Complement Regulator CD59 Protects Against Atherosclerosis by Restricting the Formation of Complement Membrane Attack Complex

Gongxiong Wu, Weiguo Hu, Aliakbar Shahsafaei, Wenping Song, Martin Dobarro, Galina K. Sukhova, Rod R. Bronson, Guo-ping Shi, Russell P. Rother, Jose A. Halperin, Xuebin Qin

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 (Apo)E, 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 overexpression 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 atheroprotective role of CD59 and suggest that inhibition of MAC formation may provide a therapeutic approach for the treatment of atherosclerosis. (Circ Res. 2009;104:550-558.)

Key Words: CD59 ▪ complement ▪ endothelial dysfunction ▪ atherosclerosis ▪ occlusive coronary atherosclerosis and vulnerable plaque

Atherosclerosis is a chronic inflammatory condition in which immune and nonimmune 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 has been historically hampered by the lack of appropriate animal models, a difficulty that was overcome by the generation of apolipoprotein (Apo)E- and LDL receptor–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 3 activation cascades known as the classic, the alternative, and the lectin pathways. These 3 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 membrane attack complex (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 nonlethal transient changes in membrane permeability allowing increased influx and/or efflux of ions4 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.7 Several lines of evidence from human and animal studies indicate that CD59 is more relevant than decay-accelerating factor in protecting red blood cells from MAC formation and MAC-induced phenomena.7 Humans have only one CD59 gene, whereas mice have 2 Cd59 genes (termed as mCd59a and mCd59b).8 mCd59a-deficient mice (mCd59a−/−) showed intravascular hemolysis9; mCd59b-deficient mice (mCd59b−/−) exhibited a complement-mediated hemolytic anemia and platelet activation,10,11 most likely attributable 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 basic fibroblast growth factor and...
platelet-derived growth factor,5,13 as well as proinflammatory 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-114 and activates signaling pathways that promote proliferation of vascular smooth muscle cells.15 Extensive clinical data showing that MAC colocalized 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 LDL.18

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. To address this question, we also used human CD59 transgenic mice (ThCD59END+/-) 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 overexpression of human CD59 in the endothelium and hematopoietic cells (ThCD59END+/−/ApoE−/−) rendered ApoE−/− mice resistant to the development of atherosclerosis. Remarkably, the development of severe atherosclerosis in
ApoE \textsuperscript{-/-} (HFD for 4m)

sclerotic lesions in both aortic root and aortic surface (atherosclerosis in the context of a favorable proatherogenic


tudinally for either 2 or 4 months (Figure 1).

complement-mediated hemolytic anemia. 24 We generated

Institutional Animal Care and Use Committee.

Materials and Methods

Animal studies were approved by the Harvard Medical School

For a detailed description of the materials and methods used in the

There were no significant differences in the lipid profiles

mCd59ab \textsuperscript{-/-} mice was reversed by C5 blockage via

mCd59ab \textsuperscript{-/-}/ApoE \textsuperscript{-/-} mice was observed in

mCd59ab \textsuperscript{-/-}/ApoE \textsuperscript{-/-} mice, with 1 animal showing histological evidence

mCd59ab \textsuperscript{-/-}/ApoE \textsuperscript{-/-} vs ApoE \textsuperscript{-/-} (χ² test).

mCd59ab \textsuperscript{-/-}/ApoE \textsuperscript{-/-} mice exhibited a much higher incidence of occlusive coronary atherosclerosis than

mCd59ab \textsuperscript{-/-}/ApoE \textsuperscript{-/-} mice (supplemental Figure II, A through C).

mCd59ab \textsuperscript{-/-}/ApoE \textsuperscript{-/-} mice were crossed
to generate mCd59ab \textsuperscript{-/-}/ApoE \textsuperscript{-/-}, and ThCd59\textsuperscript{END+/-} and

ApoE \textsuperscript{-/-} mice were used to generate ThCd59\textsuperscript{END+/-}/ApoE \textsuperscript{-/-} mice (supplemental Figure II, A through C).

Mice were fed a high-fat diet (HFD) and followed longitudinally for either 2 or 4 months (Figure 1). mCd59ab \textsuperscript{-/-}/ApoE \textsuperscript{-/-} mice developed significantly more severe atherosclerotic lesions in both aortic root and aortic surface (as evaluated by en face preparation) than ApoE \textsuperscript{-/-} mice. By contrast, transgenic endothelial and hematopoietic cell-selective overexpression of hCD59 in ThCd59\textsuperscript{END+/-}/ApoE \textsuperscript{-/-} mice significantly reduced the development of atherosclerotic lesion as compared with those of ApoE \textsuperscript{-/-} mice (Figure 1A through 1D and supplemental Figure III). There were no significant differences in the lipid profiles of mCd59ab \textsuperscript{-/-}/ApoE \textsuperscript{-/-} versus ApoE \textsuperscript{-/-} mice or of ThCd59\textsuperscript{END+/-}/ApoE \textsuperscript{-/-} versus ApoE \textsuperscript{-/-} mice (supplemental Figure IV).

Consistent with the expression of a more severe atherosclerotic phenotype, the spontaneous mortality rate among

mCd59ab \textsuperscript{-/-}/ApoE \textsuperscript{-/-} mice was significantly higher than that observed among ApoE \textsuperscript{-/-} mice. In contrast, the transgenic expression of hCD59 significantly prolonged the mean survival time of ApoE \textsuperscript{-/-} mice (Figure 1E). In addition, the body weight of mice at the 4-month time point correlated inversely with the severity of atherosclerosis (supplemental Figure IV). mCd59ab \textsuperscript{-/-}/ApoE \textsuperscript{-/-} mice exhibited a much higher incidence of occlusive coronary atherosclerosis than ApoE \textsuperscript{-/-} mice, with 1 animal showing histological evidence of myocardial infarction (Figure 2). Additionally, the plaques developing among mCd59ab \textsuperscript{-/-}/ApoE \textsuperscript{-/-} 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 \textsuperscript{-/-} mice (Figure 3A through 3E). In contrast, plaques observed in hCd59\textsuperscript{END+/-}/ApoE \textsuperscript{-/-} mice exhibited significantly smaller necrotic cores than in those found in ApoE \textsuperscript{-/-} 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 \textsuperscript{-/-} or LDLR \textsuperscript{-/-} mice unless they carry additional gene modifications.29,30

Together, these results indicate that the systemic deficiency of CD59 in the context of ApoE \textsuperscript{-/-} genetic background makes
mice more sensitive, whereas overexpression of CD59 makes them more resistant, to the development of advanced atherosclerosis.

C9 Deposition Correlates Directly With the Severity of Atherosclerosis

Because 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, whereas the density of C9 was reduced in ThCD59END+/−/ApoE−/− (Figure 4A). Histological analysis showed that mCd59ab−/−/ApoE−/− mice had significantly higher and ThCD59END+/−/ApoE−/− mice significantly lower content of inflammatory (macrophage and T cells) and apoptotic cells than ApoE−/− mice (Figure 4B through 4D). 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

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 preincubated 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 2 months resulted in a significant attenuation of the atherosclerotic lesions (Figure 5B and 5C) 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 in-
creases MAC-induced endothelial injury and dysfunction and that overexpression of hCD59 in ThCD59END+/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, and stained the aortas with Evans blue, a direct marker of increased endothelial cell membrane permeability. Levels of vWF in 6-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, whereas ThCD59END+/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 4-month-old mice fed on nonatherogenic normal chow by staining with Evans blue. As expected, there were no macroatherosclerotic lesions in any of the 3 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 ThCD59END+/−/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, to 6-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 6C and 6D).

MAC Fosters Foam Cell Formation
A characteristic pathological feature of atherosclerosis is the formation of foam cells revealing the excessive accumulation of cholesteryl esters inside macrophages. To investigate whether MAC would foster foam cell formation, we challenged a mouse macrophage cell line with Cu-oxidized LDL 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 cholesteryl esters inside and reduced cholesterol efflux from MAC-treated...
macrophages (Figure 7B and 7C).\textsuperscript{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)\textsuperscript{32} but not for scavenger receptor-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) overexpression 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\textsuperscript{34} and with the protective effect of C6 deficiency reported in a rabbit model of atherosclerosis.\textsuperscript{19} In contrast, studies in C3- and C5-deficient mice seem to contradict the above interpretation of our experimental results.\textsuperscript{20,21} In these studies, C3-deficient ApoE\(^{-/-}\)/LDLR\(^{-/-}\) mice exhibited a more severe atherogenic lipid profile than C3-sufficient ApoE\(^{-/-}\)/LDLR\(^{-/-}\) mice.\textsuperscript{21} This is most likely attributable to the concomitant absence of C3a-des-Arg, also known as acylation-stimulating protein, a critical factor for the transport of lipids into adipocytes and maintenance of metabolic homeostasis.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 more than 10
months, in a vascular smooth muscle apoptotic mouse model, or in the Akt1 deficient mouse in ApoE−/− background. 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. 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 uses ApoE−/− mice deficient in both mCd59a and mCd59b proteins. Furthermore, using ApoE−/− mice overexpressing 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 atheroclerotic lesions could be attributable to focal complement activation or downregulation of complement regulatory proteins such as CD59. CRP, currently considered a marker of the inflammatory process associated with atherosclerosis and frequently found colocalized with MAC, is a potent complement activator in humans. 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 attributable either to reduced expression of CD59, as reportedly found in both atheromas and infarcted myocardium of human subjects, or to inactivation by glycation, as we have reported, could also explain increased MAC deposition in vascular walls of patients with atherosclerosis and in all target tissues of diabetic complications. The results of our work with mCd59ab−/−/ApoE−/− 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 downregulation 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 2 complimentary experimental methods, we documented that atherosclerosis-prone mCd59ab−/−/ApoE−/− mice exhibit a significant in-
The increased inflammatory content of atherosclerotic plaques correlates with the development of foam cells. Increasing evidence indicates that foam cells are derived from macrophages that have phagocytosed oxidized LDL (8). These foam cells are further influenced by anti-inflammatory cytokines (e.g., IL-4) to become atheroma. Both foam cells and atheroma contribute to the presence and content of inflammatory cells. Thus, it is likely that the MAC is an active participant in the atherosclerotic process.

Moreover, in vitro, we demonstrated that MAC treatment of macrophages significantly increased the formation of oxidized LDL-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 associated with the increased content of inflammatory cells, and the proliferation of the atherosclerotic plaques, which are more severe in mCd59-deficient mice because their cells are unprotected from the deleterious effect of MAC formation.

Importantly, the experimental results reported herein are consistent with 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.

Figure 7. MAC promotes foam cell formation. A, MAC-induced macrophage foam cell formation. Cells were incubated with Cu-oxidized LDL 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, MAC-induced cells vs cells treated with C5b6 alone; **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, MAC-induced cells vs cells treated with C5b6 alone; **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, MAC-induced cells vs cells treated with C5b6 alone).

**References**


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ONLINE DATA SUPPLEMENT

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

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EXPANDED MATERIAL AND METHODS

Animal models and characterization of atherosclerotic lesions: We obtained \( mCd59ab^{-/-} \) mice on a C57BL/6 (B6) genetic background by backcrossing the \( mCd59ab^{-/-} \) mice (originally generated on a B6/129 mixed genetic background) to B6 mice ten times. We generated \( ThCD59^{END+/-} \) mice on a B6 genetic background \(^1\). We crossed each of the resulting two strains with \( Apoe^{-/-} \) mice (B6 background, Jackson Laboratory) to produce \( mCd59ab^{-/-}/Apoe^{-/-} \) and \( ThCD59^{END+/-}/Apoe^{-/-} \) mice, respectively. The successful generation of these compound mice was determined by both PCR methods (for genotyping as described in Online Figure II, A-C), and FACS analysis of mCd59a and mCd59b proteins in \( mCd59ab^{-/-}/Apoe^{-/-} \)’s erythrocytes (for confirming the physical absence of mCd59a and mCd59b) or human CD59 in the \( ThCD59^{END+/-}/Apoe^{-/-} \)’s platelets [for confirming the over-expression of hCD59 (Data not shown)].

To induce atherosclerosis, we fed 6-week-old mice with high fat (or atherogenic) diet (C12108; Research Diets Inc.) containing 20.1% saturated fat, 1.37% cholesterol, and 0% sodium cholate\(^2\) for 8 or 16 weeks. At each time point, we sacrificed the mice by \( CO_2 \) asphyxiation, drew the blood by heart puncture, and stored the sera at -80°C. We analyzed the entire aorta from the heart outlet to the iliac bifurcation with Oil red-O as previously described in\(^3\). Briefly, the left ventricle (LV) was cannulated, and the animals were perfused with 0.9% NaCl followed by 10% phosphate-buffered formalin. The heart was embedded in paraffin, and serial 5-mm sections were taken in the midventricular
short axis and through the aortic valve. Sections were stained with hematoxylin/eosin or Masson’s trichrome, or Oil red-O. Occlusive coronary atherosclerosis (OCA) is defined by over 50% of occlusion in the coronary artery. We stained the sections of the aortic roots with Oil red-O and counterstained with hematoxylin. The percentage of the total area with positive color for each section was recorded and two independent investigators performed all measurements in a blinded fashion with respect to the origin of the coded samples. The mice that spontaneously died showed signs of illness such as less activity, weight loss, and less food-intake 3-4 days before they died. We sacrificed and necropsied severely ill mice. The mortality rate was calculated as: spontaneous dead mice/total mice X 100%.

**Immunofluorescence and histology:** We stained the frozen sections of aortic root with rabbit anti-rat C9, which cross-reacts with mouse C9 (Kindly provided by Dr. P. Morgan, University of Wales). We characterized the cellular components in the atherosclerotic plaques (aortic root) by immunostaining with the following reagents: (1) rat anti-mouse CD68, IgG2a (clone: FA-1, AbD Serotec) for mononuclear phagocytes; and (2) rat Anti-mouse CD4, (Lou/WS1) IgG2a, κ (clone: H129.19, BD Biosciences). All the primary antibodies were detected using corresponding FITC-conjugated secondary antibodies and compared with the negative controls which were stained with each non-immune isotype antibody and FITC-conjugated secondary antibody. Two independent investigators blindly scored the samples based on the area and intensity difference between stainings by each specific Ab and non-immune isotype. We
identified apoptotic nuclei in each section using a TUNEL technique using in situ apoptosis detection kit (Chemicon International) according to the manufacture’s instruction as described in\(^4\). The number of TUNEL-positive and negative nuclei in 3 microscope (60 X) fields per sample was done in a blind fashion by two different investigators. We also stained for Masson trichrome using the Accustain staining kit (HT-15) (Sigma-Aldrich) and H&E. The features of vulnerable plaques were quantified following the method described by Clarke, M.C. et al\(^5\).

**Administration of anti-C5 antibody.** We administered 6-week old mice with anti-C5 mAb clone BB5.1 or IgG isotype control clone 135.8 (40 mg/kg/day, i.p.) on days 0–2, followed by twice a week on days 3–60, and were maintained on a HFD for 60 days. At the end of the treatment period, we analyzed atherosclerotic lesions in those mice as described above.

**Evans blue staining:** At 4 hours after CVF (i.p., 30 \(\mu\)g/mouse) or PBS injection, we examined endothelial cell damage by Evans blue staining, as previously described\(^1\). Following administration of the dye, we harvested mouse aortas, opened them along the long axis and evaluated for blue-stained areas by light microscopy 26. We quantified immunofluorescence, histological results, and Evans blue staining areas using Image ProPlus 6.0 software.

**Von Willebrand factor (vWF) measurement:** We quantified serum vWF, a biomarker for endothelial damage by ELISA as described previously\(^1\).

**Serum lipid measurement:** Serum cholesterol and triglyceride profiles were measured at the Clinical Pathology Laboratory of Children’s Hospital, Boston.
**Macrophage foam cell formation:** Cells of the macrophage RAW264.7 line were cultured in RPMI-1640 with 10% fetal bovine serum containing cupric ion-oxidized low-density lipoprotein (Cu-oxLDL; 50µg/ml) and were untreated or treated with C5b6 (24µg/ml) alone or C5b-9 assembled by the serial addition of C5b6 (at 24µg/ml, 12µg/ml, or 6µg/ml), C7 (24µg/ml), C8 (24µg/ml) and C9 (24µg/ml) for 48 hours. Cells were stained with 0.4 % Oil Red O as described\(^6\). Photographs of foam cells were taken with a phase-contrast microscope, and at least 10 microscopic fields were counted from 4 different slides for the same treatment for quantification of foam cells.

**Intracellular lipid and cholesterol efflux analysis in macrophages:** The intracellular lipid analysis procedure was as previously described\(^7\), C5b-9 was assembled as described above for the experiments on foam cell formation. Free cholesterol and total cholesterol were determined by cholesterol/cholesteryl ester Quantitation Kit (EMD Chemicals). Cholesterol ester was estimated by subtracting free cholesterol from total cholesterol. Results were normalized to total cellular protein content.

APOA1-specific cholesterol efflux to the medium was determined as previously described\(^7\). Results were normalized to cellular protein content and expressed as the percentage of 3H-cholesterol in the medium divided by the total \(^3\)H-cholesterol in the cells and medium.

**Measurement of complement activity with the hemolytic assay:** Rabbit erythrocytes were washed in PBS, and a 2% suspension was incubated with an equal volume of mouse anti-rabbit erythrocyte antiserum (1/200 dilution in PBS; 15
min at 37°C). The Ab-sensitized rabbit erythrocytes were washed with veronal buffered saline (VBS++) and resuspended in VBS++ at 1% hematocrit. Fifty microliters of the cell suspension plus 50 µl of mouse serum (10% in VBS++) were added to 96-well plates in triplicate, and the plates were incubated for 30 min at 37°C. Non-lysed cells were removed by centrifugation, and hemoglobin in the supernatant was measured as $A_{414\text{nm}}$. Percent hemolysis in each well was calculated as previously published$^4$.

**Statistical analysis:** Experimental results were shown as the mean ± s.e.m. The mean values for biochemical data from two groups were compared by 2-tailed Student’s t-test. Differences among multiple groups were analyzed by two-way ANOVA using SigmaStat software. Mortality rates were analyzed by Log-rank (Mantel-Cox) test using GraphPad Prism 5 program software. All statistical tests with $P< 0.05$ were considered significant.
Supplemental references


Online Figure I: hCD59 is expressed in the platelets of ThCD59<sup>END</sup> mice. (A) Confirmation of platelet population by FACS analysis. Red curve: goat anti-rat IgG-FITC secondary antibody staining only and black curve: anti-CD41 antibody specifically recognizing platelet marker antigen CD41 plus goat anti-rat IgG-FITC secondary antibody. (B) hCD59 is present in the platelets of ThCD59<sup>END</sup> mice. Red curve: FITC secondary antibody and black curve: anti-human CD59 antibody (Bric 229) plus FITC secondary antibody.
Online Figure II: Generation of mCd59ab−/+/Apoe−/− and hCd59END+/−/Apoe−/− mice. mCd59ab−/+/Apoe−/− or hCd59END+/−/Apoe−/− mice were generated by crossing mCd59ab−/+ or hCD59END+/− with Apoe−/− to identify the experimental mice with PCR method. (A-C) Genotyping for determining Apoe−/−, mCd59ab−/+ and hCD59END+/− respectively.
Online Figure III. Representative figures for the atherosclerosis analysis of en face aorta and aortic root with Oil red O staining in the mice on a HFD for four months.
Online Figure IV: Body weight and Lipid profile in two and four month HFD feeding mice. (A) The levels of body weigh, serum cholesterol and triglyceride among three groups in the mice on a HFD for two months. (B) The levels of body weight, serum cholesterol and triglyceride in the mice on a HFD for four months HFD.
Online Figure V: CVF-induced endothelial damage in a *mCd59ab*<sup>-/-</sup>/Apoe<sup>-/-</sup> mouse. Endothelial damage to the aorta of the 6-week-old *mCd59ab*<sup>-/-</sup>/Apoe<sup>-/-</sup> mouse produced by CVF. Low (30X) and high (90X) magnification: Thrombosis is observed in the lumina of abdominal aorta associated with endothelial injury.