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Short Communication

CD55 Deficiency Protects against Atherosclerosis in ApoE-Deficient Mice via C3a Modulation of Lipid Metabolism

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Atherosclerosis, the leading cause of death in the Western world, is driven by chronic inflammation within the artery wall. Elements of the complement cascade are implicated in the pathogenesis, because complement proteins and their activation products are found in the atherosclerotic plaque. We examined the role of CD55, a membrane inhibitor of the complement component 3 (C3) convertase, which converts C3 into C3a and C3b, in atherosclerosis. CD55-deficient (*$CD55^{-/-}$*) mice were crossed onto the atherosclerosis-prone apolipoprotein E (apoE)-deficient (*$apoE^{-/-}$*) background. High fat–fed male *$apoE^{-/-}/CD55^{-/-}$* mice were strongly protected from developing atherosclerosis compared with *$apoE^{-/-}$* controls. Lipid profiling showed significantly lower levels of triglycerides, nonesterified fatty acids, and cholesterol in *$apoE^{-/-}/CD55^{-/-}$* mice than that in controls after high-fat feeding, whereas body fat in *$apoE^{-/-}/CD55^{-/-}$* mice content was increased. Plasma levels of C3 fell, whereas concentrations of C3adesArg (alias acylation stimulating protein; ASP), produced by serum carboxypeptidase N–mediated desargination of C3a, increased in nonfasted high fat–fed *$apoE^{-/-}/CD55^{-/-}$* mice, indicating complement activation. Thus, complement dysregulation in the absence of CD55 provoked increased C3adesArg production that, in turn, caused altered lipid handling, resulting in atheroprotection and increased adiposity. Interventions that target complement activation in adipose tissue should be explored as lipid-decreasing strategies. *(Am J Pathol 2011, 179:1601–1607; DOI: 10.1016/j.ajpath.2011.06.015)*

Atherosclerosis, long considered a passive process of accumulation of lipid in blood vessel walls accompanied by smooth muscle proliferation and culminating in loss of endothelial integrity, is now recognized as an active process with immune cells and mediators accumulating in forming plaques from the earliest stages, and inflammation central to disease progression.[1,2] Both innate immunity and adaptive immunity play roles, with mediators of both arms of the immune system present in the plaque.[3] Among the innate immune components, complement (C) and its activation products are abundant and suggested to play critical roles in atherogenesis, both directly through local cell damage and indirectly by attracting and activating immune cells.[4–9] C comprises three activation pathways, alternative, classical, and lectin, and activation of each has been shown in atherosclerosis.[10–12] Effector molecules generated during C activation include anaphylactic and chemotactic fragments (C3a, C5a), opsonic fragments (C4b, C3b), and the cytotoxic membrane attack complex (MAC). C3a and C5a may promote infiltration of inflammatory cells into the plaque[13,14]; this activity is regulated by carboxypeptidase N, which clips the carboxy-terminal arginine. Although C3adesArg is inactive as an inflammatory mediator, a growing body of literature reports that it has potent adipogenic activity, promoting lipid uptake, triglyceride synthesis, and storage in adipocytes.[15,16] C3-deficient mice, which cannot generate C3adesArg, have delayed postprandial triglyceride clearance, together with higher

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levels of nonesterified fatty acids (NEFAs), and significantly reduced adiposity than do wild types.[17,18]

Animal models have contributed to establishing the relevance of C to atherosclerosis. Almost 40 years ago, studies in fat-fed C6-deficient rabbits showed that absence of C6, an essential component of the MAC, markedly inhibited plaque formation,[19] findings replicated and extended more recently.[20] Atherosclerosis-prone mouse strains back-crossed onto C-deficient strains have been used to further explore roles of C. Fat-fed apolipoprotein E (*apoE*)-deficient (*apoE*$^{-/-}$) mice lacking C6 showed significantly attenuated disease, replicating findings in rabbits, whereas absence of CD59a, the principle murine regulator of MAC assembly, exacerbated disease.[21–23] In contrast, deficiency of C5, removing the capacity to form C5a and MAC, had no effect on atherosclerosis progression in *apoE*$^{-/-}$ mice,[24] whereas deficiency of C3, but not factor B, exacerbated plaque formation and caused hyperlipidemia on *apoE*$^{-/-}$/*ldlr*$^{-/-}$ or *ldlr*$^{-/-}$ backgrounds.[25,26]

CD55 (decay accelerating factor) is a 70-kDa membrane-bound C regulator that accelerates decay of the C3 convertase. To test the effect of CD55 deficiency on progression of atherosclerosis, *CD55*$^{-/-}$ mice were back-crossed onto the *apoE*$^{-/-}$ background and fed an atherogenic diet. Informed by our findings with CD59a deficiency, we anticipated that CD55 deficiency would exacerbate disease. Instead, deficiency of CD55 was highly protective for atherosclerosis; plaques were smaller and remained structurally simple. We here show that altered lipid handling resulting from C dysregulation is responsible for reduced atherogenesis in *CD55*$^{-/-}$ mice. The demonstration that C activation products markedly affect lipid handling and plaque formation will influence future strategies for treatment of atherosclerosis.

Materials and Methods

Reagents and Animals

All chemicals were purchased from Sigma-Aldrich (Poole, UK) or Fisher Scientific (Loughborough, UK). Fatty acid and lipid standards were from Nu-Chek-Pre Inc. (Elysian, MN) and Sigma-Aldrich, respectively. Silica gel G plates were from Merck KGaA (Darmstadt, Germany).

CD55 knockout (*CD55*$^{-/-}$) mice were provided by Prof Wenchao Song (University of Philadelphia, Philadelphia, PA) and back-crossed onto C57BL/6 for nine generations. *ApoE*$^{-/-}$ mice were originally provided by J. Breslow (Rockefeller University, New York, NY). The strain background of these original mice was 71% C57BL/6 and 29% 129. The *apoE*$^{-/-}$ mice were crossed with *CD55*$^{-/-}$ mice to generate *apoE*$^{-/-}$/*CD55*$^{-/-}$ double knockouts along with *apoE*$^{-/-}$ single knockouts; these sex-, strain-, and age-matched littermates provided the appropriate controls. Mice were genotyped by polymerase chain reaction with the use of genomic DNA extracted from tail tips.

Male mice aged 8 weeks were fed a high-fat diet, containing 21% (wt/wt) pork lard and supplemented with 0.15% (wt/wt) cholesterol (Special Diet Services, Witham, UK), for 12 weeks. Animals were housed in a specific pathogen-free environment. Some mice were deprived of food for 16 hours overnight to obtain baseline levels of various parameters. All studies and protocols were approved by the institutional ethics review committee and the United Kingdom Home Office and conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996).

Histology and Immunohistochemistry

Mice were anesthetized by intraperitoneal injection of sodium pentobarbitone and weighed before exsanguination by arterial perfusion via the abdominal aorta with PBS at a constant pressure of 100 mmHg, with outflow through the incised jugular veins. Brachiocephalic arteries were removed with a piece of the aortic arch and the stump of the right subclavian artery still attached to aid orientation during processing, immediately embedded in optimum cutting temperature compound (RA Lamb Ltd, Eastbourne, UK), and snap-frozen in liquid N_2.

Serial transverse 7-μm sections were cut along the brachiocephalic artery, starting from the proximal end. Sections were stained with Miller's Elastic/Van Gieson (Sigma-Aldrich). Macrophages and smooth muscle cells were identified with anti-murine macrophage mAb (diluted 1:100; F4/80; Serotec, Oxford, UK) and anti–α-smooth muscle actin mAb (diluted 1:100; clone α−1-A4; Sigma-Aldrich), respectively. Sections were fixed in ice-cold acetone and blocked with either avidin/biotin blocking kit (Vector Laboratories, Peterborough, UK) followed by 10% goat serum or Mouse-on-Mouse kit (Vector Laboratories). Blocked sections were incubated with appropriate biotinylated secondary antibodies: goat anti-rat Ig (Vector Laboratories; 3.5 μg/mL in 10% mouse serum) or anti-mouse Ig diluted as directed (Mouse-on-Mouse kit). Staining was developed with Fluorescein-Avidin D (diluted 1:200 in 2% bovine serum albumin in PBS; Vector Laboratories), and cell nuclei were counterstained with DAPI (Sigma-Aldrich).

Immunostaining for C activation used either rat anti-mouse C3b/iC3b mAb clone 2/11 (5 μg/mL; Hycult Biotech) or affinity-purified rabbit anti-rat/mouse C9 generated in house, proven reactive with MAC in mouse tissues (2 μg/mL).[23] For C3 and C9 staining, sections were fixed in acetone at 4°C, blocked in 2% bovine serum albumin in PBS, and, after staining with primary antibody, developed with either Alexa Fluor 488–labeled goat anti-rat (20 μg/mL; Invitrogen, Carlsbad, CA) or Alexa Fluor 594–labeled goat anti-rabbit IgG (20 μg/mL; Molecular Probes, Eugene, OR) respectively. Nuclei were counterstained with DAPI.

Negative controls included replacement of primary antibody with IgG isotype control. Staining was expressed as the percentage of lesion area staining positive, assessed by computerized image analysis (Image ProPlus 4.0; Media Cybernetics, Carlsbad, CA).

Histomorphometry

Five sections were taken per mouse at the same relative positions along the brachiocephalic artery and were assessed for the presence of plaque with the use of an established method.[25] Plaque area was calculated with image analysis as above.

Measuring Serum Triglycerides, Cholesterol, and NEFAs

Mice were sacrificed between 9 AM and 11 AM, blood (1 mL) was collected into tubes with or without EDTA, and serum or plasma was separated by centrifugation. Triglyceride and cholesterol levels were measured at the Clinical Biochemistry Laboratories, University Hospital Cardiff, on an Aeroset automated analyzer (Abbott Diagnostics, Berkshire, UK). For NEFAs, lipids were extracted and separated by one-dimensional thin-layer chromatography on 10 × 10-cm silica gel G plates, double developed with toluene/hexane/formic acid (140:60:1, v/v/v) for the entire plate, followed by hexane/diethyl ether/formic acid (60:40:1, v/v/v) to half height. Plates were sprayed with 0.05% (wt/v) 8-anilino-4-naphthosulphonic acid in methanol and viewed under UV light to show lipids. Free fatty acids were scraped from the plate and were identified and quantified by gas chromatography.

Measuring C3adesArg

C3adesArg was measured in a sandwich ELISA with the use of a pair of anti-mouse C3a mAbs, one unlabeled as capture (0.2 μg/mL), the other biotinylated as detection (0.5 μg/mL), and recombinant mouse C3a (100 to 0.78 ng/mL) as standards (all from BD Pharmingen, San Diego, CA). Appropriately diluted plasma samples were included. The assay was developed with streptavidin-peroxidase (1:5000; Jackson ImmunoResearch, West Grove, PA).

Measuring Mouse C3

Serum C3 levels were measured by ELISA essentially as previously described,[27] except that rat anti-mouse C3 (2 μg/mL; clone 11H9; Hycult Biotech) was used as the capture antibody. A standard curve of known concentrations, starting from 0.5 μg/mL, was produced with the use of purified mouse C3 (a kind gift from Dr Claire Harris; Cardiff University).

Body Fat Measurement

Percentage of body fat was measured by dual-energy X-ray absorptiometry scanning of whole animals with the use of a PIXImus scanner (Lunar Corp, Madison, WI) with small animal software.

Statistical Analysis

Data are expressed as mean ± SEM, and significance was tested by two-tailed unpaired Student's *t*-test (GraphPad Prism software, version 3.0; GraphPad Software Inc., San Diego, CA), with significance assumed at $P < 0.05$.

Results

Deficiency of CD55 Protects from Atherosclerosis in apoE$^{-/-}$ *Mice*

Matched *apoE*$^{-/-}$ and *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice were sacrificed at 20 weeks of age after 12 weeks on a high-fat diet, and the extent of atherosclerosis was assessed in the brachiocephalic arteries, a known site of predilection for plaque development.[28] Plaque cross-sectional area, assessed at multiple sites along the vessel, was reduced threefold in *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice compared with *apoE*$^{-/-}$ controls ($54.7 \pm 11.2 \times 10^3$ μm^2 versus $155.2 \pm 16.8 \times 10^3$ μm^2; $P < 0.001$; Figure 1, A–C). Plaque stage and complexity were further explored by measuring smooth muscle cell content and macrophage infiltration; smooth muscle cells as a proportion of total cell number in *apoE*$^{-/-}$/*CD55*$^{-/-}$ plaques were significantly lower than in *apoE*$^{-/-}$ plaques (9.3% ± 2.0% versus 18.0% ± 2.8%; $P < 0.05$; Figure 1, D–F). Plaque macrophage content was similar in the groups (19.4% ± 6.5% versus 18.8% ± 4.2%; Figure 1, G–I).

To address whether CD55 deficiency influenced local C activation, plaques were stained for C3 fragments and MAC. C3 fragment deposition was assessed with mAb 3/26, a neoepitope-specific mAb that specifically detects C3b, iC3b, and C3c in tissues, whereas MAC was detected with affinity-purified anti-rat/mouse C9. Percentages of plaque area stained for C3b/iC3b/C3c and MAC were twofold reduced in plaques from *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice compared with *apoE*$^{-/-}$ controls (C3b/iC3b/C3c: 28.0% ± 8.1% versus 57.3% ± 7.7%; $P < 0.05$; Figure 1, J–L; MAC: 17.9% ± 3.5% versus 30.5% ± 4.0%; $P < 0.05$; Figure 1, M–O).

CD55 Deficiency Is Associated with Reduced Serum Triglyceride and Cholesterol Levels

To test whether the absence of CD55 affected lipid handling, lipid levels were measured in *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice and in *apoE*$^{-/-}$ controls at 8 weeks old on normal diet and at 20 weeks old after 12 weeks on a high-fat diet. At 8 weeks, triglyceride levels were markedly reduced in the *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice than in the *apoE*$^{-/-}$ controls (2.1 ± 0.1 mmol/L versus 5.1 ± 0.6 mmol/L; $P < 0.01$; Figure 2A); cholesterol levels were not significantly different between these groups (16.1 ± 1.4 mmol/L versus 18.2 ± 0.8 mmol/L; Figure 2B). After 12 weeks of fat feeding, triglyceride levels were little changed, as expected in the *apoE*$^{-/-}$ model,[29] and remained significantly lower in the *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice compared with the *apoE*$^{-/-}$ controls (1.7 ± 0.2 mmol/L versus 3.6 ± 0.5 mmol/L; $P < 0.01$; Figure 2A). Cholesterol levels were increased after 12 weeks of fat feeding in both groups but were significantly lower in the *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice than in the *apoE*$^{-/-}$ controls (29.0 ± 1.8 mmol/L versus 38.3 ± 2.5 mmol/L; $P < 0.01$; Figure 2B). Plasma levels of NEFAs were measured in mice before and after being fed the high-fat diet. Significant increases in NEFA concentrations were seen in both groups after 12 weeks of a

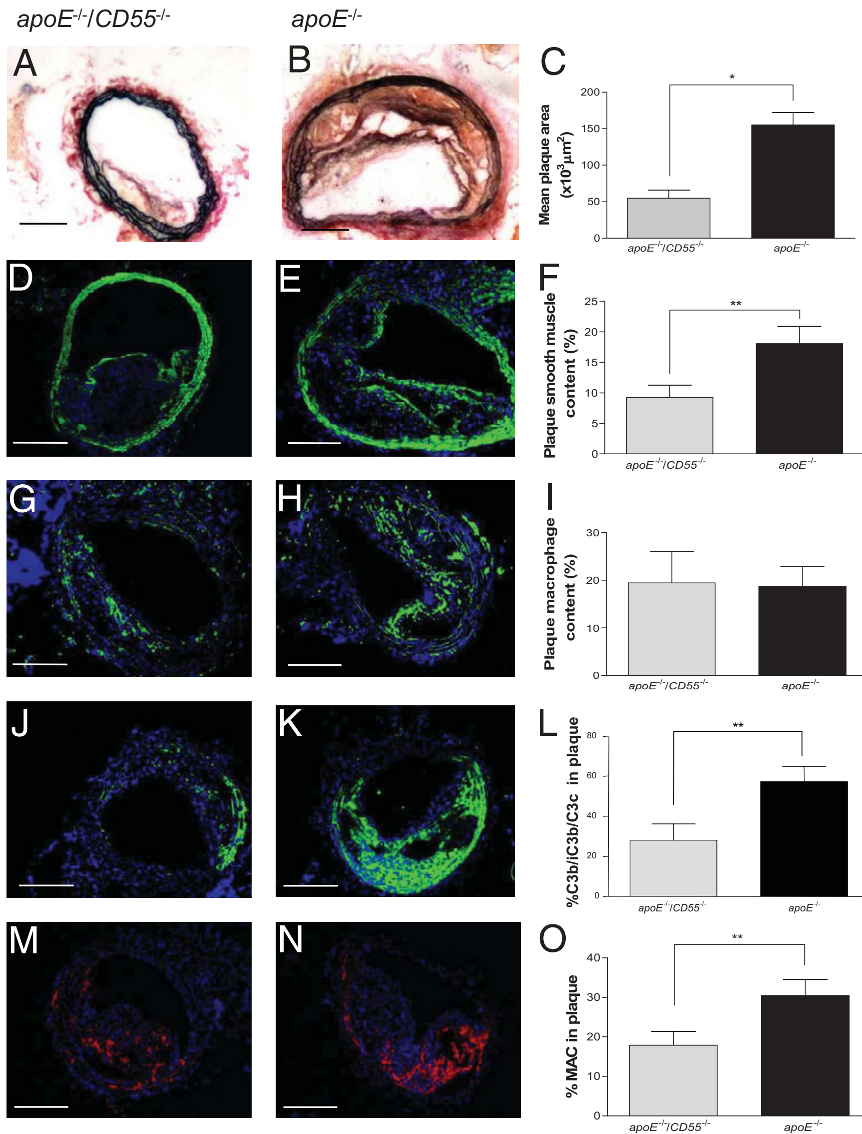

Figure 1. Assessment of atherosclerosis in brachiocephalic arteries of *apoE*$^{-/-}$/*CD55*$^{-/-}$ and *apoE*$^{-/-}$ control mice after 12 weeks of high-fat feeding. Histologic appearance of representative sections of brachiocephalic arteries from a single experiment that compared *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice (**A, D, G, J,** and **M**) with *apoE*$^{-/-}$ control mice (**B, E, H, K,** and **N**); both groups had been fed a high-fat diet for 12 weeks. Sections were stained with Miller's/Elastic Van Gieson (**A** and **B**; $n = 13$ and 12, respectively); immunostained for α-smooth muscle actin (smooth muscle cells; **D** and **E**; $n = 7$ and 7, respectively); F/480 (macrophages; **G** and **H**; $n = 8$ and 6, respectively); C3 fragments (**J** and **K**; $n = 7$ and 6, respectively), or MAC deposition (**M** and **N**; $n = 7$ and 7, respectively). Scale bars: 200 μm (**A, B, D, E, G, H, J, K, M,** and **N**). **C, F, I, L,** and **O:** Composite data from each group compiled from digital analyses of individual sections as described in *Materials and Methods*. **C:** Average plaque size for the two groups as determined from five individual sections taken from each mouse. **F, I, L,** and **O:** Mean percentage of plaque area staining for smooth muscle cells, macrophages, C3b/iC3b/C3c, and MAC, respectively. Mean values ± SEM are represented. $^{*}P < 0.0001$, $^{**}P < 0.05$.

high-fat diet (*apoE*$^{-/-}$/*CD55*$^{-/-}$: 0.153 ± 0.016 mg/mL versus 0.333 ± 0.041 mg/mL before and after the high-fat diet, respectively; $P < 0.001$; *apoE*$^{-/-}$: 0.121 ± 0.006 mg/mL versus 0.505 ± 0.046 before and after the high-fat diet, respectively; $P < 0.001$; Figure 2C). Although no significant differences were observed in NEFA levels between *apoE*$^{-/-}$*CD55*$^{-/-}$ and *apoE*$^{-/-}$ mice before fat feeding, levels were significantly lower in *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice than in *apoE*$^{-/-}$ controls after 12 weeks on a high-fat diet (0.333 ± 0.041 mg/mL versus 0.505 ± 0.046 mg/mL; *apoE*$^{-/-}$/*CD55*$^{-/-}$ and *apoE*$^{-/-}$, respectively; $P < 0.01$; Figure 2C).

CD55 Deficiency Is Associated with Increased C3 Turnover and Plasma C3adesArg Levels

C3adesArg, also known as ASP, is a stable product of C3 activation and a potent adipokine that stimulates uptake of triglycerides and NEFAs, enhances triglyceride synthesis and storage, and inhibits triglyceride lipolysis in adipose tissue.[30–32] Circulating nonfasting C3 and its activation product C3adesArg (Figure 2, D and E, respectively) were measured before and after the high-fat feeding in *apoE*$^{-/-}$/*CD55*$^{-/-}$ and *apoE*$^{-/-}$ mice. C3 levels were significantly reduced in both *apoE*$^{-/-}$/*CD55*$^{-/-}$ and *apoE*$^{-/-}$ mice after 12 weeks of high-fat feeding compared with before the high-fat feeding values (*apoE*$^{-/-}$/*CD55*$^{-/-}$: 0.42 ± 0.03 mg/mL versus 0.3 ± 0.04 mg/mL; $P < 0.01$, respectively; *apoE*$^{-/-}$ 0.48 ± 0.05 mg/mL versus 0.22 ± 0.05 mg/mL; $P < 0.05$, respectively). C3 levels were not significantly different between *apoE*$^{-/-}$/*CD55*$^{-/-}$ and *apoE*$^{-/-}$ mice at either time point (Figure 2D). Plasma C3adesArg levels were markedly lower after being deprived of food overnight compared with not being deprived of food regardless of age, diet, and genotype of the mice (compare Figure 2, E with F). Fasting C3a levels were similar in all groups of mice (Figure 2F); in contrast, nonfasting C3adesArg levels were significantly higher in *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice than in *apoE*$^{-/-}$ mice but only on the high-fat diet (Figure 2E; 1.58 ± 0.14

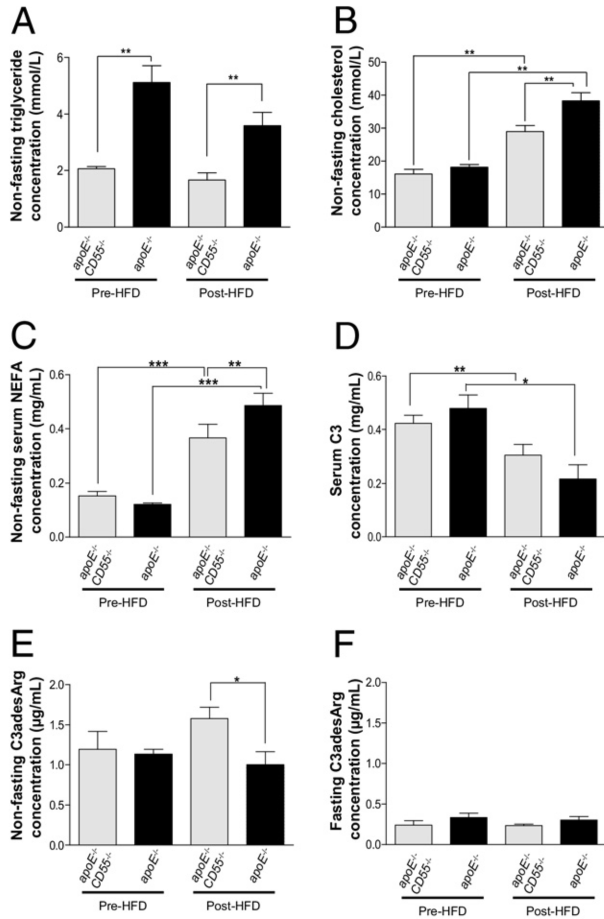

Figure 2. Assessment of lipid profile, C3 and C3adesArg levels in *apoE*$^{-/-}$/*CD55*$^{-/-}$ and *apoE*$^{-/-}$ mice. Serum levels of triglycerides, cholesterol, NEFAs, C3, and plasma C3adesArg were assayed in *apoE*$^{-/-}$/*CD55*$^{-/-}$ and *apoE*$^{-/-}$ mice at 8 weeks of age (before high-fat diet; Pre-HFD) and at 20 weeks after 12 weeks of high-fat diet (Post-HFD). **A:** Serum triglyceride levels (Pre-HFD, $n = 4$ and 6, respectively; Post-HFD, $n = 12$ and 15, respectively). **B:** Serum cholesterol levels (Pre-HFD, $n = 4$ and 6, respectively; Post-HFD, $n = 12$ and 15, respectively). **C:** Serum NEFA levels (Pre-HFD, $n = 6$ and 6, respectively; Post-HFD, $n = 12$ and 14, respectively). **D:** Total serum C3 levels (Pre-HFD, $n = 6$ and 6, respectively; Post-HFD, $n = 6$ and 4, respectively). **E:** Plasma nonfasting C3adesArg levels (Pre-HFD, $n = 6$ and 8, respectively; Post-HFD, $n = 11$ and 12, respectively). **F:** Plasma fasting C3adesArg levels (Pre-HFD, $n = 8$ and 7, respectively; Post-HFD, $n = 11$ and 11, respectively). Mean values ± SEM are represented. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.0001$.

μg/mL versus 1.00 ± 0.16 μg/mL; $P < 0.01$). The data show that high-fat feeding in *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice is associated with increased C3 turnover and higher circulating levels of C3adesArg compared with *apoE*$^{-/-}$ controls.

ApoE$^{-/-}$/CD55$^{-/-}$ *Mice Have Increased Adipose Tissue Content*

To test whether the observed changes in circulating lipid levels and C3 activation in high fat–fed *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice affected fat storage, body weight and composition were compared between the groups. The high-fat diet caused significant increases in body weight for both groups, without any significant differences between *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice and *apoE*$^{-/-}$ controls (*apoE*$^{-/-}$/*CD55*$^{-/-}$: 27.32 ± 0.6 g versus 38.16 ± 2.12 g; $P <$ 0.0001; *apoE*$^{-/-}$: 30.64 ± 0.39 g versus 41.38 ± 1.17 g; $P < 0.0001$).

Both adipose mass and percentage of body fat were measured with dual-energy X-ray absorptiometry (Figure 3, A and B, respectively). After 12 weeks of high-fat feeding CD55 deficiency was associated with increased adipose tissue mass and percentage of body fat, significant in the latter case, in *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice compared with *apoE*$^{-/-}$ controls (Figure 3B; before high-fat diet: 17.43% ± 0.64%; 17.8% ± 1.11% *apoE*$^{-/-}$/*CD55*$^{-/-}$ versus *apoE*$^{-/-}$, respectively; after high-fat diet: 28.63% ± 4.18%; 19.54% ± 1.77% *apoE*$^{-/-}$/*CD55*$^{-/-}$ versus *apoE*$^{-/-}$, respectively; $P < 0.05$).

Discussion

We here demonstrate that CD55 deficiency in fat-fed male *apoE*$^{-/-}$ mice markedly attenuated the progression of atherosclerosis. Plaques were infrequent, small, and structurally simple, having fewer smooth muscle cells and less neo-intimal thickening in comparison with their *apoE*$^{-/-}$ controls, which displayed large, advanced plaques at the same time point.

We previously showed that MAC deposition correlates with plaque stage, probably reflecting the increased amount of C-activating cell debris in advanced lesions.[23] The data show that, despite global absence of the C regulator CD55, C activation is reduced in the early, simple plaques formed in *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice. These results are surprising in that the absence of an important C regulator would be predicted to exacerbate injury in a disease characterized by C activation; indeed, deficiency of CD59a markedly exacerbated MAC formation and disease in atherosclerosis-prone mice.[21–23] Two recent studies tested effects of CD55 deficiency in atherosclerosis models. Leung et al[27] showed exacerbated disease and increased plaque size in female *CD55*$^{-/-}$/*ldlr*$^{-/-}$ mice compared with the *ldlr*$^{-/-}$ controls. This contradictory result is probably because of the different atherosclerosis-prone background; deficiency of ldlr causes a more severe metabolic derangement and deranged lipid profiles compared with apoE deficiency.[29]

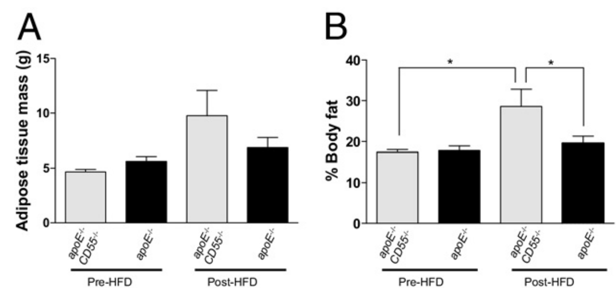

Figure 3. Measurement of adipose tissue mass and percentage of body fat in *apoE*$^{-/-}$/*CD55c*$^{-/-}$ and *apoE*$^{-/-}$ mice. Adipose tissue mass (**A**) and percentage of body fat (**B**) were measured by dual-energy X-ray absorptiometry in *apoE*$^{-/-}$/*CD55*$^{-/-}$ and *apoE*$^{-/-}$ mice at 8 weeks of age (before high-fat diet; Pre-HFD; $n = 6$ and 8, respectively) and at 20 weeks of age after 12 weeks of high-fat diet (Post-HFD, $n = 6$ and 9, respectively). Mean values ± SEM are represented. *$P < 0.05$.

An et al[33] placed male and female *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice on a high-fat diet for 8 or 16 weeks. Plaque area, measured at end point in the aortic arch, was not significantly different in *apoE*$^{-/-}$/*CD55*$^{-/-}$ and *apoE*$^{-/-}$ groups; however, when separated according to sex, there was a clear trend, not remarked on by the investigators, toward smaller plaques in male *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice after 16 weeks of the high-fat diet. Group sizes after dividing by sex were small, and the differences observed were not significant. These published findings support our observation, probably made significant in our study by the use of larger group sizes and male mice. Indeed, many inbred mouse strains, including C57BL/6, have a constitutively more active hemolytic C system because of differences in activation and lytic pathways.[34–36] Remarkably, *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice had a markedly altered lipid profile, dominated by low circulating triglyceride, NEFA, and cholesterol levels; increased triglyceride uptake into adipose tissue and fat synthesis resulted in increased adiposity. Two hypotheses were considered to explain the above findings: first, that CD55 played a direct role in lipid metabolism independent of its role in regulating C, and, second, that the absence of CD55 caused increased C3 turnover that in turn influenced lipid handling. Others have shown that C3 deficiency on atherosclerosis-prone backgrounds exacerbated hyperlipidemia and atherogenesis.[25,26] This observation favored a role for C3, provoking us to ask whether C3 turnover was altered in *CD55*$^{-/-}$ mice and contributed to the observed lipid profile and atheroprotection. C3 levels were significantly reduced in both *apoE*$^{-/-}$ and *apoE*$^{-/-}$*CD55*$^{-/-}$ mice when fed a high-fat diet, suggesting that a high-fat diet provoked C3 consumption. Remarkably, although C3adesArg levels were low in all mice after being deprived of food overnight, levels in mice not deprived of food were markedly higher and were significantly increased in high fat–fed *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice compared with *apoE*$^{-/-}$ controls. These data imply that the observed changes in lipid profile were caused by diminished capacity, in the absence of CD55, to regulate the C3 convertase, resulting in dysregulation of the ASP pathway and increased production of C3adesArg/ASP in response to circulating chylomicrons or very low density lipoprotein particles.[37–39] Although hypercholesterolemia has been the focus of most attention in atherosclerosis, elevated triglyceride and NEFA levels are recognized as independent risk factors for atherogenesis in humans and models.[40,41] Indeed, the antiatherogenic effects of lipoic acid in the *apoE*$^{-/-}$ model were shown to be due to its triglyceride-lowering properties.[42] It is therefore probable that the observed antiatherogenic effect of CD55 deficiency is due to the combined effects of lower plasma levels of triglycerides, cholesterol, and NEFAs.

These findings support *in vitro* and *in vivo* studies that implicate chylomicrons and/or very low density lipoprotein as the primary physiological trigger for C3adesArg/ASP production from adipose tissue.[43,44] *In vitro*, exposure of adipose tissue to purified chylomicrons switched on the ASP pathway, markedly increasing synthesis of precursor C3 and generation of C3adesArg, whereas, *in vivo*, chylomicronemia acutely increased plasma C3 and C3adesArg/ASP levels.[43,44] The amount of C3adesArg/ASP generated will depend on the local activity and regulation of C3 convertase, in turn depending on the presence and abundance of C regulators. CD55 is expressed on adipose cells[45,46] and will therefore contribute to local regulation of the convertase; in its absence, the convertase will persist and generate more C3adesArg/ASP.

Because C3adesArg/ASP is such a critical factor in maintaining lipid homeostasis, a persistent increase in local and circulating levels could be predicted to affect lipid profiles in precisely the manner observed in the *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice of the current study by provoking increased uptake of triglycerides and NEFAs into adipose tissue, resulting in reduced plasma levels. We chose to use male mice in this study because of proven differences in C activity and have suggested that failure to detect this effect of CD55 in previous studies was due to inclusion of female mice. A further potential confounder is the effect of sex hormones on the ASP pathway. Progesterone down-regulates the expression of C5L2, the receptor for C3adesArg, in 3T3 adipocytes, potentially rendering female mice less responsive to C3adesArg and blunting the atheroprotective effect of CD55 deficiency seen in male *apoE*$^{-/-}$/*CD55*$^{-/-}$ mice.[47]

The chain of events shown here explains both atheroprotection and increased adiposity in the mice and also provides an explanation for the enigmatic observation that C3-deficient mice, lacking the capacity to generate C3adesArg, show accelerated disease in models of atherosclerosis.[25,26] The data show that C3adesArg itself, or agents mimicking its lipid-modulating effects, might be of benefit in the treatment of atherosclerosis and related diseases.

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References

1. Glass C, Witztum J: Atherosclerosis. The road ahead. Cell 2001, 104:503–516
2. Jawień J: New insights into immunological aspects of atherosclerosis. Pol Arch Med Wewn 2008, 118:127–131
3. Hansson G, Libby P, Schönbeck U, Yan Z: Innate and adaptive immunity in the pathogenesis of atherosclerosis. Circ Res 2002, 91: 281–291
4. Kostner K: Activation of the complement system: a crucial link between inflammation and atherosclerosis? Eur J Clin Invest 2004, 34:800–802
5. Haskard D, Boyle J, Mason J: The role of complement in atherosclerosis. Curr Opin Lipidol 2008, 19:478–482
6. Meuwissen M, van der Wal A, Niessen H, Koch K, de Winter R, van der Loos C, Rittersma S, Chamuleau S, Tijssen J, Becker A, Piek J:

Colocalisation of intraplaque C reactive protein, complement, oxidised low density lipoprotein, and macrophages in stable and unstable angina and acute myocardial infarction. J Clin Pathol 2006, 59:196–201
7. Niculescu F, Niculescu T, Rus H: C5b-9 terminal complement complex assembly on apoptotic cells in human arterial wall with atherosclerosis. Exp Mol Pathol 2004, 76:17–23
8. Vlaicu R, Niculescu F, Rus H, Cristea A: Immunohistochemical localization of the terminal C5b-9 complement complex in human aortic fibrous plaque. Atherosclerosis 1985, 57:163–177
9. Yasojima K, Schwab C, McGeer E, McGeer P: Complement components, but not complement inhibitors, are upregulated in atherosclerotic plaques. Arterioscler Thromb Vasc Biol 2001, 21:1214–1219
10. Matthijsen R, de Winther M, Kuipers D, van der Made I, Weber C, Herias M, Gijbels M, Buurman W: Macrophage-specific expression of mannose-binding lectin controls atherosclerosis in low-density lipoprotein receptor-deficient mice. Circulation 2009, 119:2188–2195
11. Madsen H, Videm V, Svejgaard A, Svennevig J, Garred P: Association of mannose-binding-lectin deficiency with severe atherosclerosis. Lancet 1998, 352:959–960
12. Torzewski J, Bowyer D, Waltenberger J, Fitzsimmons C: Processes in atherogenesis: complement activation. Atherosclerosis 1997, 132: 131–138
13. Zwirner J, Werfel T, Wilken H, Theile E, Götze O: Anaphylatoxin C3a but not C3a(desArg) is a chemotaxin for the mouse macrophage cell line J774. Eur J Immunol 1998, 28:1570–1577
14. Oksjoki R, Laine P, Helske S, Vehmaan-Kreula P, Mäyränpää M, Gasque P, Kovanen P, Pentikäinen M: Receptors for the anaphylatoxins C3a and C5a are expressed in human atherosclerotic coronary plaques. Atherosclerosis 2007, 195:90–99
15. MacLaren R, Cui W, Cianflone K: Adipokines and the immune system: an adipocentric view. Adv Exp Med Biol 2008, 632:1–21
16. Sniderman AD, Cianflone K, Summers L, Fielding B, Frayn K: The acylation-stimulating protein pathway and regulation of postprandial metabolism. Proc Nutr Soc 1997, 56:703–712
17. Murray I, Sniderman A, Cianflone K: Mice lacking acylation stimulating protein (ASP) have delayed postprandial triglyceride clearance. J Lipid Res 1999, 40:1671–1676
18. Murray I, Havel P, Sniderman A, Cianflone K: Reduced body weight, adipose tissue, and leptin levels despite increased energy intake in female mice lacking acylation-stimulating protein. Endocrinology 2000, 141:1041–1049
19. Geertinger P, Soerensen H: On the reduced atherogenic effect of cholesterol feeding in rabbits with congenital complement (C6) deficiency. Artery 1977, 1:177–184
20. Schmiedt W, Kinscherf R, Deigner H, Kamencic H, Nauen O, Kilo J, Oelert H, Metz J, Bhakdi S: Complement C6 deficiency protects against diet-induced atherosclerosis in rabbits. Arterioscler Thromb Vasc Biol 1998, 18:1790–1795
21. Yun S, Leung V, Botto M, Boyle J, Haskard D: Brief report: accelerated atherosclerosis in low-density lipoprotein receptor-deficient mice lacking the membrane-bound complement regulator CD59. Arterioscler Thromb Vasc Biol 2008, 28:1714–1716
22. Wu G, Hu W, Shahsafaei A, Song W, Dobarro M, Sukhova G, Bronson R, Shi G, Rother R, Halperin J, Qin X: Complement regulator CD59 protects against atherosclerosis by restricting the formation of complement membrane attack complex. Circ Res 2009, 104:550–558
23. Lewis RD, Jackson CL, Morgan BP, Hughes TR: The membrane attack complex of complement drives the progression of atherosclerosis in apolipoprotein E knockout mice. Mol Immunol 2010, 47:1098–1105
24. Patel S, Thelander E, Hernandez M, Montenegro J, Hassing H, Burton C, Mundt S, Hermanowski-Vosatka A, Wright S, Chao Y, Detmers P: ApoE(-/-) mice develop atherosclerosis in the absence of complement component C5. Biochem Biophys Res Commun 2001, 286:164–170
25. Persson L, Borén J, Robertson A, Wallenius V, Hansson G, Pekna M: Lack of complement factor C3, but not factor B, increases hyperlipidemia and atherosclerosis in apolipoprotein E-/- low-density lipoprotein receptor-/- mice. Arterioscler Thromb Vasc Biol 2004, 24:1062–1067
26. Buono C, Come C, Witztum J, Maguire G, Connelly P, Carroll M, Lichtman A: Influence of C3 deficiency on atherosclerosis. Circulation 2002, 105:3025–3031
27. Leung V, Yun S, Botto M, Mason J, Malik T, Song W, Paixao-Cavalcante D, Pickering M, Boyle J, Haskard D: Decay-accelerating factor suppresses complement C3 activation and retards atherosclerosis in low-density lipoprotein receptor-deficient mice. Am J Pathol 2009, 175:1757–1767
28. Williams H, Johnson J, Carson K, Jackson C: Characteristics of intact and ruptured atherosclerotic plaques in brachiocephalic arteries of apolipoprotein E knockout mice. Arterioscler Thromb Vasc Biol 2002, 22:788–792
29. Joven J, Rull A, Ferré N, Escolà-Gil J, Marsillach J, Coll B, Alonso-Villaverde C, Aragones G, Claria J, Camps J: The results in rodent models of atherosclerosis are not interchangeable: the influence of diet and strain. Atherosclerosis 2007, 195:e85–e92
30. Cianflone K, Sniderman A, Walsh M, Vu H, Gagnon J, Rodriguez M: Purification and characterization of acylation stimulating protein. J Biol Chem 1989, 264:426–430
31. Germinario R, Sniderman A, Manuel S, Lefebvre S, Baldo A, Cianflone K: Coordinate regulation of triacylglycerol synthesis and glucose transport by acylation-stimulating protein. Metabolism 1993, 42:574–580
32. Maslowska M, Sniderman A, Germinario R, Cianflone K: ASP stimulates glucose transport in cultured human adipocytes. Int J Obes Relat Metab Disord 1997, 21:261–266
33. An G, Miwa T, Song W, Lawson J, Rader D, Zhang Y, Song W: CD59 but not DAF deficiency accelerates atherosclerosis in female ApoE knockout mice. Mol Immunol 2009, 46:1702–1709
34. Holt D, Botto M, Bygrave A, Hanna S, Walport M, Morgan B: Targeted deletion of the CD59 gene causes spontaneous intravascular hemolysis and hemoglobinuria. Blood 2001, 98:442–449
35. Beurskens FJ, Kuenen JD, Hofhuis F, Fluit AC, Robins DM, Van Dijk H: Sex-limited protein: in vitro and in vivo functions. Clin Exp Immunol 1999, 116:395–400
36. Tanaka S, Suzuki T, Nishioka K: Assay of classical and alternative pathway activities of murine complement using antibody-sensitized rabbit erythrocytes. J Immunol Methods 1986, 86:161–170
37. Gao Y, Gauvreau D, Cianflone K: Hormone and pharmaceutical regulation of ASP production in 3T3-L1 adipocytes. J Cell Biochem 2010, 109:896–905
38. Gao Y, Gauvreau D, Cui W, Lapointe M, Paglialunga S, Cianflone K: Evaluation of chylomicron effect on ASP production in 3T3-L1 adipocytes. Acta Biochim Biophys Sin (Shanghai) 2011, 43:154–159
39. Murray I, Sniderman A, Cianflone K: Enhanced triglyceride clearance with intraperitoneal human acylation stimulating protein in C57BL/6 mice. Am J Physiol 1999, 277:E474–E480
40. Lusis A, Mar R, Pajukanta P: Genetics of atherosclerosis. Annu Rev Genomics Hum Genet 2004, 5:189–218
41. Singh U, Zhong S, Xiong M, Li T, Sniderman A, Teng B: Increased plasma non-esterified fatty acids and platelet-activating factor acetylhydrolase are associated with susceptibility to atherosclerosis in mice. Clin Sci (Lond) 2004, 106:421–432
42. Zhang W, Bird K, McMillen T, LeBoeuf R, Hagen T, Frei B: Dietary alpha-lipoic acid supplementation inhibits atherosclerotic lesion development in apolipoprotein E-deficient and apolipoprotein E/low-density lipoprotein receptor-deficient mice. Circulation 2008, 117: 421–428
43. Maslowska M, Scantlebury T, Germinario R, Cianflone K: Acute in vitro production of acylation stimulating protein in differentiated human adipocytes. J Lipid Res 1997, 38:1–11
44. Scantlebury T, Maslowska M, Cianflone K: Chylomicron-specific enhancement of acylation stimulating protein and precursor protein C3 production in differentiated human adipocytes. J Biol Chem 1998, 273:20903–20909
45. Gronthos S, Franklin D, Leddy H, Robey P, Storms R, Gimble J: Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol 2001, 189:54–63
46. Festy F, Hoareau L, Bes-Houtmann S, Péquin A, Gonthier M, Munstun A, Hoarau J, Césari M, Roche R: Surface protein expression between human adipose tissue-derived stromal cells and mature adipocytes. Histochem Cell Biol 2005, 124:113–121
47. Wen Y, Wang H, MacLaren R, Lu H, Hu XF, Cianflone K: Sex steroid hormones induce acylation stimulating protein resistance in 3T3-L1 adipocytes. J Cell Biochem 2008, 105:404–413