Inflammatory cell recruitment in cardiovascular disease: murine models and potential clinical applications

Eileen McNEILL*, Keith M. CHANNON* and David R. GREAVES†

*Department of Cardiovascular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, U.K., and †Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, U.K.

ABSTRACT

Atherosclerosis is the pathological process that underlies the development of cardiovascular disease, a leading cause of mortality. Atherosclerotic plaque formation is driven by the recruitment of inflammatory monocytes into the artery wall, their differentiation into macrophages and the subsequent transformation of macrophages into cholesterol-laden foam cells. Models of hypercholesterolaemia such as the ApoE (apolipoprotein E)−/− mouse and the application of transgenic technologies have allowed us to undertake a thorough dissection of the cellular and molecular biology of the atherosclerotic disease process. Murine models have emphasized the central role of inflammation in atherogenesis and have been instrumental in the identification of adhesion molecules that support monocyte recruitment, scavenger receptors that facilitate cholesterol uptake by macrophages and other macrophage activation receptors. The study of mice deficient in multiple members of the chemokine family, and their receptors, has shown that chemokines play a critical role in promoting atherosclerotic plaque formation. In the present review, we will discuss novel therapeutic avenues for the treatment of cardiovascular disease that derive directly from our current understanding of atherogenesis gained in experimental animal models.

INTRODUCTION

Atherosclerosis is the underlying cause of cardiovascular diseases including heart attack and stroke. The pathology of these diseases is characterized by the formation of early arterial ‘fatty streak’ lesions that progress over many years to become more complex atherosclerotic plaques. Fatty streak formation occurs early in life, with children showing lipid-rich lesions in the aortic arch and abdominal aorta in the first decade of life [1]. Fatty streak lesions are foci of macrophage accumulation in the arterial wall trapped beneath the endothelial cells lining of the vessel. As these macrophages become bloated with cholesterol they transform to become foam cells [2]. Early lesions continue to grow and begin to recruit other cell types – both leucocytes and smooth muscle cells. As smooth muscle cells begin to proliferate within the developing lesion, it becomes an enlarged fibro-fatty

Key words: apolipoprotein E (ApoE), atherosclerosis, cardiovascular disease, chemokine, macrophage, therapy.

Abbreviations: ApoE, apolipoprotein E; CCL, CC chemokine ligand; CCR, CC chemokine receptor; CX3CL, CX3C chemokine ligand; CX3CR, CX3C chemokine receptor; CXCL, CXC chemokine ligand; CXCR, CXC chemokine receptor; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; ICAM, intracellular adhesion molecule; IL, interleukin; KC, keratinocyte-derived chemokine; LDL, low-density lipoprotein; LDLR, LDL receptor; MCP, monocyte chemoattractant protein; MI, myocardial infarction; MIP, macrophage inflammatory protein; MyD88, myeloid differentiation factor 88; oxLDL, oxidized LDL; PSGL-1, P-selectin glycoprotein ligand-1; PRR, pattern recognition receptor; RANTES, regulated upon activation, normal T-cell expressed and secreted; TLR, Toll-like receptor; TNFα, tumour necrosis factor α; VLDL, very-low-density lipoprotein.

Correspondence: Dr David R. Greaves (email David.Greaves@path.ox.ac.uk).
plaque. Although the physical size of these stable atherosclerotic lesions is of concern, with large lesions restricting blood flow and causing chronic conditions such as angina, they are otherwise relatively quiescent in nature. Unstable aggressively inflammatory lesions, characterized by increased macrophage content and decreased smooth muscle cell number, can be the cause of catastrophic acute clinical events—such as MI (myocardial infarction) or stroke. These plaques are more prone to rupture leading to platelet activation and thrombosis [3].

In the present review, we will discuss recent work using murine models of atherosclerosis that have given unique insights into the cellular and molecular mechanisms regulating monocyte recruitment and macrophage activation.

ANIMAL MODELS OF ATHEROSCLEROSIS

Seminal early studies into the pathogenesis of atherosclerosis, such as the demonstration that foam cells were derived from monocyte precursors, came from large animal models such as high-fat feeding of swine or primates [2,4,5]. Large animal models also support research into surgical interventions to treat cardiovascular disease, such as the study of smooth muscle cell biology following stent deployment in the rabbit [6]. However, genetic technologies are less commonly applied to larger animal models as they do not have the advantages of a short breeding cycle and reagent availability. Genetic intervention in small animal models has led to many of the recent insights into atherosclerosis. Feeding normal mice a high-fat diet, even for extended periods of time, leads to minimal development of disease in most strains [7]; however, the development of hyperlipidaemic mice through alteration of key molecules controlling circulating lipoprotein levels has allowed researchers to investigate mechanisms driving this disease in experiments that last a matter of weeks or months, in contrast with a disease process that occurs over many decades in humans. By combining hyperlipidaemic murine models with surgical models, researchers now have a tool kit of molecules that last a matter of weeks or months, in contrast with a disease process that occurs over many decades in humans.

The LDLR [LDL (low-density lipoprotein) receptor]°−/° mouse lacks the expression of a functional LDLR due to the insertion of a neomycin resistance cassette into exon 4 of the gene. This leads to the production of an inactive truncated protein, detected as a lower molecular-mass-immunoreactive product by Western blot analysis [8]. On a normal chow diet, these mice have only a slight elevation in circulating cholesterol levels; however, once presented with a high-fat diet these mice quickly develop severe hypercholesterolaemia. The predominant elevation of the LDL fraction of circulating cholesterol drives accelerated development of atherosclerosis.

ApoE (apolipoprotein E) is a lipoprotein that facilitates the uptake of various lipoprotein complexes by acting as a ligand for uptake receptors. The ApoE°−/° model of atherosclerosis is a more severe model of hypercholesterolaemia than the LDLR°−/° model [9]. These mice have a more pronounced elevation of plasma cholesterol on a normal chow diet and, as such, develop atherosclerotic lesions without the need for further fat feeding [10,11]. A limitation of this model is that the hypercholesterolaemia is dominated by VLDL (very-low-density lipoprotein), which does not mimic the human condition as closely as the LDLR°−/° mice [12]. Transplantation of ApoE°+/° bone marrow is sufficient to elevate plasma ApoE levels to levels that restore plasma lipids to normal levels [13].

Both the LDLR°−/° and ApoE°−/° mouse strains develop atherosclerotic lesions in a similar fashion to humans, with initial deposition of fatty streaks that gradually progress to form more complex lesions. Early characterization of ApoE°−/° mice demonstrated plaque formation throughout the arterial tree with monocyte adhesion happening within 4–8 weeks post-partum, followed by the formation of fatty streak lesions, which progressively develop into more complex and fibrous lesions [14]. To enable robust quantification of atherosclerotic plaque size, researchers concentrate on a limited number of distinct anatomical sites—routinely the aortic root (the aorta at the level of the tricuspid valves) is sectioned along its full length. This anatomical area is particularly valuable as, alongside obvious structural landmarks, it is an early site of plaque formation with fatty streak lesions easily visible at 8 weeks of age [14]. Profiling experimental atherosclerotic plaques traditionally couples immunohistochemical stains such as Oil Red O, which stains lipids red, and Masson’s Trichrome, which highlights muscle, collagen and nuclei, and immunohistochemistry to probe individual cell types such as macrophages, T-cells and smooth muscle cells. Another site of interest is the descending aorta, which can be dissected in its entirety and stained with histochemical agents such as Oil Red O to enhance the visibility of areas of plaque formation under low-power microscopy. In addition to its obvious utility as a model atherosclerosis, the ApoE°−/° mouse can also be used to profile the aggressive form of the disease resulting from vein grafting or stenting (see Table 1). Alternative sites where lesion morphometry can be followed include the brachiocephalic (also known as the innominate) artery [15]. Some of the complexities of plaque biology typically seen in the clinic are less commonly observed in experimental atherosclerosis. In particular, the formation of unstable plaques, and the occurrence and description of plaque rupture in murine models remains controversial [15–17]. The ability to probe this phenomenon...
Murine experimental models of vascular injury and atherogenesis

<table>
<thead>
<tr>
<th>Model</th>
<th>Characteristic of the model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE−/− mice</td>
<td>Lack ApoE, which facilitates the uptake of lipoprotein complexes. Have VLDL-dominated hyperlipidaemia on a normal chow diet with resulting progressive lesion formation. High-fat feeding causes extreme hypercholesterolaemia and aggressive plaque formation.</td>
<td>[10,11]</td>
</tr>
<tr>
<td>LDLR−/− mice</td>
<td>Lack a functioning LDLR, which mediates uptake of lipoprotein complexes. Minimal hypercholesterolaemia on a normal chow diet and little lesion formation. High-fat feeding causes LDLR-predominated hypercholesterolaemia and plaque formation.</td>
<td>[8]</td>
</tr>
<tr>
<td>Wire Injury</td>
<td>Insertion of a wire, typically into the femoral artery or carotid artery, via a side branch. The passage of the wire through the vessel denudes the endothelial layer. Injured arteries have rapid medial smooth muscle cell apoptosis, followed by neointimal hyperplasia causing luminal narrowing. In hypercholesterolaemic animals, atherosclerotic neointima is formed.</td>
<td>[89,90]</td>
</tr>
<tr>
<td>Carotid ligation</td>
<td>The common carotid artery is ligated near the distal bifurcation causing disrupted blood flow. Ligated vessels undergo luminal narrowing and neointima formation. In hypercholesterolaemic animals, a chow diet induces atherosclerotic neointima with a stable phenotype, whereas high-fat feeding causes disorganized lipid-rich plaque formation.</td>
<td>[91,92]</td>
</tr>
<tr>
<td>Partial carotid ligation</td>
<td>Up to three of the four caudal branches of one of the common carotid arteries are ligated causing altered blood flow and shear stress in the common carotid artery. Ligated arteries show vascular remodelling including smooth muscle proliferation and macrophage infiltration. In hypercholesterolaemic animals, atherosclerosis occurs rapidly within 2 weeks.</td>
<td>[93]</td>
</tr>
<tr>
<td>Arterial cast placement</td>
<td>A restrictive cast is placed around the carotid artery narrowing the lumen within the cast/cuff: this causes lowered shear stress upstream, elevated shear stress within the cast and oscillatory shear stress downstream of the cast. Cast placement causes vascular remodelling without damage to the endothelial layer. In hypercholesterolaemic animals, atherosclerosis forms both up- and down-stream of the cast.</td>
<td>[94]</td>
</tr>
<tr>
<td>Perivascular cuff placement</td>
<td>A non-restrictive cuff is placed around the femoral artery resulting in neointimal formation within 2 weeks. Cuffed vessels demonstrate neointimal formation without damage to the endothelial layer. In hypercholesterolaemic mice, high-fat feeding is associated with foam cell formation in the affected vessel within 3 days, with extensive near-occlusive neointima formed within 2 weeks.</td>
<td>[95,96]</td>
</tr>
<tr>
<td>Stenting and balloon angioplasty</td>
<td>Ex vivo aortic segments undergo balloon angioplasty or placement of stainless steel stents prior to carotid-interposition grafting. Alternatively self-expanding stents may be directly placed into the abdominal aorta. Vessels undergoing angioplasty alone show limited neointima hyperplasia. Thrombus formation and leucocyte adherence, followed by neointima formation and luminal restenosis, is reported to occur in stented vessels. Hypercholesterolaemic mice have enhanced neointima formation.</td>
<td>[97,98]</td>
</tr>
<tr>
<td>Vein grafting</td>
<td>Venous vessels (typically the vena cava or jugular vein) are carotid-interposition grafted in an end-to-end manner. Vessels undergo wall thickening within the first week, followed by the loss of smooth muscle cells and monocyte infiltration. Hypercholesterolaemic animals develop foam-cell-laden atheromatous lesions within the grafted tissue.</td>
<td>[99,100]</td>
</tr>
</tbody>
</table>

experimentally is of clear benefit in the testing of potential therapeutics and warrants further study.

A key feature of mouse models of atherosclerosis is that they allow assessment of the cellular origin of pro- or anti-atherogenic mediators in transgenic mice. The reconstitution of the immune system with that of a syngeneic population of leucocytes of a different genotype allows an almost absolute determination of whether a given phenotype is driven by haemopoietic or non-haemopoietic cells.

LEUCOCYTE TRAFFICKING

Appropriate trafficking of leucocytes to a site of infection or tissue damage is an essential facet of the immune system (Figure 1). As a general paradigm, inflammatory stimuli caused by infection or tissue damage activate local resident cells such as fibroblasts, mast cells and resident macrophages. Production of cardinal pro-inflammatory mediators, such as TNFα (tumour necrosis factor α), amplifies the expression of directional signals for leucocytes in the form of chemical messengers such as chemokines (chemotactic cytokines). These form a concentration gradient leading to the site of injury [18]. Another feature of sites of inflammation are activated endothelial cells, which begin to express adhesion molecules on their surface. Leucocytes in the circulation undergo an initial tethering via selectins (and some integrins) and their glycoprotein ligands allowing the cells to slow and begin to roll along the endothelial surface [19]. In doing so, the leucocytes encounter
In order to migrate to the site of inflammation, leucocytes first become tethered to the inflammatory endothelium. The tethering process typically occurs by binding of selectins to their carbohydrate ligands, although there is some evidence this can occur via integrins in experimental atherosclerosis. Tethering brings the leucocyte chemokine receptors into close contact with chemokines immobilized upon endothelial cells, whilst they roll along the endothelial surface. Activation of the leucocytes via chemokine receptors leads to a firmer adhesion to the endothelium via integrin binding. Leucocytes then migrate to intracellular junctions where they extravasate out through the vessel wall following chemoattractant gradients. An animated version of this Figure can be found at http://www.ClinSci.org/cs/118/0641/cs1180641add.htm

The immobilized chemokine gradient on the endothelial cell surface, leading to cellular activation via GPCRs (G-protein coupled receptors). This in turn results in an increase in the binding capacity of cell-surface adhesion molecules, such as β2 integrins, by molecular clustering and a switch to a high-affinity state [20,21]. Interaction of leucocyte integrins with their targets on the endothelial cell surface allows the cells to firmly adhere and undergo transendothelial migration [22]. In the case of fatty streak formation, monocytes, non-terminally differentiated macrophage progenitor cells, follow chemokines to migrate from the circulation to the inflamed vasculature.

The localization of experimental atherosclerosis within the arterial tree to distinct predictable plaque-prone areas means it is possible to map the endothelial dysfunction that correlates with these sites even before plaque formation. Early studies observed that, in hyperlipidaemic animals, atherosclerosis-prone sites showed up-regulation of the adhesion molecule VCAM-1 (vascular cell adhesion molecule-1) by en-face immunohistochemical staining of the aortic arch [23,24]. This expression was not seen in control mice and was detected prior to lesion formation [24]. ICAM (intracellular adhesion molecule)-1 was also observed to be up-regulated in lesion-prone areas, although interestingly this was not associated with hypercholesterolaemia, as normal control mice also had ICAM-1 up-regulation and these sites were identified as sites of turbulent blood flow [24]. This phenomenon seems to imply that endothelial activation occurs endogenously at sites of turbulent blood flow and that, in the presence of elevated plasma cholesterol, this activation is enhanced, leading to a pathological elevation of other adhesion molecules supporting the recruitment of leucocytes to the subendothelial space.

The therapeutic potential of reducing leucocyte recruitment using anti-adhesion strategies has already been explored in other disease areas; however, this approach has not been without problems. Monoclonal antibodies that block the function of integrins have been licensed for use in treating other inflammatory diseases – multiple sclerosis and psoriasis. Natalizumab (Tysabri) binds to α4 integrin and is used to reduce pathogenic T-lymphocyte recruitment to the central nervous system in multiple sclerosis patients. Efalizumab (Raptiva) is an anti-CD11a [LFA-1 (lymphocyte function-associated antigen-1)] antibody used to treat psoriasis. However,
both patients have been associated with a small number of patients suffering PML (progressive multifocal leucoencephalopathy) as a result of the JC virus [25]. Therapeutics that target leucocyte migration have clear utility in the treatment of other inflammatory conditions, such as vascular disease. However, further clinical trials and careful patient monitoring will be required to limit off-target immune effects and identify the best treatment regimes for this new class of anti-inflammatory drugs. This information will allow us to assess whether anti-integrin drugs will have an application in cardiovascular disease and whether this treatment would be long-term or applicable in short-term acute events.

MACROPHAGE ACTIVATION: TLRs (TOLL-LIKE RECEPTORS) AND INNERNATE IMMUNE ACTIVATION

One of the defining cell types present in all types of atherosclerotic lesion is the macrophage or its progenitor cell the monocyte [2]. Macrophages are highly phagocytic cells that play an essential role in clearing pathogens and apoptotic debris, which in atherosclerotic plaques can become bloated with lipid and transform into foam cells. They express a multitude of ‘pattern recognition receptors’ that allow the innate immune system to sense invading pathogens or apoptotic cell debris for fast and effective removal, utilizing the phagocytic potential of these cells [26]. In addition to this cardinal role in the early phases of an inflammatory response, they also act at an interface between the innate and adaptive immune response, being able act as potent antigen-presenting cells [27].

TLRs are an important family of receptors that recognize PAMPs (pathogen-associated molecular patterns) [26]. These PRRs (pattern recognition receptors) form the basis of the innate immune system’s ability to sense a wide range of pathogens, and synergy between this class of PRRs and the scavenger receptors may contribute to the pathological activation of macrophages in atherosclerosis. The ability of receptors so intimately linked with microbial recognition to have a strong disease-modifying effect hints that there may be a role for pathogens in the initiation of atherosclerosis; however, many TLR family members also recognize endogenous markers of tissue damage. The study of TLR expression in atherosclerotic plaques has shown the expression of TLR1, TLR2, TLR4 and TLR5 in diseased vessels [28,29]. Indeed, it has been shown that oxLDL (oxidized LDL) can up-regulate TLR4 mRNA expression in human blood-derived macrophages in vitro [29]. The study of ApoE−/− mice which also lacked the MyD88 (myeloid differentiation factor 88) intracellular adaptor protein, which is essential for the function of most members of the TLR family, revealed a likely role for this family of proteins in atherogenesis [30]. Aortic sinus lesions in MyD88−/−/ApoE−/− mice fed a Western-type (high-fat) diet were 40–65% (depending on gender) smaller than their ApoE−/− controls. This was despite the unexpected finding in that particular study that MyD88−/−/ApoE−/− mice have higher plasma cholesterol levels. Although these findings strongly implicate a role for TLR signalling in atherogenesis, it is not absolutely conclusive, as MyD88 also transduces intracellular signals from the IL (interleukin)-1 family cytokine receptors.

Studying mice deficient for individual TLR family members has shown that deletion of TLR4 on an ApoE−/− background causes a significant decrease in aortic atherosclerosis, aortic sinus lipid accumulation and macrophage infiltration [31]. The ability of TL4 to mediate strong effects on plaque formation in the absence of any overt bacterial infection strongly suggests that endogenous ligands for this receptor may be driving the disease process in vivo in this experimental model. A similar anti-atherogenic effect of TLR2 deficiency in LDLR−/− mice shows that a pro-atherogenic role for TLR agonists in atherosclerosis is not limited to TLR4 ligands, although in this case the cell type responsible for this effect was non-haemopoietic in origin [32]. Further analysis of TLR2-deficient animals using cell trafficking studies showed that the absence of TLR2 in the endothelial cell layer resulted in a decrease in leucocyte recruitment to atherosclerosis-prone sites and a decrease in lipid accumulation [33]. The treatment of LDLR−/− mice with a TLR2 ligand, PAM3CysSK4 [tripalmitoylcysteinylseryl-(lysyl)4], caused a dramatic enhancement of atherosclerosis in the descending aorta, which was largely absent when TLR2-deficient mice were treated [32]. The ability of TLRs to modulate atherosclerosis underlines the possibility that infectious disease could be pro-atherogenic.

Further evidence for the role of the TLR family in human cardiovascular disease comes from studies of human TLR4 polymorphisms and the detection of specific pathogens in atherosclerotic plaques. However, these studies to date have not shown a definitive link. An initial study found that individuals carrying a Asp299Gly missense mutation in TLR4, which had been associated previously with LPS (lipopolysaccharide) hyporesponsiveness, had reduced carotid atherosclerosis [34]. Asp299Gly was an uncommon polymorphism; however, the study did indicate that individuals carrying this variant had a lower prevalence of cardiovascular disease [34]. Further genetic analyses of associations linking infection with the severity of atherosclerosis [35]. However, subsequent genetic analyses of associations between TLR polymorphisms and cardiovascular disease susceptibility have been unable to confirm the initial positive findings reported by Kiechl et al. [34] (genetic
Key chemokines and chemokine receptors in atherogenesis

<table>
<thead>
<tr>
<th>Chemokine/chemokine receptor</th>
<th>Experimental evidence in atherosclerosis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR2 ApoE−/− mice</td>
<td>Decreased macrophage accumulation and decreased plaque formation. Mice also have decreased hypercholesterolaemia-induced monocytes</td>
<td>[39,73,74]</td>
</tr>
<tr>
<td>CCR1 ApoE−/− mice</td>
<td>Increased aortic root lesion area and exaggerated T-cell infiltration.</td>
<td>[41,42]</td>
</tr>
<tr>
<td>CCR5 ApoE−/− mice</td>
<td>Decreased plaque area and very decreased macrophage infiltration. Competitive antagonist for the CCR5 ligand RANTES (met-RANTES) reduces plaque formation. Protective in the wire injury model. Supports migration of both major monocyte subsets into plaque.</td>
<td>[41,42,74,75]</td>
</tr>
<tr>
<td>CCR7 Function of the two CCR7 ligands CCL19 and CCL21 is required for regression in a transplantation model of atherosclerosis regression.</td>
<td>[82]</td>
<td></td>
</tr>
<tr>
<td>CKCR2 bone marrow transplantation into LDLR−/− mice</td>
<td>Causes a significant decrease in plaque formation and macrophage infiltration. The CKCR2 ligand KC (CCL21) is expressed by the endothelium in lesion-prone areas. KC blockade in the ApoE−/− wire injury model causes increased plaque formation and delayed re-endothelialization.</td>
<td>[47,48,50]</td>
</tr>
<tr>
<td>CKCR6 Knockout animals on an ApoE−/− background have a decreased plaque formation associated with reduced T-cell and macrophage content in the plaque. Knockout of the ligand CCL16 causes exacerbated lesion formation.</td>
<td>[55,56]</td>
<td></td>
</tr>
<tr>
<td>CXCR1−/− animals on both an ApoE−/− and LDLR−/− background, particularly in the brachiocephalic artery. Supports the migration of Ly-6CLO cells, but not Ly-6CHI monocytes, into plaques.</td>
<td>[61,62,74]</td>
<td></td>
</tr>
</tbody>
</table>

analyses of polymorphisms in the TLR genes are summarized in [36]).

The ligands responsible for TLR activation in atherosclerosis, be they exogenous or endogenous, may prove to be very informative in the development of new therapeutic strategies to reduce cardiovascular disease. A previous review by Yan and Hansson [35] provides a broader overview of the roles for innate immune receptors in macrophage activation in atherosclerosis.

The analysis of TLR-dependent processes is an exciting emerging theme in the biology of cardiovascular disease. Simple targeting of the TLRs themselves is unlikely to be therapeutically effective as this family of receptors is of unquestioned importance to the innate immune system. However, as greater detail about the involvement of these receptors in the pathogenesis of cardiovascular disease becomes known, there may be scope for modulation of their function. Perhaps more likely still this field may be informative about some of the earliest steps in atherogenesis and provide insight into the effects of infectious diseases on cardiovascular disease.

CHEMOKINES AND ATHEROSCLEROSIS

Chemokines are small 8–12 kDa proteins that can be divided into four main groups according to the position of highly conserved cysteine residues: CC, CXC, CX3C and XC [37]. Chemokines bind their GPCRs causing G-protein activation and downstream signalling, leading to leucocyte activation, cytoskeletal re-arrangements and chemotaxis. This system, however, shows marked functional redundancy, with individual chemokines binding to multiple receptors and individual receptors having multiple high-affinity ligands. This complexity has made elucidating the classical chemokine signals regulating macrophage trafficking to the developing atherosclerotic plaque a less than straightforward process. Mice deficient in the expression of one or more chemokine or receptor give researchers insights into the biology of chemokines and their receptors [38]. Crossing these mice on to a hyperlipidaemic background has enabled researchers to dissect the process of inflammatory cell recruitment in atherosclerosis one building block at a time. The chemokine and chemokine receptors discussed in the present review are summarized in Table 2.

The first paper to demonstrate the anti-atherogenic effects of reducing chemokine-controlled cell migration in atherosclerosis utilized the CCR2 (CC chemokine receptor 2)−/− mouse on an ApoE−/− background [39]. Aortic root sections from these mice have a significant decrease in macrophage accumulation in the subendothelial space after 5 weeks of a high-fat diet. At later time points, this manifested itself as a significant decrease in plaque formation after 9–13 weeks of a high-fat diet. That study highlighted the role of chemokines
and their receptors in atherosclerosis, and the therapeutic potential of targeting molecules of this type to treat cardiovascular disease. Since this first confirmation of the role of chemokines in atherosclerosis, further detail and complexity in the process has been revealed by the study of further genetic models.

The detection of the chemokines RANTES [regulated upon activation, normal T-cell expressed and secreted; CCL5 (CC chemokine ligand 5)], MIP (macrophage inflammatory protein)-1α (CCL3) and MIP-1β within atherosclerotic plaques implicates the receptors of these molecules in leukocyte recruitment to developing plaques [40]. These chemokines bind and signal at the CCR1 and CCR5 receptors. Studies utilizing the ApoE-deficient knockouts of these receptors highlighted that interfering with leukocyte trafficking can have pro- and anti-atherogenic effects. ApoE−/−CCR1−/− mice had an increased lesion area in the aortic root, which is linked to an exaggerated T-cell infiltration into the plaque [41]. Conversely ApoE−/−CCR5−/− animals had a decrease in lesion area in both aged chow-fed animals and animals subjected to a high-fat ‘Western type’ diet. In two independent strains, atherosclerosis was reduced by at least 50% on both a chow and high-fat diet [41]. Even more strikingly, when the composition of the lesions was examined there was a 77% reduction in macrophage content. The utility of anti-chemokine strategies in having effects greater than just reducing the number of cells which can migrate into a plaque underlines by ApoE−/−CCR5−/− mice having increased smooth muscle cell content, a property of more stable atherosclerotic plaques, and increased levels of the anti-inflammatory cytokine IL-10 [41]. A similar pattern of protection from vascular inflammation by CCR5, but not CCR1, was found in the model of neointima formation following wire injury [42]. These experiments highlight CCR5 blockade as a clear target to reduce atherosclerosis as opposed to targeting CCR1. This also underlines the need to balance the potential for pro- and antiatherogenic effects of chemokine-targeted interventions.

When assessing a phenotype resulting from germline chemokine receptor/ligand deletions caution needs to be exercised in drawing conclusions about these molecules as potential therapeutic targets. The question that arises is can we ever achieve such a complete or exquisitely targeted effect with pharmacological agents? In the case of RANTES, the efficacy of interrupting this receptor–ligand interaction pharmacologically has been shown by the treatment of LDLR−/− mice with the RANTES antagonist met-RANTES. Animals were treated for 14 weeks resulting in a 43% reduction in plaque in the aortic root and a 58% reduction in the thoracic aorta [43]. These animals had a lower expression of CCR2 and CCR5 in atherosclerotic tissue, greater than that which could be accounted for by reduced leukocyte infiltration alone. This underlines the potential ability of anti-chemokine strategies to re-programme atherosclerotic-prone areas to a less inflammatory phenotype.

Work from our group has demonstrated robust inhibition of atherosclerosis following therapeutic inhibition of CC chemokine activity. Vaccinia viruses encodes a 35 kDa pan-CC chemokine-binding protein, vCCl (viral chemoline inhibitor)/35K, which binds many CC chemokines with high affinity making it a very efficient broad-spectrum inhibitor of CC chemokine activity. Expression of the 35K molecule in vivo using both short-term high-level adenoviral delivery or long-term lentiviral delivery causes a potent inhibition of atherogenesis [44,45]. Additionally, this molecule is effective in ameliorating vein-graft disease in a murine model [46]. Our results indicate that treatments targeting CC chemokine activity can be as effective as the germline ablation of members of the CC chemokine family and their receptors in experimental models of cardiovascular disease. These findings are very encouraging for the prospects of chemokine-targeted therapies being effective in treating human vascular disease.

Members of the CXC family of chemokines have also been implicated in the pathogenesis of atherosclerosis. CXCL (CXC chemokine ligand)–CXCR (CXC type chemokine receptor) interactions are primarily associated with the control of neutrophil and T-lymphocyte trafficking. Cells expressing CXCR2, the receptor for IL-8 homologues, have been identified in human coronary artery lesions [47]. Transfer of CXCR2−/− bone marrow into LDLR−/− animals caused a significant decrease in the mean aortic root lesion area in excess of 50%, with a striking reduction in macrophage accumulation [47]. The macrophages in CXCR2+/− lesions were shown to co-localize with areas of CXCR2 immunoreactivity. The CXCR2 ligand KC (keratinocyte-derived chemokine; CXCL1) was identified as a mediator of monocyte arrest on the endothelium in a later study [48]. In that study, the CXC chemokine KC was identified as being presented on the endothelium along with MCP (monocyte chemoattractant protein)-1 at lesion-prone sites. Murine blood monocytes were shown to adhere to these regions during ex vivo perfusion experiments. This adhesion was found to be reversible by treatment with CXCR2-blocking antibodies. These experiments would appear to imply that strategies to block this interaction could be useful in treating vascular disease. Knockout of KC in LDLR−/− animals similarly caused a reduction in atherosclerotic lesion area, but this was about half that of the decrease seen in the CXCR2-deficient animals [49]. This observation is consistent with other ligands for CXCR2 mediating a pro-atherogenic role. When extrapolating these results to human disease, it should be noted that there are some important differences in murine compared with human biology of IL-8 homologues; humans have two receptors for this subfamily of CXC
Increased expression of CXCL16 RNA in ApoE−/− immunohistochemistry of human atherosclerotic lesions. This expression pattern was confirmed by tissue as measured by RT (reverse transcription)–PCR analysis [54]. This interaction can have pathological consequences [50]. Monocytes traffic to the site of endothelial damage in this model and contribute to neointima formation, so by analogy to the LDLR−/− studies it might be expected that neointimal atherosclerosis would be reduced by a KC-blocking antibody. Contrary to expectations, an aggravation of disease was observed, with increased plaque area and delayed re-endothelialization. These findings underline a secondary role of the subset of chemokines containing the ELR motif, which are known to be pro-angiogenic chemokines. CXCR2 ligands are also potent neutrophil chemokines, the uncompromised function of which is absolutely crucial to host defence against bacteria. Effective therapy in cardiovascular disease based on inhibition of the CXCR2–CXC chemokine axis will rely on the ability to separate anti-atherogenic effects from those in host defence, wound healing and re-endothelialization [51,52].

Another member of the CXCR family found to be expressed in human plaques is CXCR6 and its ligand CXCL16. CXCL16 is an unusual chemokine as it can exist as a transmembrane and a soluble chemokine. More unusually this chemokine was originally identified as an uptake receptor for oxLDL and apoptotic bodies [53]. Human carotid plaques have a higher expression of both CXCR6 and CXCL16 than control vascular tissue as measured by RT (reverse transcription)–PCR analysis [54]. This expression pattern was confirmed by immunohistochemistry of human atherosclerotic lesions. Increased expression of CXCL16 RNA in ApoE−/− lesions was found to associate with increases in the macrophage marker CD68 [54]. CXCR6−/−ApoE−/− mice have an ∼40 % reduction in plaque size after 17 weeks on a high-fat diet and a greater reduction of ∼55 % in chow-fed animals (41 weeks) [55]. Analysis of these mice shows a reduced recruitment of T-cells to the aortic wall and a reduced presence of macrophages. Experiments targeting the CXCL16 gene have more complex effects. Rather than showing a reduction in leucocyte recruitment and plaques, the CXCL16−/−ApoE−/− mouse has an exacerbation of experimental atherosclerosis [56]. Macrophages from these mice have a reduced ability to uptake oxLDL, as was seen with cells deficient in CD36 or SR-A (scavenger receptor-A) [57,58]. However, ablation studies of those molecules, although slightly complicated, did ultimately reveal a pro-atherogenic role. This would lead us to conclude that either the scavenger receptor function of CXCL16 is atheroprotective or that the net effect of the loss of both cholesterol uptake and chemokine functions of CXCL16 is pro-atherogenic. Before we can consider interventions in this receptor–ligand axis, further work is required to dissect the relative pro- and anti-atherogenic properties of the CXCL16 chemokine and its receptor.

Studies of transgenic animals with altered chemokine function, as well as identifying leucocyte recruitment signals, have also highlighted interesting differences in the control of plaque formation at different anatomical sites in murine models of disease. Fractalkine is another unusual chemokine with a markedly different structure from other chemokines. This protein is a transmembrane chemokine, having both a chemokine-like domain and a mucin-like transmembrane stalk. The membrane-associated form of this molecule acts as a cell adhesion molecule, but can be cleaved to release the soluble molecule which acts as a classical chemokine [59]. The fractalkine receptor [CX3CR1 (CX3C chemokine receptor 1)] is a seven-transmembrane-spanning GPCR similar to other chemokine receptors [60]. CX3CR1 was shown to be expressed by lesional macrophages, endothelial cells and smooth muscle cells, but immunohistochemical staining of the aortic root plaque from ApoE−/− mice had a particularly high level of expression in plaque-associated smooth muscle cells [61]. Although ApoE−/−CX3CR1−/− mice had decreased plaque formation in both the aorta and aortic root [61], CX3CL1 (CX3C ligand 1)−/−ApoE−/− and CX3CL1−/−LDLR−/− mice both had a consistently greater reduction in the brachiocephalic artery compared with the aortic root (ApoE−/−, 85 % reduction compared with 30 %; and LDLR−/−, 50 % reduction compared with 35 %) [62]. It will be interesting to determine whether similar variation in lesion development at different anatomical sites is seen when targeting other chemokine ligands in animal models. Regional variation in atherogenesis would have important implications for therapeutic targeting of chemokines in human atherosclerosis.

Although the critical role of chemokines in atherogenesis is clear, it has been proposed that targeting chemokines or their receptors would not be sufficiently specific to avoid off-target immune effects, given the role of these proteins in the function of the immune system. As a means to avoid this potential problem, recent work has proposed targeting the formation of disease-associated chemokine heteromers resulting from intra-chemokine binding. Koenen et al. [63] reported that peptide inhibitors of a CCL5–CXCL4 interaction, formed by the deposition of platelet chemokines on activated endothelium, reduced monocyte adhesion to atherosclerotic endothelium, total plaque burden and macrophage infiltration. This work provides an intriguing possibility for disease-targeted moieties if other disease-associated chemokines heteromers can be targeted in a similar fashion [64].
Murine monocytes can be divided into two main subtypes: inflammatory and patrolling/resident phenotypes. These cell types are identified by expression of multiple myeloid cell markers. The cell-surface markers and receptors commonly referred to in the literature [88] are summarized. The detailed phenotyping of murine monocytes and identification of analogous populations and their role in human disease is ongoing.

**MONOCYTE SUBSETS AND THE CONTROL OF PERIPHERAL MONOCYTOSIS**

Recent evaluation of the blood of ApoE<sup>−/−</sup> mice has demonstrated dysregulation of monocyte function, and that macrophages and macrophage-derived foam cells in murine experimental plaques primarily come from recruited circulating monocytes, rather than from migration of local resident cells. This evidence comes from models such as the rapid formation of atherosclerosis following arterial injury [65]. Adult mice were reconstituted with bone marrow from mice that are otherwise identical, except for expression of an alternative isoform of CD45 (which causes no immunological effect). Following full engraftment of the bone marrow transplant, atherosclerosis was induced by arterial ligation [65]. Histological examination of the resultant plaques revealed the majority of monocytic/macrophage cells originated from the donor animal, rather than from the resident myeloid cell population. Similarly, active recruitment of adoptively transferred radioactively labelled monocytes to the aorta is seen in ApoE<sup>−/−</sup> animals on a high-fat diet, which can be reduced by statin treatment [66,67].

Hypercholesterolaemic mice have a peripheral blood monocytosis that develops over time in both fat-fed and chow-fed ApoE<sup>−/−</sup> animals [72]. Dissection of this phenomena showed that the Ly-6C<sup>HI</sup> (analogous to the Gr-1<sup>+</sup>), ‘inflammatory’ monocyte subset exhibit a far more marked monocytosis compared with the ‘patrolling’ Ly6C<sup>LO</sup> (Gr-1<sup>−</sup>) population. This monocytosis was shown to be due to increased numbers of monocytes in the bone marrow pool, and increased survival and continued proliferation of the Ly-6C<sup>HI</sup> cells. The pro-atherogenic nature of this monocyte subset is inferred from their increased adhesion to activated endothelium and the presence of Ly-6C<sup>HI</sup> monocytes in plaques [72]. High-fat-fed ApoE<sup>−/−</sup> mice have a striking doubling of this monocyte population in blood with every month on a high-fat diet. Although this phenomena correlates highly significantly with plasma cholesterol levels and was depressed by statin treatment,
Further studies have since implicated chemokines and their receptors as critical players in this process.

The first study to implicate chemokines in hypercholesterolaemia-induced monocytosis utilized CCR2\(^{-/-}\) animals to show that initiation of high-fat feeding in CCR2\(^{-/-}\) mice caused no induction of monocytosis even over 45 weeks of feeding, whereas there was an evident increase in circulating monocytes in the fat-fed wild-type animals [73]. When the CCR2\(^{-/-}\) animals were crossed on to an ApoE\(^{-/-}\) background, there was again a profound reduction in the circulating monocyte population in the CCR2\(^{-/-}\) animals, which was coupled to a modest, but statistically significant, increase in the bone marrow. These results imply that CCR2 is involved in the trafficking of monocytes from the bone marrow. Analysis of multiple CCR2-ligand-knockout mice revealed MCP-1 and MCP-3 as the ligands responsible for the control of monocyte numbers in the blood of wild-type animals [73].

Further analysis of the role of different chemokines in monocyte trafficking in ApoE\(^{-/-}\) mice showed a differential expression of chemokine receptors on the cell surface of the two major monocyte subsets. Tacke et al. [74] transplanted atherosclerotic aortic arches into host animals that were deficient in chemokine receptor expression to determine which chemokine receptors supported the migration of monocyte subsets into atherosclerotic plaques. By in vivo labelling the different monocyte subsets using a latex-bead-loading protocol, they could follow the trafficking of the chemokine-deficient monocytes into the transplanted aortic arch. Using this model they demonstrated that Ly-6CHI monocytes utilize both CCR2 and CX3CR1 to migrate into plaques. The Ly-6CLO monocytes, which do not express CCR2, as might be expected, had no reliance on CCR2-mediated signalling for trafficking into the aortic plaque. Surprisingly, given that the Ly-6CLO population express higher levels of CX3CR1, these monocytes had no requirement for CX3CR1 expression to support their migration into atherosclerotic plaque. Gene array data and cell-surface labelling comparing the different monocyte subsets indicated that the Ly-6CLO monocytes had a higher expression of CCR5, and the authors hypothesized that this may support monocyte migration into plaques. Inhibition of CCR5-mediated migration was achieved using a CCR5-blocking antibody. This treatment significantly reduced the trafficking of Ly-6CHI monocytes to atherosclerotic plaques despite their low expression of CCR5; the treatment more potently blocked the migration of Ly-6CLO monocytes into plaques, indicating that CCR5 ligands direct the migration of both monocyte subsets into the plaque.

The combined role of CCR2, CCR5 and CX3CR1 in monocyte trafficking was confirmed by experiments that showed reduced circulating monocyte numbers in CCL2\(^{-/-}\)/CX3CR1\(^{-/-}\)/ApoE\(^{-/-}\) mice, which could be reduced further by RANTES blockade with met-RANTES, and that this blockade lead to a further decrease in aortic root plaque burden [75]. The gradation of circulating monocyte numbers produced allowed the correlation of monocytosis with lesion area and directly implicated inflammatory monocyte (discriminated using 7/4\(^{HI}\), which is analogous to Ly-6C\(^{HI}\)) recruitment in atherosclerotic lesion progression, at least in the ApoE\(^{-/-}\) model of atherosclerosis.

The ability of the different monocyte subsets to selectively traffic to sites of inflammation has also highlighted some differences in the expression of adhesion molecules by monocytes. PSGL-1 (P-selectin glycoprotein ligand-1) is an adhesion molecule that interacts with selectins and has higher surface expression on Ly-6CHI monocytes compared with Ly-6CLO cells (see Figure 2). Lack of PSGL-1 in hyperlipidaemic mice manifested as a reduced recruitment of Ly-6CHI cells to atherosclerotic plaques and wire-injury-induced neointima, with a concordant reduced size of plaques in both models [76]. CD11c has also been shown to support adhesion of monocytes, with Ly6CLO cells being the predominant monocyte subtype expressing the molecule [77]. CD11c\(^{-/-}\)/ApoE\(^{-/-}\) mice had decreased macrophage accumulation and plaque area [77].

The role of the different monocyte subsets in cardiovascular disease is not limited to atherogenesis and neointima formation. A recent publication has characterized the role of both of the major monocyte populations in the healing myocardium post-MI [69]. It has been shown that early post-MI, the inflammatory Ly-6CHI population is recruited to sites of experimental MI where they can digest damaged tissue. Later in the healing response, there is a recruitment of Ly-6CLO monocytes which support the healing process by promoting angiogenesis, collagen deposition and myofibroblast accumulation. The ability to fine tune this biological response may provide an unexpected therapeutic opportunity in the treatment of patients post-MI.

The description and control of monocytosis in experimental models of atherosclerosis has been a ‘hot’ topic in cardiovascular research in recent years. However, the presence and importance of this process in human cardiovascular disease is yet to be clarified. Should similar pathological mechanisms be found in patients with cardiovascular disease, then the potential for therapeutic targeting of human monocyte subpopulations in cardiovascular disease is an exciting prospect. The ability to target the inflammatory monocyte populations whilst leaving the more homoeostatic resident populations unaffected appears an attainable target given the differential regulation of their trafficking to experimental atherosclerotic plaques. The different programme of chemokines the monocyte subsets use to migrate provides another aspect of the disease process in which chemokine-directed therapies may have great utility.
Atherosclerotic Plaque Regression Models

Although many studies using experimental models of atherosclerosis have demonstrated reduced atherosclerotic plaque formation, this is not the ultimate aim for clinical therapy. Clinical presentation of cardiovascular disease is nearly always the result of advanced plaques. Therapy that reduces atherosclerotic plaque size, rather than just halts plaque progression, would provide a clear clinical benefit. To facilitate the design of strategies that can achieve plaque regression in the clinic, a good experimental model of this process is required [78]. It has been shown previously that transplantation of a segment of plaque-laden aorta from a hyperlipidaemic animal into a mouse with normal lipid levels brings about the rapid regression of atherosclerosis [79]. Rapid egress of foam cells within 3 days to 1 month was observed from early fatty streak lesions and complex late lesions [80,81]. Chemokine regulation of macrophage function has been shown to have a pivotal role in this process [82]. The egress of foam cells from the lesions could be retarded by treatment with anti-CCL19- and anti-CCL21-blocking antibodies. This implicates the important homoeostatic chemokine receptor CCR7 in the process of regression and highlights the induction or enhancement of this chemokine-mediated pathway as a potential mechanism for switching on foam cell egress. This experimental animal model highlights the potential for chemokines to be key players in controlling pro-resolution pathways in atherosclerosis.

Other non-surgical methods to dramatically improve the lipoprotein profile in animal models have reduced existing plaque size. Induced inactivation of the microsomal triacylglycerol (triglyceride) transfer protein leads to a sustained reduction in circulating cholesterol levels [83]. Induced re-expression of the ApoE model is curative in knockout animals either by gene therapy or by activation of transgenic ApoE expression [84,85].

Examining the basic biology of plaque regression models could yield insight into the cellular and molecular processes, and may provide us with targets that actively encourage the process of regression as opposed to retarding the process of plaque progression. The ability to cause plaque regression following anti-inflammatory intervention in experimental models would be the next step in demonstrating the clinical utility of these approaches.

Chemokines: Clinical Perspectives

We have reviewed several different processes that control macrophage activation and inflammatory cell recruitment to the atherosclerotic plaque. One of the targets that we believe holds great promise to yield new therapeutics to treat cardiovascular disease over the next 5–10 years is the chemokine/chemokine receptor system. Why do we believe this to be the case, how might this be achieved and which patient groups could benefit most from this approach?

It is now over 10 years since the first publication of work showing that CCR2+/− hyperlipidaemic mice have reduced atherosclerosis [39]. Research has highlighted an extended network of chemokines that can control experimental atherogenesis. Therapeutic inhibition of this pathway is capable of recapitulating the effects of germ-line deletions of key family members. It is justified to have concerns regarding the potential negative impacts of this strategy arising from the role of the chemokine system in the control of infection and wound healing. A useful comparison can be made with the initial concerns about targeting ‘cardinal regulators’ of the innate immune response such as TNFα in the treatment of rheumatoid arthritis. Reactivation of latent infectious disease (e.g. tuberculosis) is a complication in a small percentage of patients treated with anti-TNFα drugs, but many more patients have seen significant clinical benefits from the introduction of this class of anti-inflammatory drugs [86].

How can we target chemokines and chemokine receptors for therapeutic benefit in cardiovascular disease? We envisage five potential routes to chemokine inhibition in vivo: (i) small-molecule inhibitors acting as classical GPCR antagonists or inverse agonists at chemokine receptors; (ii) blocking antibodies targeted against pro-inflammatory chemokines or their receptors; (iii) chemokine-binding proteins – we have shown efficacy of this approach using the pan-CC chemokine-binding protein 35K in the ApoE−/− model [45]; (iv) decoy chemokine ligands – these molecules ligate chemokine receptors, but do not cause receptor activation, e.g. met-RANTES [43]; and (v) chemokine-oligomerization-targeting peptides and small molecules. So far traditional approaches to altering chemokine function have yielded a number of promising targets in drug development programmes (reviewed in [87]).

Statin treatment has been successful in the long-term control of cardiovascular disease in large patient populations with relatively few side effects. So how might anti-chemokine therapies be deployed in the treatment of patients with cardiovascular disease? (i) Long-term treatment with an anti-chemokine agent in patients with stable disease does not seem a likely clinical scenario – the potential for off-target effects and/or biological compensation would probably outweigh any clinical benefit over and above that which can already be achieved clinically with statins. (ii) A more likely arena for the deployment of new anti-chemokine therapies might be found in the treatment of patients with advanced disease and/or presenting acutely with unstable plaque, manifesting as acute coronary syndromes, MI or stroke.
In this case a shorter-term more aggressive therapy to regress and stabilize plaque would have utility. (iii) Anti-chemokine/chemokine receptor therapies may also find application in the control of vascular inflammation following acute vascular injury. Anti-chemokine drugs could be used to alter cell trafficking during interventions such as stenting, angioplasty and bypass grafting.

**CONCLUSIONS**

Treatments to control cardiovascular disease, such as statins to reduce plasma cholesterol levels, have yielded great improvements in patient health over the past two decades. However, despite their efficacy, there is still an unmet clinical need for disease-modulating therapies that can stabilize or, better yet, regress more advanced or unstable atherosclerotic plaques. The in-depth understanding of the basic biology of atherosclerosis gained from murine hyperlipidaemic models since their first publication in the early 1990s has highlighted several aspects of monocyte/macrophage biology that we believe are underutilized as therapeutic targets. We expect promising new pharmacological agents that target these cellular pathways to be the focus of research in this area in the short- to medium-term future.

**FUNDING**

E.M., D.R.G. and K.M.C. all receive funding from the British Heart Foundation [grant number RG/05/011], and the NIHR Biomedical Research Centre.

**REFERENCES**


Received 23 September 2009/4 January 2010; accepted 22 January 2010
Published on the Internet 9 March 2010. doi:10.1042/CS20090488