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Complement regulator CD59 protects against atherosclerosis by restricting the formation of complement membrane attack complex

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Abstract

Complement is a central effector system within the immune system and is implicated in a range of inflammatory disorders. CD59 is a key regulator of complement membrane attack complex (MAC) assembly. The atherogenic role of terminal complement has long been suspected, but is still unclear. Here, we demonstrate that among mice deficient in apolipoprotein E (Apoe), the additional loss of murine CD59 (*mCd59ab*$^{-/-}$*/Apoe*$^{-/-}$) accelerated advanced atherosclerosis featuring occlusive coronary atherosclerosis, vulnerable plaque, and premature death, and that these effect could be attenuated by over-expression of human CD59 in the endothelium. Complement inhibition using a neutralizing anti-mouse C5 antibody attenuated atherosclerosis in *mCd59ab*$^{-/-}$*/Apoe*$^{-/-}$ mice. Furthermore, MAC mediated endothelial damage and promoted foam cell formation. These combined results highlight the atherogenic role of MAC and the athero-protective role of CD59, and suggest that inhibition of MAC formation may provide a therapeutic approach for the treatment of atherosclerosis.

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Keywords

CD59; complement; complement regulation; endothelial dysfunction; atherosclerosis; occlusive coronary atherosclerosis and vulnerable plaque

Introduction

Atherosclerosis is a chronic inflammatory condition in which immune and non-immune mechanisms induce endothelial dysfunction, the first step in atherogenesis[1]. Despite significant progress in the past decade, the cellular and molecular pathogenesis of atherosclerosis is still not fully understood. Research in this field had been historically hampered by the lack of appropriate animal models, a difficulty that was overcome by the generation of Apoe- and LDLR-deficient mice (*Apoe*$^{-/-}$ and *LDLR*$^{-/-}$), which recapitulate most aspects of human atherosclerosis and are now the established models of the disease. However, the potential atherogenic role of the complement system, a main effector arm of immunity and inflammation, remains to be determined.

The complement system consists of ≈ 30 proteins that interact with one another in three activation cascades known as the classical, the alternative, and the lectin pathways. These three pathways eventually converge at the level of C3 and the formation of a C5 convertase. Enzymatic cleavage of C5 generates C5b which initiates the terminal complement cascade leading to polymerization of C9 and insertion of MAC into cell membranes[2, 3]. MAC is a transmembrane pore that in a rigid irreversible conformation leads to swelling and lysis of the target cells. In a reversible conformation, MAC can also induce non-lethal transient changes in membrane permeability allowing increased influx and/or efflux of ions[4] and biologically active molecules[5], resulting in activation of cell signaling cascades[6]. An array of complement regulatory proteins including CD59 has evolved to protect autologous cells from the deleterious effect of complement activation and MAC formation[3]. Several lines of evidence from human and animal studies indicate that CD59 is more relevant than decay-accelerating factor (DAF) in protecting red blood cells from MAC formation and MAC-induced phenomena[7]. Humans have only one *CD59* gene while mice have two *Cd59* genes (termed as *mCd59a* and *mCd59b*) [8]. *mCd59a* deficient mice (*mCd59a*$^{-/-}$) showed intravascular hemolysis [9]; *mCd59b* deficient mice (*mCd59b*$^{-/-}$) exhibited a complement-mediated hemolytic anemia and platelet activation [10, 11], most likely due to the absence of mCd59b function combined with downregulation of mCd59a [2,12].

Work from our laboratory showing that MAC insertion into endothelial cell membranes results in the release of growth factors such as bFGF and PDGF [5, 13] as well as pro-inflammatory and prothrombotic cytokines such as interleukin-1 established a connection between MAC formation and focal cell proliferation as seen in proliferative disorders including atherosclerosis. Others have shown that the MAC also induces the release of monocyte chemotactic protein-1 (MCP-1)[14] and activates signaling pathways that promote proliferation of vascular smooth muscle cells[15]. Extensive clinical data showing that MAC co-localized with other complement activation products and immunoglobulins in human atheromas support the notion that MAC may play a pathogenic role in human

atherosclerosis[16]. In the vascular wall, complement can be activated to form MAC by bound immunoglobulins, C-reactive protein (CRP)[17], and cholesterol crystals or cholesterol-containing lipids and enzymatically modified low-density lipoprotein (E-LDL)[18]. In animals, however, evidence for an atherogenic role of the MAC is more controversial. Complement C6-deficiency protects against fat-induced atherosclerosis in rabbits [19]. The absence of C3 in *Apoe*$^{-/-}$ and *LDLR*$^{-/-}$ double knockout (*Apoe*$^{-/-}$/*LDLR*$^{-/-}$) mice or of C5 in *Apoe*$^{-/-}$ mice did not protect against atherosclerosis, although other confounding factors, such as the profound hyperlipidemia leading to a more severe proatherogenic lipid profile observed with C3 deficient-*Apoe*$^{-/-}$/*LDLR*$^{-/-}$ mice could also contribute to these negative observations[20, 21].

Recently, Yun, et al demonstrated that the deficiency of mCd59a in *LDLR*$^{-/-}$ mice sensitizes *LDLR*$^{-/-}$ mice to develop atherosclerosis[22]. Although mCd59b is considered to play less relevant role for restricting MAC formation in mice than mCd59a[12, 23], mCd59b is expressed at lower level in hematopoietic cells and testes[2, 24], and has anti-MAC activity in the mouse, especially in the mCd59a-deficient condition[10, 12, 24]. To fully demonstrate the protective role of CD59 in atherogenesis, we used mCd59a and mCd59b double knockout mice (*mCd59ab*$^{-/-}$) in this study[24]. Moreover, the underlying mechanism by which CD59 plays a protective role in the pathogenesis of atherosclerosis, remains unclear. In order to address this question, we also used human CD59 transgenic mice (*ThCD59*^{END}), and anti-C5 antibodies in combination with *mCd59ab*$^{-/-}$ to define the role of MAC in atherosclerosis. Briefly, CD59 ablation in the *Apoe*$^{-/-}$ background (*mCd59ab*$^{-/-}$/*Apoe*$^{-/-}$) increased the severity of atherosclerosis as characterized by the development of occlusive coronary atherosclerosis with vulnerable plaques associated with extensive C9 deposits in the atheromas. Conversely, selective over-expression of human CD59 in the endothelium and hematopoietic cells (*ThCD59*$^{END+/-}$/*Apoe*$^{-/-}$) rendered *Apoe*$^{-/-}$ mice resistant to the development of atherosclerosis. Remarkably, the development of severe atherosclerosis in *mCd59*$^{-/-}$/*Apoe*$^{-/-}$ mice was reversed by C5 blockage via the administration of a neutralizing anti-C5 monoclonal antibody. Together, these results establish a role of the MAC in the pathogenesis of atherosclerosis and provide experimental evidence that restriction of complement activation is a novel avenue for the treatment of atherosclerosis.

Materials and Methods

Animal studies were approved by the Harvard Medical School Institutional Animal Care and Use Committee. A detailed description of the materials and methods use is provided in the online-only Data Supplement. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

CD59 deficiency induces advanced atherosclerosis with occlusive coronary disease and vulnerable plaques

We previously generated *mCd59ab*$^{-/-}$, which exhibits complement-mediated hemolytic anemia [24]. We generated human CD59 (hCD59) transgenic mice that selectively express the transgene in erythrocyte (*ThCD59*^{RBC}) or in endothelium and some hematopoietic cells such

as platelets (Supplemental Figure I), neutrophils, and monocytes (*ThCD59^{END}*) as previously described [25, 26]. In these transgenic strains, over-expression of hCD59 is effective in providing additional protection against mouse complement[25, 26]. To study the role of CD59 and complement in atherosclerosis in the context of a favorable pro-atherogenic environment, *mCd59ab$^{-/-}$* and *Apoe$^{-/-}$* mice were crossed to generate *mCd59ab$^{-/-}$/Apoe$^{-/-}$*, and *ThCD59$^{END+/-}$* and *Apoe$^{-/-}$* mice to generate *ThCD59$^{END+/-}$/Apoe$^{-/-}$* mice (Supplemental Figure II, A-C online).

Mice were fed a high fat diet (HFD) and followed longitudinally for either two or four months (Figure 1). *mCd59ab$^{-/-}$/Apoe$^{-/-}$* mice developed significantly more severe atherosclerotic lesions in both aortic root and aortic surface (as evaluated by en face preparation) than *Apoe$^{-/-}$* mice. By contrast, transgenic endothelial and hematopoietic cell-selective over-expression of hCD59 in *ThCD59$^{END+/-}$/Apoe$^{-/-}$* mice significantly reduced the development of atherosclerotic lesion as compared with those of *Apoe$^{-/-}$* mice (Figure 1, A-D and Supplemental Figure III). There were no significant differences in the lipid profiles of *mCd59ab$^{-/-}$/Apoe$^{-/-}$ vs. Apoe$^{-/-}$* mice or of *ThCD59$^{END+/-}$/Apoe$^{-/-}$ vs. Apoe$^{-/-}$* mice (Supplemental Figure IV).

Consistent with the expression of a more severe atherosclerotic phenotype, the spontaneous mortality rate among *mCd59ab$^{-/-}$/Apoe$^{-/-}$* mice was significantly higher than that observed among *Apoe$^{-/-}$* mice. In contrast, the transgenic expression of hCD59 significantly prolonged the mean survival time of *Apoe$^{-/-}$* mice (Figure 1E). In addition, the body weight of mice at the four-month time point correlated inversely with the severity of atherosclerosis (Supplemental Figure IV). *mCd59ab$^{-/-}$/Apoe$^{-/-}$* mice exhibited a much higher incidence of occlusive coronary atherosclerosis than *Apoe$^{-/-}$* mice, with one animal showing histological evidence of myocardial infarction (Figure 2). Additionally, the plaques developing among *mCd59ab$^{-/-}$/Apoe$^{-/-}$* mice had classic features of vulnerable plaque[27, 28], including larger necrotic cores with thinner fibrous caps containing less collagen and more inflammatory cells, as compared with plaques among *Apoe$^{-/-}$* mice (Figure 3, A-E). In contrast, plaques observed in *hCD59$^{END+/-}$/Apoe$^{-/-}$* mice exhibited significantly smaller necrotic cores than in those found in *Apoe$^{-/-}$* mice (Figure 2B). These findings are remarkable because occlusive coronary artery disease with myocardial infarction, the hallmark of atherosclerotic heart disease in humans, is rarely seen in *Apoe$^{-/-}$* or *LDLR$^{-/-}$* mice unless they carry additional gene modifications[29, 30].

Together, these results indicate that the systemic deficiency of CD59 in the context of *Apoe$^{-/-}$* genetic background makes mice more sensitive, while overexpression of CD59 makes them more resistant, to the development of advanced atherosclerosis.

C9 deposition correlates directly with the severity of atherosclerosis

Since inhibition of MAC formation is the only known function of CD59, the previous data imply that MAC may contribute to the atherogenic phenotype of *mCd59ab$^{-/-}$/Apoe$^{-/-}$* mice. Staining of the aortic roots with anti-C9 specific antibodies revealed that *mCd59ab$^{-/-}$/Apoe$^{-/-}$* mice had significantly more extensive deposits of C9 associated with higher C9 staining intensity than *Apoe$^{-/-}$* mice, while the density of C9 was reduced in *ThCD59$^{END+/-}$/Apoe$^{-/-}$* (Figure 4A). Histological analysis showed that *mCd59ab$^{-/-}$/*

Apoe$^{-/-}$ mice had significantly higher and *ThCD59*$^{END+/-}$*/Apoe*$^{-/-}$ mice significantly lower content of inflammatory (macrophage and T-cells), and apoptotic cells than *Apoe*$^{-/-}$ mice (Figure 4, B-D). These results are consistent with a pathogenic role of the MAC in the atherosclerotic phenotype of our experimental mice.

Inhibition of MAC formation attenuates atherosclerosis

In order to establish conclusively the atherogenic role of the MAC we used an anti-mouse C5 monoclonal antibody raised in C5-deficient mice that has been used extensively to block activation of the terminal complement cascade and MAC formation[31]. Both mouse sera pre-incubated with the anti-C5 antibody and mouse sera extracted from experimental mice injected with the antibody exhibited a significant reduction of complement activity assessed in a standard sensitized rabbit erythrocytes hemolytic assay (Figure 5A). Administration of the anti-C5 antibody to *mCd59ab*$^{-/-}$*/Apoe*$^{-/-}$ mice in parallel with a HFD for two months, resulted in a significant attenuation of the atherosclerotic lesions (Figure 5, B and C) and was associated with a parallel decrease in C9 staining area and intensity (Figure 5D).

Complement activation mediates endothelial dysfunction

It is widely accepted that endothelial dysfunction is the first and critical step in atherosclerosis. It is conceivable that in *mCd59ab*$^{-/-}$*/Apoe*$^{-/-}$ mice the loss of CD59 activity increases MAC-induced endothelial injury and dysfunction and that overexpression of hCD59 in *ThCD59*$^{END+/-}$*/Apoe*$^{-/-}$ protects against the deleterious effect of the MAC on the endothelium. To assess endothelial damage in our experimental mice, we measured serum levels of Von Willebrand factor (vWF), an established biomarker of endothelial injury[25], and stained the aortas with Evans blue, a direct marker of increased endothelial cell membrane permeability [25]. Levels of vWF in six-week old mice on a normal diet were similar among the different experimental groups (Figure 6A). Once fed a HFD, *mCd59ab*$^{-/-}$*/Apoe*$^{-/-}$ mice had a significantly higher, while *ThCD59*$^{END+/-}$*/Apoe*$^{-/-}$ mice a significantly lower level of vWF than *Apoe*$^{-/-}$ mice (Figure 6A). To establish whether endothelial damage precedes the development of atherosclerosis, we evaluated the integrity of the aortic walls of four-month-old mice fed on non-atherogenic normal chow by staining with Evans blue. As expected, there were no macro-atherosclerotic lesions in any of the three experimental groups. *mCd59ab*$^{-/-}$*/Apoe*$^{-/-}$ mice had significantly larger Evans blue stained aortic area, as compared with *Apoe*$^{-/-}$ mice (Figure 6B). Transgenic expression of hCD59 in *ThCD59*$^{END+/-}$*/Apoe*$^{-/-}$ protected against the endothelial injury revealed by Evans blue staining. To evaluate further whether complement activation can mediate endothelial injury and the protective effect of CD59, we injected cobra venom factor (CVF), an activator of the alternative pathway[10] to six-week old mice from each of the experimental groups. Four hours after CVF injection, *mCd59ab*$^{-/-}$*/Apoe*$^{-/-}$ mice had a significantly higher level of vWF and larger Evans blue stained aortic areas than *Apoe*$^{-/-}$ mice, and the transgenic expression of hCD59 protected against this endothelial injury (Figure 6, C and D). We also found a directly acute CVF-induced endothelial damage associated with thrombosis in a *mCd59ab*$^{-/-}$*/Apoe*$^{-/-}$ mouse (Supplemental Figure V).

MAC fosters foam cell formation

A characteristic pathological feature of atherosclerosis is the formation of foam cells revealing the excessive accumulation of cholesteryl esters (CE) inside macrophages[1, 32]. To investigate whether MAC would foster foam cell formation, we challenged a mouse macrophage cell line with Cu-oxidized LDL (Cu-oxLDL) in the absence or presence of the MAC assembly. This in vitro experiment demonstrated that terminal complement components significantly increased formation of foam cells in a dose-dependent fashion for C5b6 and only when added in a sequence that leads to MAC formation (Figure 7A). This effect could not be mediated by individual C7, C8 or C9 alone (Data not shown). MAC-induced foam cell formation was associated with increased accumulation of CE inside and reduced cholesterol efflux from MAC-treated macrophages (Figure 7, B and C)[33]. Furthermore, MAC-treated macrophages expressed an increased number of mRNA transcripts encoding for CD36, a scavenger receptor implicated in the accumulation of oxidatively modified lipoproteins (Figure 7D) [32], but not for scavenger receptor-A (SR-A) (Data not shown).

Discussion

This study demonstrates that: 1) systemic deficiency of CD59 renders *Apoe*$^{-/-}$ mice much more sensitive to the atherogenic effect of a HFD; 2) over-expression of CD59 in endothelial and some of hematopoietic cells such as platelets renders *Apoe*$^{-/-}$ mice resistant to the atherogenic effect of a HFD, 3) inhibition of MAC formation attenuates HFD-induced atherosclerosis, and 4) The severity of atherosclerosis correlates strongly with C9-deposition in atherosclerotic plaques developing in molecularly engineered mice. Collectively, these results provide strong support for a critical role of the MAC in atherogenesis. This conclusion is consistent with previous reports of MAC deposition in human atherosclerotic plaques[34], and with the protective effect of C6 deficiency reported in a rabbit model of atherosclerosis[19]. In contrast, studies in C3- and C5-deficient mice seem to contradict the above interpretation of our experimental results[20, 21]. In these studies, C3-deficient *Apoe*$^{-/-}$/*LDLR*$^{-/-}$ mice exhibited a more severe atherogenic lipid profile than C3-sufficient *Apoe*$^{-/-}$/*LDLR*$^{-/-}$ mice[21]. This is most likely due to the concomitant absence of C3a-des-Arg, also known as acylation-stimulating protein (ASP), a critical factor for the transport of lipids into adipocytes and maintenance of metabolic homeostasis[35]. In addition, it has been reported that compared to *C3*$^{+/+}$ mice, *C3*$^{-/-}$ mice have higher activity of plasma thrombin, which substitutes for the C3-dependent C5 convertase, thereby leading to the formation of MAC[36]. The atherosclerosis studies in C5-deficient mice were conducted on a B10 rather than a B6 genetic background, which is widely accepted for studying atherosclerosis. In addition, the MAC deposition in atherosclerotic lesions was not investigated in that study[20]. These or other model-specific differences may account for the discrepant results observed in our experiments as compared with those in C3- or C5-deficient mice.

Furthermore, *mCd59ab*$^{-/-}$/*Apoe*$^{-/-}$ mice on a HFD died prematurely and developed advanced atherosclerosis featuring occlusive coronary atherosclerosis and vulnerable plaques. As early as 2 months on a HFD, *mCd59ab*$^{-/-}$/*Apoe*$^{-/-}$ mice developed vulnerable plaques similar to those found in *Apoe*$^{-/-}$ mice fed a HFD for over ten months[37], in a

vascular smooth muscle apoptotic mouse model[28], or in the Akt1 deficient mouse in *Apoe*$^{-/-}$ background [30]. These results combined with the increased mortality rate in *mCd59ab*$^{-/-}$/*Apoe*$^{-/-}$ mice strongly indicate that the MAC plays an active role in both the development of severe atherosclerosis with occlusive coronary disease and vulnerable plaques, although the actual cause of premature death in *mCd59ab*$^{-/-}$/*Apoe*$^{-/-}$ mice deserves further investigation. Yun, et al recently reported the protective role of CD59 in the pathogenesis of atherosclerosis using mCd59a single knock out deficient mice [22]. However, the underlying mechanism, by which mouse CD59 protects against the development of atherosclerosis, has not been investigated. Our study is more comprehensive in that it employs *Apoe*$^{-/-}$ mice deficient in both mCd59a and mCd59b proteins. Furthermore, using *Apoe*$^{-/-}$ mice over-expressing hCD59, as well as anti-C5 antibody treatment, we demonstrate that MAC plays a critical role in the pathogenesis of atherosclerosis.

Increased MAC deposition in atherosclerotic lesions could be due to focal complement activation or down-regulation of complement regulatory proteins such as CD59. CRP, currently considered a marker of the inflammatory process associated with atherosclerosis and frequently found co-localized with MAC, is a potent complement activator in humans[38]. Thus, the association of CRP and MAC immunostaining in atheromas could represent the histological evidence of increased focal complement activation. On the other hand, decreased CD59 anti-MAC activity due either to reduced expression of CD59, as reportedly found in both atheromas and infarcted myocardium of human subjects [39, 40], or to inactivation by glycation, as we have reported[41, 42], could also explain increased MAC deposition in vascular walls of patients with atherosclerosis [34] and in all target tissues of diabetic complications 42-44. The results of our work with *mCd59ab*$^{-/-}$ mice reported herein provide strong experimental evidence that reduced CD59 function and the consequent increase in MAC deposition fosters atherosclerosis. Furthermore, the presence of vulnerable plaque seen in *mCd59ab*$^{-/-}$/*Apoe*$^{-/-}$ mice suggests that increased MAC deposition, which occurs under conditions of CD59 deficiency, plays a critical role in the formation of vulnerable plaque. Together, these results from human and experimental studies indicate that increased MAC formation, which may result from either abnormal complement activation or down-regulation of the complement regulatory proteins such as CD59, contributes to the development of atherosclerosis and diabetic complications.

The endothelium is particularly vulnerable to complement proteins which are present in the plasma of all vertebrates from fish to mammals. Both basal “tick over” activation of complement occurring in the normal circulation as well as complement activation by different “stressors” present a serious threat to normal endothelium. Using two complimentary experimental methods we documented that atherosclerosis-prone *mCd59ab*$^{-/-}$/*Apoe*$^{-/-}$ mice exhibit a significant increase in endothelial damage as early as 2 months after initiation of a HFD. This early endothelial damage is 1) associated with increased MAC deposition, 2) recapitulated by injection of the acute complement activator CVF, and 3) significantly decreased by the transgenic expression of hCD59 in the endothelium. Furthermore, the administration of an anti-C5 antibody dramatically attenuated the development of atherosclerosis in *mCd59ab*$^{-/-}$/*Apoe*$^{-/-}$ mice. These results

categorically establish the role of MAC-induced endothelial damage in the development of atherosclerosis.

Moreover, *in vitro*, we demonstrated that MAC-treatment of macrophages significantly increased the formation of oxLDL-induced foam cells. Thus, the MAC is likely to actively contribute to the increased content of inflammatory cells, and the proliferative phenotype of the atherosclerotic plaques, which are more severe in *mCd59*-deficient mice because their cells are unprotected from the deleterious effect of MAC formation.

Important among the experimental results reported herein is the significant attenuation of atherosclerosis obtained by either over-expression of CD59 in the endothelium or the injection of anti-C5 specific antibodies. Basal “tick over” activation of complement that occurs in all animal cells exposed to the circulation provides the basis for the potentially catastrophic damage to self cells that may result from the amplification of the complement cascades in response to either exogenous threats such as pathogens or endogenous activators such as antibodies, CRP or ox-LDL. For this reason, a complex array of complement regulators has evolved to prevent complement attack to the “self”. The delicate balance between complement activation and restriction can be broken by decreased protection, as in the experiments comparing *Apoe*$^{-/-}$ mice with *mCd59ab*$^{-/-}$/*Apoe*$^{-/-}$ mice. Conversely, the broken balance between complement activation and restriction could be restored by either pharmacological inhibitors of complement activity, as in the experiments with anti-C5 antibodies, or increased expression of complement regulators, as in the experiments with transgenic mice expressing hCD59 in the endothelium and hematopoietic cells. The significant attenuation of the atherosclerotic phenotype we observed under either experimental approach strongly suggests that inhibition of complement is a novel avenue for the treatment of atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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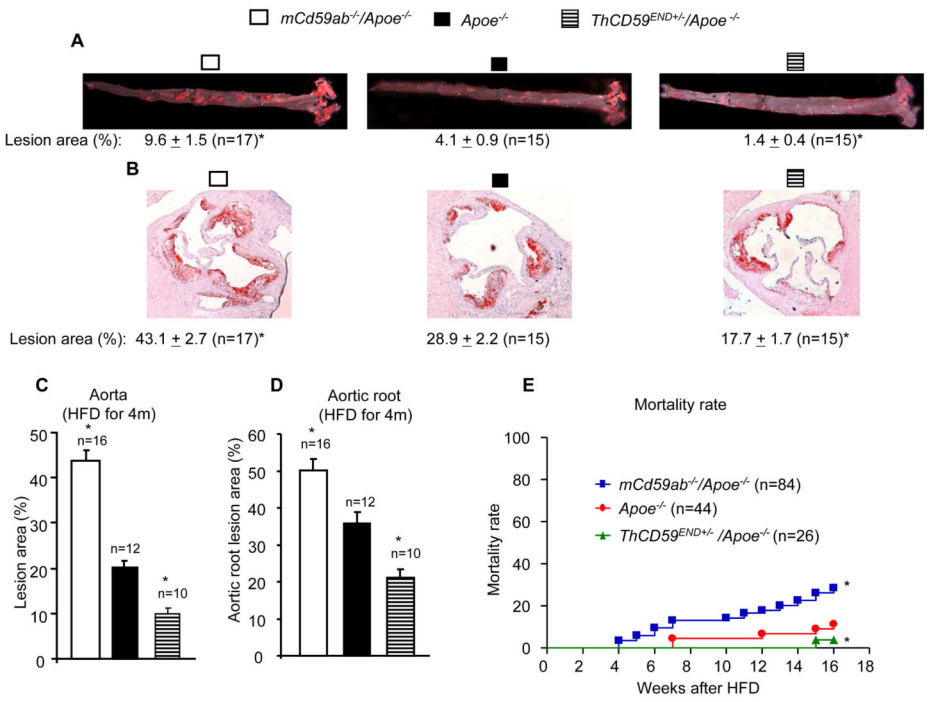

Figure 1. CD59 prevents against atherosclerosis

(**A**) Atherosclerosis analysis of *en face* aorta with Oil red O staining in the mice fed with a HFD for two months. Statistical significance ($P < 0.0001$ *vs.* $Apoe^{-/-}$) is indicated with an asterisk. Lesion area (%) is [Oil red O staining area / aortic area] × 100. (**B**) Atherosclerosis analysis of aortic root in the mice on a HFD for two months. Statistical significance ($P < 0.0001$ *vs.* $Apoe^{-/-}$) is indicated with an asterisk. Lesion area (%) is [Oil red O staining area / ventricle area] × 100. (**C**) Atherosclerosis analysis of the aorta in mice fed on a HFD for four months. Statistical significance ($P<0.0001$ *vs.* $Apoe^{-/-}$) is indicated with an asterisk. (**D**) Atherosclerosis analysis of the aortic root in mice fed on a HFD for four months. Statistical significance ($P<0.0001$ *vs.* $Apoe^{-/-}$) is indicated with an asterisk. (**E**) Mortality rate among $mCd59ab^{-/-}/Apoe^{-/-}$, $ThCd59^{END+/-}/Apoe^{-/-}$ and $Apoe^{-/-}$ mice. Asterisk indicates statistical significance ($P<0.05$) *vs.* $Apoe^{-/-}$ mice evaluated using a Log-rank (Mantel-Cox) test (GraphPad Prism 5 software).

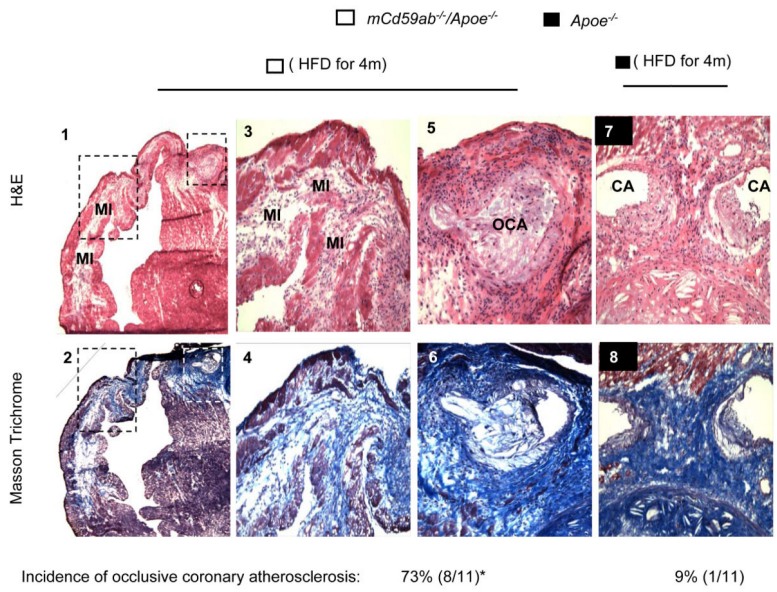

Figure 2. Occlusive coronary atherosclerosis in *mCd59ab*$^{-/-}$/*Apoe*$^{-/-}$ mice

Low magnification of H&E staining shows that the central portion of the wall of the right ventricle has severe edema and loss of myocardial fibers. The coronary artery is occluded as also shown in high magnification. Masons trichrome staining shows the replacement of myocardial fibers (red) by collagen (blue) in the infarct. High magnification (H&E) of the coronary artery (CA) shows that it is occluded by loose connective tissue (trichrome staining) and lipid. High magnification of the myocardial infarction (MI) by H&E staining shows the loss of cardiomyocytes in the middle of the wall and replacement by the loose connective tissue (Trichrome staining). Occlusive coronary atherosclerosis (OCA) is defined by over 50% of occlusion in the coronary artery. *P< 0.01 (The Chi-Square Test) *vs.* *Apoe*$^{-/-}$.

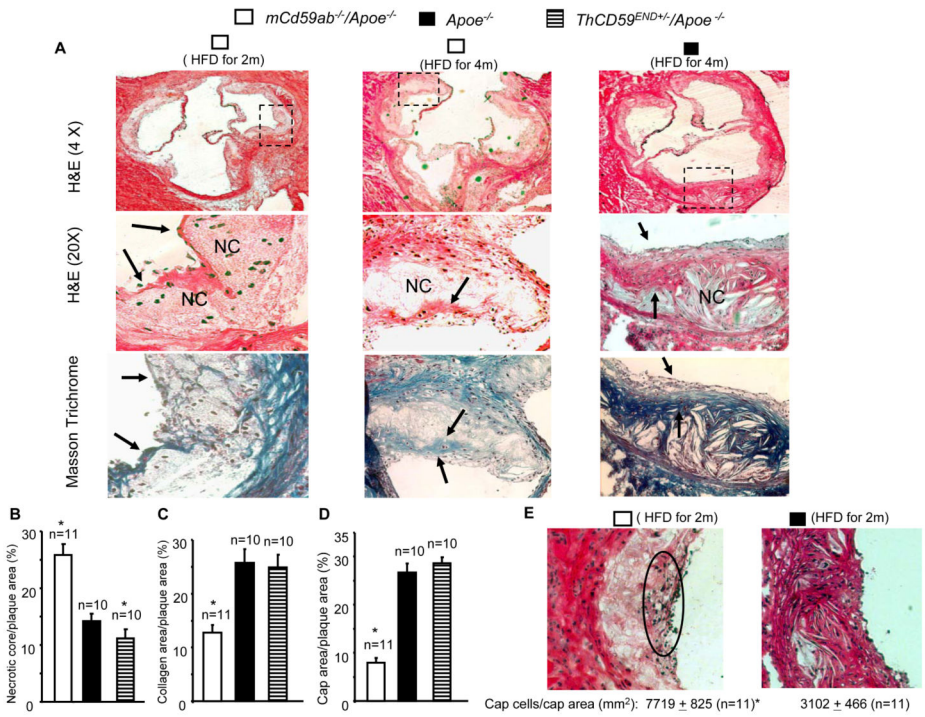

Figure 3. Myocardial infarction, and vulnerable plaque in *mCd59ab*$^{-/-}$/*Apoe*$^{-/-}$ mice

(**A**) H&E stain (top two panels) and masons trichrome (bottom panel) stain show that *mCd59ab*$^{-/-}$/*Apoe*$^{-/-}$ mice on HFD for either two or four months had a larger necrotic core, thinner fibrous cap and less collagen compared with *Apoe*$^{-/-}$ mice. (**B-E**) Quantitative analysis of the necrotic core (**B**), collagen (**C**), fibrous cap of plaques (**D**) and cap cells (**E**), indicative of inflammatory cells. **P*< 0.05 *vs. Apoe*$^{-/-}$.

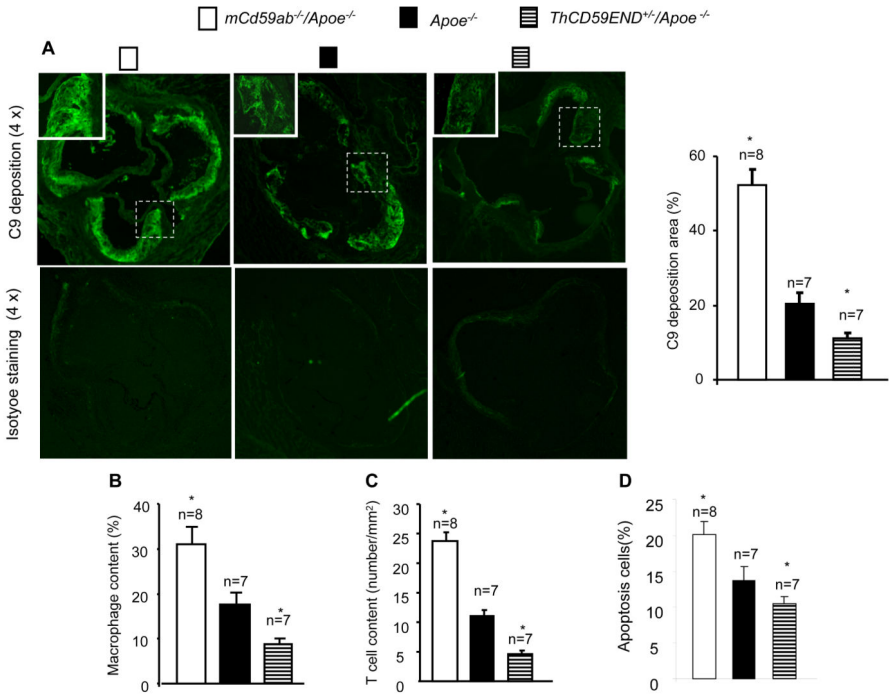

Figure 4. Characterization of atherosclerosis lesions

(**A,** *left panels*) C9 deposition in the atherosclerotic lesions of mice. (**A**, right panel) Levels of C9 deposition (percentage of positive area *vs.* lesion area) detected in atherosclerotic lesions. (**B**-**E**) Percentages of lesion areas staining for macrophages and T cells (percentage of positive area *vs.* lesion area) and apoptotic cells (percentage of apoptotic cells *vs.* total cells) in lesion areas. Statistical significance ($P < 0.01$ *vs.* $Apoe^{-/-}$) is indicated by an asterisk. We analyzed the mice fed with a HFD for two months.

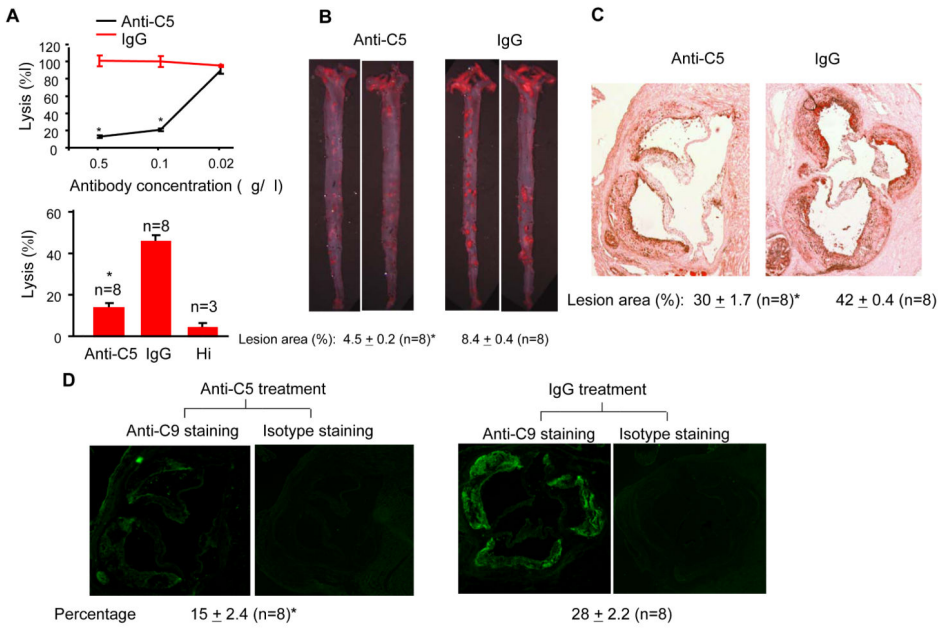

Figure 5. Control of complement activation influences the development of atherosclerosis in *mCd59ab*$^{-/-}$/*Apoe*$^{-/-}$ mice

(**A**) Functional analysis of anti-C5 antibody-mediated MAC inhibition in vitro (top panel) and complement activity of the sera obtained from the *mCd59ab*$^{-/-}$/*Apoe*$^{-/-}$ mice treated with anti-C5 antibody or IgG isotype control (bottom panel). Top panel: Complement activity was measured in a hemolytic assay with commercial mouse serum pretreated with the indicated concentrations of anti-C5 or IgG isotype control on ice for 2 hours. Statistical significance (P<0.01 *vs.* corresponding IgG treated cells (from 6 different experiments)) is indicated with an asterisk. Bottom panel: The complement activity of the sera from the mice administered anti-C5 or IgG isotype control. Statistical significance (P<0.01 *vs.* IgG control-treated mice) is indicated with an asterisk. (**B-D**) Atherosclerosis analysis in aorta (**B**) and aortic root (**C**) and C9 deposition in aortic root (**D**) in anti-C5 or IgG isotype antibody-treated *mCd59ab*$^{-/-}$/*Apoe*$^{-/-}$. Lesion area (%) is [Oil red O staining area / aortic area] × 100. Percentage is [C9 staining area/lesion area] × 100. Statistical significance (P < 0.01 *vs.* IgG treated mice) is indicated with an asterisk.

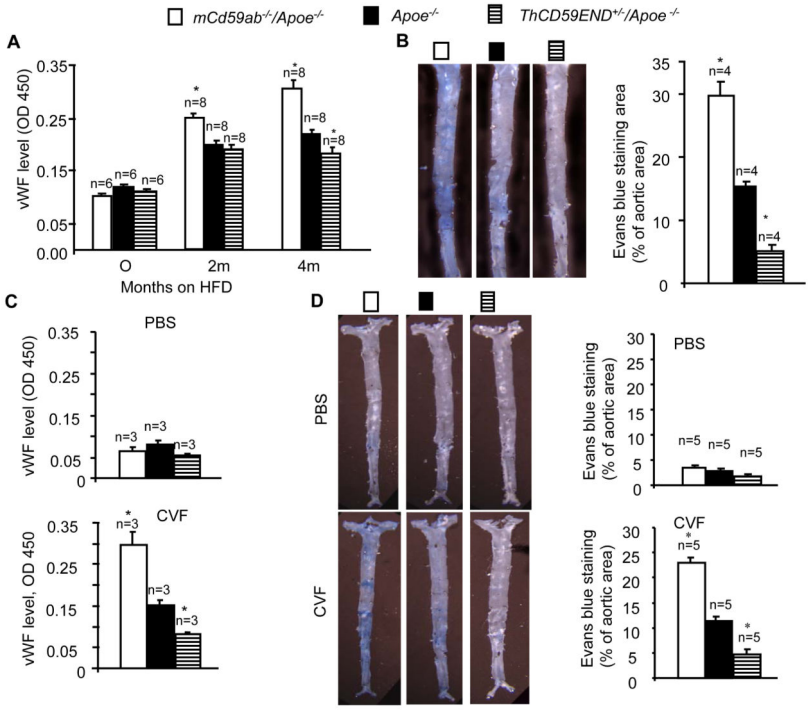

Figure 6. Complement activation mediates endothelial dysfunction

(**A**) Comparison of vWF levels among the three groups of mice fed on a HFD for 0, 2, and 4 months. HFD was initiated when mice reached 6-weeks of age. (**B**) Comparison of Evans blue staining area of the aorta. Mice were 4 months of age on normal chow. (**C**) The levels of vWF in six-week old mice 4 hours after i.p. administration of PBS (Top panel) or CVF (Bottom panel). (**D**) Evans blue staining of the aorta of the six-week old mice after i.p. administration of PBS (Top panel) or CVF (Bottom panel). Statistical significance ($P < 0.01$ *vs.* $Apoe^{-/-}$.) is indicated by an asterisk.

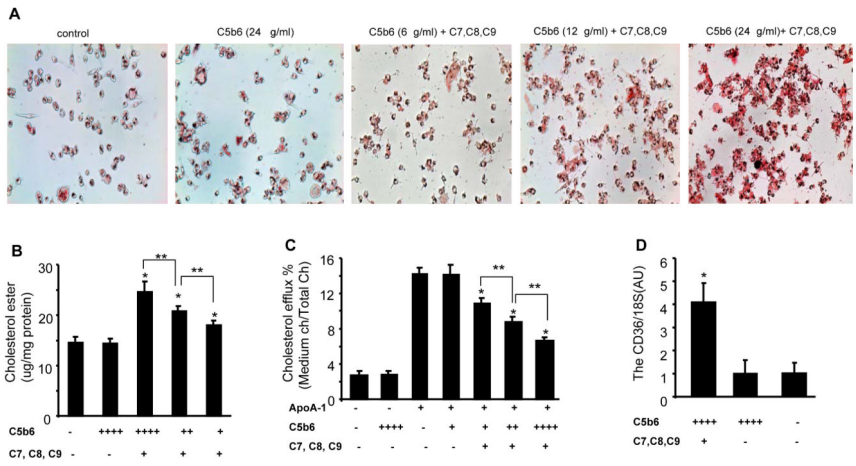

Figure 7. MAC promotes foam cell formation and up-regulates transcripts of growth factors and cytokines

(**A**) MAC-induced macrophage foam cell formation. Cells were incubated with Cu-oxLDL in the absence or presence of the indicated complement components as described in the methods section. (**B**) The cholesterol ester level of macrophage foam cells. Statistical significance ($P<0.01$ of MAC-induced cells *vs.* cells treated with C5b6 alone is indicated by (*); **$P<0.01$. (**C**) Cholesterol efflux of macrophage foam cells cultured in the absence or presence of the indicated complement components. Statistical significance ($P<0.01$ of MAC-induced cells *vs.* cells treated with C5b6 alone is indicated by (*); **$P<0.01$. (**D**) Real-time PCR analysis of CD36 transcripts in macrophage foam cells cultured in the absence or presence of the indicated complement components. Statistical significance ($P<0.01$ of MAC-induced cells *vs.* cells treated with C5b6 alone is indicated by (*). + for C5b6: 6 μg/ml and + for C7, C8 or C9: 24 μg/ml.