Materials and Methods

Materials

RPMI-1640 was obtained from Mediatech (Herndon, VA); fetal bovine serum (FBS) and Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+} (DPBS) and without Ca^{2+} and Mg^{2+} were obtained from Invitrogen (Carlsbad, CA); 6-well, 12-well, and 10-cm CellBIND culture plates and dishes were obtained from Corning (Corning, NY); T-75 CELLSTAR® tissue culture flasks were obtained from Greiner Bio One (Monroe, NC); human macrophage colony-stimulating factor (M-CSF), human interleukin-10, and mouse M-CSF were obtained from PeproTech (Rocky Hill, NJ); acetylated low-density lipoprotein (AcLDL) was obtained from Intracel (Frederick, MD); TO901317 (TO9) was obtained from Cayman Chemical (La Jolla, CA); egg sphingomyelin was obtained from Avanti Polar Lipids (Alabaster, AL); glycerol-gelatin mounting media, penicillin-streptomycin, L-glutamine, and bovine serum albumin (BSA) (catalogue #A7906) were obtained from Sigma (St. Louis, MO); mouse anti-cholesterol microdomain Mab 58B1 IgM in ascites was produced as previously described [1], mouse anti-*Clavibacter michiganense* Mab (clone 9A1) IgM in ascites was obtained from Agdia (Elkhart, IN); paraformaldehyde was obtained from Polysciences (Warrington, PA); biotinylated goat anti-mouse IgM, and Vectashield hard set mounting medium with 4'-6-diamidino-2-phenylindole (DAPI) were obtained from Vector Laboratories (Burlingame, CA); and Streptavidin Alexa Fluor 488 Conjugate, 0.25% trypsin- ethylenediaminetetraacetic acid (EDTA) solution (25200056),

EDTA solution (AM9260G), 2.5% trypsin solution (15090046), and trypsin inhibitor solution (R-007-100) were all obtained from Invitrogen (Grand Island, NY).

ApoA-I mimetic peptide and ApoA-I

Preparation of the 37 amino acid ApoA-I mimetic peptide 5A and control peptide EE were described previously [2, 3]. Amino acids phenylalanine-18 and tryptophan-21 of peptide 5A are substituted by amino acid glutamic acid in peptide EE. In contrast to peptide 5A, peptide EE does not bind the phospholipid, 1-palmitoyl, 2-oleoyl phosphatidylcholine or solubilize dimyristoyl phosphatidylcholine vesicles [2]. Peptide 5A-sphingomyelin (SPH) complexes were prepared by a co-lyophilization procedure as previously described using a peptide to sphingomyelin ratio of 1:1.25 w/w [4]. The peptide 5A-SPH complexes were highly homogeneous discoidal shaped recombinant HDL of 8-12 nm diameters. In experiments comparing peptide 5A to peptide 5A-SPH complexes, peptide 5A-SPH was added such that equal peptide 5A concentrations were attained. SPH added alone was added at the same concentration (125 µg/ml) as would occur with 5A-SPH complexes that were added to incubations.

When added to macrophages, 5-times more of ApoA-I mimetic peptide was added on a weight basis compared with ApoA-I taking into account that ApoA-I has 10 amphipathic helices and the ApoA-I mimetic peptide has 2 amphipathic helices (5:1 amphipathic helical content). Amphipathic helices are one factor that

determines the amount of phospholipid that can be bound by ApoA-I and ApoA-I mimetic peptide. The amount of bound phospholipid in turn determines the amount of cholesterol that the ApoA-I- and ApoA-I mimetic peptide-phospholipid complexes can bind.

To prepare ApoA-I, 40 milligrams of delipidated HDL from a donor was fractionated at 5°C on a Sephacryl S-200 superfine 2.5 x 186 cm column (GE Healthcare Bio-Sciences, Pittsburg, PA) in a buffer containing 6 M urea, 0.5 M NaCl, 50 mM glycine, and 2 mM NaOH (pH 8.8). The fractions that contained ApoA-I were ascertained by SDS/polyacrylamide slab gel electrophoresis. The eluted fractions containing ApoA-I were pooled and dialyzed against 0.01 M ammonium bicarbonate (pH 8.2).

Mice

Female ABCA1-/- mice were generated from DBA/1-*Abca1*^{tm1Jdm}/J mice (#003897) obtained from Jackson Laboratory (Bar Harbor, ME). These mice were of mixed genetic background. The ABCA1 mutation was transferred to a C57BL/6N background by 10 consecutive crossings with C57BL/6N. Wild-type C57BL/6 control mice were sex and age-matched to ABCA1-/- mice.

Culture of mouse bone marrow-derived macrophages

Femurs and tibias were isolated from mice and muscle was removed. Both ends of the bones were cut with scissors and then flushed with 3.5 ml of RMPI-1640

medium with a 25-gauge needle. Bone marrow cells were centrifuged and resuspended at a concentration of 4 to 6 $x10^6$ cells/ml in 1 ml of freezing media containing 90% FBS and 10% DMSO [5]. Cells were stored at -80°C overnight and then in liquid nitrogen until use.

On the day of use, 4 to 6 $x10^6$ cells were thawed and added to 25 ml RPMI 1640 medium containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, 10% FBS, and 50 ng/ml mouse M-CSF (complete medium). The cell suspension was then placed into a 75-cm^2 culture flask and incubated in a 37°C cell culture incubator with 5% CO_2/95% air. After 24 hours, cultures were rinsed 3 times with RPMI 1640 medium containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, and then cultured in fresh complete medium. Medium was changed every 2 days until sufficient macrophages had grown in the flask, which usually occurred by the 7th day.

Experiments were initiated by harvesting macrophages at 37°C with 10 ml 0.25% trypsin-EDTA solution. After about 20-30 minutes, macrophages rounded but mostly remained attached. Trypsinization was stopped by addition of 10 ml RMPI 1640 medium containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine and 10% FBS. A cell lifter was used to retrieve macrophages from the culture surface. The cell suspension was centrifuged 5 minutes at 300 *g* and the resulting cell pellet was resuspended in 1 ml complete medium. Macrophages were counted with a hemocytometer. For immunostaining

experiments, 1 x10^5 macrophages per well were seeded in 12-well CellBIND culture plates containing 1.5 ml of complete medium per well, and for cholesterol efflux experiments, 3 x10^5 macrophages/per well were seeded in 6-well CellBIND culture plates containing 3 ml of complete medium per well. Macrophages were incubated overnight before experiments were initiated with 1 ml of complete medium and the indicated additions but without FBS. Experimental incubations were carried out for 4 days with the medium and additions refreshed after 2 days.

Animal studies were conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and were approved by the NHLBI Institutional Animal Care and Use Committee.

Culture of human monocyte-derived macrophages

Mononuclear cells were obtained from human donors by monocytopheresis and subsequently purified using counterflow centrifugal elutriation as previously described [6]. Monocytopheresis was carried out under a human subjects research protocol approved by a National Institutes of Health institutional review board. The monocytes were centrifuged at 300 *g* for 5 min at room temperature. Then, 25 x 10^6 monocytes were resuspended in 25 ml of complete medium (RPMI 1640 medium with 2 mM L-glutamine, 50 ng/ml human M-CSF, 25 ng/ml interleukin-10, and 10% FBS) and seeded into a 75 cm^2 cell culture flask. Cultures were incubated in a 37°C cell culture incubator with 5% CO_2/95% air for 48 hr. Next, the cultures were rinsed 3 times with 10 ml RPMI 1640 medium. Following

rinsing, fresh complete medium was added and medium was changed every 2 days until monocytes differentiated and proliferated sufficiently to become confluent. This required about 1 week of culture.

Experiments were initiated by rinsing the differentiated macrophages in the flask 3 times with 10 ml Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+}, adding 10 ml 0.25% trypsin-EDTA solution, and incubating the flask at 37°C for 10-15 min to detach the macrophages. Then, 10 ml of RMPI 1640 medium containing 10% FBS was added to stop trypsinization. The macrophage cell suspension was centrifuged, resuspended in 1 ml of complete medium, counted, and seeded into culture plates as described above for mouse bone marrow-derived macrophage immunostaining experiments. Macrophages were incubated overnight to allow cell attachment before initiating experiments the next day with complete medium minus FBS.

Determination of cell viability

Cell viability was determined for all conditions using the Pierce LDH cytotoxicity assay kit (Pierce Biotechnology, Rockford, IL, number 88953) following the manufacturer instructions. Release of macrophage LDH into the medium relative to total culture LDH gives an estimate of the cell viability. All determinations were based on triplicate wells. Macrophage viability was greater than 88% for all conditions (Supplemental Table I).

Removal of human monocyte-differentiated macrophages from culture wells

When required, human monocyte-derived macrophages were removed from culture wells after they were cholesterol-enriched by incubation with 50 µg/ml AcLDL. Then, cultures were rinsed 3 times with DPBS (without Ca^{2+} and Mg^{2+}) at room temperature. Next, macrophage cultures were incubated with 1 ml of DPBS (without Ca^{2+} and Mg^{2+}) containing 5 mM EDTA at 37°C for 20-30 minutes. This was followed by flushing the macrophages from the culture surface by pipetting the EDTA solution up and down over the culture surface. Removal of the released macrophages was carried out with 3 rinses of DPBS (without Ca^{2+} and Mg^{2+}). Lastly, the culture wells, now without macrophages, were incubated with RPMI 1640 medium and the indicated additions.

Immunostaining of macrophages

Fixation, immunostaining, and microscopy were all performed with macrophages in their original 12-well CellBIND culture plates, and all steps were carried out at room temperature. Macrophage cultures were rinsed 3 times (5-minutes each time) in DPBS, fixed for 10 minutes with 4% paraformaldehyde in DPBS, and then rinsed an additional 3 times in DPBS. Macrophages were then incubated 60 minutes with 5 µg/ml purified mouse anti-cholesterol microdomain Mab 58B1 IgM diluted in DPBS containing 0.1% BSA. Control staining was performed with 5 µg/ml of an irrelevant purified mouse anti-*Clavibacter michiganense* Mab (clone 9A1) IgM diluted in DPBS containing 0.1% BSA. Both antibodies were purified

with the ImmunoPure IgM purification kit from Pierce (Rockford, IL). Then, cultures were rinsed 3 times (5 minutes each) in DPBS, followed by a 30-minute incubation with 5 µg/ml biotinylated goat anti-mouse IgM diluted in DPBS containing 0.1% BSA. After 3 rinses in DPBS (5 minutes each), cultures were incubated 10 minutes with 10 µg/ml Streptavidin Alexa Fluor 488 diluted in DPBS. After rinsing 3 times with DPBS, the cultures were mounted in Vectashield hard-set mounting medium with DAPI nuclear stain in preparation for digital imaging using an Olympus IX81 fluorescence microscope. Because macrophages were not permeabilized, Mab 58B1 staining represents cell surface or extracellular staining. No staining was observed when the control Mab was substituted for the anti-cholesterol microdomain Mab.

Microscopic analysis

Cells were identified using phase-contrast microscopy, or by locating DAPI-stained nuclei. The pattern and intensity of Mab 58B1 staining were assessed with cultures for each experimental parameter, and these data were compared with one another. We considered Mab 58B1-labeling cellular if it was located within cell membrane boundaries, as identified on the corresponding phase-contrast view. Labeling was considered extracellular if it was located outside the cell membrane boundaries seen on phase-contrast view. Different planes of focus were visualized before acquiring images to confirm that only a monolayer of cells was present, thereby ensuring that labeling seen outside cell membrane boundaries did not represent cellular labeling from cells lying in a different plane

of focus. The immunostained cells shown in figures are representative of a minimum of 5 microscopic fields viewed in one culture well.

CD14 immunostaining of macrophage cultures

Where indicated macrophages were removed from the culture wells as described above. Fixation, immunostaining, and microscopy were all performed with macrophages in their original 12-well CellBIND culture plates, and all steps were carried out at room temperature. Macrophage cultures were rinsed 3 times (5-minutes each time) in DPBS, fixed for 10 minutes with 4% paraformaldehyde in DPBS, and then rinsed an additional 3 times in DPBS. Macrophages were then incubated 60 minutes with 1 µg/ml purified mouse anti-CD14 IgG2a Mab (AB181470, Abcam, Cambridge, MA) diluted in DPBS containing 1.0% BSA. Control staining was performed with 1 µg/ml of an isotype-matched purified mouse Mab (RS-90G2A, ICL, Portland, OR) diluted in DPBS containing 1.0% BSA. Then, cultures were rinsed 3 times (5 minutes each) in DPBS, followed by a 30-minute incubation with 5 µg/ml biotinylated goat anti-mouse IgG (BA-9200, Vector Laboratories, Burlingame, CA) diluted in DPBS containing 0.1% BSA. After 3 rinses in DPBS (5 minutes each), cultures were incubated 10 minutes with 5 µg/ml Streptavidin Alexa Fluor 488 diluted in DPBS. After rinsing 3 times with DPBS, the cultures were mounted in Vectashield hard-set mounting medium with DAPI nuclear stain. Because macrophages were not permeabilized, anti-CD14 immunostaining represents cell surface or extracellular staining. No

immunostaining was observed when the control Mab was substituted for the anti-CD14 Mab.

Quantification of macrophage cholesterol

After culture and incubation in 6-well CellBIND culture plates, macrophage cultures were rinsed 3 times in DPBS. Then, macrophages were harvested from wells by scraping into 1 ml distilled water. Thus, cholesterol measurements represent both macrophage- and extracellular matrix-associated cholesterol. Lipid was extracted from the resulting cell suspension using the Folch method [7], and quantities of esterified and unesterified cholesterol were determined using the method previously described by Gamble et al. [8]. Total cholesterol content is shown in Figures 2 and 6. Protein quantification was performed on an aliquot of cell lysate using the Lowry method with BSA as a standard [9].

Data are presented as the mean ± SEM. Means were determined from 3 replicate wells. Significant differences were determined with one-way ANOVA analysis and Tukey's multiple comparisons test. A p-value ≤ 0.5 was considered significant.

Isolation of cholesterol microdomains

One-week-old human M-CSF differentiated monocyte-derived macrophage cultured from an initial seeding of $12x10^5$ monocytes in each of six 10-cm CellBind dishes were incubated 2 days with 50 µg/ml AcLDL to induce

macrophage deposition of extracellular cholesterol microdomains. Then, macrophages were removed from the culture with 5 mM EDTA, and the cultures were rinsed twice with DPBS without Ca^{2+} and Mg^{2+}. This was followed by release of the cholesterol microdomains from the extracellular matrix using 2 ml of 2.5% trypsin solution per dish incubated for 30 minutes at 37°C. Then, the trypsin was neutralized by addition of 2 ml of trypsin inhibitor solution.

The resulting cholesterol microdomain-containing solution was centrifuged in a polypropylene tube at 1000 x*g* for 2 minutes, and the supernatant was transferred to another tube. Then, the supernatant was concentrated to 1 ml with a centrifugal filter (4304, EMB Millipore, Danvers, MA). Next, the concentrate was subjected to density gradient centrifugation to separate the cholesterol microdomains from other lipids (e.g., AcLDL) that might have been released from the extracellular matrix. Density gradient centrifugation was carried out similar to that described previously [10]. The gradient was constructed in a 14x89 mm polyallomer centrifuge tube from bottom to top with 2.5 ml of 1.10 g/ml, 2.5 ml of 1.061 g/ml, 2.0 ml of 1.019 g/ml, and 2.0 ml of 1.016 g/ml NaCl solutions. Lastly, 1 ml of sample was applied to the top of the gradient. All solutions contained 0.1% EDTA. The initial discontinuous gradient was centrifuged to form a continuous gradient in an SW41Ti rotor at 170,000 x*g* for 22 hours (4°C) using a Beckman ultracentrifuge (Optima L-100XP, Beckman Coulter, Indianapolis, IN)[11]. Upon completion, 1 ml fractions were collected from top to bottom with the pellet resuspended in the last fraction.

Cholesterol was analyzed in the fractions using the cholesterol assay method described above for macrophages. Density was determined by weighing the fractions. Lastly, a sample (20 µl) from each of the 3 cholesterol-enriched density gradient regions was applied to a microscope slide, and after the sample dried, it was immunostained with the anti-cholesterol microdomain Mab 58B1 as described above for immunostaining of macrophage cultures.

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