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Scavenger Receptor Function of Mouse Fcγ Receptor III Contributes to Progression of Atherosclerosis in Apolipoprotein E Hyperlipidemic Mice

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Recent studies showed loss of CD36 or scavenger receptor-AI/II (SR-A) does not ameliorate atherosclerosis in a hyperlipidemic mouse model, suggesting receptors other than CD36 and SR-A may also contribute to atherosclerosis. In this report, we show that apolipoprotein E (apoE)-CD16 double knockout (DKO; apoE-CD16 DKO) mice have reduced atherosclerotic lesions compared with apoE knockout mice. In vivo and in vitro foam cell analyses showed apoE-CD16 DKO macrophages accumulated less neutral lipids. Reduced foam cell formation in apoE-CD16 DKO mice is not due to change in expression of CD36, SR-A, and LOX-1. This led to a hypothesis that CD16 may have scavenger receptor activity. We presented evidence that a soluble form of recombinant mouse CD16 (sCD16) bound to malondialdehyde-modified low-density lipoprotein (MDALDL), and this binding is blocked by molar excess of MDA-modified BSA and anti-MDA mAbs, suggesting CD16 specifically recognizes MDA epitopes. Interestingly, sCD16 inhibited MDALDL binding to macrophage cell line, as well as soluble forms of recombinant mouse CD36, SR-A, and LOX-1, indicating CD16 can cross-block MDALDL binding to other scavenger receptors. Anti-CD16 mAb inhibited immune complex binding to sCD16, whereas it partially inhibited MDALDL binding to sCD16, suggesting MDALDL binding site may be in close proximity to the immune complex binding site in CD16. Loss of CD16 expression resulted in reduced levels of MDALDL-induced proinflammatory cytokine expression. Finally, CD16-deficient macrophages showed reduced MDALDL-induced phosphorylation. Collectively, our findings suggest scavenger receptor activity of CD16 may, in part, contribute to the progression of atherosclerosis.

Oxidative modification of native low-density lipoprotein (nLDL), resulting in the generation of oxidized low-density lipoprotein (oxLDL), enhances lipid accumulation and chronic inflammation in the arterial intima, leading to development of atherosclerotic lesions (1, 2). Under hyperlipidemic conditions, reactive aldehydes including malondialdehyde (MDA) formed during low-density lipoprotein (LDL) lipid oxidation form adducts with ε-amino group of lysine in apolipoprotein B, a protein backbone of nLDL, to generate MDA-modified LDL (MDALDL) (3, 4). Recent studies have revealed that MDALDL level is increased in a number of diseases including coronary artery disease (5–9), diabetes, and hypertriglyceridemia (10). Moreover, increased levels of MDALDL were reported in patients with acute coronary syndrome (5, 6). These findings suggest a causal link between MDALDL and cardiovascular disease.

Oxidatively modified LDL binds to a group of transmembrane receptors called scavenger receptors. Currently scavenger receptors can be broadly classified into eight classes, A–H (11, 12), and based on a recent report on scavenger receptor nomenclature, it may be increased to 10 classes (A–J) (13). Of these scavenger receptors, functions of scavenger receptor-AI/II (SR-A) and CD36 class A and B scavenger receptors, respectively, have been implicated to contribute to the progression of atherosclerosis (12, 14). Earlier studies have reported that SR-A or CD36 deficiency in hyperlipidemic apolipoprotein E (apoE) knockout (KO) mice had reduced atherosclerotic lesions (15, 16). However, a recent study by Moore et al. (17) showed loss of CD36 or SR-A in apoE KO mice did not alleviate atherosclerotic lesions. Moreover, overexpression of human SR-A in bone marrow–derived cells did not result in the anticipated increase in atherosclerotic lesions in a hyperlipidemic mouse model (18, 19). Similarly, LDL receptor (LDLR)-CD36 double KO (DKO) mice did not show difference in lesions compared with LDLR KO mice (20). Collectively, these findings suggest a role for other cell-surface receptors expressed on macrophages with scavenger receptor functions contributing to foam cell formation and atherosclerosis.
In mice, four different classes of FcyRs have been recognized: FcyRI, FcyRIIa, FcyRIIb, and FcyRIII (21). FcyRIII, FcyRIII, and FcyRIV (CD32b, CD16, and CD16.2, respectively) are low-affinity receptors for monomeric IgG (21), whereas FcyRI (CD64) is the only high-affinity FcyR. Functionally, FcyRs can be classified into the activating (CD64, CD16, and FcyRIV) and inhibitory (CD32b) receptors. Fcy-chain is the signaling subunit with ITAM for all the activating FcyRs (22, 23). Assembly and cell-surface expression of the activating FcyRs (CD16, CD64, and FcyRIV) require the coexpression of Fcy-chain. FcyR plays an important role in inflammatory cell activation, clearance, presentation of Ag, and maintenance of IgG homeostasis (21). In addition to its binding to immune complex (IC), FcyRs have been shown to bind to non-IgG ligands. Stein et al. (24) have presented evidence that human CD64 and CD32 bind to human CRP. Murine CD32b expression has been shown to bind to a non-IgG ligand expressed on mouse thymic stromal cells (25). Recent studies have presented evidence that murine CD16 binding to an Escherichia coli component (26) negatively regulates the function of macrophage receptor with collagenous structure (MARCO), a class A scavenger receptor implicated in host defense by promoting pathogen clearance (27, 28). Notably, mouse CD32b has been shown to bind oxLDL (29); however, functional implications of oxLDL binding function of mouse CD32b have not been explored. Findings from these reports indicate that FcyRs can bind to non-IgG ligands. In search of receptors other than CD36, SR-A, and LOX-1 to bind to oxLDL, we investigated whether murine CD16 specifically binds to oxLDL. We hypothesize that murine CD16 expressed constitutively on monocytes and macrophages binds oxLDL and subsequently leads to inflammatory responses. In this study, we demonstrated that deficiency of CD16 in a hyperlipidemic apoE KO mouse model showed attenuated atherosclerotic lesions, reduced foam cell formation, without affecting the expression of other scavenger receptors. We also present evidence that CD16 recognized MDA epitopes in MDALDL and CD16–MDALDL interaction resulted in induction of proinflammatory cytokine and chemokines.

### Materials and Methods

#### Recombinant proteins

A soluble form of recombinant mouse CD16 (sCD16) prepared by fusing cDNA encoding extracellular domain of CD16 in E. coli expressed with 10-histidine (His) tag at the C-terminal end and purified from supernatant of myeloma cells was purchased from R&D Systems. Similarly, soluble forms of recombinant mouse SR-A and LOX-1 (SR-A and LOX-1, respectively) were prepared by fusing cDNA encoding extracellular domains of these receptors (SR-A and LOX-1, respectively) with 9-10-His tag at the N-terminal end (R&D Systems). Soluble form of CD36 (sCD36) was constructed by fusing cDNA encoding extracellular domain of CD36 (cDNA encoding extracellular domain of CD36) with human IgG1 (IgG1) with a six-amino acid linker in between CD16 and human IgG1 (R&D Systems). Growth and differentiation factor-associated serum protein-1 (GASP-1), a His-tag protein (R&D Systems), was used as a negative control.

#### Abs and chemicals

Anti-CD16/CD32 mAbs (also known as FcBlock) clone 2.4G2 (rat IgG1), and clone 93 (rat IgG2a) and corresponding isotype controls (rat IgG1 and IgG2a) were purchased from BD Biosciences and Biologend, respectively. Other Abs used in this study include PE-conjugated anti-CD36 (JC63.1, mIgA; Cayman Chemical), PE-conjugated anti–SR-A (clone 268318, rat IgG2a) and corresponding isotype controls (rat IgG1 and IgG2a) were purchased from BD Biosciences and Biolegend, respectively. Other Abs used in this study include PE-conjugated anti–LOX-1 (clone 214012, rat IgG2a; R&D Systems), anti–SR-A (clone 268318, rat IgG2a), anti–SR-A (clone 268318, rat IgG2a) and corresponding isotype controls (rat IgG1 and IgG2a) were purchased from BD Biosciences and Biolegend, respectively. Antibody-purified anti-MDA IgG and anti-OVA IgG were purchased from Academic Biomediicals and MyBioSource, respectively. Substances such as alkaline phosphatase, streptavidin-PEN, streptavidin-HRP, peroxidase/anti-peroxidase IC (PAP-IC) and peroxidase-conjugated donkey anti-rabbit IgG, and F(ab′)2 goat anti-rat IgG were purchased from Jackson ImmunoResearch (West Grove, PA). In some experiments, PAP-IC was bio- coupled using EZ-link NHS biotin labeling kit (Pierce, Rockford, IL) and used as a soluble IC (sIC) ligand. Total Syk (#2712) and phospho- Syk (#4941) Abs were purchased from Cell Signaling Technologies (Danvers, MA). Syk inhibitor III (3,4-methylenedioxy-β-nitrostyrene) was purchased from Calbiochem. All other chemicals were purchased from Sigma.

#### Animals

C57BL/6 (wild type [WT]), apoE KO (stock #002052), CD16 KO (stock #003171), and transgenic mice expressing enhanced GFP (C57BL/6-GFP, stock #004355) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in microisolator cages with filter tops and maintained on a 12-h light/dark cycle in a temperature-controlled room. To generate apoE–CD16 DKO mice, we mated apoE KO and CD16 KO mice on a C57BL/6 background, and we mated F1 progeny (apoE–CD16 heterozygous) to generate apoE–CD16 DKO mice. Genotype analyses of apoE–CD16 were done as described previously (30), and CD16 were done using the following primers (comprises, 5′-GTGGCTGAAAAGTTGTGCTG- CGT-3′; mutant, 5′-GCACAGGACTAGTGAGACGTG-3′; WT, 5′-CTA- CATCCTCCATCCTTTAG-3′) and by separated PCR method as described on the Jax Web site. The WT and mutant CD16 will yield 238 and 500 bp, respectively. apoE KO and apoE–CD16 DKO mice (5 wk of age) were fed a high-fat Western diet (TD 05576; Harlan Laboratories, Madison, WI). A diet for 10 wk. Macrophages were isolated from apoE KO, and apoE–CD16 DKO mice were fed normal chow or high-fat diets. Thioglycollate-elicited mice peritoneal mononuclear macrophages were obtained 3 d after injection of 4% thioglycollate (1 ml/mouse). The peritoneal cells were plated in RPMI 1640 supplemented with 10% (v/v) FBS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate (complete medium). Nonadherent cells were removed (after 48 h). This study was reviewed and approved by the Institutional Animal Care and Use Committee at University of Pittsburgh. Part of the animal studies was carried out at University of Arkansas for Medical Sciences and was approved by the Institutional Animal Care and Use Committee at University of Arkansas for Medical Sciences.

### Determination of atherosclerotic lesions

Mice fed a high-fat diet were anesthetized with isoflurane and euthanized at 15 wk of age, and blood was collected by cardiac puncture into heparin-coated tubes. Plasma was separated and stored at −80°C until further analysis. The heart was excised and fixed in PBS/4% formalin/30% sucrose overnight before mounted in optimal cutting medium and frozen at −70°C. Aortic sinus cross-sections (10 μm) were stained with Oil Red O (31). For quantitative analysis of atherosclerosis, the percent lesion area in each of five sections from each mouse was obtained.

#### Plasma lipid analyses

Concentrations of plasma total cholesterol and high-density lipoprotein cholesterol were determined by enzymatic methods using kits from BioVision (Mountain View, CA) as described earlier (31).

#### Lipoprotein modifications

nLDL isolated from human plasma was purchased from Kalen Biomedical (Montgomery Village, MD). nLDL was biotinylated using Cholainol bitionylation kit (Solulin, San Diego, CA). MDALDL (MDALDL) and MDALDL were prepared by incubating nLDL with freshly prepared MDA (32). In brief, MDA was generated by incubating 1,1,3,3-tetramethoxypropane malonaldehyde bis-dimethyl acetal (704 μM) with 4N HCl (96 μl) and water 3.2 ml for 10 min at 37 °C. MDA solution was adjusted to pH 7.4 with 1 M NaOH. Then nLDL (0.1 mg) was incubated with freshly prepared MDA (100 μl, 0.5 M) at 37 °C for 3 h. Free MDA was removed by extensive dialysis in PBS. To confirm CD16 binding to MDALDL prepared in the laboratory, we also purchased MDALDL from two different commercial sources (Academy Biomedical, Houston, TX, and Kalen Biomedical). MDALDL (low and high MDA modification) was purchased from Kalen Biomedical. The extent of MDA modification was assessed by the electrophoretic mobility of MDALDL and nLDL using TITAN (Helena Labs, Houston, TX) agarose gel electrophoresis (33). Differentially oxLDL was prepared by incubating nLDL with 5 μM CuSO4 at different time period as indicated. nLDL treated with CuSO4 was used as control. Oxidation of LDL was confirmed by thiobarbituric acid-reactive substance (TBA-RS). Czarnik-Chemical high anti-MDA IgG (Academy Biomedical) binding, and gel electrophoresis as described previously (33). Lipoproteins were used for experiments within 3 wk after preparation. All the reagents used to prepare MDALDL were endotoxin-free.
free, and MDALDL was tested for the presence of endotoxin using endotoxin assay kit (GenScript). MDALDL preparation with endotoxin levels >50 pg/mg was not used.

**ELISA to determine MDA epitope in MDALDL and oxLDL**

MDA epitopes in MDALDL, oxLDL, and differentially oxLDL were determined by ELISA using affinity-purified rabbit anti-MDA IgG (1 μg/ml; Abcam; Cambridge, MA) and saturating concentrations of goat anti-rabbit IgG (H+L) followed by streptavidin-HRP.

**Cytokine analysis**

Cytokine secretion (TNF-α, MCP-1 and RANTES) in supernatants from MDALDL- or IC-stimulated macrophages was determined by cytokine bead array in a BD Fortessa and analyzed using FCAP array CBA software (BD Biosciences) or MCP-1 DuoSet ELISA kit (R&D Systems).

**Quantitative real-time RT-PCR**

Total RNA was isolated from macrophages and macrophage cell lines using RNeasy kit (Qiagen) according to the manufacturer’s instructions and treated with RNase-free DNase. RNA was quantified using Nanodrop spectrophotometer (Nanodrop Products, Wilmington, DE). Gene expression was measured by quantitative RT-PCR after reverse transcription of total RNA. PCR primer pairs were purchased from SA-Biosciences (Frederick, MD). Two-step PCR with denaturation at 95˚C for 15 s and annealing and extension at 60˚C for 1 min for 40 cycles was conducted in a CFX96 (Bio-Rad). Expression of β-actin was determined for each sample as a reference gene.

**Cell culture and small interfering RNA transfection**

RAW264.7 and J774, murine macrophage cell lines, were maintained in complete media. Cells were cultured at 37˚C in a humidified atmosphere containing 5% CO₂. CD16 silencing was done using SMARTpool ON-TARGETplus CD16 small interfering RNA (siRNA) and nontarget control siRNA (Dharmacon) as control. Transfections were performed using INTERFein (Polyplus, New York, NY) with siRNA (1 nM), following manufacturer’s protocol. siRNA transfection was done twice to knock down CD16 protein expression; the second transfection was done 48 h after the first transfection. Knockdown efficiency for mRNA and protein expression was confirmed by quantitative RT-PCR and FACS analyses, respectively. CD16 primers (sense, 5'-CCGAGGAGCCGGCAAGTG-3'; antisense, 5'-CTAGAGCGACCAGTACCGG-3') were used to determine CD16 knockdown. CD36, SR-A, LOX-1, β-actin, and GAPDH primer sets were purchased from SA-Biosciences.

**Immune complexes**

MDALDL or OVA (10 μg/ml) was incubated at 4˚C for 18–24 h with affinity-purified rabbit anti-MDA IgG or rabbit anti-OVA IgG at 30 μg/ml to prepare soluble MDALDL-IC and OVA-IC. sIC was centrifuged at 10,000 rpm for 20 min to remove any particulate IC. To prepare insoluble BSA-IC, we incubated IgG-free BSA (10 μg/ml in PBS, Sigma A0281) with rabbit anti-BSA antisera (300 μl of 2 mg/ml anti-BSA IgG, Sigma B1520) for 20 min at 37˚C. Insoluble BSA-IC was centrifuged at 5000 rpm for 2 min, and pellet-containing insoluble BSA-IC was washed with PBS three times and resuspended in 310 μl serum-free RPMI 1640. BSA-IC (15 μlI × 10⁶ cells/well) was used to activate macrophages. In all the assays, both soluble and insoluble ICs were prepared fresh and used within 48 h.

**MDALDL binding to sCD16**

sCD16 (50 μl of 2.5 μg/ml in PBS) was coated on 96-well ELISA white plate (Corning 2592) overnight at 4˚C. Plates were washed twice with PBST and blocked with PBS-Tween-protein free blocking buffer (cat. no. 37570; Pierce) for 1 h at room temperature (RT). Biotinylated ligands (MDALDL or nLDL) were added to the plate at indicated concentration and incubated for 1 h at RT. Plates were washed twice with PBST and a saturating concentration of streptavidin–alkaline phosphatase (1:20000 dilution), and incubated further for 1 h at RT. Plates were washed three times with PBST, and Lumi-Phos 530 (Lumigen, Southfield, MI), a luminescent phosphatase substrate, was added to the plate and luminescence was measured in a Bio-Tek or Tecan microplate reader. Controls used in this assay include wells coated with CD16 alone without ligands and wells blocked with blocking buffer that received ligands. All of the control wells showed very low luminescence values, indicating minimal nonspecific binding of MDALDL to BSA-coated wells. CD16 alone did not bind to streptavidin–alkaline phosphatase detecting reagent. To simulate polyvalent ligands, in the indicated experiments, we performed reverse binding assay in which ligands (such as MDALDL) were coated on the plate followed by the addition of sCD16. CD16 binding was detected using anti-poly His IgG (R&D Systems) and streptavidin–alkaline phosphatase.

**Surface plasmon resonance studies of binding kinetics**

Surface plasmon resonance (SPR) experiments were performed using a BIAcore 3000 on CM5 biosensor chips (BIAcore Life Sciences, GE Healthcare, Piscataway, NJ). Anti-His IgG was captured to the experimental and reference flow cells on CM5 chip surfaces using a BIAcore/GE Healthcare His capture kit, by standard amine coupling chemistry, according to manufacturer’s instructions. The surface density of the sCD16 was tested empirically and optimized for each binding interaction based on standard SPR kinetic considerations. All experiments were carried out in duplicate at 25˚C. Two-fold dilutions starting at 100 nM of rat anti-mouse CD16 mAb (2.4G2; BD Biosciences) were injected at a flow rate of 30 μl/min. Association was measured for 3 min, followed by 10 min of dissociation in HBS-EP running buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20; GE Healthcare). Association of BSA-IC with sCD16 was measured using 2-fold serial dilutions (starting at 50 nM) at a flow rate of 40 μl/min for 3 min. Dissociation was allowed to occur for 10 min in HBS-EP buffer alone. sCD16 binding with MDALDL was examined using 1.5-fold serial dilutions of the MDALDL starting at 230 nM at a flow rate of 60 μl/min. Three minutes of association was followed by 5 min of dissociation in HBS-EP buffer. In all three binding interaction studies, surfaces were regenerated upon completion of dissociation with 10 mM glycine-HCl, pH 1.5. All measurements were double referenced by subtracting out the response to the reference flow cell surface, as well as a buffer-only injection. Binding isotherms were analyzed using BIAevaluation 4.1.1 software (BIAcore/GE Healthcare). sCD16 binding to anti-CD16 mAb was fit to a 1:1 Langmuir binding model. The heterogeneous nature of the IC and MDALDL did not allow for curves that fit the Langmuir kinetic analysis with sensitivity to see IC determined for IC and MDALDL interactions, with sCD16 using the general fit model of steady-state kinetics and response equilibrium versus concentration plots of binding curves.

**Cell binding**

RAW264 cells (5 × 10⁵) were incubated with MDALDLs (at indicated concentration) at 4°C for 1 h. MDALDL binding was detected using saturation of concentrations of anti-ICPE/MDALDL. sCD16 (10 μg/ml) was preincubated with sCD16 (5 μg/ml) for 2 h at 22°C to determine whether sCD16 cross-block MDALDL binding to macrophage cell line. MDALDL treated with irrelevant His-tag protein (GASP-1) was used as negative control. Cells were washed and fixed in PBS/1% formalin, acquired in a FACSCalibur flow cytometer equipped with CellQuest Pro software (BD Biosciences, San Jose, CA), and analyzed using FlowJo (Tree Star, Ashland, OR). Alternatively, this protocol was labeled as being fluorescent-labeled macrophage cell adhesion to MDALDL-coated plates was performed as described previously (33, 34). In brief, nLDL or MDALDL (50 μl of 10 μg/ml in borate buffer/10 mM EDTA pH 8.5) was coated onto an ELISA plate for 16 h at 4°C. After blocking the plates with binding buffer A (HBSS Ca/Mg free/5 mM EDTA/0.5% BSA) for 2 h, J774 cells (5 × 10⁵/ml) were labeled with 2 μM calcine-AM (Molecular Probes), and the adhesion of labeled cells (1 × 10⁵/well) at 4°C was performed as described earlier (33, 34). The fluorescent intensity of the cells adhered to the plates was measured in a Synergy microplate reader (BioTek) at Ex480/Em530 nm. BSA-coated wells were used as a blank. Adhesion assay was performed in the absence (buffer) or presence of sCD16 or sCD36. GASP-1 and human IgG1 added wells were used as controls. Cells adhered to nLDL and BSA-coated wells were used as negative controls.

**MDALDL-induced Syk activation**

Thioglycollate-elicted peritoneal macrophages from apoe KO and apoe-CD16 DKO mice were obtained 3 d after injection of thioglycollate. Macrophages (3 × 10⁵ cells/well) in RPMI 1640 medium supplemented with 10% (v/v) FBS, 2 mM l-glutamine, penicillin, streptomycin, and sodium pyruvate were plated in 24-well plates. Nonadherent cells were removed after 2 h, and adherent cells were used. Macrophages were incubated with MDALDL (40 μg/ml) for the indicated time. For CD16 cross-linking, macrophages were incubated on ice for 30 min with anti-CD16 mAb (2.4G2, 10 μg/ml). After washing, cells were treated with...
prewarmed cross-linking F(ab’), goat anti-rat IgG (10 μg/ml) for 30 min. Unstimulated cells incubated on ice followed by 37°C in complete media were used as a control. Macrophages incubated with LPS (100 ng/ml) for 5 min were used as an additional control. Cells were washed with cold PBS containing 1 mM sodium vanadate and lysed with ice-cold lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% [v/v] Triton X-100, 1 mM Na3VO4, 1 mM β-glycerophosphate, 1 mM PMSF, protease inhibitor, and phosphatase inhibitors) for 10 min. Supernatants were separated by centrifugation at 13,200 rpm for 10 min at 4°C, and protein was estimated using DC protein assay kit (Bio-Rad). Aliquots of total lysates (10 μg) were separated on 8% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). The blots were subjected to immunoblotting with phosphoSyk Tyr525/526 (1:2000) and total Syk (1:2000) antibodies (Jackson Immunoresearch) and detected using ECL prime Western blot Detection Reagent (GE Healthcare), and pixel density was determined using QuantityOne software (Bio-Rad). To determine the effect of Syk on MDA-LDL-induced MCP-1 secretion, we preincubated macrophages with Syk inhibitor III (5 μM) for 30 min followed by the addition of ligands (nLDL or MDA-LDL at 40 μg/ml) or soluble MDA-LDL-IC (20 μg/ml) or insoluble BSA-IC (15 μg/well) for 24 h. MCP-1 levels in the supernatant were determined using DuoSet MCP-1 ELISA kit (R&D Systems).

Foam cell assay

Thioglycollate-elicited macrophages from apoE KO and apoE-C16 DKO mice fed high-fat diet for 6 wk were plated on glass coverslips for 2 h. Nonadherent cells were removed after 2 h, and cells were stained with Oil Red O the next day to determine diet-induced foam cells. To determine MDA-LDL-induced in vitro foam cell formation, we plated thioglycollate-elicited macrophages from WT and CD10 KO mice (fed chow diet) as described earlier. Macrophages were exposed to MDA-LDL (10 μg/ml) for 24 h, stained with Oil Red O and DAPI to determine MDA-LDL-induced in vitro foam cell formation. To determine the effect of CD16 deficiency on in vivo foam cell formation, we generated apoE KO expressing GFP (apoE KO-GFP) mice by crossing apoE KO with C57BL/6-GFP. Colonies were unexpected, to confirm sCD16-mediated inhibition of MDALDL binding to J774 cells (Fig. 1A). Because this finding was unexpected, to confirm sCD16-mediated inhibition of MDALDL binding to macrophages, we generated apoE KO expressing GFP (apoE KO-GFP) mice by crossing apoE KO with C57BL/6-GFP; colonies were confirmed by genomic PCR, and GFP expression in macrophages was confirmed by flow cytometric analysis. ApoE KO-GFP mice were fed a high-fat diet for 6 wk and used as recipient mice. Thioglycollate-elicited macrophages (20 × 10⁶) from WT or CD16 KO mice were transferred to apoE KO-GFP+ mice (total n = 6, 3/group) as recipients. Peritoneal cells were collected from apoE KO-GFP+ recipient mice and analyzed for foam cells after staining macrophages with Oil Red O and counterstaining with DAPI. An aliquot of peritoneal cells from apoE KO-GFP+ recipient mice was used to determine GFP+ cells by flow cytometry. Macrophages from WT (GFP+) and apoE KO-GFP+ mice were used as negative and positive controls, respectively, for FACS analysis.

Statistical analyses

Values are expressed as mean ± SD. Differences between the groups were considered significant at p < 0.05 using the two-tailed Student t test. All data were analyzed using InStat version 3.1a for Macintosh (GraphPad, San Diego, CA).

Results

Mouse sCD16 blocks MDA-LDL binding to macrophages

sCD36 was evaluated for its ability to block MDA-LDL binding to J774, a mouse macrophage cell line. To address the specificity of sCD36-mediated blocking, we used human IgG1 and sCD16 as negative controls. J774 cells did not adhere to nLDL-coated wells, although it adhered to MDA-LDL-coated wells (Fig. 1A). As expected, sCD36 blocked mouse macrophage binding to MDA-LDL (Fig. 1A), whereas human IgG1 did not block the binding. Surprisingly, sCD16 also inhibited MDA-LDL binding to the macrophage cell line (Fig. 1A). Because sCD16 has 10-His tag at the C-terminal end, another irrelevant recombinant protein GASP-1 with His tag was used as a control. GASP-1 did not inhibit MDA-LDL binding to J774 cells (Fig. 1A). Because this finding was unexpected, to confirm sCD16-mediated inhibition of

FIGURE 1. Mouse CD16 blocks MDA-LDL binding to macrophages. (A) CD16 inhibits J774 adhesion. MDA-LDL-coated ELISA plates were treated with indicated reagents (at 2 μg/ml) or buffer for 30 min. Calcine-labeled J774 cells were added to the plates and incubated at 4°C for 30 min. After removing nonadherent cells by inverting the plates in PBS for 5 min, cell adhesion (%) was calculated by dividing fluorescence before and after washing the plate to remove nonadherent cells. (B) Dose-dependent MDA-LDL binding to RAW264 cells. RAW264 cells were incubated with MDA-LDL biotin at indicated concentration for 1 h at 4°C and binding followed by streptavidin-PE. Cells incubated without MDA-LDL biotin were used as a negative control. MDA-LDL binding was determined by FACS analysis. (C) CD16 inhibits MDA-LDL binding to RAW264 macrophages. MDA-LDL biotin (10 μg/ml) was preincubated without (buffer) or with sCD16 (5 μg/ml) for 2 h followed by its binding to RAW264 cells. Recombinant sCD16 blocks MDA-LDL binding to CD16. sCD16 or indicated soluble scavenger receptors were coated onto ELISA white plate. MDA-LDL biotin was preincubated with 2.5 μg/ml sCD16 or buffer for 2 h and added to sCD16-(D) or sCD36- (E), sLOX-1- (F) or sSR-A (G)-coated plates. Binding was detected using streptavidin-alkaline phosphatase and Lumiphos 530 luminescence substrate as described in Materials and Methods. In all these experiments, values are means ± SD in triplicate wells. Shown is a representative of three independent experiments. **p < 0.01, ***p < 0.001, compared with buffer-treated wells (no competitor).
MDALDL binding to J774 cells, we determined MDALDL binding to RAW264 cells, another macrophage cell line. RAW264 macrophages bound to MDALDL dose dependently (Fig. 1B), and preincubation of MDALDL with sCD16 inhibited MDALDL binding to cells (Fig. 1C). Similar findings were observed using macrophages from WT mice (data not shown). We then determined whether sCD16 inhibits MDALDL binding to membrane-expressed CD16. J774, RAW264 (mouse macrophage cell lines), and macrophages constitutively express CD16, and other scavenger receptors: CD36, SR-A, and low levels of LOX-1 (data not shown). Hence we investigated whether sCD16 cross-blocks MDALDL binding to other scavenger receptors. MDALDL binding to individual scavenger receptors was determined using ELISA plates coated with sCD36, sSR-A, or sLOX-1. Dose-dependent binding assay showed the saturation of MDALDL binding to scavenger receptors is ∼1–3 μg/ml (data not shown). Preincubation of MDALDL with sCD16 blocked ∼50% MDALDL binding to sCD16-coated plates (Fig. 1D). Interestingly, preincubation of MDALDL with sCD16 also inhibited MDALDL binding to sCD36 (Fig. 1E), sLOX-1 (Fig. 1F), and sSR-A (Fig. 1G). These findings suggest that mouse CD16 binds to MDALDL and cross-blocks MDALDL binding to scavenger receptors including CD36, SR-A, and LOX-1.

Mouse sCD16 directly binds to MDALDL

Based on its ability to block MDALDL binding to macrophages, we characterized the MDALDL, a non-IgG alternative ligand, binding function of CD16 by two approaches. In the first approach, mouse sCD16 was added to MDALDL-coated plates (plate bound) and the binding was detected using anti-polystyrene IgG. Mouse sCD16 bound to MDALDL with no detectable binding to nLDL or BSA (Fig. 2A). GASP-1, an irrelevant His-tag protein, did not bind to MDALDL. Because plate-bound MDALDL represents a polyvalent ligand, we investigated whether sCD16 could bind to MDALDL in solution. In this assay, sCD16 was coated onto the

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Mouse CD16 specifically binds to MDALDL. (A) CD16 binds to plate-bound MDALDL. MDALDL (10 μg/ml) was coated on ELISA plate overnight. CD16-His (2 μg/ml) was added to the plates and incubated for 1 h at 22˚C. CD16 binding was detected using HRP-conjugated anti-polyHis IgG followed by the addition of HRP substrate. Wells coated with nLDL or BSA were used as controls. Human GASP-1, a His-tag protein, was used as negative control. ***p < 0.001, compared with MDALDL binding to GASP-1–coated wells. (B) Dose dependence and specificity of MDALDL binding of sCD16. CD16 was coated on ELISA white plate (2.5 μg/ml). MDALDL was added at different concentration, and binding was detected by luminescence assay (relative light units [RLU]). Specific MDALDL binding to sCD16 was calculated by subtracting RLU values in the absence (buffer) from RLU values in the presence of molar excess of unlabeled MDALDL (+MDALDL). (C) Steady-state affinity analysis of MDALDL and sCD16. SPR was used to evaluate sCD16 binding kinetics and affinity for MDALDL. Binding surfaces were constructed on CM5 chips as detailed in Materials and Methods. Duplicate response equilibriums (Req) of serial dilutions (gray dots) are plotted as a function of their respective increasing log10 concentrations and fit (x² = 4.1) to the general fit model of steady-state affinity (black line). Inset isotherm is a representative of the doubled-referenced binding data (gray traces) of RU over time. (D) MDA-BSA competes for MDALDL binding to CD16. MDALDL (3 μg/ml) binding to sCD16 was inhibited by molar excess of unlabeled MDALDL and MDA-BSA (30 μg/ml). Fucoidan (40 μg/ml) added to sCD16-coated wells was also used as a competing reagent to block MDALDL binding to sCD16. nLDL and no competitor (buffer)-treated wells were used as controls. ***p < 0.001, compared with buffer-treated wells (no competitor). (E) Anti-MDA IgM mAb inhibits MDALDL binding to CD16. MDALDL (3 μg/ml) was preincubated with mouse anti-MDA IgM mAb (E012) or control mouse IgM (G8G7) at 1:25 or 1:50 dilution for 2 h followed by the addition to CD16-coated plates. **p < 0.01, compared with control mIgM. (F) Anti-MDA IgG pAb inhibits MDALDL binding to CD16. MDALDL (3 μg/ml) was preincubated with affinity-purified rabbit IgG (whole molecule) or F(ab')2 fragment or buffer, followed by detecting binding to CD16. MDALDLs treated with anti-OVA IgG (whole molecule or F(ab')2 fragment) were used as controls. In all these experiments, values are mean ± SD of triplicate wells. Representation of two to three independent experiments is presented. ***p < 0.001, compared with buffer-treated wells (no competitor).
ELISA plate and MDALDL\textsubscript{b} was used as a ligand. MDALDL bound to sCD16 in dose-dependent manner, and the binding was detected as low as MDALDL concentration of 1 \mu g/ml (Fig. 2B). Moreover, addition of unlabeled MDALDL inhibited MDALDL\textsubscript{b} binding to sCD16. The relative affinity of MDALDL binding to plate-bound sCD16 is estimated to be \sim 20 \mu M compared with anti-CD16 mAb at 300 pM (Supplemental Fig. 1A) and OVA-IC at 90 nM (Supplemental Fig. 1B). To confirm the non-Ig ligand binding to sCD16, we determined binding affinity of the sCD16 to MDALDL (Fig. 2C), IC, and anti-CD16 mAb (Supplemental Fig. 1C, 1D) using SPR spectroscopy. Interaction analysis was designed to achieve 1:1 binding interaction where possible. Anti-CD16 mAb bound to sCD16 at a high affinity of 248 pM. IC and MDALDL binding interactions were not capable of 1:1 Langmuir analyses, and steady-state kinetic analyses indicated IC and MDALDL bound to sCD16 at low affinities of 78 nM and 25 \mu M, respectively.

\textit{CD16 recognize MDA epitope in MDALDL}

Next, we investigated the specificity of sCD16 recognizing MDA epitopes in MDALDL in a competition immunoassay. Unlabeled MDALDL and MDA-modified BSA (MDA-BSA) inhibited MDALDL\textsubscript{b} binding to sCD16, whereas nLDL and BSA did not inhibit the binding (Fig. 2D). Interestingly, fucoidan also inhibited MDALDL binding to sCD16 (Fig. 2D). The specificity of mouse CD16 binding to MDA epitopes was further confirmed using anti-MDA polyclonal Ab (pAb) and mAb that specifically recognizes MDA epitope. Anti-MDA IgM mAb and anti-MDA IgG specifically bound to MDALDL, whereas binding to nLDL was very minimal (Supplemental Fig. 2), indicating the specificity of anti-MDA Abs. Anti-MDA–specific IgM mAb (E012) inhibited mouse sCD16 binding to MDALDL (Fig. 2E), whereas isotype-specific mouse IgM (G8G7) did not inhibit MDALDL binding to sCD16. Similarly, rabbit anti-MDA IgG (whole molecule) inhibited MDALDL binding to sCD16 (Fig. 2F), whereas rabbit anti-OVA IgG did not. Because the natural ligand for CD16 is IgG, F(ab\textsuperscript{\prime})\textsubscript{2} fragment of anti-MDA IgG was prepared and used in the binding assay. F(ab\textsuperscript{\prime})\textsubscript{2} fragment of anti-MDA IgG also inhibited MDALDL binding to sCD16 (Fig. 2F). However, under similar conditions, rabbit anti-OVA IgG (whole molecule and F(ab\textsuperscript{\prime})\textsubscript{2} fragment) did not inhibit. These findings indicate that CD16 specifically recognize MDA epitopes present in MDALDL.

\textit{Mouse sCD16 does not bind to oxLDL}

Because MDALDL is one of the modifications that occur during the oxidation of LDL (3, 4, 8), we then determined whether sCD16 can bind to oxLDL. Interestingly, sCD16 bound poorly to oxLDL (Fig. 3A). To confirm whether oxLDL used in this immobilization is functionally active, we repeated the binding experiment using other scavenger receptors. OxLDL bound to sCD36, sSR-A, and sLOX-1 (Fig. 3B–D). These findings suggest that mouse CD16 does not recognize epitopes in oxLDL. The selective binding of sCD16 to MDALDL is surprising because oxLDL has been reported to contain MDA epitopes (3, 4, 8). One possibility is that MDA epitopes either may be masked or reduced in the extensively oxLDL. To address this possibility, we repeated CD16 binding assay using differentially oxLDL. nLDL was incubated with Cu\textsuperscript{2+} for the indicated time to prepare differentially oxLDL. To confirm differentially oxLDL is indeed oxidized, we determined nLDL and differentially oxLDL electrophoretic mobility. The electrophoretic mobility of differentially oxLDL increased over time of LDL oxidation (Fig. 3E). Kinetics of differentially oxLDL and subsequent binding of differentially oxLDL in solution to sCD16-

\textbf{FIGURE 3.} Mouse CD16 does not bind to oxLDL. MDALDL\textsubscript{b} or oxLDL\textsubscript{b} (3 \mu g/ml) was added at sCD16 (A), sCD36 (B), or sSR-A (C) or sLOX-1 (D)-coated plates. Binding was detected by luminescence assay. \textbullet\textbullet\textbullet\textbullet < 0.001, compared with nLDL binding. (E) Electrophoretic mobility of differential oxidation of LDL. nLDL\textsubscript{b} (200 \mu g/ml) was incubated with CuSO\textsubscript{4} for the indicated time, and reaction was stopped by the addition of 0.1 mM EDTA. An aliquot (1 \mu l) was loaded on agarose gel and stained with Fat Red 7B. nLDL (Kalen Biomedical) and nLDL\textsubscript{b} without the addition of copper (0 h) were used as controls. (F) Decreased binding of differential oxLDL\textsubscript{b} binding to sCD16-coated plates determined by luminescence-based assay using streptavidin-alkaline phosphate. (G) Decreased sCD16 binding to plate-bound differential oxLDL. Differential oxLDL was prepared as described in (F), using unlabeled nLDL. Differential oxLDL (5 \mu g/ml) was coated onto white ELISA plates for 16 h at 4°C. After blocking the plate with blocking buffer, sCD16 (3 \mu g/ml) was added and sCD16 binding was detected using anti-poly His IgG\textsubscript{b} followed by the addition of streptavidin-alkaline phosphate in a luminescence assay. (H) Loss of MDA epitopes in extensively oxLDL. Differential oxLDL (5 \mu g/ml) in PBS/1 mM EDTA was coated on ELISA plates. Affinity-purified rabbit anti-MDA IgG binding was detected by ELISA using goat anti-rabbit IgG-HRP and peroxidase substrate. OxLDL (copper oxLDL for 24 h) from a commercial source (Kalen Biomedical) was used as an additional control. In all of these assays, values are mean \pm SD of triplicate wells. Representation of two to three independent experiments is presented. \textbullet p < 0.05, compared with 2-h oxLDL.
coated plates showed oxLDL binding to sCD16 was higher at early oxidation of LDL, and this binding to sCD16 decreased considerably with increasing time of LDL oxidation (Fig. 3F). We used differentially oxLDL as a soluble ligand, hence it is possible that sCD16 binding may be increased if differentially oxLDL is presented as polyvalent ligand in a plate-bound form. To address this possibility, we developed a reverse binding assay in which differentially oxLDL was coated on the plate followed by the addition of sCD16. Reverse binding assay also showed sCD16 bound to the early oxidation product of LDL better than the products of extensive oxidation of LDL (Fig. 3G). These findings collectively suggest that MDA epitopes in the extensively oxLDL may either not be accessible for binding to CD16 or MDA epitopes are lost during the extensive oxidation of LDL. To address this possibility, we determined rabbit anti-MDA IgG (specific to MDA epitopes) binding to differentially oxLDL. Anti-MDA IgG binding was higher at early time (1–4 h) of LDL oxidation, whereas anti-MDA IgG binding decreased in the extensive oxidation (24 h) of LDL (Fig. 3H). To confirm this finding, we also used oxLDL (24-h oxidation) from a commercial source (Kalen Biomedical), which also showed reduced anti-MDA IgG binding (Fig. 3H). These findings collectively suggest that early oxidation of LDL may have either more MDA epitope and/or MDA epitope is more accessible to anti-MDA IgG binding. Based on these findings, we investigated whether the extent of MDA modification influences sCD16 binding. MDALDL was purchased from Kalen Biomedical with different levels of MDA. Electrophoretic mobility analyses showed more MDA modification resulting in faster mobility of MDALDL, whereas LDL modified with low amount of MDA showed slower mobility (Supplemental Fig. 3A). These findings indicate that electrophoretic mobility of differentially MDALDL is dependent on the extent of MDA modification. CD16 binding to differentially modified MDALDL showed sCD16 binding was dependent on the extent of MDA modification (Supplemental Fig. 3B). These findings suggest that CD16 binding to MDALDL is dependent on the extent of MDA modification.

**MDALDL may bind to IC binding domain in CD16**

Thus far we have presented evidence that CD16 binds to MDALDL, a non-Ig ligand. Because IC is the natural ligand for CD16 (35), we investigated whether MDALDL and IC binds to similar or overlapping domains in mouse CD16. Pretreatment of sCD16 with anti-CD16 mAb (two different mAbs), which has been reported to block IC binding, completely inhibited IC binding to sCD16 (Fig. 4A). Interestingly, pretreatment of sCD16 with blocking anti-CD16 mAb partially inhibited sCD16 binding to MDALDL (Fig. 4B). Because IC is the natural Ig-containing ligand for CD16, we then determined whether sIC could block MDALDL binding to sCD16. sIC addition partially inhibited the MDALDL binding to sCD16 (Fig. 4B), whereas it completely inhibited soluble PAP-IC binding to CD16 (Fig. 4A). Moreover, MDALDL and MDA-BSA competitively inhibited IC binding to sCD16 (Fig. 4C). Collectively, these findings suggest that MDALDL may either share IC binding domain or the MDALDL binding domain could be at close proximity to the IC binding domain in mouse CD16.

**ApoE-CD16 DKO mice show reduced atherosclerotic lesions**

If mouse CD16 has MDALDL binding function, we hypothesize that deficiency of CD16 may result in attenuated atherosclerotic lesions. We tested this hypothesis using apoE-CD16 DKO mice. To determine the role of CD16 in the progression of atherosclerosis, we generated CD16 deficiency in apoE KO background. Genotype analyses showed complete KO for CD16 in the apoE KO background (Fig. 5A). Atherosclerotic lesions in apoE KO and apoE-CD16 DKO female mice fed a high-fat diet showed a 40% reduction in lesions (p < 0.001, Student t test) in apoE-CD16 DKO mice compared with apoE KO mice (Fig. 5B, 5C). Male apoE-CD16 DKO mice also showed a similar reduction in lesions (Fig. 5D, 5E), suggesting there is no effect of sex on the extent of lesions. These findings suggest that CD16, one of the activating FcγRs, contribute to the progression of atherosclerosis.

**Mechanisms contributing to attenuated lesions in apoE-CD16 DKO mice**

To determine the molecular mechanisms contributing to the decreased atherosclerotic lesions in apoE-CD16 DKO mice, we determined plasma total cholesterol levels. There were no significant differences in plasma total cholesterol levels in apoE-CD16 DKO mice (1113 ± 172 versus 1073 ± 213 mg/dL) compared with apoE KO mice fed a high-fat diet. Similarly, high-density lipoprotein cholesterol levels were also similar in apoE KO and apoE-CD16 DKO mice (data not shown). These data suggest that the reduced lesions in apoE-CD16 DKO mice were not due to changes in plasma lipid levels.

**Reduced foam cell formation in apoE-CD16 DKO macrophages**

Because foam cell formation is an early event in atherosclerosis (36, 37), we subsequently investigated whether reduced foam cell differences in plasma total cholesterol levels in apoE-CD16 DKO mice fed a high-fat diet. Interestingly, pretreatment of sCD16 with blocking anti-CD16 mAb partially inhibited sCD16 binding to MDALDL (Fig. 4B). Because IC is the natural Ig-containing ligand for CD16, we then determined whether sIC could block MDALDL binding to sCD16. sIC addition partially inhibited the MDALDL binding to sCD16 (Fig. 4B), whereas it completely inhibited soluble PAP-IC binding to CD16 (Fig. 4A). Moreover, MDALDL and MDA-BSA competitively inhibited IC binding to sCD16 (Fig. 4C). Collectively, these findings suggest that MDALDL may either share IC binding domain or the MDALDL binding domain could be at close proximity to the IC binding domain in mouse CD16.

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**FIGURE 4.** MDALDL binds to IC binding domain in CD16. sCD16 was coated on white ELISA plate. Soluble PAP-IgG (2 μg/ml (A) or MDALDL at 3 μg/ml (B) binding to sCD16-coated plates was performed by luminescence-based assay. Anti-CD16 blocking mAbs (2 μg/ml, FcBlock 1 or 2) were added to sCD16-coated wells before the addition of ligands. CD16-coated wells incubated with unlabeled PAP-IC and isotype control rat IgG were used as positive and negative controls, respectively. sCD16-coated well without blocking reagent (buffer) shows the total ligand (PAP-IC) binding. (C) MDA-modified proteins inhibit sIC binding to CD16. Soluble PAP-IgG (2 μg/ml) binding to sCD16-coated wells was performed in the absence (buffer) or presence of unlabeled MDA-BSA or MDALDL (at 20 μg/ml) by luminescence-based assay. nLDL (20 μg/ml) was used a negative control. Values are mean ± SD of triplicate wells. Representation of two independent experiments is presented. **p < 0.01, ***p < 0.001, compared with buffer-treated (no competitor) wells.
formation by CD16-deficient macrophages contributed to the attenuated lesions in apoE-CD16 DKO mice. ApoE KO and apoE-CD16 DKO mice were fed a high-fat diet for 6 wk to determine diet-induced foam cell formation in vivo by staining elicited macrophages with Oil Red O. Macrophages from apoE-CD16 DKO mice showed attenuated diet-induced foam cell formation compared with macrophages from apoE KO mice (Fig. 6A, 6B). To confirm the attenuated diet-induced foam cell formation in apoE-CD16 DKO mice, we treated elicited macrophages with MDALDL to determine foam cell formation in vitro. WT macrophages treated with MDALDL showed 50% foam cell formation (Fig. 6C, 6D). CD16 single KO (CD16 KO) macrophages showed reduced (<20%) foam cell formation (Fig. 6C, 6D). Spann et al. (38) suggested that in vivo foam cell formation reflects lipid-laden macrophage at the lesion site in vivo. Hence we investigated whether CD16 deficiency could result in reduced in vivo foam cell formation using high-fat-fed apoE KO-GFP mice as recipient, and macrophages from WT and CD16 KO as donors. WT macrophages transferred to recipient apoE KO-GFP mice fed with high-fat diet showed >50% foam cell formation (Fig. 6E, 6F). However, transfer of CD16-deficient macrophages to hyperlipidemic apoE KO-GFP mice showed reduced in vivo foam cell formation (Fig. 6E, 6F). FACS analysis showed the recovered peritoneal macrophages from apoE KO-GFP recipient mice were negative (>95%) for GFP expression (Fig. 6G), indicating that contribution by resident macrophages from the apoE KO-GFP recipient mice was minimal. Because other scavenger receptor expression in CD16 KO macrophages could influence MDALDL-induced foam cell formation, expression of CD36 and SR-A was determined. FACS analysis of macrophages showed CD16 KO macrophages have no expression of CD36 (Fig. 6H), and low levels of expression observed is due to the anti-sCD16/CD32 mAb recognizing CD32 expression. CD36 and SR-A expression in CD16 KO macrophages were similar to WT macrophages (Fig. 6H), suggesting that decreased foam cell formation in CD16 KO is independent of CD36, SR-A, and LOX-1 expression. These findings also suggest that CD16 contributes to foam cell formation.

**CD16 deficiency results in reduced MDALDL-induced chemokine secretion**

We investigated functional implications of scavenger receptor–like function of CD16. For this purpose, initially, CD16 expression in J774 macrophages was silenced using a Smartpool siRNA specific for CD16. Smartpool CD16 siRNA efficiently reduced CD16 mRNA expression by 90%, whereas control nontarget siRNA did not have any effect (Supplemental Fig. 4A). CD16 protein expression also reduced by >60% in CD16 siRNA-transfected cells (data not shown). CD16 silencing did not affect CD36, SR-A, and LOX-1 mRNA (Supplemental Fig. 4A) and protein expression...
CD16 silencing reduced MDALDL and sIC binding to J774 cells (Supplemental Fig. 4B). Then we determined the effect of CD16 silencing on MDALDL-induced proinflammatory cytokine/chemokine expression. Nontarget siRNA-transfected J774 cells treated with MDALDL (10 μg/ml) for 24 h, and percent foam cells (D) was determined after staining cells with Oil Red O and DAPI. n = 4. **p < 0.01, compared with apoE KO mice. (E) CD16 KO macrophages show reduced foam cell formation in vivo. Thioglycollate-elicited peritoneal macrophages (3 × 10⁶) from WT or CD16 KO (donor, GFP⁺) mice (n = 3/strain) were injected to apoE KO-GFP⁺ recipient mice (n = 6) fed high-fat diet for 6 wk. Three days after injecting macrophages, peritoneal cells were plated and stained with Oil Red O to determine in vivo foam cell formation (F). FACS analyses showed 3 d after injecting donor macrophages (WT) (1–3) or CD16 KO (4–6) into apoE KO-GFP⁺ recipient mice, >95% recovered peritoneal macrophages were GFP⁺ (G). WT and apoE KO-GFP⁺ (top panel) were used as controls for FACS analysis. Values are means ± SD. **p < 0.01, compared with apoE KO (A and B); ***p < 0.001, compared with WT (C-F) mice. (H) Scavenger receptor expression was not altered in CD16 KO macrophages. Thioglycollate-elicited peritoneal macrophages from WT or CD16 KO mice were stained with biotin-conjugated anti-CD16, anti-CD36, or anti-SR-A mAb (closed histogram) followed by streptavidin-PE. Cells stained with corresponding isotype control IgG (open histogram) were used as a control. Stained cells were analyzed in FACScalibur flow cytometer. (A, C, and E) Original magnification ×20. Number indicates mean fluorescence.

**FIGURE 6.** Reduced foam cell formation in apoE-CD16 DKO macrophages. (A) High-fat diet–induced foam cell formation is reduced in apoE-CD16 DKO mice. ApoE KO and apoE-CD16 DKO mice were fed high-fat diet for 6 wk. Resident peritoneal macrophages were collected, plated on a cover glass, and stained with Oil Red O and DAPI (nuclear stain), and foam cells (%) were determined (B). n = 7–8. **p < 0.01, compared with apoE KO mice. (C) Attenuated MDALDL-induced foam cell formation in vitro in CD16 KO macrophages. Thioglycollate-elicited macrophages from WT or CD16 KO mice were treated with MDALDL (10 μg/ml) for 24 h, and percent foam cells (D) was determined after staining cells with Oil Red O and DAPI. n = 4. **p < 0.01, compared with apoE KO mice.

(data not shown). CD16 silencing reduced MDALDL and sIC binding to J774 cells (Supplemental Fig. 4B). Then we determined the effect of CD16 silencing on MDALDL-induced proinflammatory cytokine/chemokine expression. Nontarget siRNA-transfected J774 cells treated with MDALDL showed increased secretion of TNF-α (Fig. 7A), MCP-1, (Fig. 7B), and RANTES (Fig. 7C). However, MDALDL-induced proinflammatory cytokine and chemokine secretion in CD16 silenced J774 macrophages were significantly decreased (Fig. 7A–C). To confirm that the attenuated cytokine expression is due to CD16 silencing, we treated J774 (nontarget and CD16 siRNA transfected) cells with BSA-IC or MDALDL-IC to determine IC-mediated chemokine expression. Nontarget siRNA-transfected J774 cells treated with BSA-IC or MDALDL-IC, natural ligand for CD16, also induced TNF-α (Fig. 7D), MCP-1 (Fig. 7E), and RANTES (Fig. 7F). However, in CD16 silenced cells, BSA-IC– and MDALDL-IC–induced cytokine chemokine expression was reduced (Fig. 7D–F).

To confirm the findings from the macrophage cell line, we repeated experiments using primary macrophages from CD16 KO mice. MDALDL and sIC binding were lower in CD16 KO macrophages compared with WT macrophages (Fig. 7G), and there was no difference in oxLDL binding between WT and CD16 KO macrophages (Fig. 7G). WT macrophages exposed to MDALDL-IC induced MCP-1 secretion (Fig. 7H), whereas CD16 KO macrophages showed reduced response. Interestingly, CD16 KO macrophages exposed to MDALDL showed significantly reduced MCP-1 secretion compared with WT macrophages (Fig. 7H). These findings collectively suggest that scavenger receptor–like function of sCD16 contributes to MDALDL-induced chemokine secretion.

**MDALDL-induced Syk phosphorylation**

Activation of Syk is essential for CD16-induced macrophage activation (22, 23, 39). We were therefore keen to understand whether the MDALDL binding to CD16 is able to transduce signals similar to those described for IC binding to CD16 (22, 23,
CD16-mediated Syk phosphorylation was examined using macrophages derived from CD16 KO mice. CD16 cross-linking with anti-CD16 mAb induced Syk phosphorylation in WT macrophages (Fig. 8A, 8B). However, anti-CD16 mAb–induced Syk phosphorylation is significantly reduced in CD16 KO macrophages (Fig. 8A, 8B). MDALDL binding to WT macrophages also induced Syk phosphorylation (Fig. 8A, 8B). Interestingly, MDALDL-induced Syk phosphorylation is reduced in CD16 KO macrophages (Fig. 8A, 8B) without affecting total Syk expression (Fig. 8A, 8C). We also determined whether blocking Syk activation would affect MDALDL-induced MCP-1 secretion. Preincubation of macrophages with selective inhibitors of Syk activity (40, 41) inhibited MDALDL– and IC-induced MCP-1 secretion (Fig. 8D), confirming that CD16 binding to MDALDL induced Syk phosphorylation and subsequent MCP-1 secretion.

Discussion

In this study, we tested the hypothesis that non-IgG scavenger receptor function of the CD16, one of the activating FcγRs, contributes to the progression of atherosclerosis. We presented evidence that mouse CD16 blocks MDALDL binding to macrophages and cross-blocks MDALDL binding to CD36, SR-A, and LOX-1. We also showed that sCD16 binds selectively to MDALDL, and MDALDL binding site may be either shared and/or in close proximity to IC binding domain of CD16. We showed a significant reduction in arterial lesions in apoE-CD16 DKO mice after a high-fat diet. Remarkably, the reduction in atherosclerotic lesion progression in CD16 deficiency in hyperlipidemic apoE KO mouse model was associated with reduction in foam cell formation and MDALDL-induced inflammatory cytokine and chemokine expression and Syk activation.

The role of SR-A and CD36 in the progression of atherosclerosis is not resolved because earlier studies have reported that apoE-SR-A DKO or apoE-CD36 DKO mice (mixed genetic background 129sv/B6) had reduced atherosclerotic lesions (15, 16). However, a recent study showed loss of CD36 or SR-A in apoE KO mice (B6 congenic) did not alleviate atherosclerotic lesions (17). The difference in genetic background was attributed to the differences in the findings from both studies. Similar findings were also observed in LDLR-CD36 DKO mice fed a high-fat diet (20). Moreover, bone marrow transplantation of human SR-A over-
A. Thioglycollate-elicited macrophages from apoE KO and apoE-CD16 DKO mice were plated and incubated with MDALDL (40 µg/ml) at indicated time. Macrophages incubated with anti-CD16 mAb (2-G2, 5 µg/ml) followed by cross-linking with F(ab')2 goat anti-rat IgG (10 µg/ml) was used as a positive control. LPS (100 ng/ml)-treated cells were used as an additional control. Phospho-Syk (B) and total Syk (C) levels were determined by Western blot using specific Abs, and band intensities (pixel density) were determined in Bio-Rad Quantity One software. (D) Syk inhibitor blocked MDALDL-induced MCP-1 secretion. Macrophages were preincubated with Syk inhibitor III (5 µg/ml) for 30 min, followed by the addition of MDALDL (40 µg/ml) or MDALDL-IC (20 µg/ml) for 18 h. Cells incubated with BSA-IC (15 µg/ml) or nLDL (40 µg/ml) were used as positive and negative controls, respectively. Cells without the addition of Syk inhibitor or indicated reagents (media) were used to determine basal level of MCP-1 secretion. MCP-1 levels were determined by DuoSet ELISA kit from R&D Systems. Representation of two independent experiments is presented.

B. Staining of spleen macrophages from control and apoE-CD16 DKO mice with anti-CD16 mAb and their binding to mouse sCD16 was competitively inhibited by MDA-LDL. Specificity analyses showed MDALDL also inhibited IC binding to sCD16. These findings suggest that mouse CD16 binding to MDALDL may occur via domains that have been reported to bind to IC (natural ligand). However, amino acids present in the IC-binding domain that specifically recognize MDA epitope need to be established. Notably, CD16 has been shown to recognize non-IgG ligands, which includes thymic stromal cell Ag (44), an uncharacterized ligand expressed on NK target cells (45), E. coli K12 (26). All of these non-IgG ligands have been shown to bind to IC binding domain of mouse CD16.

Several reports have presented evidence that oxidation of nLDL results in complex mixture of products including MDALDL, minimally modified LDL (46), oxidized phospholipids (47, 48), and oxidized polysaturated fatty acids such as hydroxyoctadecadienoic acid and hydroxyeicosatetraenoic acid (48). This raises the question of what is the clinical significance of MDALDL and its binding to CD16. Using mAbs that specifically recognize oxLDL or MDALDL, clinical studies have shown that plasma oxLDL and MDALDL levels were increased in patients with coronary artery disease (6–9). Interestingly, elevated plasma MDALDL level has been associated with myocardial infarction (5, 6), and elevated plasma MDALDL levels have been suggested to be a biomarker for myocardial infarction. Moreover, the significance of MDALDL contributing to the progression of atherosclerosis is supported by the presence of MDA epitopes in atherosclerotic lesions of rabbits and human (1). Collectively, these reports suggest that MDALDL binding to cell-surface receptors such as CD16 expressed on monocytes, neutrophil, dendritic cells, and macrophages could lead to inflammatory responses.

Clinical studies have suggested a strong positive correlation between CD16 polymorphisms and a higher incidence of inflammatory diseases in humans including coronary artery disease (49). Earlier studies using CD16 deficiency in LDLR, another hyperlipidemic mouse model, have shown attenuated lesions, and the reduced lesions were attributed to increased IL-10 secretion by T cells (50). However, in the same studies, the authors have also reported that T cells from CD16-LDLR DKO mice have higher IFN-γ levels (50). Although the relative contributions by anti-inflammatory IL-10 (Th2) and proinflammatory IFN-γ (Th2) were not addressed, it was suggested that macrophages and innate cells could also be contributing to attenuated lesions. Hence it is possible that the attenuated lesions in CD16-deficient mice could also be mediated by mechanisms other than in addition to altered T cell immune response. In this study, we showed that apoE-CD16 DKO mice have reduced lesions and diet-induced foam cell formation. In addition, CD16 KO macrophages also showed reduced MDALDL-induced foam cell formation. CD16 binding to E. coli has been shown to bacterial clearance functions of MARCO (26), a class A scavenger receptor implicated in bacterial clearance and host defense (27, 28). Because MARCO can also bind to modified LDL, it is conceivable that the attenuated lesions and foam cell formation in apoE-CD16 DKO mice may be mediated by MARCO function in CD16 KO mice. This possibility is

expr. bone marrow–derived cells to apoE KO or LDLR KO did not show increase in atherosclerotic lesions (18, 19). These reports raise a possibility that other cell-surface receptors expressed on macrophages with scavenger receptor functions may also contribute to foam cell formation and atherosclerosis. Interestingly, during the course of our study to determine the efficacy of sCD36 to block MDALDL-induced macrophage function, we made a surprising finding that sCD16 (used as a negative control) also blocked MDALDL binding to macrophages. This led to the present study to investigate the scavenger receptor–like function of mouse CD16. We demonstrated that mouse CD16 bound to MDALDL, one of the molecular species generated during LDL lipid peroxidation (3, 4, 8). Specificity analyses showed MDALDL binding to mouse sCD16 was competitively inhibited by MDA-
not likely because CD16 has been shown to negatively regulate bacterial clearance function of MARCO without affecting MARCO expression (26). Based on these reports, one should expect deficiency of CD16 would result in increased MARCO-dependent modified LDL binding and subsequent inflammatory response. However, our data show that MDALDL binding is reduced in CD16 KO macrophages, and decreased MDALDL binding to CD16 KO macrophages is also associated with blunted inflammatory cytokine response. Because expression of CD36, SR-A, and LOX-1 were not altered in CD16 KO and apoE-CD16 DKO macrophages, the reduction in lesions and foam cell formation is independent of previously characterized scavenger receptor expression.

In macrophages, FcγRs including CD16 associate with the ITAM-containing common Fcγ-chain, and this association is necessary for downstream signaling (22, 23). IC binding to activating FcγRs including CD16 initiates signaling events via activation of Src family tyrosine kinases, resulting in rapid and transient phosphorylation of the ITAMs on the associated Fcγ-chain of CD16 (39, 51–53). Phosphorylated ITAM creates docking sites for Syk and subsequent phosphorylation of Syk (54, 55). A number of studies have shown that Syk activation play a critical role in promoting FcγR-mediated inflammatory responses and phagocytosis (39, 56–58). To examine the functional significance of MDALDL binding to CD16, we investigated whether CD16 binding to MDALDL increased Syk tyrosine phosphorylation and MCP-1 secretion. The results reported in this article demonstrate that MDALDL-induced Syk activation resulting in Syk phosphorylation in CD16 WT macrophages. However, MDALDL-induced phosphorylation of Syk is significantly reduced in CD16-deficient macrophages. We also showed MDALDL-induced MCP-1 secretion is reduced in macrophages from CD16 KO mice. Moreover, Syk inhibitor significantly inhibited MDALDL-induced MCP-1 secretion. Collectively, the findings from this study suggest CD16 expressed in monocytes/macrophages has an alternative function to bind MDALDL, and the scavenger receptor–like function of CD16 expressed in monocytes/macrophages may have functional significance in inflammatory processes associated with atherosclerosis.

In summary, our investigation demonstrated that murine CD16 binds to MDALDL, and scavenger receptor activity of CD16, in part, contributed to the abridged lesions in CD16-deficient mice in hyperlipidemic conditions. Our results strongly suggest that scavenger receptor function of CD16 resulting in binding to MDALDL induced chemokine secretion, which may promote monocyte migration and progression of atherosclerosis. In addition to hypercholesterolemic condition, increased formation of MDA-protein adducts have been suggested to contribute to acceleration of lupus in lupus-prone MRL/lpr mice (59) and systemic lupus erythematosus patients (60). Similarly, MDA adducts have also been implicated in the progression of experimental autoimmune encephalitis (61) and alcohol-induced liver disease (62). These clinical and preclinical observations suggest a possibility that MDA-adducts formed in autoimmune and alcohol liver diseases may bind to constitutively expressed CD16 on inflammatory cells resulting in proinflammatory responses and subsequent progression of autoimmune diseases. These studies further suggest broader implication of scavenger receptor function of CD16. It will also be important to determine whether human CD16 has similar scavenger receptor–like function.

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References


