
Xueting Jin, Denis Sviridov, Ying Liu, Boris Vaisman, Lia Addadi, Alan T. Remaley, Howard S. Kruth

Objective—We examined the function of ABCA1 (ATP-binding cassette transporter A1) in ApoA-I (apolipoprotein A-I) mobilization of cholesterol microdomains deposited into the extracellular matrix by cholesterol-enriched macrophages. We have also determined whether an ApoA-I mimetic peptide without and with complexing to sphingomyelin can mobilize macrophage-deposited cholesterol microdomains.

Approach and Results—Extracellular cholesterol microdomains deposited by cholesterol-enriched macrophages were detected with a monoclonal antibody, 58B1. ApoA-I and an ApoA-I mimetic peptide 5A mobilized cholesterol microdomains deposited by ABCA1<sup>−/−</sup> macrophages but not by ABCA1<sup>+/−</sup> macrophages. In contrast, ApoA-I mimetic peptide 5A complexed with sphingomyelin could mobilize cholesterol microdomains deposited by ABCA1<sup>−/−</sup> macrophages.

Conclusions—Our findings show that a unique pool of extracellular cholesterol microdomains deposited by macrophages can be mobilized by both ApoA-I and an ApoA-I mimetic peptide but that mobilization depends on macrophage ABCA1. It is known that ABCA1 complexes ApoA-I and ApoA-I mimetic peptide with phospholipid, a cholesterol-solubilizing agent, explaining the requirement for ABCA1 in extracellular cholesterol microdomain mobilization. Importantly, ApoA-I mimetic peptide already complexed with phospholipid can mobilize macrophage-deposited extracellular cholesterol microdomains even in the absence of ABCA1. (Arterioscler Thromb Vasc Biol. 2016;36:2283-2291. DOI: 10.1161/ATVBAHA.116.308334.)

Key Words: apolipoprotein A-I ■ atherosclerosis ■ cholesterol ■ extracellular matrix ■ macrophages

Atherosclerotic plaques show accumulation of both intracellular and extracellular lipid. Our earlier studies show that much unesterified cholesterol accumulates within the extracellular matrix of human atherosclerotic plaques.¹⁻³ Thus, strategies to decrease lipid accumulation by stimulating reverse cholesterol transport from plaque cholesterol-containing cells should also address how to mobilize extracellular cholesterol deposits.

Recently, we have shown that macrophages contribute to extracellular deposits of unesterified cholesterol by depositing excess cholesterol into the extracellular matrix.⁴⁻⁶ Depending on the macrophage type and culture conditions, macrophages enriched with cholesterol show development of cholesterol microdomains that are associated with the plasma membrane or that macrophages deposit into the extracellular matrix.⁴⁻⁶ We have visualized these cholesterol microdomains with a unique monoclonal antibody that immunolabels an ordered array of cholesterol molecules.⁷ Both ABCA1 (ATP-binding cassette transporter A1) and ABCG1 (ATP-binding cassette transporter G1) mediate extracellular deposition of the cholesterol microdomains by mouse bone marrow–derived macrophages, whereas ABCA1 mediates their deposition by human macrophage colony-stimulating factor–differentiated monocyte-derived macrophages.⁴⁻⁵ This previously unrecognized pool of extracellular cholesterol microdomains can be mobilized by high-density lipoprotein, cyclodextrin, and ApoA-I (apolipoprotein A-I).⁴⁻⁶ However, in contrast to high-density lipoprotein, ApoA-I mobilization of the cholesterol microdomains depends on the presence of macrophages.⁶ The fact that the cholesterol microdomains deposited by macrophages can be mobilized by high-density lipoprotein and ApoA-I shows that the extracellular cholesterol microdomains can potentially function in the reverse cholesterol transport pathway.

There has been much interest in the development of ApoA-I mimetic peptides that could be administered to patients as treatment for atherosclerosis.⁸⁻¹⁰ ApoA-I mimetics would hopefully promote reverse cholesterol transport and
thus decrease cholesterol build-up in atherosclerotic plaques. The advantage of administering ApoA-I mimetic peptides compared with administration of ApoA-I itself would be a lesser cost to produce the mimetic peptides and the possibility that these peptide mimetics could be administered orally. To date, clinical trials of some early ApoA-I mimetics have shown limited efficacy and unexpected toxicities. However, refinement of the peptides may allow for enhancement of their cholesterol mobilization properties and diminished toxicity.

One such refined ApoA-I mimetic peptide is 5A, which has been shown to stimulate cholesterol efflux from a mouse macrophage cell line and to decrease atherosclerosis in a mouse model of atherosclerosis. Peptide 5A is a 37-amino acid–long birec, helical amphipathic peptide that contains an 18A α-helix sequence in the first helix as described previously, joined by a proline with a modified 18A sequence containing 5 Ala substitutions in the second helix. Similar to ApoA-I and other ApoA-I mimetic peptides, peptide 5A can be complexed with phospholipids such as sphingomyelin to increase their potential to stimulate cellular cholesterol efflux independent of ABCA1.

To further investigate the potential efficacy of peptide 5A for human therapy of atherosclerosis, we have examined whether this ApoA-I mimetic peptide can mobilize the extracellular cholesterol microdomains deposited by cholesterol-enriched human monocyte-derived macrophages. Also, we have tested whether ApoA-I and peptide 5A require ABCA1 function in order for these agents to mobilize the extracellular cholesterol microdomains deposited by macrophages. Last, we compared the efficacy of ApoA-I and peptide 5A with peptide 5A complexed with sphingomyelin in mobilization of extracellular cholesterol microdomains. Our findings indicate that while ApoA-I and peptide 5A depend on ABCA1 function for their efficacy in mobilizing extracellular cholesterol microdomains, peptide 5A complexed with sphingomyelin can mobilize extracellular cholesterol microdomains independent of ABCA1 function.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

**ApoA-I and ApoA-I Mimetic Peptide 5A Mobilize Extracellular Cholesterol Microdomains Deposited by Macrophages**

Human macrophage colony-stimulating factor–differentiated monocyte-derived macrophages were cholesterol enriched by 1-day incubation with acetylated low-density lipoprotein (50 μg/mL). During this incubation, macrophages deposited cholesterol (detected with monoclonal antibody [Mab]58B1) into the extracellular matrix surrounding the macrophages as we reported previously. After rinsing of the cultures and subsequent 1-day incubation with peptide 5A, peptide 5A–sphingomyelin, or ApoA-I, Mab58B1 immunostaining was eliminated consistent with mobilization of the extracellular cholesterol microdomains (Figure 1). In contrast, cholesterol was not mobilized when macrophages were incubated with control peptide EE or with no addition. The effect of peptide 5A was concentration dependent with a maximal effect observed at a concentration of 40 μg/mL (Figure I in the online-only Data Supplement). On the contrary, peptide EE sometimes (Figure 1), but not always (Figure I in the online-only Data Supplement) increased extracellular cholesterol microdomain immunostaining for reasons that are not clear to us.

We also assessed the cholesterol content of cholesterol-enriched macrophages after incubation with ApoA-I and the...
ApoA-I mimetic peptide. Peptide 5A, peptide 5A–sphingomyelin, and ApoA-I all caused large and similar decreases in cholesterol content of the cholesterol-enriched human macrophages (=170–190 nmoles cholesterol/mg cell protein equivalent to ≈70% of the accumulated cholesterol; Figure 2). Sphingomyelin added alone without having been complexed with peptide 5A did not decrease macrophage cholesterol content. Peptide EE showed some decrease in macrophage cholesterol content, but the amount (39±8 nmoles cholesterol/mg cell protein) was much less than that of peptide 5A.

Uncomplexed With Sphingomyelin, ApoA-I Mimetic Peptide 5A Does Not Mobilize Extracellular Cholesterol Microdomains in Absence of Macrophages

Previously, we reported that ApoA-I could not mobilize extracellular cholesterol microdomains when macrophages were removed from the culture. Therefore, we examined whether peptide 5A could mobilize extracellular cholesterol microdomains in the absence of macrophages. First, human macrophages were cholesterol enriched by incubation with acetylated low-density lipoprotein as described above except that incubation was for 2 days with the liver X receptor agonist, TO901317 (TO9), added to induce maximally ABCA1 and ABCG1 that we previously showed to mediate macrophage deposition of extracellular cholesterol microdomains. Then, macrophages were removed from one set of cultures leaving only the extracellular macrophage-deposited cholesterol. Macrophages were not removed in a duplicate set of cultures. Both sets of cultures without and with macrophages were then incubated 1 day without and with the various peptides followed by Mab58B1 immunostaining of cholesterol microdomains.

Without the presence of macrophages during the incubation with peptides or ApoA-I, only peptide 5A–sphingomyelin mobilized cholesterol from the extracellular matrix (Figure 3). However, as compared with its effect with macrophages present, some patches of extracellular Mab58B1 immunostaining remained. This experiment with intensified deposition of cholesterol microdomains showed that in the presence of macrophages, peptide 5A–sphingomyelin completely eliminated Mab58B1 immunostaining, whereas peptide 5A and ApoA-I only partially decreased Mab58B1 immunostaining. This is contrast to the experiment above (Figure 1), where peptide 5A, peptide 5A–sphingomyelin, and ApoA-I completely eliminated extracellular Mab58B1 immunostaining in the presence of macrophages.

The extracellular cholesterol microdomains that underlie cholesterol-enriched macrophages were not simply cell debris left behind on the culture surface. This was the case because CD14, a macrophage plasma membrane marker, while present on the macrophage surface, was not present in the extracellular matrix (Figure II in the online-only Data Supplement).

Uncomplexed With Sphingomyelin, ApoA-I Mimetic Peptide 5A Fails to Mobilize Extracellular Cholesterol Microdomains Deposited by ABCA−/− Mouse Macrophages

Peptide 5A mobilization of macrophage-deposited cholesterol microdomains depended on the continued presence of macrophages, whereas peptide 5A–sphingomyelin complex mobilization of extracellular cholesterol microdomains did not depend on macrophages. This suggests that macrophages were necessary for peptide 5A efficacy possibly by providing ABCA1-mediated complexing of macrophage phospholipid with peptide 5A as occurs with ApoA-I. We examined this possibility.

First, we determined the degree of mobilization of macrophage-deposited cholesterol microdomains during a 2-day incubation with peptide 5A. In contrast to the 1-day incubation with peptide 5A as shown above in Figure 3, where Mab58B1 immunostaining was only partially decreased, a 2-day incubation showed complete elimination of Mab58B1 immunostaining (Figure 4). Peptide 5A–sphingomyelin and ApoA-I (but not peptide EE) showed a similar elimination of Mab58B1 immunostaining. However, this was not the case when ABCA−/− mouse macrophages were tested. In this case, only peptide 5A–sphingomyelin could mobilize extracellular cholesterol microdomains but peptide 5A and ApoA-I could not (Figure 5). Thus, ABCA1 was necessary for peptide 5A and ApoA-I mobilization of extracellular cholesterol microdomains.

We next assessed the importance of ABCA1 for macrophage cholesterol mass efflux mediated by peptides 5A and 5A–sphingomyelin. Similar to human macrophages, peptides 5A and 5A–sphingomyelin caused a substantial decrease in
cholesterol content (218±15 and 309±25 nmoles cholesterol/mg cell protein, respectively) in cholesterol-enriched ABCA1+/+ (wild type) mouse macrophages (Figure 6). Also, similar to human macrophages, the control peptide EE decreased cholesterol content substantially less (61±18 nmoles cholesterol/mg cell protein) than the ApoA-I mimetic peptide 5A. However, only peptide 5A–sphingomyelin significantly decreased the cholesterol content of cholesterol-enriched ABCA1−/− mouse macrophages (Figure 6). This showed that peptide 5A required macrophage ABCA1 to stimulate macrophage cholesterol efflux.

**Isolated Cholesterol Microdomains Contain Cholesterol that Is Unestified**

The anticholesterol microdomain Mab58B1 shows the presence of cholesterol in the extracellular deposits, and we previously showed that the deposits do not stain with Oil Red O indicating the lack of cholesteryl ester. However, to confirm that the cholesterol microdomains contain cholesterol in a predominantly unestified form, we analyzed the cholesterol composition of the extracellular cholesterol microdomains. We isolated the cholesterol microdomains by first removing macrophages from culture wells using EDTA and then releasing the microdomains from the extracellular matrix by trypsin treatment. This was followed by density gradient centrifugation of the released cholesterol microdomains (Figure III in the online-only Data Supplement). The density gradient showed 3 regions of cholesterol at densities ≤1.005, 1.030 to 1.044, and ≥1.091 g/mL showing 51%, 91%, and 95% of cholesterol that was unesterified, respectively. The cholesteryl ester in the density ≤1.005 region is likely extracellular matrix-bound acetylated low-density lipoprotein that floats after loss of protein after exposure to protease. Mab58B1 immunostaining of the isolated cholesterol particles from each cholesterol-rich density gradient region showed that the density 1.030 to 1.044 g/mL cholesterol region was most enriched with cholesterol microdomains that stained with the anticholesterol microdomain Mab58B1 (green), and nuclei were imaged with DAPI fluorescence staining (blue). To the right of each fluorescence image is the corresponding, phase-contrast microscopic image. f/b indicates followed by. Bar=50 µm and applies to all.

**Figure 3.** Unless complexed with sphingomyelin, ApoA-I (apolipoprotein A-I) mimetic peptide 5A does not mobilize extracellular cholesterol microdomains in the absence of macrophages. One-wk-old human macrophage colony-stimulating factor–differentiated monocyte-derived macrophage cultures were incubated 2 d with 50 µg/mL acetylated low-density lipoprotein (AcLDL)+5 µm TO901317 (TO9) to induce macrophage deposition of extracellular cholesterol microdomains. After rinsing, macrophages remained in a one set of cultures (left) and were removed from a second set of cultures (right). Next, both sets of culture wells were incubated 1 d with the indicated additions (20 µg ApoA-I, 100 µg/mL peptide, and 125 µg/mL sphingomyelin complexed with 100 µg/mL peptide 5A [5A-SPH]) without AcLDL. Cholesterol microdomains were visualized by fluorescence microscopy using anticholesterol microdomain Mab58B1 (green), and nuclei were imaged with DAPI fluorescence staining (blue). To the right of each fluorescence image is the corresponding, phase-contrast microscopic image. f/b indicates followed by. Bar=50 µm and applies to all.
ApoA-I and ApoA-I mimetic peptide 5A mobilization of extracellular cholesterol microdomains depended on ABCA1. The cholesterol microdomains deposited by macrophages could not be directly solubilized by peptide 5A or ApoA-I. This is presumably because ABCA1 mediates complexing of cell-derived phospholipid with ApoA-I and ApoA-I mimetic peptides, and it is the phospholipid that solubilizes cholesterol. Under certain conditions, ApoA-I and ApoA-I mimetic peptides can directly solubilize phospholipid and form complexes without ABCA1. However, this depends on the phospholipid type and the amount of cholesterol associated with the phospholipid. The fact that the cholesterol microdomains could not be directly solubilized by ApoA-I or the ApoA-I mimetic peptide suggests that the cholesterol microdomains are not associated with a cholesterol-solubilizing lipid that is susceptible to amphipathic apolipoprotein-induced micellarization.

Interestingly, an ApoA-I–binding site has been described in the extracellular matrix underlying macrophages. Thus, this binding site localizes ApoA-I to the same location where the extracellular cholesterol microdomains deposit. Because the ApoA-I–binding site mediates ABCA1-dependent cholesterol efflux, this binding site could be functioning in mobilization of the macrophage-deposited cholesterol microdomains.

The cholesterol pool that is mobilized by ApoA-I interaction with ABCA1 has been postulated to be present within the plasma membrane, intracellularly within late endosomes/lysosomes, and within the extracellular matrix as we have shown here. It is possible that efflux from all these cholesterol pools could be occurring simultaneously or individually depending on the cell type under study. Also, intracellular
cholesterol pools may be a source of cholesterol that is available for efflux within the plasma membrane or extracellular matrix. In this regard, ABCA1 redistributes intracellular cholesterol to cholesterol oxidase-sensitive pools of cholesterol that could be located within either the plasma membrane or the extracellular cholesterol microdomains we have described.4,34

Figure 6. ApoA-I (apolipoprotein A-I) mimetic peptide 5A substantially decreases ABCA+/+ but not ABCA−/− mouse macrophage cholesterol content. One-wk-old mouse macrophage colony-stimulating factor– differentiated bone marrow–derived macrophage cultures were incubated 2 d with 50 µg/mL acetylated low-density lipoprotein (AcLDL)+5 µM TO901317 (TO9) to enrich macrophages with cholesterol. After rinsing, macrophage cultures were incubated 2 d with the indicated additions (100 µg/mL peptide and 125 µg/mL sphingomyelin alone or complexed with 100 µg/mL peptide 5A [5A-SPH]) without AcLDL. Then, the macrophage total cholesterol content was determined, and the decrease in macrophage cholesterol content induced by the added peptide was calculated. Cholesterol contents before and after incubation with AcLDL, respectively, were 107±14 nmoles cholesterol/mg cell protein (10±2% cholesteryl ester) and 453±27 nmoles cholesterol/mg cell protein (70±0% cholesteryl ester) for ABCA+/+ macrophages and 146±26 nmoles cholesterol/mg cell protein (25±10% cholesteryl ester) and 571±7 nmoles cholesterol/mg cell protein (75±1% cholesteryl ester) for ABCA−/− macrophages. For ABCA1+/+ macrophages, 5A and 5A-SPH were significantly different than EE and SPH. For ABCA1−/− macrophages, 5A-SPH was significantly different than 5A, SPH, and EE. EE indicates peptide EE; and f/b, followed by.

Previous investigations of ApoA-I–mediated cellular cholesterol efflux have suggested that cholesterol is concurrently effluxed with the phospholipid that complexes with ApoA-I (one-step process).23,30,35,36 Simultaneous complexing of phospholipid and cholesterol with ApoA-I has been suggested to result from microsolubilization of plasma membrane microdomains.37 Both nonraft and raft microdomains, the latter characterized by sphingomyelin and cholesterol enrichment, may contribute to such ApoA-I–lipid complexes.38–40

Alternatively, ApoA-I could complex with phospholipid before the complex acquiring cholesterol from the cell (2-step process).35,41–44 The findings that ABCA1-mediated phospholipid efflux can occur without cholesterol efflux and that cholesterol efflux can be regulated independent of phospholipid efflux in some cell systems could be interpreted to support a 2-step process.33,35,41–47 Our findings show that in the case of extracellular cholesterol microdomains, a 2-step process mediates cholesterol efflux from the extracellular matrix. First, macrophages excrete cholesterol microdomains into the extracellular matrix, and subsequently interaction of ApoA-I with macrophage ABCA1 mobilizes the cholesterol microdomains.

We have shown previously that ABCA1 and ABCG1 mediate macrophage deposition of cholesterol microdomains into the extracellular matrix.4,5 Thus, ABCA1 shows 2 separate functions: one that mediates deposition of the extracellular cholesterol microdomains and another that mediates ApoA-I’s mobilization of the extracellular microdomains.

Dependence on ABCA1 to mediate complexing of phospholipid with ApoA-I and ApoA-I mimetic peptides could be a rate limiting process in reverse cholesterol transport from atherosclerotic plaques because ABCA1 expression in human atherosclerotic plaques is diminished. Studies have shown that ABCA1 expression in human atherosclerotic plaques is limited because of the differentiation state of intimal smooth muscle cells48 and in the case of macrophages possibly because of the fact that ABCA1 is downregulated by various cytokines likely present within atherosclerotic plaques.49 Moreover, much of the cholesterol within human atherosclerotic plaques accumulates in relatively acellular plaque regions where there would not be any cellular expression of ABCA1.50 In these acellular regions, there is no possibility to locally complex either Apo-I or Apo-I mimetic peptides with cell-derived phospholipid.

Given that much of the cholesterol in human atherosclerotic plaques accumulates within the extracellular space,1–3,6 mobilizing extracellular cholesterol microdomains may be as important as mobilizing cholesterol stored within atherosclerotic plaque cells. Because of dependence on ABCA1 as discussed above, peptide 5A and ApoA-I could not mobilize extracellular cholesterol microdomains deposited by macrophages when macrophages were removed from the cultures or when macrophages lacked ABCA1. In contrast, peptide 5A complexed with sphingomyelin could mobilize extracellular cholesterol microdomains under these conditions showing the potential usefulness of preformed ApoA-I mimetic–phospholipid complexes in mobilizing extracellular cholesterol microdomains even in the absence of ABCA1.
The choice of sphingomyelin as the phospholipid complexed to peptide 5A should be optimal for mobilization of cholesterol as this phospholipid has a relatively high affinity for cholesterol as compared with other common phospholipids.  However, sphingomyelin alone did not show any mobilization of extracellular cholesterol microdomain deposits or stimulate a decrease in macrophage cholesterol content.  The sphingomyelin was added as liposomes.  Such liposomes previously have been shown to be poor acceptors of cholesterol, most likely because of size constraints limiting access of these relatively large particles to sites of potential cholesterol efflux.  In this regard, efficient delivery of cholesterol acceptors to sites of cholesterol accumulation within atherosclerotic plaques is also an obstacle to attaining plaque reverse cholesterol transport.  Efficient delivery of cholesterol acceptors may not only be limited by acceptor size but also by loss of acceptor efflux potential as the acceptor passes through the blood and picks up cholesterol effluxed from other sites besides plaques such as liver and the spleen that processes cholesterol from degraded red cells.

ApoA-I and ApoA-I mimetic peptides show a beneficial effect on the development of atherosclerosis in experimental animal models.  The mechanism of this effect may be multifactorial, including anti-inflammatory effects in addition to stimulating reverse cholesterol transport by mobilizing cellular and, as we have demonstrated here, extracellular cholesterol microdomains.  If not mobilized, extracellular cholesterol microdomains could promote atherogenesis by promoting inflammation.  The antibody used here to detect extracellular deposited cholesterol does not bind individual cholesterol molecules.  Rather, this antibody labels a structural domain of organized cholesterol molecules as can occur for example in a cholesterol crystal.  Although the physical form of the extracellular cholesterol microdomains deposited by macrophage has not yet been determined, high concentrations of cholesterol in crystals or liposomes can stimulate complement formation and thereby promote inflammation.  Thus, future studies of the potential inflammatory properties of the extracellular cholesterol microdomains is warranted.

In conclusion, previously we showed that both ABCA1 and ABCG1 mediate macrophage deposition of extracellular cholesterol microdomains.  Our finding here that ABCA1 is necessary for mobilization of this cholesterol demonstrates an interesting concerted action of ABCA1, both to deposit and then effect mobilization of extracellular cholesterol microdomains.  However, ApoA-I and ApoA-I mimetic peptides already complexed with phospholipid may show better efficacy for both cellular and extracellular plaque cholesterol removal when ABCA1 expression is limited.

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Disclosures

Peptide 5A has been licensed by the National Institutes of Health to KineMed for clinical development. The others report no conflicts.

References

efflux by the ABCA1 transporter. *J Pharmacol Exp Ther.* 2013;344:50–58. doi: 10.1124/jpet.112.198143.


Highlights

- Cholesterol-enriched macrophages deposit cholesterol microdomains into the extracellular matrix.
- ApoA-I (apolipoprotein A-I) and ApoA-I mimetic peptide can mobilize these extracellular cholesterol microdomains by a process mediated by macrophage ABCA1.
- In the absence of macrophage ABCA1, ApoA-I mimetic peptide can mobilize extracellular cholesterol microdomains if the peptide is first complexed with sphingomyelin.
- Recognition that macrophages not only store excess cholesterol intracellularly but also deposit this cholesterol extracellularly is important to our further understanding of reverse cholesterol transport.

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ABCA1 transporter

ApoA-I + phospholipid (nascent HDL)

cholesterol deposition into extracellular matrix

ABCA1 transporter

ApoA-I + phospholipid + cholesterol

plasma membrane

cholesterol microdomains
SUPPLEMENTAL MATERIAL

Supplemental Figures

[Images of cellular images for Peptide 5A and Peptide EE at different concentrations]
Supplemental Figure I. Effect of ApoA-I mimetic peptide 5A concentration on mobilization of extracellular cholesterol deposited by human macrophages. One-week-old human M-CSF differentiated monocyte-derived macrophage cultures were incubated 1 day with 50 µg/ml AcLDL to induce macrophage deposition of extracellular cholesterol microdomains. Following rinsing, macrophage cultures were incubated 1 day with the indicated concentration of either ApoA-I mimetic peptide 5A or control peptide EE without AcLDL. For each peptide, the left-hand column shows cholesterol microdomains visualized by fluorescence microscopy using anti-cholesterol microdomain Mab 58B1 (green), and nuclei imaged with DAPI fluorescence staining (blue). To the right of each fluorescence image is the corresponding phase-contrast microscopic image. Bar = 50 µm and applies to all.
Supplemental Figure II. One-week-old human M-CSF differentiated monocyte-derived macrophage cultures were incubated 2 days with 50 µg/ml AcLDL + 5 µm TO9 to induce macrophage deposition of extracellular cholesterol microdomains. Then, macrophages were removed from one culture and remained in a second culture as labeled. In the upper row, cultures were immunolabeled using either anti-CD14 mouse Mab (left panel-green) or anti-cholesterol microdomain Mab (right panel-green), and nuclei were imaged with DAPI fluorescence staining (blue). In the lower row, macrophages were visualized using phase-contrast microscopy. Upper and lower rows show corresponding sets of microscopic fields. Note that the regions of cholesterol microdomains that surround the attached macrophages and underlie the removed macrophages do not show anti-CD14 immunostaining. Bar = 50 µm and applies to all.
Supplemental Figure III. One-week-old human M-CSF differentiated monocyte-derived macrophage cultures 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 EDTA. This was followed by recovery of the cholesterol microdomains from the extracellular matrix using trypsin treatment. The cholesterol microdomain material was subjected to density gradient centrifugation to separate the microdomains from other lipids (e.g., AcLDL) that might have been released from the extracellular matrix. Bar = 12.5 µm and applies to all.
### Supplemental Table I

Viability of macrophages

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<th>% Viability ± SEM</th>
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AcLDL, acetylated low-density lipoprotein; f/b, followed by; ApoA-I, apolipoprotein A-I; SPH, sphingomyelin; 5A-SPH, peptide 5A-sphingomyelin complex; SEM, standard error of the mean.
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. 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. 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. 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. 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-cm² 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 RPMI 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, $1 \times 10^5$ 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, $3 \times 10^5$ 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, $25 \times 10^6$ 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\(^\circ\)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-\textit{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, and quantities of esterified and unesterified cholesterol were determined using the method previously described by Gamble et al. 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.

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 $12 \times 10^5$ 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 xg 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 xg 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.

References


ApoA-I + phospholipid

cholesterol microdomains

cholesterol deposition into extracellular matrix (nascent HDL)

phospholipid

cholesterol

ApoA-I

ApoA-I (nascent HDL)

ABCA1 transporter

ABCA1 transporter

plasma membrane