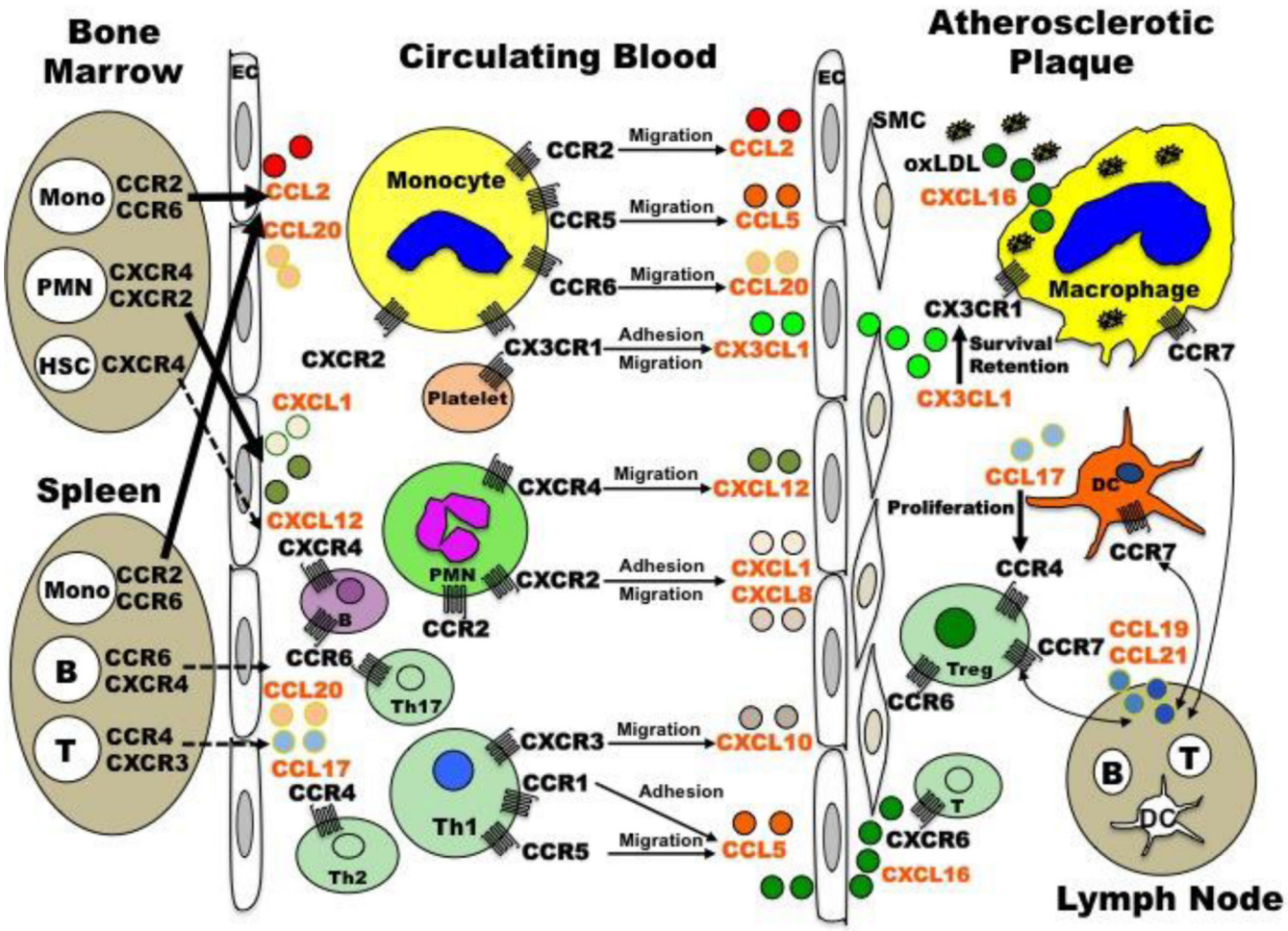

Figure 1. Schematic representation of chemokines/chemokine receptors and their target cells involved in the progress of atherosclerosis

CCL2-CCR2 and **CCL20-CCR6** induce the egress of monocytes from the bone marrow and the spleen into the circulating blood (arrows), and then together with **CCL5-CCR5** and **CX3CL1-CX3CR1** they mediate the arrest of monocytes on the endothelium and their migration into the atherosclerotic plaque. In the plaque, monocytes differentiate into macrophages (foam cells) after digestion of oxLDL through **CXCL16** and other scavenger receptors. **CX3CL1-CX3CR1** is also important for the survival/retention of macrophages in the plaque and the formation of monocyte-platelet complex during atherogenesis. The egress of neutrophils from the bone marrow and their recruitment into the vessel wall are mediated by **CXCL1/8-CXCR2** and **CXCL12-CXCR4**. The adhesion and migration of Th1 cells are controlled by **CXCL10-CXCR3** and **CCL5-CCR1/5**, while the recruitment of Th2 and Th17 cells relies on **CCR4** and **CCL20-CCR6** respectively. The proliferation of Tregs in the plaque is controlled by **CCR4** and its ligand **CCL17**, which is secreted by DCs. The recruitment of HSCs, B cells and T cells may depend on their specific chemokine receptors (dashed arrow). Macrophages, DCs and Tregs all express **CCR7** and during atherosclerosis regression these cells may egress into the lymph node by signaling through **CCL19** and **CCL21**.*Abbreviations*: DCs, dendritic cells; EC, endothelial cell; HSCs, hematopoietic stem

cells; Mono, monocytes; oxLDL, oxidized low density lipoprotein; PMN, polymorphonuclear leukocytes (neutrophils); SMC, smooth muscle cell; Tregs, T regulatory cells.

Table 1

Different effects of chemokines/chemokine receptors in atherogenesis

Effect in Atherosclerosis	Chemokines / Chemokine Receptors	References
Pathogenic	CCL2-CCR2	Boring et al. 1998; Dawson et al. 1999; Gosling et al. 1999; Gu et al. 1998; Guo et al. 2003
	CCL5-CCR5	Braunersreuther et al. 2007; Potteaux et al. 2006; Quinones et al. 2007
	CCR6	Doran et al. 2012; Wan et al. 2011
	CCL17	Weber et al. 2011
	CX3CL1-CX3CR1	Combadiere et al. 2003; Lesnik et al. 2003; Liu et al. 2008; Teupser et al. 2004
	CXCL1-CXCR2	Boisvert et al. 1998; Boisvert et al. 2006; Huo et al. 2001
	CXCL10-CXCR3	Veillard et al. 2005; Heller et al. 2006
	CXCR6	Galkina et al. 2007
Protective	CCR1	Braunersreuther et al. 2007; Potteaux et al. 2005
	CXCR4	Zernecke et al. 2008
Controversial	CXCL16	Aslanian and Charo 2006; Yi et al. 2011
	CCR7	Trogan et al. 2006; Luchtefeld et al. 2010; Potteaux et al. 2011; Feig et al. 2011

ytes (neutrophils); SMC, smooth muscle cell; Tregs, T regulatory cells.

Table 2

Antagonists to inhibit chemokines/chemokine receptors in atherogenesis

Chemokines / Chemokine Receptors	Antagonists	Atherosclerotic lesion change after treatment	References
CCR2	GSK1344386B	No reduction in mice	Olzinski et al. 2010
	INCB-3344	No reduction in mice	Aiello et al. 2010
	MLN1202	Reduced plasma CRP levels in patients	Gilbert et al. 2011
CCL2	11K2	Reduced in mice	Lutgens et al. 2005
	PA508	Reduced in mice	Liehn et al. 2010
CCR5/CXCR3	TAK-779	Reduced in mice	van Wanrooij et al. 2005
CCL5	Met-RANTES	Reduced in mice	Combadiere et al. 2008; Veillard et al. 2004
	[^{44}AANA^{47}]-CCL5	Reduced in mice	Braunersreuther et al. 2008
CCL5/CXCL4	MKEY	Reduced in mice	Koenen et al. 2009
CXCR2	SB-5177785-M	Reduced arteriolar leukocyte recruitment in rats	Nabah et al. 2007
CXCR3	NBI-74330	Reduced in mice	van Wanrooij et al. 2008
CXCR4	AMD-3465	Increased in mice	Zernecke et al. 2008
Chemokines	Evasin-3	Reduced post-ischemic infarct size in mice	Montecucco et al. 2010
	M-T7	Reduced intimal hyperplasia and aortic allograft vasculopathyin rats and mice	Liu et al. 2000; Liu et al. 2004; Dai et al. 2010
	NR58-3.14.3	No reduction in mice	Reckless et al. 2005
	35K	Reduced in mice	Ali et al. 2005; Bursill et al. 2004