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Absence of CD36 protects against atherosclerosis in ApoE knock-out mice with no additional protection provided by absence of scavenger receptor A I/II

Sai Kuchibhotla[1†], Difernando Vanegas[1†], David J. Kennedy[1], Ella Guy[2], George Nimako[1], Richard E. Morton[1], and Maria Febbraio[1*]

[1]*Department of Cell Biology, Cleveland Clinic, 9500 Euclid Ave., NC-10, Cleveland, OH 44195, USA; and* [2]*Department of Medicine, Weill Medical College of Cornell University, New York, NY 10021, USA*

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Aims The role of scavenger receptors in atherogenesis is controversial as a result of conflicting reports and a recent hypothesis suggesting that scavenger receptor absence would enhance the pro-inflammatory, pro-atherogenic milieu. This study addresses the effect of combined absence of scavenger receptors CD36 and SRA I/II on atherosclerosis lesion development in the apolipoprotein E knock-out (apoE°) model.
Methods We created background-related strains of apoE°, scavenger receptor A I/II knock-out (SRA°)/apoE°, CD36 knock-out (CD36°)/apoE°, and CD36°/SRA°/apoE° mice that were >99% C57Bl/6. Four-week-old mice were fed a Western diet for 12 weeks and were assessed for lesion burden/morphology, risk factors for atherosclerosis, inflammatory mediators, and macrophage function.
Results There was a 61 and 74% decrease in total aortic lesion area in CD36°/apoE° males and females, respectively, compared with apoE° controls. The absence of SRA was protective (32% decrease in lesion) in female mice. The combined absence of CD36 and SRA provided no further protection in either gender. Macrophages from mice lacking CD36 had decreased pro-inflammatory characteristics and less migration to a pro-inflammatory stimulus. Plasma levels of cytokines/chemokines showed that CD36°/apoE° and CD36°/SRA°/apoE° mice had a less pro-inflammatory phenotype compared with apoE° and SRA°/apoE° mice. Oblivious mice in the apoE° background ruled out potential 'passenger gene' effects in the case of CD36.
Conclusion These results provide new insights into the pro-atherogenic mechanisms of CD36 by implicating processes other than modified lipoprotein uptake.

1. Introduction

Scavenger receptors represent a diverse group of receptors expressed by macrophages with roles in normal homeostasis, including recognition of repeated pattern molecules on pathogens, apoptotic cells, and aberrant lipoproteins.[1] In this study, we addressed the hypothesis that combined absence of the two major scavenger receptors involved in the recognition of pro-atherogenic low-density lipoprotein (LDL), CD36, and scavenger receptor A I/II (SRA) would lead to greater protection against atherosclerosis than absence of either alone. This hypothesis was based on previous data demonstrating that CD36 and SRA were involved in uptake of pro-atherogenic-modified forms of LDL and led to foam cell formation, and that the absence of these receptors was protective in at least some models of murine atherosclerosis.[2–4] It had also been previously shown that the two receptors had affinity for different forms of modified LDL.[5] Thus, it was reasoned, since the *in vivo* ligand had not been precisely characterized, absence of both receptors might achieve a level of protection greater than either alone.

A recent report by Moore *et al.*[6] suggested that scavenger receptors were anti-atherogenic, a hypothesis in contrast to much of the data supporting a pro-atherogenic role. They further hypothesized that the absence of scavenger receptors would lead to a more pro-inflammatory milieu that would promote atherogenesis as a result of lack of uptake of modified LDL.[6] In this study, we obtained SRA knock-out (SRA°)/CD36 knock-out (CD36°) and SRA°/apolipoprotein E knock-out (apoE°) mice from Moore *et al.* to create background-related strains of apoE°, SRA°/apoE°, CD36°/apoE°, and CD36°/SRA°/apoE°. Four-week-old mice were

†These authors contributed equally.
*Corresponding author. Tel: +1 216 445 5605; fax: +1 216 444 9404.
E-mail address: febbram@ccf.org

fed a Western diet for 12 weeks, and indices of atherosclerosis and inflammation were compared. We characterized the morphology of lesions and probed macrophage function. The results provide further insight into the role of scavenger receptors in atherogenesis. To rule out the effect of potential 'passenger genes', we obtained oblivious (OBL) mice[7] that were created by exposure of pure C57Bl/6 mice to the chemical carcinogen *N*-ethyl-*N*-nitrosurea, and were found to harbour a mutation in CD36 that renders them null.[7] These mice further demonstrated the pro-atherogenic nature of CD36, and ruled out potential overriding passenger gene effects.

2. Methods

2.1 Animals and diets

Mice were housed in an AAALAC accredited facility, with access *ad libitum* to food and water. All procedures conform to the *Guide for the Care and Use of Laboratory Animals* (NIH publication 85–23, revised 1996). SRA°/CD36° and SRA°/apoE° mice (seven times backcrossed to C57Bl/6)[3] were provided by M. Freeman (MGH), and were mated to derive CD36°/SRA°/apoE° mice. We crossed CD36°/SRA°/apoE° mice to apoE° mice to derive apoE° and CD36°/apoE° mice. OBL mice were generously provided by B. Beutler (Scripps Institute), and were crossed to apoE° mice (Jackson Laboratory, 100% C57Bl/6). Offspring were genotyped by polymerase chain reaction (PCR),[7] and littermate-derived apoE° OBL wild-type (apoE°/OBL WT) and apoE°/OBL mice (CD36 null) strains were obtained. All mice were >99% C57Bl/6. Mice were screened by PCR,[8] and protein absence confirmed by western blot (CD36) or cholesterol assay (apoE); the absence of SRA could not be confirmed at the protein level due to the fact that the antibody available does not recognize SRA from C57Bl/6 mice.[9] Mice were weaned at 4 weeks, and placed on a Western diet for 12 weeks (Harlan Teklad 88137; this diet does not contain cholate).

2.2 Plasma parameters

Tail vein blood was collected 1 week prior to sacrifice after an overnight fast in 38 mM/L citric acid (trisodium salt), 75 mM/L sodium citrate, and 136 mM/L glucose (ACD; 8:1 dilution blood:anticoagulant), and centrifuged to isolate plasma. Non-esterified fatty acids (NEFA) and triacylglycerol were assayed as described previously.[8,10] Blood was collected at sacrifice (pentobarbital overdose, 200 mg/kg) from non-fasted mice by cardiac puncture into ACD for cholesterol assay.[8,10] The results are expressed as mg/dL ± SE.

2.3 Lipoprotein analysis

Mice were fasted overnight, sacrificed by pentobarbital overdose, and blood was collected into ACD by cardiac puncture midway (6 weeks) through the study. Plasma lipoprotein profiles were determined by fast protein liquid chromatography using 10 μL aliquots on two Superose 6 columns with continuous online detection of cholesterol in the eluant.[11,12] Mean results ± SE are expressed as percentage distribution of the specific lipoprotein relative to the total.

2.4 Glucose tolerance testing

Glucose tolerance testing was performed on overnight fasted mice after 11 weeks on Western or normal chow diets as described previously.[12] The results ($n = 6$–12 per genotype/gender) are expressed as mean ± SE.

2.5 Morphometry, histology and immunohistochemistry

Aorta dissection was performed as described previously.[8,10,13] Lesion area was expressed as percentage of total aortic area ± SE. We also divided the aorta into three parts (aortic arch, descending aorta, and bifurcation and below) and calculated the percentage lesion area at these sites ± SE and expressed the results as a function of total lesion area. Statistical significance was determined by one-way ANOVA, followed by Bonferroni's multiple comparison test (for greater than two groups), or Mann–Whitney test (groups of two).

Aortic sinus lesions were obtained and stained with oil red O as described previously,[10] or hematoxylin and eosin (H&E), or Masson's trichrome by standard methods. For detection of free cholesterol, paraformaldehyde-fixed aortic sinus sections were stained for 2 h with 0.05 mg/mL filipin solution (Sigma).

Some sections were used for immunohistochemistry to detect apoptotic cells [dUTP terminal nick end-labelling, TUNEL assay (Roche)] and macrophages (anti-mouse CD68 antibody, 1:200, Serotec); nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). Tissues were mounted in Fluorsave (Calbiochem) and visualized under a fluorescent microscope using appropriate filters. Photographs were taken at ×100 or ×200 magnification, and in some cases, overlaid to create a composite.

2.6 Peritoneal macrophages

Thioglycollate-elicited macrophages were collected by peritoneal lavage into PBS after sacrifice by carbon dioxide (CO_2) asphyxiation 4 days after intraperitoneal injection of 1 mL sterile 4% thioglycollate broth.

2.7 Real-time reverse transcriptase polymerase chain reaction

Total RNA was extracted using the RNAeasy kit (QIAGEN). Five micrograms of total RNA were used for reverse transcription using oligo dt (Invitrogen). Primers and probes were purchased from Applied Biosystems and real-time reverse transcriptase–PCR (RT–PCR) carried out on an Icycler (Biorad). PCR reactions were performed using 20× Assays on Demand gene expression assay mix and Taqman universal PCR master mix (Applied Biosystems). PCR conditions were 40 cycles of 95°C, 10 min, 95°C, 1 min, and 62°C, 1 min. Experiments were performed in duplicate on cells pooled from three mice, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. Results for receptor expression are expressed as mean fold value ± SD over GAPDH × 10^2. The results for cytokine expression are expressed as mean fold value ± SD over GAPDH × 10^3.

2.8 Detection of reactive oxygen species

Thioglycollate-elicited macrophages were plated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). Non-adherent cells were removed by washing, and cells were incubated overnight at 37°C, 5% CO_2. Cells were treated with 50 μg/mL oxLDL (copper oxidized LDL, prepared as described previously,[14] TBARS $>2<10$) in media containing 1% FBS for 24 h. 5-Carboxy-2′7′-dichlorodihydrofluorescein diacetate (DCF, Molecular Probes) was prepared as a 0.5 mM/L stock in dimethyl sulfoxide (DMSO). Cells were loaded with DCF at a final concentration of 1 μM/L in PBS at 37°C for 30 min (or incubated with DMSO alone). The percentage of fluorescent cells was determined in ten 20× fields in two separate experiments. In a separate assay, intracellular reactive oxygen species (ROS) was further evaluated using the Fc-Oxyburst assay (Molecular Probes) and LDL oxidized with the myeloperoxidase, hydrogen peroxide, nitric oxide generating system (NO_2LDL) as described previously.[14] In a third assay, we measured nitrotyrosine as a marker for peroxynitrite

by ELISA (Northwest Life Sciences Specialties, LLC). Thioglycollate-elicited macrophages were harvested and pooled from apoE° and CD36°/apoE° mice fed a Western diet for 2 weeks. Cells were incubated with NO_2LDL or native LDL for 24 h and peroxynitrite was measured in triplicate and expressed as per milligram protein.

2.9 Cytokine/chemokine detection

Levels of IL-1α in culture media were determined using a Quantikine kit specific for mouse (R&D Systems). The results are expressed as mean ± SD. Levels of other cytokines/chemokines were compared using the Proteome Profiler Mouse Cytokine Array, Panel A Array

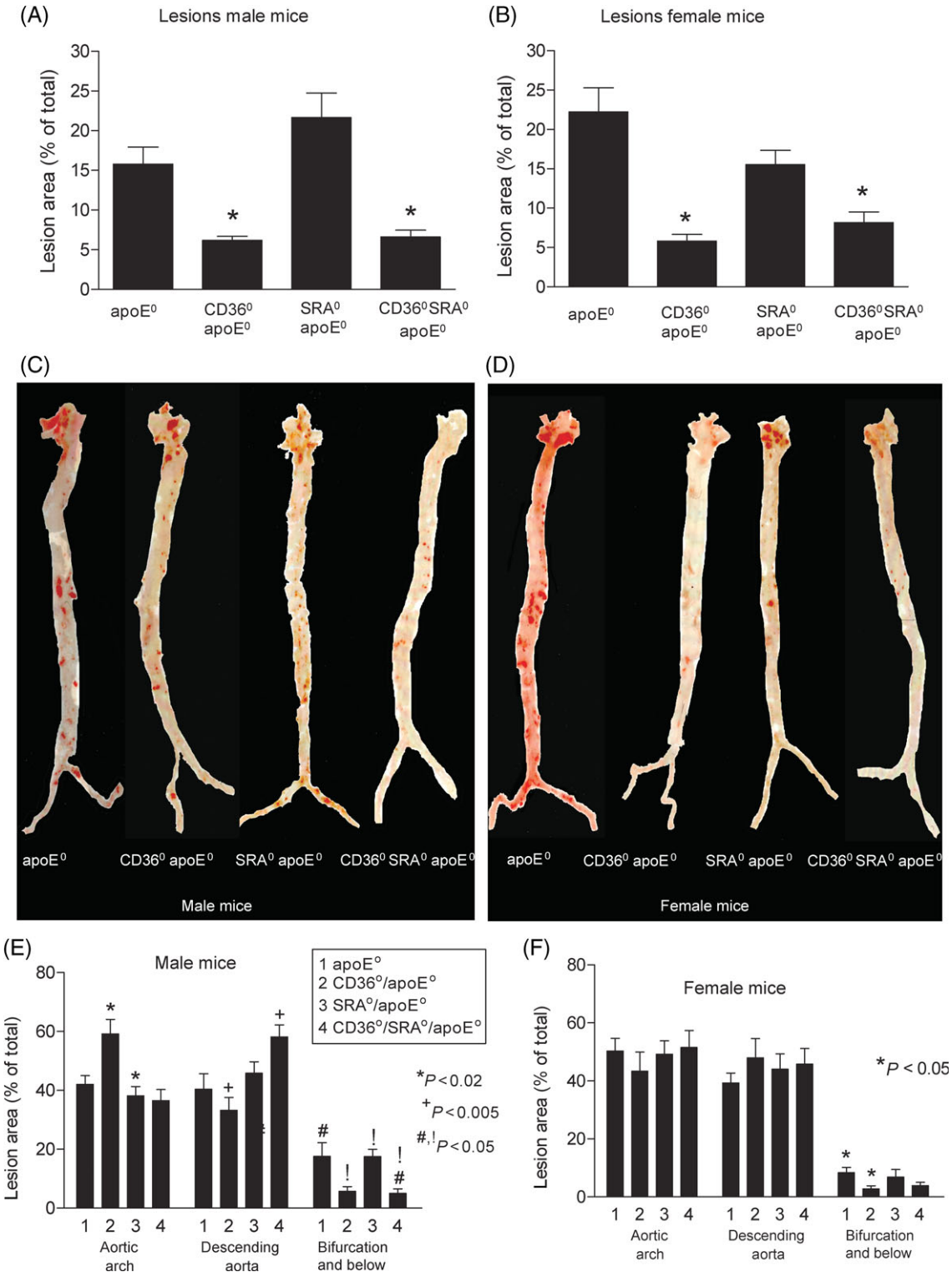

Figure 1 Aortic lesion area expressed as percentage of total aortic area in male (*A*) and female (*B*) apoE°, CD36°/apoE°, SRA°/apoE°, and CD36°/SRA°/apoE° mice. Aortas were stained with oil red O, opened longitudinally, and scanned. Strains lacking CD36 (CD36°/apoE° and CD36°/SRA°/apoE°) showed significant decrease in lesion area when compared with apoE°. Aortae from male mice (*C*) and female mice (*D*) are shown. Aortic lesion area at different sites along the aortic tree in male (*E*) and female mice (*F*). The results are expressed as percentage of total lesion area.

Kit (R&D Systems), as per the manufacturer's instructions. Pooled plasmas from 3–6 female mice of each genotype were compared in duplicate for relative expression. Values are based on density of infrared signal assessed using the Licor Odyssey for analysis.

2.10 Migration assay

A total of 1.5×10^5 thioglycollate-elicited macrophages were added to the upper chamber of a modified Boyden chamber in DMEM media. The lower chamber contained DMEM + 10 ng/mL macrophage chemoattractant protein (MCP)-1 (recombinant mouse MCP-1, Biosource) with or without 50 μg/mL oxLDL. After 16 h incubation, the transwell filter was stained with DAPI and migrated cells counted (blindly) in ten 20× fields. The experiment was repeated three times and the results are expressed as mean ± SE.

2.11 Statistical analysis

Student's unpaired *t*-test or ANOVA was used; $P < 0.05$ was considered significant.

3. Results

As shown in *Figure 1A* and *B*, there was dramatically less atherosclerotic lesion area in both males and females of mouse strains which lacked CD36. Compared with apoE° males ($n = 11$), there was a 60.8 and 58% decrease in lesion area in CD36°/apoE° ($n = 18$) and CD36°/SRA°/apoE° males ($n = 17$), respectively. Compared with apoE° females ($n = 20$), there was a 73.9 and 63.3% decrease in lesion area in CD36°/apoE° ($n = 11$) and CD36°/SRA°/apoE° females ($n = 18$), respectively. Aortas from each genotype are shown in *Figure 1C* and *D*. We observed no significant difference in lesion area between apoE° and SRA°/apoE° males ($n = 12$). SRA°/apoE° females ($n = 12$) had a 30.2% reduction in lesion area compared with apoE° females that was significant at the 0.05 level when analysed with the less conservative Newman–Keuls multiple comparison post test.

When we compared different sites of the aorta for lesion predilection, we observed that in male CD36°/apoE° mice, the greatest percentage of lesion area was in the aortic arch (59.14 ± 4.97%, *Figure 1E*). CD36°/SRA°/apoE° had the greatest percentage of lesion area in the descending aorta. Interestingly, male mice lacking CD36 were protected against lesion development at the bifurcation and below. In female mice, percent lesion area was similar in all strains at all sites except at the bifurcation and below. Here, CD36°/apoE° female mice were significantly protected from lesion development; CD36°/SRA°/apoE° also had less percentage of lesion area but it did not reach statistical significance. Protection at this site is consistent with our first report on lesion development in male and female CD36°/apoE° mice.[10]

Because the mice are not of homogenous background and there is the potential for effect(s) from 'passenger genes' that are closely linked to the targeted allele, we obtained mice that are null for CD36 created in the C57Bl/6 background (OBL mice),[7] and crossed them into the apoE°. After a 12 week Western diet regiment, apoE°/OBL male mice had significantly less lesion area compared with littermate-derived apoE°/OBL WT mice [*Figure 2*; 11.59 ± 1.19% ($n = 15$) vs. 6.56 ± 0.97% ($n = 17$), $P = 0.0005$]. Female apoE°/OBL mice were also protected against lesion development [*Figure 2*; 15.58 ± 2.26% ($n = 17$) vs. 5.11 ± 0.57 ($n = 16$), $P < 0.0001$]. Although a passenger gene effect cannot be ruled out for the SRA allele, it did not appear to exert a dominant effect on lesion development since the results for CD36°/SRA°/apoE° and CD36°/apoE° were similar.

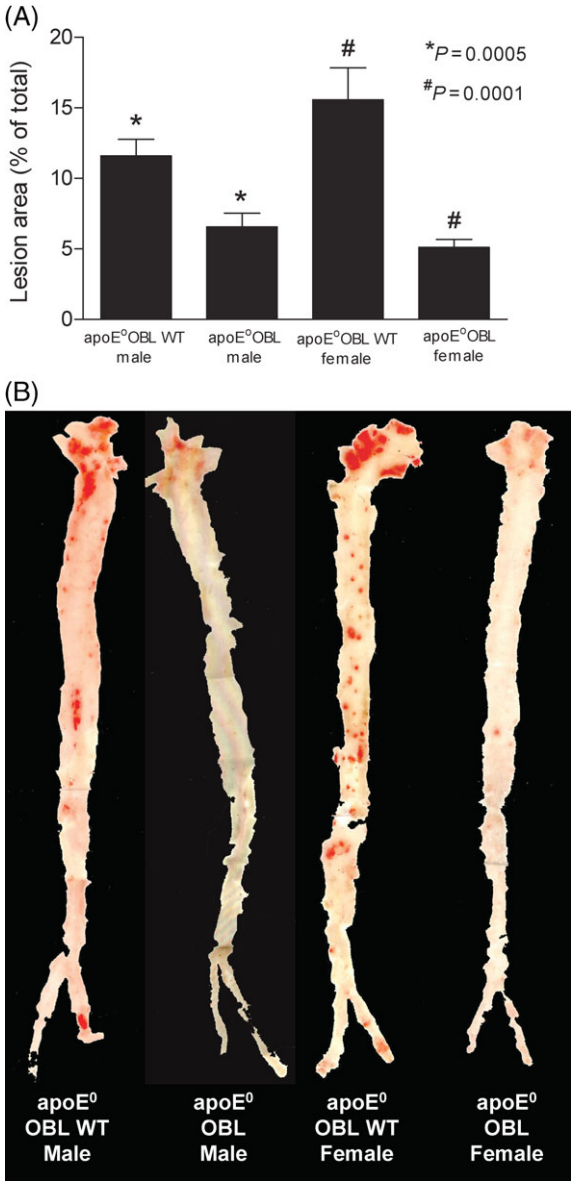

Figure 2 (*A*) Aortic lesion area expressed as percentage of total aortic area in male and female apoE°/OBL wild type and apoE°/OBL mice. (*B*) Representative aortae from male and female mice.

Microscopic analysis of lesions stained with oil red O at the level of the aortic sinus (*Figure 3A–D*) revealed highly complex lesions (containing cholesterol clefts and regions of acellularity and necrosis) and lesions of less complexity (primarily macrophage foam cells) in all genotypes. Although this location is not linear for lesion development over time in Western diet fed mice,[15] it provides an area to compare lesion morphology. Sections were stained with Masson's Trichrome to determine whether there were differences in lesion collagen content. Collagen deposition was dependent on lesion stage, and thus similarly staged lesions were compared. No differences were discerned (data not shown). Further analysis using an antibody specific

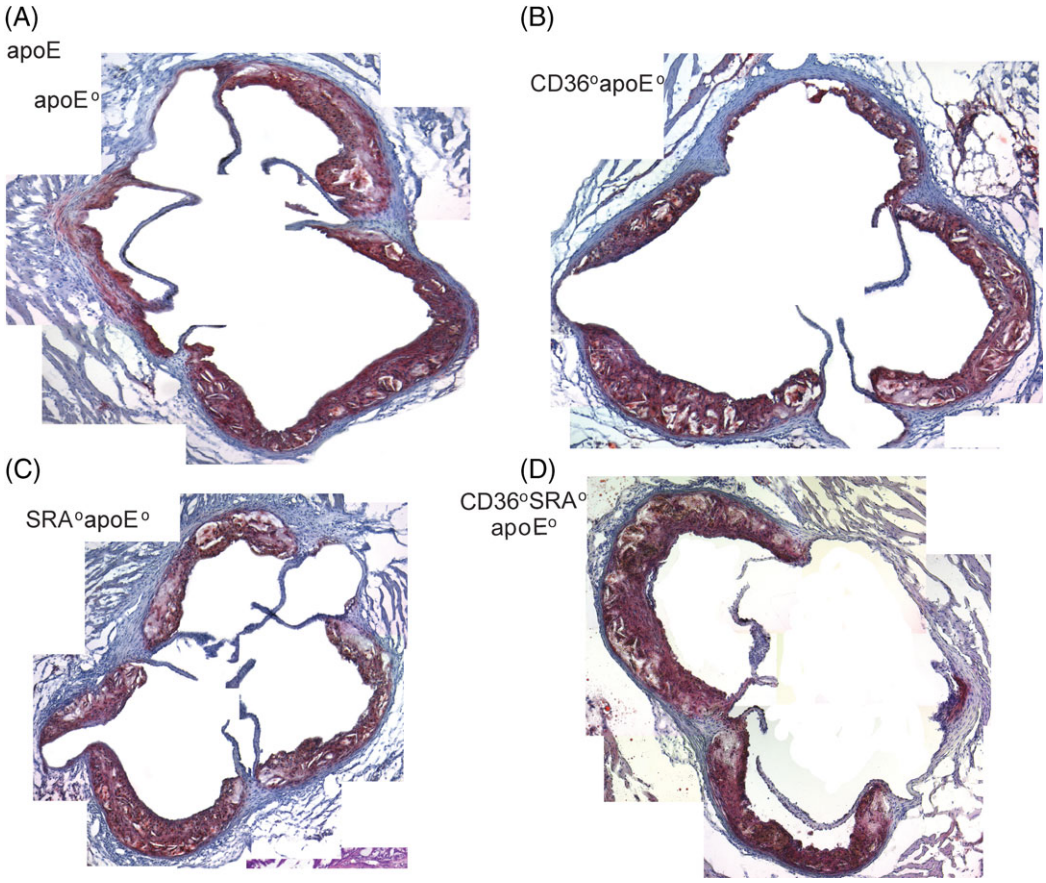

Figure 3 Lesion morphology at the aortic sinus. Composite sections (×100 magnification) from female (*A*) apoE°, (*B*) CD36°/apoE°, (*C*) SRA°/apoE°, and (*D*) CD36°/SRA°/apoE° mice are shown. Complex lesions, consisting of macrophage rich foam cells (oil red O intense areas), cholesterol clefts, acellular areas, and areas of lipid deposition and necrosis, characterize all four genotypes.

for macrophages (anti-CD68) revealed intense luminal staining of the lesions (*Figure 4A*). High power examination also showed macrophages within the lesion, and this varied in extent in accordance with the stage of progression of the lesion (*Figure 4B*). Using the TUNEL assay (*Figure 4C*), the greatest density of apoptotic cells was noted in regions with apparent necrotic cores. We assessed free cholesterol deposition because of reports that associated increased free cholesterol with apoptosis in atherosclerotic lesions.[16,17] SRA was shown to be essential for this effect.[18] Filipin staining for free cholesterol revealed intense staining at the medial muscle layer, which has been observed previously[17] (*Figure 4D*). Macrophage dense areas atop established lesions were positive in all genotypes, particularly in apoE° mice. Filipin staining also appeared nearby to areas of necrosis. Mice expressing SRA did not have more TUNEL positive areas or cells compared with SRA°, but this may be because the lesions are not significantly advanced.[19] The apparent higher level of free cholesterol in lesions of apoE° mice is consistent with reports that these macrophages accumulate greater amounts of both free and esterified cholesterol compared with macrophages lacking CD36 or SRA.[6]

Neither male nor female mice demonstrated significant differences in total cholesterol (*Tables 1* and 2). However, lipoprotein analysis (*Table 3*) revealed significantly greater very low-density lipoprotein (VLDL) cholesterol levels (20–21% higher, depending on comparison genotype) in apoE° mice. Because chylomicron remnants, the pro-atherogenic particles in the plasma of apoE° mice, segregate in this fraction, this suggests an increase in atherosclerosis risk. High-density lipoprotein (HDL) cholesterol levels were lowest in apoE° mice, but irrespective of genotype or gender, the HDL cholesterol fraction was $<1\%$ of the total cholesterol, and thus unlikely to impact on lesion development. Differences in the levels of triacylglycerol and NEFA did not appear to correlate specifically with aortic lesion area.

Because CD36 has been linked to insulin resistance,[20] and insulin resistance is a risk factor for cardiovascular disease, we performed glucose tolerance tests in conscious mice. Before diet treatment, all genotypes of mice cleared a bolus of glucose similarly within 2 h (data not shown). After diet intervention, the greatest area under the curve was measured in apoE° males and females (*Figure 5A* and *B*; similar results were obtained with apoE°/OBL WT and apoE°/OBL mice but are not shown). This may contribute to their risk of lesion development.

To determine whether there was altered regulation of lipid influx genes that might account for our results, we performed real-time RT–PCR on macrophages isolated from the four genotypes. ApoE° mice had 206.5 ± 9.2- and 10.6 ± 2.8-fold increases in expression of the scavenger receptors *CD36* and *SRA*, respectively. Compared with expression on macrophages from chow fed apoE° mice, these results show approximately a two-fold increase in *CD36* expression

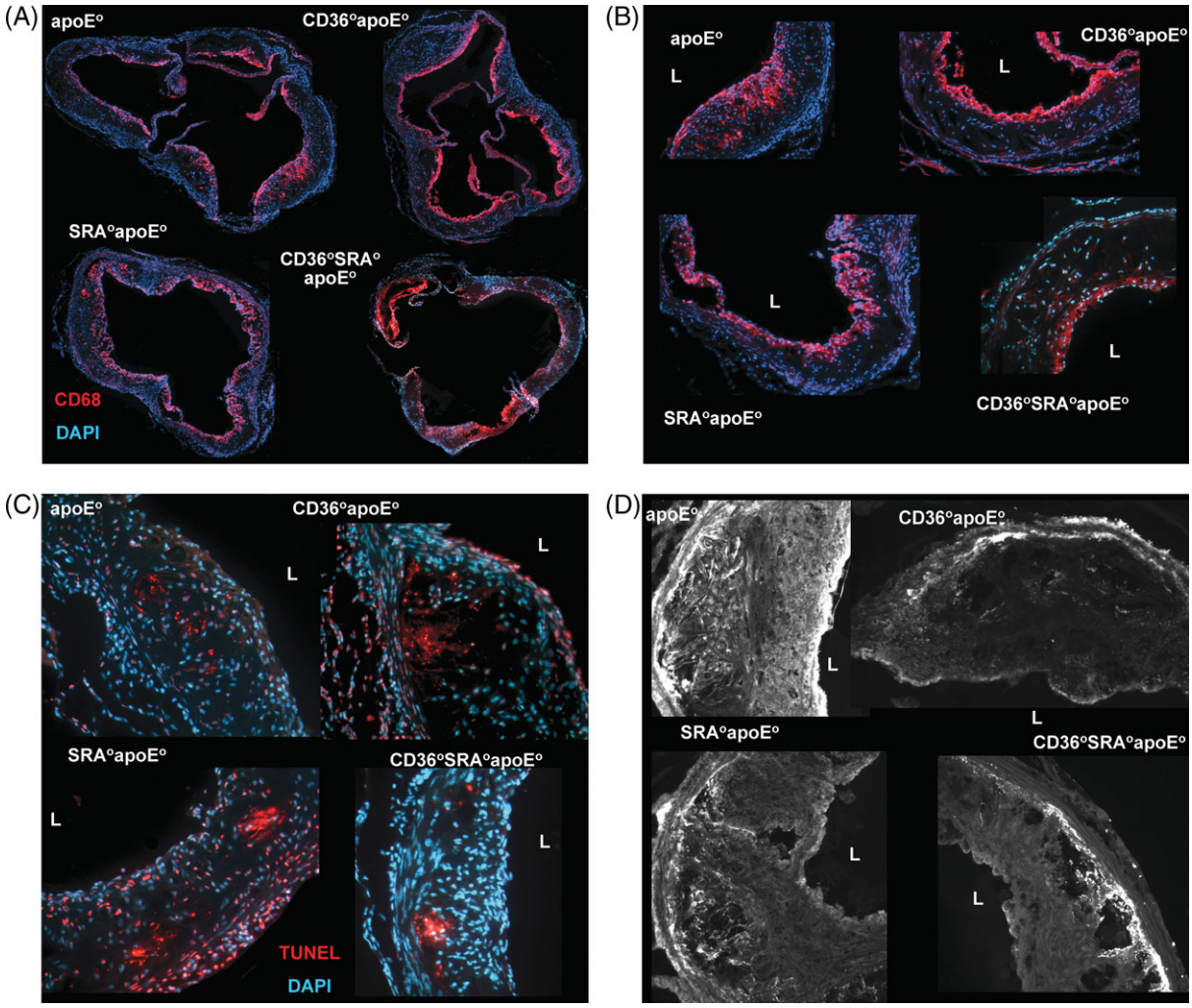

Figure 4 Morphological features of aortic sinus lesions from female apoE°, CD36°/apoE°, SRA°/apoE°, and CD36°/SRA°/apoE° mice. (*A*) Sections were incubated with anti-CD68 antibody to identify macrophages and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Composite sections (×100 magnification) are shown. Macrophages were found prominently in areas that stained most intense with oil red O, and were localized atop complex lesions. (*B*) Close-up inspection of the lesions in (A), however, demonstrates macrophages throughout. Intralesion macrophage density correlated best with lesion progression—more complex lesions, in general, had fewer macrophages and fewer cells overall. (*C*) TUNEL staining to identify apoptotic cells. Apoptotic cells were prominently within acellular, necrotic areas of the lesions. In some cases, apoptotic cells were noted within other areas, but overall, there were no differences in apoptotic cell number ($n = 3$ aortic sinus lesions/group). (*D*) Filipin staining to demonstrate free cholesterol. Greater amounts of free cholesterol were observed in lesions from apoE° mice. In addition to staining at the intima-media junction, there was prominent staining in macrophage foam cells at the luminal surface. This did not correlate with an increase in apoptotic cells in that region.

Table 1 Plasma values, male mice

	apoE°	CD36°/apoE°	SRA°/apoE°	CD36°/SRA°/apoE°	apoE°/OBL WT	apoE°/OBL
Total cholesterol (mg/dL)	1139 ± 113.3 ($n = 11$)	992.7 ± 52.31 ($n = 11$)	1168 ± 95.91 ($n = 13$)	1240 ± 83.38 ($n = 17$)	815.4 ± 53.51 ($n = 15$)	894.3 ± 57.34 ($n = 17$)
Triacylglycerol (mg/dL)	129.6 ± 14.73 ($n = 12$)	92.49 ± 10.84 ($n = 6$)*	159.2 ± 13.96 ($n = 8$)*	121.7 ± 9.72 ($n = 12$)	102.3 ± 15.54 ($n = 15$)**	140.5 ± 8.98 ($n = 14$)†
Non-esterified fatty acids (nM)	0.94 ± 0.07 ($n = 12$)	0.96 ± 0.04 ($n = 6$)	1.03 ± 0.18 ($n = 8$)	0.83 ± 0.04 ($n = 12$)	1.105 ± 0.11 ($n = 15$)	1.26 ± 0.09 ($n = 14$)

Values shown are mean ± SE.
*$P < 0.05$, **$P < 0.05$ (apoE°/OBLWT and apoE°/OBL were compared with each other only since they are of a different background from the other strains).

and a 12-fold decrease in *SRA* expression. SRA°/apoE° macrophages had a 187 ± 2.3-fold increase in *CD36* expression and CD36°/apoE° macrophages showed a 15.4 ± 6.9-fold increase in *SRA*. The values for *SR-BI*, *ABCA1*, and *ABDG1*, the major lipid efflux genes, were similar among the genotypes (data not shown).

Table 2 Plasma values, female mice

	apoE°	CD36°/apoE°	SRA°/apoE°	CD36°/SRA°/apoE°	apoE°/OBLWT	apoE°/OBL
Total cholesterol (mg/dL)	903.8 ± 45.75 ($n = 20$)	1010 ± 83.96 ($n = 11$)	922.8 ± 76.27 ($n = 11$)	1039 ± 68.64 ($n = 18$)	887.9 ± 49.79 ($n = 18$)	824.8 ± 49.12 ($n = 14$)
Triacylglycerol (mg/dL)	60.73 ± 4.1 ($n = 5$)*,†,‡	87.77 ± 3.79 ($n = 10$)‡	111.8 ± 2.6 ($n = 6$)*	108.9 ± 10.22 ($n = 9$)†	85.53 ± 12.54 ($n = 12$)§	134.4 ± 15.74 ($n = 13$)§
Non-esterified fatty acids (nM)	0.83 ± 0.03 ($n = 5$)*,†	1.51 ± 0.17 ($n = 10$)*,†	0.6 ± 0.02 ($n = 6$)†	0.88 ± 0.04 ($n = 9$)†	1.01 ± 0.18 ($n = 12$)	1.28 ± 0.09 ($n = 13$)

Values shown are mean ± SE.
$^{*,\ddagger,\S}P < 0.05$, $^{\dagger}P < 0.01$ (apoE°/OBLWT and apoE°/OBL were compared to each other only since they are of a different background from the other strains).

Table 3 Lipoprotein analysis

Percentage of total cholesterol	apoE° ($n = 4$)	CD36°/apoE° ($n = 4$)	SRA°/apoE° ($n = 4$)	CD36°/SRA°/apoE° ($n = 4$)
VLDL	67.6 ± 1.2	56.1 ± 3.7*	56.5 ± 1.9*	56 ± 1.2*
IDL/LDL	33.2 ± 1.2	42.5 ± 1.8*	43 ± 1.7*	41.7 ± 1.9*
HDL	0.04 ± 0.4	0.5 ± 0.3	0.5 ± 0.2	0.5 ± 0.1

*$P < 0.05$ compared with apoE°.

We profiled cytokines/chemokines in Western diet fed (2 weeks) female mice of each genotype. We chose this time point because lesions would be similar in stage and the mice would be hyperlipidemic, which, we reasoned, would induce a pro-inflammatory response. MCP-1, macrophage colony stimulating factor (M-CSF), complement component 5a (C5a), KC, soluble intracellular adhesion molecule-1 (sICAM-1), CXC ligand 9 (CXCL9), and triggering receptor expressed on myeloid cells-1 (TREM-1) were expressed similarly in all genotypes (*Table 4*). IL-1 receptor antagonist (IL-1Ra) was increased in expression by three- to four-fold in SRA°/apoE°, CD36°/apoE°, and CD36°/SRA°/apoE° strains (*Table 4*). In apoE° mice, compared with strains lacking CD36, we observed greater than two-fold increased expression of other pro-inflammatory cytokines/chemokines, including IL-1α, IL-1β, IL-2, IL-4, IL-12, interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), macrophage inflammatory protein-1α (MIP-1α), and macrophage inflammatory protein-2 (MIP-2) (*Table 4*). Interestingly, SRA°/apoE° mice were similar to mice lacking CD36, except for expression of IL-1α, IL-1β, MIP-1α, and MIP-2; for these, they showed expression levels similar to or greater than apoE° mice (*Table 4*).

We also measured IL-1α levels in thioglycollate-elicited macrophages in culture. We found that macrophages from apoE° mice secreted ∼1.5-fold more IL-1α compared with CD36°/apoE° macrophages when exposed to 50 μg/mL NO_2LDL overnight (2061.8 ± 69.3 vs. 1373.8 ± 124.6 pg/mL, $P < 0.01$). This was consistent with real-time RT–PCR data, which showed that apoE° macrophages (not cultured) had the greatest IL-1α mRNA levels (5.95 ± 0.64 vs. 2.3 ± 0.99).

Next, we isolated macrophages from apoE° and CD36°/apoE° mice and measured ROS generation. In response to native LDL, there was no difference in the number of cells positive for intracellular ROS (5.6 vs. 4%, apoE° vs. CD36°/apoE°). In response to a 1 h incubation with 50 μg/ml oxLDL, we detected ROS in ∼3.8-fold more apoE° macrophages than CD36°/apoE° macrophages (18.1 vs. 4.8%, $P < 0.0001$). We also isolated macrophages from Western diet fed mice (2 weeks) and incubated them with 50 μg/mL native or NO_2LDL for 30 min. Again, while there was no difference in response to native LDL, there were ∼2.3-fold more ROS positive macrophages from apoE° mice (9.7%) compared with macrophages from CD36°/apoE° (4.3%) mice. In this same study, 3.5% of macrophages from CD36°/SRA°/apoE° mice were positive for ROS. We next measured nitrotyrosine in macrophages from mice fed a Western diet for 2 weeks, after exposure to NO_2LDL or native LDL. Nitrotyrosine is a protein adduct that can be measured as a surrogate for peroxynitrite, which is a powerful oxidant and nitrating agent that can have harmful effects on proteins. There was 13% more nitrotyrosine in lysates from apoE° compared with CD36°/apoE° macrophages after incubation with native LDL (1.15 vs. 1.02 nM/mg). After exposure to NO_2LDL, there was 1.49- and 1.48-fold increase in nitrotyrosine in lysates from apoE° compared with CD36°/apoE° macrophages (two experiments). These data give relevance to the increase in ROS generation in apoE° macrophages by also showing increased oxidation-dependent protein damage in apoE° macrophages.

Finally, we noticed differeces in degree of spreading of the different macrophages after 10 days in culture (*Figure 6A*), and we explored migration as a functional effect. We found robust migration of apoE° cells to MCP-1 (10 ng/mL). There was a 30–40% decrease in migration as a result of absence of CD36, SRA, or both (*Figure 6B*). OxLDL strongly inhibited migration in all genotypes tested. These data suggest that macrophage recruitment to lesion sites could contribute to the increased lesion burden in apoE° mice, and that the presence of modified, pro-atherogenic LDL could trap these macrophages in the subintimal layer.

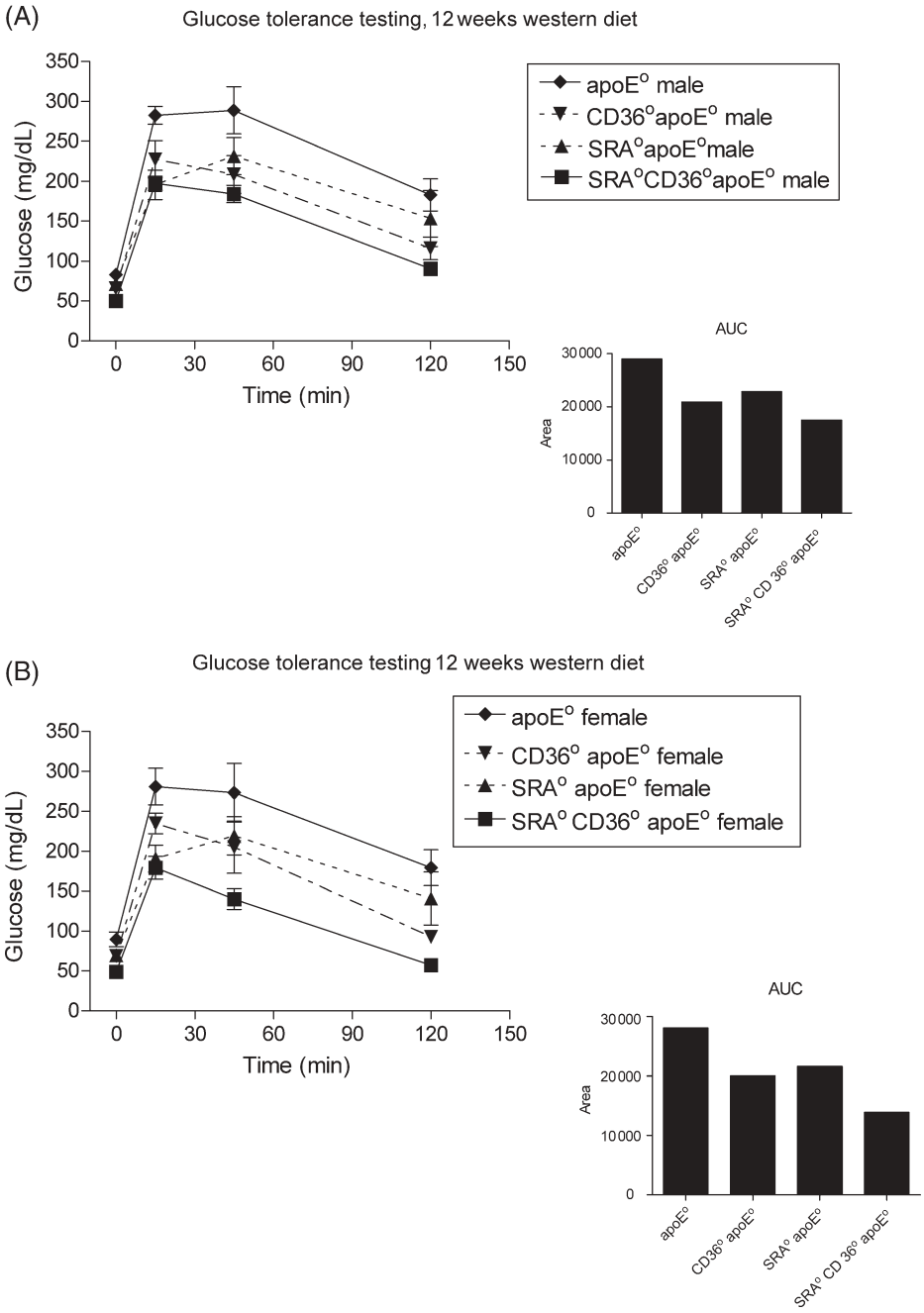

Figure 5 Glucose tolerance testing of male (*A*) and female (*B*) mice after 11 weeks Western diet. Area under the curve, a measure of insulin resistance, is shown in the insets. The greatest area under the curve was noted in apoE° male and female mice, which may increase risk for atherosclerosis.

4. Discussion

The major finding of this report is that in mice of >99% C57Bl/6 background, combined absence of CD36 and SRA provided no greater protection against atherosclerosis than absence of CD36 alone. The absence of CD36 afforded a high degree of protection, however, consistent with previous studies,[8,10,13] and the protection was not due to 'passenger gene effects', since we observed similar results using mice that were pure C57Bl/6. These results strongly support a pro-atherogenic role for CD36. The role of SRA remains somewhat equivocal and may be influenced by environmental and genetic factors.

Early in the pathogenesis of atherosclerosis (2 weeks of Western diet feeding), we found expression of cytokines linked to atherogenesis in all strains of mice. But, we also found specifically pronounced increased expression of pro-inflammatory cytokines, including IL-1α, IL-1β, IL-2, IL-4, MIP-2, MIP-1α, IFN-γ, and TNF-α in apoE° mice compared with CD36°/apoE° and CD36°/SRA°/apoE° strains. SRA°/apoE° mice had a less consistent expression pattern with some cytokines/chemokines expressed similarly to CD36°/apoE° and CD36°/SRA°/apoE° strains and others similarly expressed or increased in expression compared with apoE° mice. Mechanistically, we found that macrophages from apoE° mice were more often positive for intracellular

Table 4 Comparison of pooled plasmas from female mice of each genotype

	apoE° ($n = 6$)	CD36°/apoE° ($n = 6$)	SRA°/apoE° ($n = 3$)	CD36°/SRA°/apoE° ($n = 6$)
MCP-1	21 511	21 925	24 013	16 747
M-CSF	29 399	34 458	34 468	31 835
C5a	37 169	37 622	39 901	32 831
KC	14 280	14 432	14 480	12 719
sICAM-1	48 827	40 578	44 501	39 910
CXCL9	11 056	8943	8085	9382
TREM	9269	6208	8454	6135
IL-1Ra	6938	23 297	30 806	21 126
IL-1α	11 532	4834	21 072	3664
IL-1β	7946	2026	20 685	3541
IL-2	7717	1904	2001	1700
IL-4	11 624	3182	5991	3918
IL-12	16 627	8228	7001	6730
IFN-γ	12 574	6263	5209	3189
TNF-α	11 896	3996	4625	3924
MIP-1α	6867	2901	8010	2660
MIP-2	6729	3660	17 010	3156

Pooled plasmas from female mice of each genotype were compared in duplicate for relative expression of cytokines/chemokines using the Mouse Cytokine Array (R&D Systems). Values shown are average arbitrary values based on density of infrared signal assessed using the Licor Odyssey for analysis.

ROS, which is associated with increased inflammation and apoptosis, and also expressed and secreted more of the pro-inflammatory cytokine IL-1α, in response to incubation with pro-atherogenic forms of LDL. The increase in ROS led to an increase in nitrotyrosine, a surrogate marker for peroxynitrite, which can have deleterious effects on proteins. Thus, in contrast to the hypothesis of Moore *et al.*, our data suggest that macrophages expressing CD36 have a more pro-inflammatory phenotype when compared with those lacking CD36 or CD36 and SRA. This phenotype is likely because of direct and indirect signalling events which occur as a result of interaction and uptake of modified pro-atherogenic forms of LDL.[21–28] We further found that apoE° mice had higher levels of the pro-atherogenic fraction of lipoproteins, and decreased glucose tolerance, two more risk factors for atherosclerosis.

Macrophages lacking SRA and/or CD36 showed a less spread phenotype. A defect in macrophage spreading has previously been reported in SRA° macrophages.[29,30] We investigated macrophage migration as a potential functional consequence of this phenotype. In *in vitro* migration assays, macrophages from apoE° mice had the greatest response to MCP-1, a potent macrophage chemoattractant found in atherosclerotic lesions. Macrophages from CD36°/apoE° and CD36°/SRA°/apoE° mice demonstrated significantly impaired migration; SRA°/poE° macrophages also had reduced migration, but this did not reach statistical significance. These results support the observation of Moore *et al.*[6]; in their study, they noted 40% fewer macrophages per total aortic sinus lesion area in CD36°/apoE° mice. The data presented herein suggest that the absence of CD36 is not only protective at the level of decreasing lipid accumulation in macrophages, but also by decreasing secretion of pro-inflammatory cytokines and ROS, and by affecting macrophage migration in response to factors promoting lesion growth. Our present study thus is important in that it addresses the controversial hypothesis of Moore *et al.* with experimental data.

The hypothesis that scavenger receptors were anti-atherogenic put forth by Moore *et al.*[6] was based on data showing no difference or an increase in lesion area at the level of the aortic sinus in SRA°/apoE° and CD36°/apoE° mice compared with apoE° mice fed a Western diet for 8 weeks. Measurement of lesion area in the entire aorta of SRA°/apoE° males and females showed no significant differences, whereas female CD36°/apoE° mice had a statistically significant 38–40% decrease in lesion area.[6] Although not statistically significant, lesion area in male CD36°/apoE° mice was also decreased.[6] After a series of studies showing conflicting results at the level of the aortic sinus in Western diet fed mice of the same genotype, a recent editorial concluded that lesion development in the aortic sinus under such conditions is not linear with time, and thus is not an accurate measure of lesion burden, nor is it an accurate comparison of lesion burden between strains.[15] Our results, using a 12 week Western diet regimen, confirm the observations of Moore *et al.* pertaining to reduced lesion burden throughout the aortic tree in apoE° mice lacking CD36, and, further, are consistent with previous works, demonstrating that the interaction of ligand with CD36 is pro-inflammatory and leads to increased production of ROS.[26–28] Inhibition of ROS, e.g. by feeding tungsten or glutathione in liposomes, has been shown to decrease atherosclerosis in apoE° mice, and thus may be an important aspect of the CD36-dependent pathogenesis.[31,32]

The protection observed in strains lacking CD36 was in good agreement with our previous work, where we found that CD36°/apoE° male and female mice (four times backcrossed to C57Bl/6) had an 82 and 69% decrease in aortic tree lesion area, respectively.[10] Stem cell transplant revealed a specific role for haematopoietic-derived CD36 expressing cells in protection against atherosclerosis. In this study, a 74 and a 61% decrease in aortic tree lesion area in male and female apoE° mice receiving CD36°/apoE° bone marrow, respectively, was observed.[8] More recently, we have reported the results of

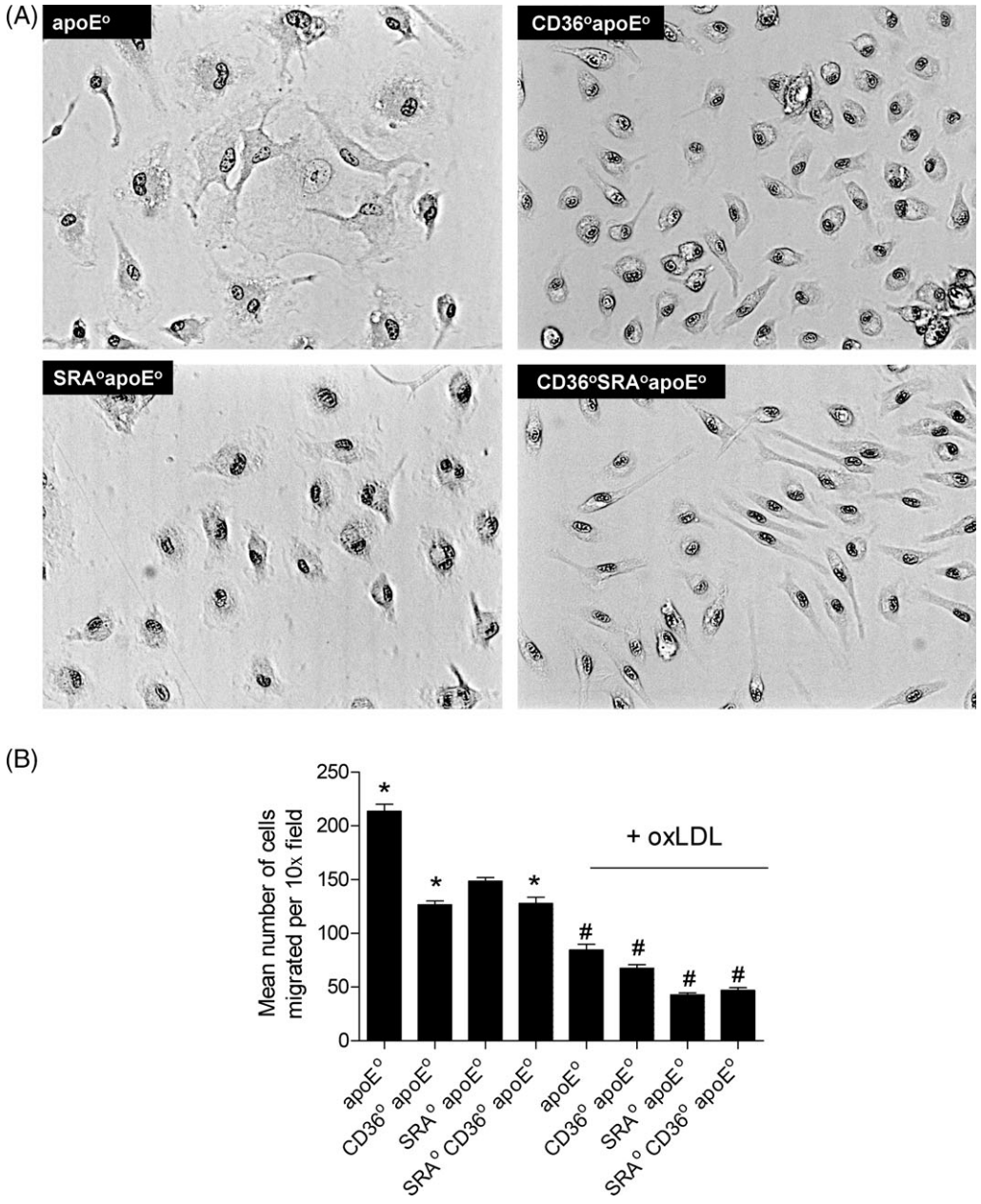

Figure 6 (*A*) Images of macrophages from apoE°, °CD36°/apoE°, SRA°/apoE°, and CD36°/SRA°/apoE° mice after 10 days in culture (×100 magnification). Macrophages from apoE° mice showed the greatest degree of spreading, and those from CD36°/SRA°/apoE° showed the least. (*B*) The results from modified Boyden chamber migration assays. Macrophages from apoE° mice showed the most robust migration in response to 10 ng/mL MCP-1. In comparison, macrophages from CD36°/apoE° and CD36°/SRA°/apoE° demonstrated a significant decrease in migration. Macrophages isolated from all four groups were significantly inhibited in their migration to MCP-1 when co-cultured with 50 μg/mL oxLDL.

another study using background-matched CD36°/apoE° and apoE° male mice backcrossed six times to C57Bl/6.[13] These mice were fed a Western diet for 20 and 35 weeks. At 20 weeks, we found that there was a 24.97% decrease in aortic tree lesion area in CD36°/apoE° mice ($P = 0.052$, Mann–Whitney test), demonstrating sustained protection afforded by absence of CD36.[13] At 35 weeks, we noted a statistically significant 35.1% decrease in lesion area in CD36°/apoE° mice.[13] More compelling, however, were the morphological differences in the aortae. Those from apoE° mice were filled with friable lesions that rendered the aortae thin and sclerotic. Aortae from CD36°/apoE° mice had more punctate lesions and greater elasticity.

The result that combined absence of CD36 and SRA provided no further protection than absence of CD36 alone, while disappointing, is in agreement with two recent studies: one demonstrating that CD36 is responsible for most of the uptake and degradation of oxidatively modified LDL and LDL modified by the myeloperoxidase, hydrogen peroxide, nitric oxide generating system, and the other showing that CD36 has a higher affinity than SRA for the atherogenic ligand of apoE° mice.[3,5] We also observed decreased lesion area in the absence of SRA in female mice that was statistically significant when a less conservative post test was used. In agreement with Moore *et al.*,[6] we observed no protection in male SRA° mice. These data strongly suggest that there are

pro-atherogenic pathways independent of SRA and CD36, which may involve other forms of modified pro-atherogenic LDL and/or other pathways of lipid uptake. The CD36°/SRA°/apoE° mouse provides a unique tool to explore these other mechanisms of atherogenesis.

A pro-atherogenic role for CD36 has been supported by other recent mechanistic findings. Not only is CD36 a receptor for modified LDLs that are pro-atherogenic, it initiates a specific signalling cascade involving *Src* and mitogen-activated protein kinases and ending with activation of *jnk2*, which leads to foam cell formation.[21] Pharmacological or small peptide inhibitors directed against *jnk2* drastically decreased macrophage lipid accumulation in *in vitro* and *in vivo* models, and this was shown to be CD36 dependent by utilizing cells from CD36° mice.[21] These effects were independent of toll-like receptor-2, a CD36-associated protein that also impacts atherogenesis.[21,33] A decrease in CD36 expression on macrophages was also found to be associated with signal transducer and activator of transcription (STAT) 1 deficiency, and bone marrow transplant of STAT1°/apoE° macrophages into apoE° mice resulted in significant inhibition of atherosclerosis.[34] STAT1 is a major modulator of the response of interferons, which are involved in atherogenesis.[35–37]

In summary, the absence of CD36 resulted in decreased lesion area in the aorta of apoE° mice fed a Western diet for 12 weeks. Combined absence of SRA and CD36 provided no added benefit. The absence of SRA showed a trend towards protection in female mice, but this gene apparently is affected by other factors such that results are not unequivocal. The combined absence of both SRA and CD36 did not lead to accelerated atherosclerosis, nor did it completely block lesion development. However, the significant decrease in lesion burden could have significant clinical relevance, which makes CD36 an attractive target for therapeutic intervention.

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