The Role of Complement in the Development and Manifestation of Murine Atherogenic Inflammation: Novel Avenues

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Abstract

Atherosclerosis is a chronic progressive inflammatory disease which manifests in the arterial vascular tree. It is a major cause of cardiovascular morbidity and contributes significantly to mortality in the developed world. Triggers for this inflammatory process are elevated levels of cholesterol, bacterial infection and obesity. The immune response in atherosclerosis is essentially pro-atherogenic, leading to lipid accumulation and cellular changes within the arterial wall. Small-animal models of atherosclerosis are used to study the relevance of candidate factors (cells, genes, diets) in the development and progression of lesions. From a multidisciplinary viewpoint, there are challenges and limitations to this approach. Activation of complement determines or modifies the outcome of acute and chronic inflammation. This review dissects the role of complement in the early development as well as the progressive manifestation of murine atherosclerosis and the advances in knowledge provided by the use of specific mouse models. It gives a critical overview of existing models, analyses seemingly conflicting results obtained with complement-deficient mouse models, highlights the importance of interrelationships between pro-coagulpant activity, adipose tissue, macrophages and complement, and uncovers exciting avenues of topical research.

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Introduction

Atherosclerosis is a metabolically induced and genetically influenced chronic inflammatory condition of the arteries. The characteristic pathologic manifestation is the atheroma, i.e. arterial intima thickened by a fatty deposit and inflammatory cells. Oxidatively modified low-density lipoproteins (oxLDLs) are intimately involved in this process. Their presence is often associated with hyperlipoproteinaemia (elevated levels of lipoproteins in blood), a recognised risk factor for the development of arteriosclerosis. The prevalence of hyperlipoproteinaemia in the population is high, and it may be secondary to diabetes, gout and obesity or of hereditary origin. Bound by scavenger receptors, oxLDLs activate endothelial cells to produce pro-inflammatory mediators, leading to adhesion of leukocytes. Oxidation of LDL, mediated in the arterial wall and aggravated by dietary intake of oxidised
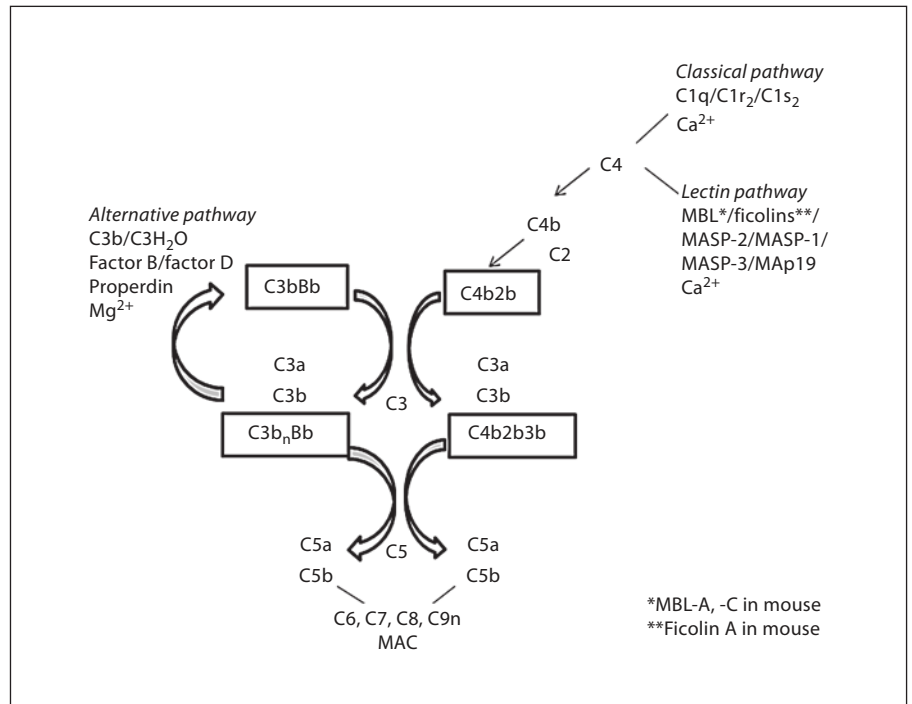
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Fig. 1. Pathways of complement activation. MASP = MBL-associated serine protease.

fatty acid and cholesterol [1], leads to the formation of foam cells (macrophages laden with cholesterol) and interferes with the activity of an endothelial coagulation inhibitor [through modification and degradation of tissue factor (TF) pathway inhibitor], thereby promoting local coagulation [2].

There is no single target to ameliorate this disease; once atherosclerosis is diagnosed, attempts are made to limit the progress of the disease, such as treating co-morbidities (dyslipidaemia, hypertension and diabetes mellitus), influencing other factors known to precipitate disease (platelet aggregation) and managing complications of the disease (stroke, myocardial infarction, embolism and aneurysm). Because atherosclerosis is the leading cause of death in the developed world and of growing importance in the developing world, novel treatment approaches are sought, using genomic studies and expression profiling in cardiovascular disease. Expression studies in humans have documented an inflammatory component of atherosclerotic disease that may be modulated by cholesterol-lowering drugs [3, 4]. Currently, selective targeting of chemokines and their receptors is being discussed for pharmacologic treatment of atherosclerosis [5].

Innate and adaptive immune mechanisms play a role in both the initiation and progression of atherosclerotic disease. This review focuses on the contribution made by complement in the development and manifestation of murine atherosclerosis.

Complement comprises defined zymogens which are activated in a hierarchical cascade on foreign or altered self surfaces via the classical pathway (CP), lectin pathway (LP) or alternative pathway (AP) [6]. Activation is triggered by binding of C1 to bound immunoglobulins (Igs) (CP) or binding of the lectin recognition molecule mannose-binding lectin (MBL) or ficolins to surface carbohydrates (LP), leading to cleavage of C4 and C2, a necessary step in the activation of C3, which is a central feature of the complement system because of the potency of C3 activation fragments. The AP (with zymogens factors B and D and positive regulator properdin) primarily amplifies the activation of C3 (fig. 1). Opsonisation and phagocytosis are mediated by C3 fragments and their cellular receptors (such as CR1, the integrins CR3 and CR4, and the C3b-binding receptor of the Ig superfamily CRIg). Complement-mediated chemoattraction occurs as a consequence of the cleavage of C3 and subsequently C5 via the release of C3a and C5a and their desArg components ($C3a^{desArg}$, $C5a^{desArg}$) and relevant receptors, C3aR, C5aR and C5L2, on inflammatory cells. Non-enzymatic assembly of C5b, C6, C7, C8 and C9 leads to insertion into membranes (in the absence of down-regulators) as the membrane attack complex (MAC; fig. 1).

With regard to atherosclerotic lesions, complement anaphylatoxins C3a and C5a bind to receptors expressed by plaque intima macrophages, T cells, mast cells, endothelial cells and medial smooth muscle cells and therefore may have a role in orchestrating the inflammatory component of atherosclerosis. In vitro, oxLDL seems to activate complement directly, as measured by the production of sC5b-9, involving AP and CP activation [7, 8]. The net effect of complement activation distal of protein C3 was thought to promote maturation of atherosclerotic lesions, based on immunohistochemical findings obtained with an LDL receptor (LDLR)$^{-/-}$/C3$^{-/-}$ mouse line showing lower smooth muscle and collagen content of atherosclerotic lesions [9]. A non-specific complement inhibitor has been tested in mice with the aim of limiting the disease phenotype; treatment of high-fat diet (HFD)-fed apolipoprotein E (ApoE)$^{-/-}$/LDLR$^{-/-}$ mice with recombinant *Vaccinia virus* complement control protein (VCP; a secreted viral protein which has been shown to block progression of the AP, LP and CP of complement activation) produced a significant reduction in the aortic lesion area compared to control HFD-fed ApoE$^{-/-}$/LDLR$^{-/-}$ mice when administered once a week for 7 weeks [10]. The mode of action of the purified protein was not investigated, so while interference with complement activation is one possible mechanism via which the extent of the lesion is limited, binding of VCP to heparin is another, because this interaction may competitively inhibit binding of chemokines to endothelial cells. VCP, through its resemblance to host proteins containing complement control protein modules (such as CD55, C4bBP, CD46), is thought to be of low immunogenicity, so it appears unlikely that antibodies raised to VCP are confounders in the positive outcome of this treatment [11].

Blockers which interfere with complement activation or signalling could thus be suitable tools to target features of atheroma development.

ApoE- and LDLR-deficient mice were generated by gene-specific targeting to study the pathogenesis of atherosclerotic disease [12]. LDLR is a cell surface receptor which mediates the endocytosis and hepatic clearance of LDL, whereas ApoE transports lipoprotein and cholesterol and has a high affinity for the LDLR and ApoE receptor. Both ApoE and LDLR are necessary for the clearance of lipoproteins. ApoE$^{-/-}$ mice have an increased level of total plasma cholesterol, which is exaggerated on a cholesterogenic Western-type diet (21% fat, HFD). The development of atherosclerosis is primarily linked to the extent of hypercholesterolaemia [13]. Very low-density lipoprotein and intermediate-density lipoprotein fractions are increased, and mice demonstrate an early onset of atheromatous lesions [14], which manifest throughout the arterial tree. These lesions are significantly more pronounced in ApoE$^{-/-}$ mice than in the HFD-fed wild-type strain C57BL/6 [15]. LDLR$^{-/-}$ mice have increased levels of intermediate-density lipoprotein and LDL on normal chow [16] and produce a milder atherosclerotic phenotype when fed an HFD [15]. ApoE*3-Leiden mice represent the third model used to study the development of atherosclerotic lesions and are transgenic for a human ApoE mutant (ApoE*3) which binds defectively to LDLR, leading to hyperlipoproteinaemia.

In the majority of study protocols (HFD for up to 40 weeks), the murine models allow investigation of atheromatous plaque development but not of progression to rupture of the fibrous cap [15]. This remains a limitation of the mouse models because it is the late phase of atherosclerosis which causes life-threatening symptoms in humans. However, one study which analysed ApoE$^{-/-}$ mice in an extended feeding protocol (HFD for 59 weeks) showed significant rupture and sudden death, which most likely was due to thrombotic embolism [17].

Infections exacerbate the development of atherosclerosis [18], and mouse models are starting to be used to analyse the infectious contribution to the atherosclerotic phenotype. However, it may be the overall lifetime pathogen burden that determines the development and progression of human atherosclerotic disease rather than episodic events replicated in the animal models. Because of the evidence of *Chlamydia pneumoniae* within atheromatous lesions [19] and elevated anti-*C. pneumoniae* titres in patients with cardiovascular disease, the influence of long-term antibiotic treatment on the incidence of cardiovascular events was investigated in patients. Overall, large-scale clinical trials over at least 10 years did not support a wide application of this treatment approach [20]. Common, functional gene polymorphisms for MBL, which binds to *C. pneumoniae*, may be relevant disease modifiers.

Mouse Models of Atherosclerosis Reveal a Role of Complement

The use of LDLR$^{-/-}$ and ApoE$^{-/-}$ mouse models in combination with complement-deficient mouse lines (or cells) has advanced the understanding of the role of complement in the development of atheromatous lesions. Different Western-type HFDs have been used to generate atherosclerotic lesions in these strains. ApoE*3-Leiden

mice fed a high-cholesterol diet had up-regulated expression of complement C1q, C1qR, C3aR and C9 [21], and LDLR$^{-/-}$ mice fed an HFD showed a significant increase in the expression of complement C3, factor D and properdin compared to LDLR$^{-/-}$ mice fed normal chow [22]. Table 1 gives an overview of phenotypic observations in complement-deficient mice on an ApoE$^{-/-}$, LDLR$^{-/-}$ or ApoE$^{-/-}$/LDLR$^{-/-}$ compound-deficient background. The types of diet and lengths of studies vary and complicate a direct comparison. Nevertheless, the following conclusion may be drawn: in the development of lesions, the presence of C1q (recognition component of the CP), MBL (recognition component of the LP), C3 (central component of the complement activation cascades) and CD59 (called protectin because it inhibits insertion of MAC) may be protective [23–28]. While the membrane-bound down-regulator of complement activation CD55 (decay accelerating factor, dissociates C3 and C5 convertases) regulates lesional lipid deposition in LDLR$^{-/-}$ mice on an HFD [29], development of lesions is attenuated in ApoE$^{-/-}$ mice on an HFD in the absence of CD55 [30].

By contrast, C6 (part of MAC) [23], C3a receptor C3aR [31] and factor B (Bf; of the AP) [32] appear to exacerbate disease during HFD. In studies analysing an early and later time point, the effect of complement deficiency on an atherosclerosis-prone genetic background manifests within the first 10–16 weeks of an HFD. Lesions may then progress [24], stagnate [23–25, 33] or approach the wild-type phenotype [33]. Therefore, the interpretation of phenotypes caused by different diet regimens is restricted to the particular time points of observation.

The principle dual functionality of complement working either in a predominately atheroprotective or proatherogenic manner has previously been concluded from mice lacking specific complement components and also from analyses of immunohistochemical staining of atherosclerotic lesions and levels of complement components in patients [34].

The lesion-limiting effect of CD59 compared to C1q is noted earlier in the disease (8 vs. 12 weeks) in studies which use similar diets (21% pork lard and 0.15% cholesterol vs. normal chow with 0.15% cholesterol and 20% butter fat). The contribution of complement C5 to disease development and manifestation appears to be small [35]. To deduce the role of MBL-A and -C, LDLR$^{-/-}$ mice chimeric for MBL-A/-C$^{-/-}$ bone marrow were generated and compared to LDLR$^{-/-}$ mice injected with MBL-A/-C$^{+/+}$ bone marrow cells [27]. MBL-A/-C$^{-/-}$ macrophages show decreased apoptotic clearance or less phagocytic ability compared to wild-type macrophages and may co-

determine the greater plaque area with the former cells. C1q may be involved in an atheroprotective way via two mechanisms, namely apoptotic body removal and binding to natural antibodies, which are protective in the development of atherosclerosis because of their ability to inhibit the uptake of oxLDL by macrophages [36]. Serum IgM-deficient/LDLR$^{-/-}$ mice have larger aortic lesions compared to C1q$^{-/-}$ mice but display a similar phenotype with regard to cholesterol accumulation and smooth muscle cell proliferation; therefore, the protective effect of IgM may be exerted only in part via C1q [37]. There is a long history of the study of natural antibodies in atherosclerosis, and they are reactive with an epitope that is found on apoptotic cell surfaces, oxLDL and bacterial wall components. ApoE$^{-/-}$ mice develop high titres of anti-oxLDL antibody [38], and LDLR$^{-/-}$ mice have readily detectable anti-oxLDL IgM [39]; these antibodies block the uptake of oxLDL by CD36-positive macrophages.

Overall, triglycerides and total cholesterol in complement-deficient mice appear not to be different from controls on either an ApoE$^{-/-}$ or LDLR$^{-/-}$ background alone (which already present with hyperlipidaemia) with two exceptions; Bf-deficient (Bf$^{-/-}$)/LDLR$^{-/-}$ mice have significantly reduced cholesterol levels on an HFD compared with Bf-sufficient LDLR$^{-/-}$ mice [32], and C3$^{-/-}$/ApoE$^{-/-}$/LDLR$^{-/-}$ mice on normal chow show significantly increased levels of serum triglycerides and cholesterol compared to C3-sufficient ApoE$^{-/-}$/LDLR$^{-/-}$ controls [33]. Although the postprandial clearance of triglycerides was the same between C3$^{-/-}$/ApoE$^{-/-}$/LDLR$^{-/-}$ and C3-sufficient ApoE$^{-/-}$/LDLR$^{-/-}$ controls, C3$^{-/-}$/ApoE$^{-/-}$/LDLR$^{-/-}$ mice weighed significantly less than their C3-sufficient controls with a marked decrease in adipose tissue. Previous work using C3$^{-/-}$ mice demonstrated that triglyceride and cholesterol levels in plasma in mice fed a normal diet were not altered in the absence of C3 [40], which is the precursor of C3a^{desArg} (acylation stimulatory protein, ligand for C5L2, a stimulatory receptor for triglyceride synthesis), although postprandially they did demonstrate delayed triglyceride clearance [41, 42]. This shows the importance of using atherosclerosis-prone mouse lines to investigate the pathogenesis of diet-induced atherosclerosis and of measuring relevant parameters. It also demonstrates that atherosclerotic phenotypes dependent on complement, such as C3 and Bf, may be markedly influenced by concomitant metabolic changes, such as hypo- and hypercholesterolaemia, respectively.

Table 2 collates the measurements performed in models using the aforementioned transgenes for complement

Table 1. Overview of in vivo studies investigating the contribution of complement to murine ApoE$^{-/-}$ and LDLR$^{-/-}$ phenotypes

Ref. No.	Complement	Background	Sex	Model	Control	Diet	End week	Effects on lipids	Effects on lesion
23	C6$^{-/-}$	C57BL/6	♂	C6$^{-/-}$/ApoE$^{-/-}$	ApoE$^{-/-}$	HFD (0.15% C)	16/20	not determined	↓
	CD59$^{-/-}$		♂	CD59$^{-/-}$/ApoE$^{-/-}$				no difference	↑
31	C3aR1$^{-/-}$	B6	♂, ♀	C3aR1$^{-/-}$/ApoE$^{-/-}$	ApoE$^{-/-}$	HFD (0.15 % C, 42% fat)	24	no difference	♀ size ↓, ♂ no difference
24	CD55$^{-/-}$	C57BL/6J	♂, ♀	DAF$^{-/-}$/ApoE$^{-/-}$	ApoE$^{-/-}$	HFD (0.15% C, 20% fat)	14	not determined	no significant difference
							22	not determined	no significant difference
	CD59$^{-/-}$		♂, ♀	CD59$^{-/-}$/ApoE$^{-/-}$	ApoE$^{-/-}$		14	↑ total serum C in ♀	more extensive lesion area and size ♀
							22	slight ↑ total serum C in ♀	even more extensive lesion area only ♀
25	CD59ab$^{-/-}$	C57BL/6	♂, ♀	CD59$^{-/-}$/ApoE$^{-/-}$	ApoE$^{-/-}$	HFD (1.37% C, Ch-free, 20.1% fat)	14/22	no difference	↑
26	CD59a$^{-/-}$	C57BL/6	♀	CD59$^{-/-}$/LDLR$^{-/-}$	LDLR$^{-/-}$	LFD (no C, 5.2% fat)	22	no difference in C, T, lipoprotein profile	lesion ↑
						HFD (0.25% C, Ch-free, 16% fat)		no difference in C, T, lipoprotein profile	lesion ↑, more complex
29	CD55$^{-/-}$	C57BL/6	♀	CD55$^{-/-}$/LDLR$^{-/-}$	LDLR$^{-/-}$	LFD	22	no difference in C, T, lipoprotein profile	↑ lesion, ↑ lipid deposition
						HFD	22	no difference in C, T, lipoprotein profile	↑ lipid deposition
30	CD55$^{-/-}$	C57BL/6/ 129Ola	♂	CD55$^{-/-}$/ApoE$^{-/-}$	ApoE$^{-/-}$	HFD (0.15% C, 21% fat)	20	↓ serum T and cholesterol	↓ lesion
37	C1qa$^{-/-}$	C57BL/6	♀	sIgM$^{-/-}$/C1q$^{-/-}$/ LDLR$^{-/-}$	LDLR$^{-/-}$	LFD (5.2% fat)	22	not determined	4.1-fold ↑, more complex
						HFD (0.25% C, Ch-free, 16% fat)			33% ↑ lesion size
				C1q$^{-/-}$ LDLR$^{-/-}$		LFD (5.2% fat)			2.8-fold ↑ in lesions
						HFD (0.25% C, Ch-free, 16% fat)			no difference
28	C1qa$^{-/-}$	C57BL/6	♀	C1qa$^{-/-}$/LDLR$^{-/-}$	LDLR$^{-/-}$	LFD	22	no difference in lipoprotein profile	3-fold ↑
						HFD (0.25% C, Ch-free)		no difference in lipoprotein profile	10-fold ↑ vs. LFD controls, no difference vs. HFD controls
27	MBL-A/-C$^{-/-}$	C57BL/6, BM chimera	♀	MBL-A/-C$^{-/-}$/ LDLR$^{-/-}$	LDLR$^{-/-}$/ MBL-A/ -C$^{+/+}$	HFD (0.15% C, Ch-free, 16% fat)	10	no difference in T or C	30% ↑ plaque surface compared to wild-type mice
9	C3$^{-/-}$	C57BL/6J × 129BL6	♂, ♀	C3$^{-/-}$ LDLR$^{-/-}$	C3$^{+/-}$/ LDLR$^{-/-}$	HFD (1.25% C)	15	no difference in serum lipid content	↑ lipid deposition, ↑ lesion
33	C3$^{-/-}$	C57BL/6J/ 129Ola	♂	C3$^{-/-}$/ApoE$^{-/-}$/ LDLR$^{-/-}$	ApoE$^{-/-}$/ LDLR$^{-/-}$	normal chow	16	58% ↑ in serum T; C not affected	84% ↑, more advanced lesions
		C57BL/6J/ 129Ola					26	79% ↑ in serum T; C not affected	no difference in lesion size
	Bf$^{-/-}$	C57BL/6J/ 129Ola	♂	Bf$^{-/-}$/ApoE$^{-/-}$/ LDLR$^{-/-}$	ApoE$^{-/-}$/ LDLR$^{-/-}$	normal chow	16	no difference in T or C	no difference in lesion size
		C57BL/6J/ 129Ola					26	no difference in T or C	no difference in lesion size
32	Bf$^{-/-}$	C57BL/6	♀	Bf$^{-/-}$/LDLR$^{-/-}$	LDLR$^{-/-}$	LFD	22	no difference	no difference in lesion area
						HFD		↓ in serum C (VLDL, LDL)	no difference in lesion area; lesion size ↓
35	C5$^{-/-}$, B10.D2-H2	C57BL/6J × C57BL/10SnJ	♂	C5$^{-/-}$/ApoE$^{-/-}$	ApoE$^{-/-}$	HFD (0.15% C)	22	no difference in T or C	no difference in lesion area
			♀						no difference in lesion area, but ↓ than ♂

T = Triglycerides; C = cholesterol; Ch = cholate (cholate facilitates fat and cholesterol absorption); LFD = low-fat diet; VLDL = very low-density lipoprotein; ↑ = increase; ↓ = decrease.

Table 2. Methodological characterisations of the atherosclerotic phenotype in ApoE$^{-/-}$ and LDLR$^{-/-}$ models

Descriptors	Method	Ref. No.
Lesion size		
Plaque, lumen, media and vessel area	morphometry	23, 28
Histological features		
Aorta en face and/or staining by aortic root section	Sudan IV/Oil Red O/Mayer hematoxylin/TOPRO-3	9, 23, 24, 25, 26, 28, 29, 31, 32, 33, 37
Lipid content	Oil Red O	28, 35
Endothelial damage	Evans blue staining	25
Collagen deposition	Picrosirius Red	9, 24, 27, 29
Fibrosis, cholesterol clefts, acellular areas and fibrous cap	hematoxylin and eosin	31, 33
Foam cells	Oil Red O	25
Elastin	Miller/Van Gieson	25
Cellular and humoral characterisations		
Complement deposition, binding	C3, C3d, C9/MAC, C5, C5b-9, DAF, CD59 antibodies	9, 23, 25, 26, 29, 31, 32, 37
Macrophage content	CD68, MOMA-2, Mac3 antibodies	23, 25, 26, 31, 32, 33, 37
Vascular smooth muscle cell content	α-smooth muscle actin antibody	25, 26, 32, 33, 37
T cell content	CD3, CD4 and CD8 antibodies	25, 37
Apoptotic cells	anti-cleaved caspase 3 antibody, terminal uridine nick-end labelling	25, 26, 31, 37
B cell content	CD19 antibody	26
IgG/IgM deposition	confocal microscopy	37
Foam cells	CD11b antibody	35
Triglyceride levels and clearance	enzymatic assays	27, 33
Complement activation: C3, C3a levels	ELISA	29, 32
Lipoprotein profile and cholesterol	gel filtration liquid chromatography/enzymatic assay	9, 23, 25, 29, 32, 33, 35, 37
IgM and IgG levels, auto-antibodies for oxLDL/modified LDL	ELISA	9, 31, 33
ssDNA, dsDNA, chromatin, histone	ELISA	28, 37
Endothelial damage	ELISA (for vWF in serum)	25

proteins and LDLR$^{-/-}$ and/or ApoE$^{-/-}$ mice. Constituents of atherosclerotic lesions (lipids, collagen, monocytes and smooth muscle cells) are key parameters to characterise the atherosclerotic phenotype, but these analyses are not carried out in every study. A complex lesion is defined by an increase in smooth muscle cell and collagen content, a concomitant decrease in macrophages and the development of a fibrous cap. The quantification of atherosclerosis in mice involves two principal methods, en face (lesion area) and aortic root (lesion size), with en face measurement normally requiring higher cholesterol consumption [43]. Other common measurements relate to general physiological parameters (body weight, body fat, heart-body weight ratio).

Procoagulant Activity and Complement in Atherosclerosis

Platelets are essential in thrombus formation over dysfunctional and eroded or fissured atherosclerotic lesions and play an important role in the development and progression of atherosclerosis. Thrombosis is a response mechanism to vascular damage including, typically, the late stage of atherosclerotic plaque rupture. Endothelial cell injury induces platelet adhesion to the vessel wall and secretion of von Willebrand factor (vWF), which promotes platelet recruitment. A rise in vWF levels has been shown in CD59ab$^{-/-}$/ApoE$^{-/-}$ mice on an HFD, which demonstrate a high incidence of occlusive coronary atherosclerosis and mortality [25]. Interestingly, vWF was

shown to co-purify with properdin, possibly interacting through its thrombospondin-like domains [44], but the relevance of this finding has not yet been studied in vivo. Platelet activation promotes activation of the coagulation cascade; oxLDL stimulates macrophage-mediated activation of the intrinsic coagulation pathway, and TF, important for the activation of the extrinsic coagulation pathway, is expressed in macrophages of atheromatous plaques [45]. Risk factors for the development of atherosclerosis, such as hypertension and dyslipidaemia, can elevate TF expression in atherosclerotic lesions and contribute to the thrombotic phenotype of disease, but the role of TF in murine atherogenesis models is still unclear [46]. Interestingly, C5a is a sufficient stimulus to induce expression of TF in endothelial cells [47] and therefore would appear to be relevant in inflammation-associated atherosclerosis. Apart from their role in progressive disease, platelets have also been shown to adhere to endothelial cells in ApoE$^{-/-}$ mice before lesion development [48]. Platelet microparticles, membranous vesicles released from activated platelets, provide surfaces which are conducive to complement activation via CP and AP, even in the presence of membrane-bound regulators of complement activation [49]. Platelet microparticles express P-selectin, which binds C3b and supports Bf-dependent complement activation (AP) [50]. Importantly, platelet microparticles are detectable in mice and are thought to facilitate adhesion of monocytes to inflamed endothelium, leading to the formation of foam cells, but have not yet been studied in the existing compound complement/LDLR/ApoE-deficient mouse models.

Adipose Tissue Produces Complement Factors and Sustains Inflammation That Aggravates Atherosclerosis

Aside from being a lipid storage organ, adipose tissue is an endocrine organ active in the secretion of a variety of adipokines. The extent of its contribution to systemic immunity and inflammation has not yet been quantified, though expression of pro-inflammatory genes [e.g. interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1] and plasticity within the tissue have been documented. Adipocytes express a pattern of Toll-like receptors (TLRs) which depends on their state of differentiation, meaning that a different response to inflammation is exhibited by pre-adipocytes and mature adipocytes. Adipose tissue is seen as an immune responsive organ based on TLR signalling and secretion of inflammatory markers such as cytokines, growth factors and also complement components (factors B, D and C3) [51]. The exact cellular origin of these mediators, including complement proteins, from within the adipose tissue remains an issue of debate. While there is certainty regarding the capacity of adipocytes to act as immune cells [52], it has been reported that separation of cells from murine white adipose tissue produces lipid-rich floating adipocytes and a fraction containing stroma-vascular cells, a mixture of pre-adipocytes, endothelial cells and various immune cells [53]. Expression of inflammation genes [tumor necrosis factor (TNF)-α, macrophage inflammatory protein-1α and MCP-1] was found in the Mac-1$^+$/F4/80$^+$/CD68$^+$ stroma-vascular fraction but not the leptin-positive adipocyte fraction [53]. A study investigating the production of adipokines from human adipose explants (containing adipocytes and stroma-vascular cells) and from isolated adipocytes in parallel found that adipokines such as IL-8, IL-6, vascular endothelial growth factor and TNF-α (but not adiponectin and leptin) are produced by non-fat cells (present in adipose tissue explants but not in adipocyte cultures) [54]. Therefore, studies investigating gene expression of the entire tissue, comprising a mixture of adipocytes, pre-adipocytes, macrophages, mast cells and endothelial cells, may provide a more useful insight into the potential of cells within the adipose context to modulate systemic disease [55]. In our hands, methodological variability, especially the duration of collagenase digestion, accounted for varying purity of the adipose and stroma-vascular subpopulations prepared from mouse epididymal adipose tissue.

Nevertheless, it is clear that adipokines directly influence endothelial function, platelets and monocytes [56] and for this reason are underestimated modulators of systemic inflammation, together with adipose tissue-resident macrophages, which express a phenotype (M2 type) determined by the adipose environment [57]. Adipose tissue-derived leptin, adiponectin and IL-6 have a direct role in endothelial dysfunction and platelet activation [58]. Adiponectin is protective against early atherosclerosis by suppressing the production of adhesion molecules and cytokines (e.g. TNF-α) from endothelial cells and macrophages, respectively. It also binds C1q and factor H (inhibitor of C3 and C5 convertases of the AP of complement activation), leading to a less pro-inflammatory engagement of complement activation [59]. Mast cell-derived cytokines are integral modulators of white adipose tissue and energy expenditure [60]. Adipose tissue-resident mast cells express complement properdin [61], but its importance in the generation of C3a^{desArg} as well as in

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sustaining the inflammatory capacity of adipose tissue has not yet been investigated. Adipsin, a serine protease identical to human complement factor D, is produced by adipose tissue and secreted into the blood; genetically obese rats and mice show a substantial reduction of adipsin mRNA in their adipose tissue [62].

A role for perivascular adipose tissue in the pathogenesis of atherosclerosis was proposed based on its chemoattractive capacity [63]. Since then, studies have concluded that coronary perivascular adipose tissue is related to coronary artery disease in humans but also that perivascular adipose tissue in mice is composed of white as well as brown fat with regional differences (thoracic vs. abdominal aorta) and plasticity on an HFD [64]. Adipose tissue, macrophages and mast cells in periarterial locations all contribute to thrombogenicity and plaque progression.

Macrophages Express Complement Factors and Are Pathogenic in Vessel Wall and Adipose Tissue

Monocytes adhere to endothelial cells and infiltrate the arterial intima where they mature into macrophages and internalise modified LDL to become foam cells, characterising early arteriosclerotic lesions. oxLDL acts as endogenous ligand for signalling via TLR2 and TLR4, resulting in the release of pro-inflammatory TNF-α by macrophages [65]. Advanced atherosclerotic lesions exhibit hypoxic areas; accumulating macrophages respond to hypoxia by up-regulating a number of genes required for angiogenesis and cell survival. Plaque destabilisation is brought about not only by matrix metalloproteinase (MMP)-expressing macrophages but also by mast cells. The levels and activity of MMP-1 and MMP-9 in human monocyte-derived macrophages were increased in vitro by the anaphylatoxin C5a, and C5a as well as MMP-1 and MMP-9 were found to be co-expressed in human atherectomy specimens [66].

Macrophages express most complement components and receptors but are a heterogenous and dynamic cell group characterised by differential expression of markers, which are not uniformly used in immunohistochemical analyses of lesion material.

In aortas of ApoE$^{-/-}$ mice (fed normal chow), significantly more F4/80$^+$/C5aR$^+$ macrophages were observed at 25 weeks of age compared to wild-type controls (C57BL/6J). Administration of the C5a antagonist PMX53 from 5 to 30 weeks of age led to a significant reduction in lipid area and size in this treated group compared to the untreated ApoE$^{-/-}$ group, though the treatment did not alter the lesional content of CD68$^+$ macrophages [67]. By contrast, in the absence of C1q or CD55, HFD-fed LDLR$^{-/-}$ mice showed fewer lesional MOMA-2$^+$ macrophages compared to LDLR$^{-/-}$ controls [28, 29], and the content of Mac-3$^+$ (LAMP-2$^+$) macrophages was higher in HFD-fed C3$^{-/-}$/LDLR$^{-/-}$ mice compared to LDLR$^{-/-}$ controls [9]. These changes relate to the increased severity of the lesion phenotype in each of these compound complement/LDLR-deficient models.

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine which attracts macrophages to adipose tissue in LDLR$^{-/-}$ mice [68]; its significance in disease progression was deduced from studies investigating MIF$^{-/-}$/LDLR$^{-/-}$ mice on normal chow over 52 weeks, which showed a significant reduction in the atherosclerotic lesion area compared to LDLR$^{-/-}$ controls [68]. In HFD-fed mice, the number of macrophages in adipose tissue is increased, and they contribute to the increase in C3aR expression as described elsewhere [69]. There is a greater increase in pro-inflammatory CD11c$^+$ M1-type macrophages (secretion of inducible nitric oxide synthase and TNF-α) than CD206$^+$ M2-type macrophages (involved in tissue remodelling via secretion of MMPs) in epididymal fat tissue [70], but this shift may critically depend on the caloric value of the HFD [71]. How complement activation-derived factors are involved in the process of macrophage and immune cell recruitment to adipose tissue during the development of obesity is an area that remains to be explored, although it is clear that the proximal complement pathway (and production of C3a^{desArg}) is functional, with demonstration of increases in C3a^{desArg} production in HFD-fed mice [72].

The importance of M1- and M2-type macrophages in relation to the development of murine atheromatous plaques has been investigated in models using M1/M2 ex vivo polarised bone marrow-derived macrophages and ApoE$^{-/-}$ mice. Of greater immediate relevance, however, are perivascular macrophages which, via TNF-α secretion, may activate vascular smooth muscle cells to express a phenotype consistent with lymphoid tissue organising cells [73]. This is important because tertiary lymphoid organs are local immune environments, which lead to perpetuation of any chronic inflammatory disease.

$F_c\gamma$ receptors ($F_c\gamma$Rs) are IgG-binding receptors expressed by myeloid cells, which trigger cellular responses such as macrophage phagocytosis and endothelial dysfunction [74]. $F_c\gamma$RIIB inhibits the development of atherosclerosis in LDLR$^{-/-}$ mice, even when fed a diet of 0.15% cholesterol and 21% fat [75]. Although cooperation

between $F_c\gamma Rs$ and complement has been described [76, 77], this has not yet been studied for atherosclerosis, even though anti-oxLDL antibodies are present and macrophage activation is a hallmark in the development of plaques. IL-1, IL-12, IL-18, MIF, TNF-α, interferon-γ and macrophage colony-stimulating factor are pro-atherogenic cytokines, as deduced from a wealth of mouse models. Unfortunately, their levels have not been studied in the complement-deficient mouse models, which are increasingly being used to identify a role of complement regulation and activation in the process of atheromatous plaque development. In this context, IL-12 seems of particular interest; it is produced by adipocytes during inflammation, it is one of the key drivers in Th1-dominated development of atheromatous lesions, and complement activation leads to an increase in IL-12 production via macrophage-expressed complement receptor CR3 and release of complement C5a [78, 79].

Fig. 2. Complement – an agent in activation of cells and intercellular communication.

Perspective

Using genetically engineered mice, evidence for the differential involvement of complement in progressive atherogenic inflammation has been provided. Many questions remain. One limitation, as can be seen from the compilation of studies in table 1, is the variety of backgrounds, use of male or female mice, feeding protocols and the scope of measurements. To maximise the knowledge gained through these laborious in vivo studies, consensus in the community on the choice of protocol would be desirable. Further frontiers remain; no model yet has addressed the role of infection, platelet function or adipose tissue together with complement in the context of atherosclerotic disease. Because complement has humoral as well as cellular effects, acting systemically as well as locally (fig. 2), the dissection of its role in complex, non-acute disease such as atherosclerosis is bound to be difficult, in spite of the availability of specific gene-targeted experimental mice.

As part of the normal recognition of patterns, the complement system and the TLR-mediated response have so far been investigated singly in order to ascertain their individual roles and importance within innate immune defence. More recently, the implications of the overlap in activation of these two pattern-effector systems have begun to be investigated. Joint activation of complement and TLR leading to a cellular response which, driven by IL-6, develops into a Th17-driven phe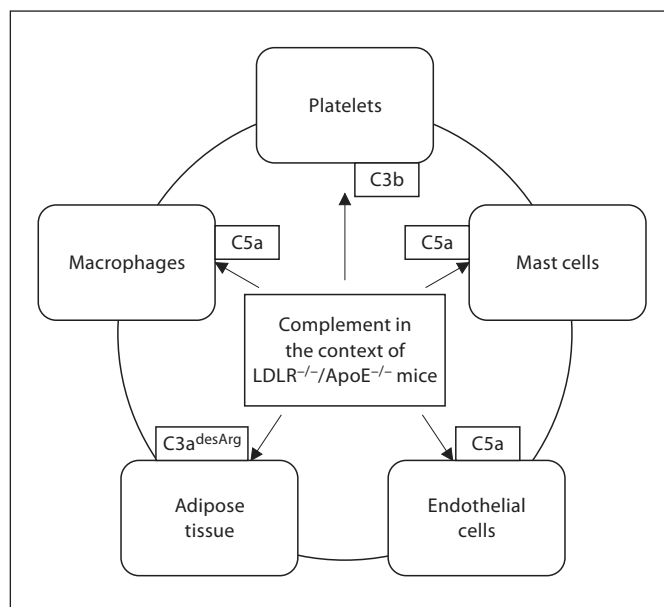notype [80], is likely to play a role in atherosclerosis models incorporating an infectious precipitant. IL-17 was found to be significantly elevated in the walls of atherosclerotic vessels of HFD-fed ApoE$^{-/-}$ mice by immunohistochemistry and RT-PCR. Further, CD4$^+$/IL-17$^+$/interferon-γ^+ (Th1/Th17) T cells were elevated in the spleens of HFD-fed ApoE$^{-/-}$ mice at the late stage, and their numbers correlated with the plaque area [81]. Therefore, it will be of interest in future to follow the modulation of this cellular phenotype in complement-deficient experimental mouse models of atherosclerosis.

Current therapeutics target risk factors for cardiovascular events, which are associated with atherosclerosis, such as platelet aggregation and elevated cholesterol and triglyceride levels [5]. Since its discovery as a targetable, significant mediator of mortality due to sepsis, inhibition of C5a-mediated signalling has been pursued in pre-clinical and clinical trials [82]. A C5a antagonist was not found to reverse the complexity of atherosclerotic histopathology; rather it significantly reduced plaque lipid areas [83]. Others see an application of complement activation inhibitors in the acute phases of chronic disease [84]. The aim of the experimental mouse model work is to identify complement factors which, if significantly important in the pathogenesis of disease, could be targeted in order to interfere with the natural progression of atheroma development. However, mechanisms by which these individual factors exert their significant actions are
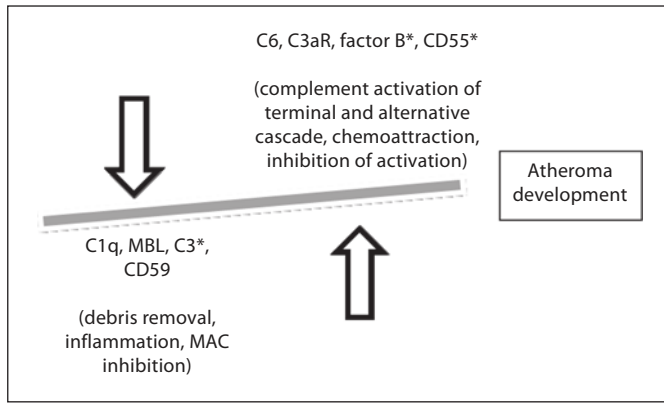
Francescut/Steiner/Byrne/Cianflone/Francis/Stover

C6, C3aR, factor B*, CD55*

(complement activation of terminal and alternative cascade, chemoattraction, inhibition of activation)

Atheroma development

C1q, MBL, C3*, CD59

(debris removal, inflammation, MAC inhibition)

Fig. 3. Complement – a balancing act. * Effects on lipid metabolism as deduced from the use of gene-targeted mice: factor B and C3 in LDLR$^{-/-}$ mice have antagonistic effects on lipids; while the former raises cholesterol levels, the latter lowers triglycerides. CD55 in LDLR$^{-/-}$ mice leads to elevated triglyceride and cholesterol levels.

not yet clear (fig. 3). Assessing the contribution of complement by investigating complement-deficient mice at a protracted endpoint may miss these important temporal factors; the effect of complement might well be to sustain the pathologic process over time, but complement may also have a more instructive role early on in disease development (such as M1/2 biasing [85]). Alternatively, as the disease progresses, there may be a qualitative change in the involvement of complement, such as when shear stress of endothelial cells (relevant in areas of plaque formation) increases properdin expression [86].

So far, complement has been viewed in a mainly pro-inflammatory capacity; its relevance in lipid metabolism is only beginning to be investigated more thoroughly. In fact, its role in atheroprotection/atherogenesis may operate via regulation of lipid metabolism; the most recent findings using a CD55$^{-/-}$/ApoE$^{-/-}$ model suggest that C3a^{desArg} levels may provide the missing link between diet, blood biochemistry and vascular changes [30].

The approach, namely to dissect into small parts those components which we seek to understand in detail, to then put together as a greater whole, i.e. to synthesise, in order to gain fuller understanding of the experiments and reality, reminds us of the Cartesian method of science. It is becoming clear that in order for this process to be meaningful physiologically, present avenues in atherosclerosis research need to be extended to include, for example, the role of complement in adipose tissue enhanced platelet/leukocyte aggregates (a connection between the latter two entities has been made [87]) as well as the role of complement in the interactions between renal failure and adipose tissue [88]. Great advances are to be expected from a multidisciplinary approach.

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