Published in final edited form as:
Circ Res. 2013 May 10; 112(10): 1345–1354. doi:10.1161/CIRCRESAHA.111.300581.

Apolipoprotein A-I and HDL Have Anti-Inflammatory Effects on Adipocytes via Cholesterol Transporters: ATP-Binding Cassette (ABC) A-1, ABCG-1 and Scavenger Receptor B-1(SRB-1)

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Abstract

Rationale—Macrophage accumulation in adipose tissue associates with insulin resistance and increased cardiovascular disease risk. We previously have shown that generation of reactive oxygen species (ROS) and monocyte chemotactic factors after exposure of adipocytes to saturated fatty acids (SFAs) such as palmitate occurs via translocation of NADPH oxidase 4 (NOX4) into lipid rafts (LRs). The anti-inflammatory effects of apolipoprotein A-I (apoA-I) and HDL on macrophages and endothelial cells appears to occur via cholesterol depletion of LRs. However, little is known concerning anti-inflammatory effects of HDL and apoA-I on adipocytes.

Objective—To determine whether apoA-I and HDL inhibit inflammation in adipocytes and adipose tissue, and whether this is dependent on LRs.

Methods and Results—In 3T3L-1 adipocytes, apoA-I, HDL and methyl-β-cyclodextrin inhibited chemotactic factor expression. ApoA-I and HDL also disrupted LRs, reduced plasma membrane cholesterol content, inhibited NOX4 translocation into LRs, and reduced palmitate-induced ROS generation and monocyte chemotactic factor expression. Silencing ABCA-1 abrogated the effect of apoA-I, but not HDL, while silencing ABCG-1 or SRB-1 abrogated the effect of HDL but not apoA-I. In vivo, apoA-I transgenic mice fed a high fat, high sucrose, cholesterol-containing diet showed reduced chemotactic factor and pro-inflammatory cytokine expression and reduced macrophage accumulation in adipose tissue.

Conclusion—ApoA-I and HDL have anti-inflammatory effects in adipocytes and adipose tissue similar to their effects in other cell types. These effects are consistent with disruption and removal

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DISCLOSURES
None

of cholesterol from LRs, which are regulated by cholesterol transporters such as ABCA-1, ABCG-1 and SRB-1.

Keywords

Adipocytes; ABC transporters; cholesterol; HDL; Apolipoprotein A-I

INTRODUCTION

Obesity, especially visceral obesity, is accompanied by adipose tissue inflammation, which in turn is associated with insulin resistance and an increased risk of cardiovascular disease [1, 2]. A hallmark of adipose tissue inflammation is the accumulation of macrophages [3, 4] and other immune cells [5–7], that are recruited to adipose tissue by chemotactic factors such as monocyte chemotactic protein-1 (MCP-1) [8–10] and serum amyloid A3 (SAA3) [9, 10]. Reactive oxygen species (ROS) are generated by adipocytes in a NOX4-dependent fashion after exposure to excess glucose and certain saturated fatty acids (SFAs) in vitro, and the generation of ROS is linked to NFκB activation and expression of monocyte chemotactic factor genes [9, 10]. Moreover, these chemotactic factors are expressed and macrophages accumulate in adipose tissue when mice are exposed to a diet rich in saturated fat and sucrose [9].

HDL and apolipoprotein A-I (apoA-I) are believed to protect against the development of atherosclerosis, in part due to their role in promoting cholesterol efflux and reverse cholesterol transport (reviewed in [11]). However, in addition to these properties, HDL and apoA-I have anti-inflammatory properties [reviewed in [12–14]]. The most studied of these is its ability to inhibit adhesion molecule expression by endothelial cells [15, 16], which reduces monocyte adhesion [17]. At the level of the artery wall, this property of HDL would reduce recruitment of monocyte-macrophages and inhibit atherogenesis. HDL and apoA-I also have been shown to have anti-inflammatory effects on monocytes [18] and macrophages [19, 20]. However, little is known concerning their effect on adipocytes.

HDL and apoA-I can exert an anti-inflammatory effect on macrophages via ABCA-1[ATP-binding cassette (ABC) A-1] and ABCG-1-mediated cholesterol efflux from plasma membrane microdomains known as lipid rafts (LRs), which are enriched in cholesterol, glycosyl-phosphatidylinositol-linked proteins, glycosphingolipids, cholesterol and caveolin [21]. Free cholesterol accumulation in LRs is associated with increased signaling via proteins concentrated in these microdomains. NADPH oxidase 4 (NOX4) is the source of ROS after exposure of adipocytes to palmitate in vitro, and its activity is increased by its translocation into LRs following exposure to palmitate. Cholesterol efflux occurs via two main pathways; first, free cholesterol simply diffuses from the plasma membrane through the aqueous medium to HDL, and second, cholesterol efflux is mediated by transporters in the plasma membrane [ABCA-1, ABCG-1 and scavenger receptor (SRB-1)] [22, 23 24]. ABCA-1 transports free cholesterol from plasma membrane to lipid-poor apoA-I, and ABCG-1 transports cholesterol to mature HDL particles. These transporters regulate the cholesterol content of LRs by efflux to HDL and apoA-I. In turn, disruption and depletion of cholesterol in LRs blocks signal transduction[25]. However, whether and how HDL and apoA-I affects inflammation in adipocytes are unknown.

To test the hypothesis that HDL has anti-inflammatory effects [12, 13] on adipocytes analogous to other cell types [18–20], we first exposed fully differentiated 3T3-L1 adipocytes to normal human HDL and apoA-I in vitro to test their effect on palmitate-induced inflammation. We found that HDL and apoA-I inhibited palmitate-induced ROS generation and chemotactic factor expression by decreasing LR formation and translocation of NOX4 into LRs.

Moreover, these effects of HDL and apoA-I were dependent on cholesterol transporters in the plasma membrane. To determine whether HDL inhibited adipose tissue inflammation in vivo, we evaluated the effect of increased HDL levels that resulted from overexpression of the human apoA-I transgene on adipose tissue inflammation induced by a high fat, high sucrose, cholesterol-containing diet in mice. We found that adipose tissue inflammation also was ameliorated in human apoA-I transgenic mice. Our finding suggest that HDL and apoA-I have anti-inflammatory properties on adipocytes and adipose similar their effects in other cell types.

METHODS

Reagents and other detailed methods are described in Supplemental Methods.

Preparation of fatty acid–albumin complexes

Free fatty acids (FFAs) were prepared by conjugation with albumin, as described previously [10].

Multiplex real-time quantitative reverse-transcription polymerase chain reaction

(RT-PCR) was performed using the TaqMan Master kit (Applied Biosystems) in the ABI prism 7900HT system [27, 28] (Supplemental Methods).

Quantification of cholesterol levels in cellular membranes of 3T3L-1 adipocytes

Cellular membranes were isolated from 3T3L-1 adipocytes as described previously [29]. Membrane preparations were resuspended in 100μl of ethanolic potassium hydroxide (1mol/L) and cholesterol-d7 was added as the internal standard. After saponification, the lipid fractions were extracted from the membrane preparations with hexane and dried under nitrogen gas. Total cholesterol levels were determined after derivatization using liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS) as described previously [30].

In vitro ABCA-1, ABCG-1 and SRB-1 gene silencing

For experiments in which we tested the roles of ABCA-1, ABCG-1 and SRB-1 in mediating ROS generation and the expression of SAA3 and MCP-1, two days after completion of the differentiation protocol 3T3-L1 adipocytes were transiently transfected with small interfering RNA (siRNA) duplexes for ABCA-1, ABCG-1 and SRB-1 or scrambled sequences, which were synthesized and purified by Ambion using the DeliverX system (Panomics), as described previously [10, 29]. Briefly, the cells were incubated with 300μl of siRNA/siRNA transfection reagent complex for 3min. After that, 300μl of serum free media was added and incubated for 24h. Four days later mRNA and protein were analyzed for confirm the efficiency of silencing by RT-PCR and Western blotting.

Quantification of reactive oxygen species (ROS)

ROS generation was assessed as CM-H_2DCFDA (Molecular Probes) fluorescence, which was monitored by FACS (FACSCanto, Becton-Dickinson) as described previously [10, 29].

Visualization of lipid rafts

To detect lipid rafts in plasma membranes, Alexa Fluor 594 conjugated cholera toxin subunit β (CTB) was used to stain 3T3-L1 adipocytes. Briefly, cultured 3T3-L1 adipocytes were incubated with 1μg/ml of Alexa Fluor 594 conjugated CTB for 15 min at 4 °C. After washing twice with cold PBS, cells were fixed in 4% paraformaldehyde for 20 min at 4 °C. Fixed cells were photographed by fluorescent microscopy (Nikon Eclipse 80i).

Detergent-free lipid raft fractionation

Lipid raft (LR) and non-LR fractions from adipocytes were obtained by Optiprep gradient centrifugation using a detergent-free protocol [31] (Supplemental methods).

Animals, diet, and tissue collection

To investigate the role of HDL and apoA-I in adipose tissue inflammation, adult (ten week-old) male human apoA-I overexpressing transgenic (apoA-I$^{tg/tg}$) and control C57BL/6 mice were fed either a high fat, high sucrose, cholesterol-containing (HFHSC diet, 35.5% calories as fat and 36.6% as carbohydrate, 0.15% added cholesterol, BioServ No.F1850) or chow (control) diets for 24 weeks [32]. In a parallel experiment, ten-week-old male LDL receptor (LDLR)-deficient and human apoA-I overexpressed transgenic (LDLR$^{-/-}$ apoA-I$^{tg/tg}$) mice, and control LDLR$^{-/-}$ mice also were fed either HFHS or chow diets for 16 weeks. ApoA-I$^{tg/tg}$ and LDLR$^{-/-}$ mice were bought from the Jackson laboratory, and LDLR$^{-/-}$ apoA-I$^{tg/tg}$ mice were generated from these homozygous mice. At sacrifice, adipose tissues were either fixed in 10% formalin for macrophage staining using a Mac2 antibody[33], or snap-frozen at −70°C for isolation of total RNA. Metabolic variables were measured in blood samples obtained from the retro-orbital sinus after a 4h fast. Cholesterol and triglycerides in plasma and fast-phase liquid chromatography (FPLC) fractions were measured using colorimetric assay kits. Lipoproteins were separated from pooled plasma samples by FPLC. Plasma insulin and SAA levels were measured using ELISA as described previously [27]. All experimental procedures were undertaken with approval from the Institutional Animal Care and Use Committee of the University of Washington.

Statistical analysis

Statistical significance was determined by Student's t-tests (two tailed and paired). All data are shown as means ± SD of three independent experiments performed in triplicate. $P<0.05$ was considered significant.

RESULTS

ApoA-I and HDL disrupt lipid rafts and inhibit palmitate-induced generation of chemotactic factors

We previously showed that exposure of differentiated 3T3-L1 adipocytes to palmitate (250μmol/L) increased chemotactic factor gene expression[10]. We also showed that chemotactic factor generation was linked to the translocation of NOX4 into LRs, which led to ROS generation [29]. Cholesterol is an essential component of LRs. Depletion of cholesterol in the plasma membrane disrupts LRs and blocks the assembly of proteins, resulting in inhibition of signal transduction [25]. Therefore, we investigated whether modulation of membrane cholesterol using methyl-β-cyclodextrin (MβCD), a compound that depletes membrane cholesterol and disrupts LRs [25, 34], affects palmitate-induced chemotactic factor gene expression. MβCD totally blocked palmitate-induced *Saa3* and *Mcp-1* gene expression (Figure 1*A* and *B*). Moreover, adding back cholesterol reversed the effect of MβCD on *Saa3* and *Mcp-1* gene expression (Figure 1*A* and *B*).

When cells were pre-exposed to simvastatin (10μmol/L), an inhibitor of 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase, for 24h to reduce cholesterol biosynthesis, we also found that palmitate-induced *Saa3* and *Mcp-1* gene expression was blocked (data not shown). Since apoA-I and HDL have the ability to remove cholesterol from cell membranes, we also investigated whether apoA-I and HDL could inhibit this effect of palmitate. We first determined whether apoA-I and HDL could decrease the cholesterol level of membranes in 3T3L-1 adipocytes. Indeed, apoA-I and HDL decreased membrane cholesterol levels before as well as after exposure of adipocytes to palmitate (Supplemental figure I). Palmitate

exposure alone led to a significant increase in membrane cholesterol (Supplemental figure I). Both apoA-I and HDL blocked the increase of *Saa3* and *Mcp-1* gene expression induced by palmitate in a dose-dependent manner (Figure 1*C–F*), although *Mcp-1* expression appeared to be more sensitive to the effects HDL and apoA-I possibly because the promoter of the *Mcp-1* gene might be more sensitive to the anti-inflammatory effects of HDL and apoA-I than the *Saa3* gene.

Moreover, co-treatment with apoA-I and HDL synergistically inhibited these chemotactic factor expression induced by palmitate (Supplemental figure II). Since a trace of HDL in the serum used for adipocyte cells culture could potentially affect these results, we also performed experiments using lipoprotein-deficient serum and found no differences between these two conditions (data not shown). To determine whether apoA-I and HDL disrupt LR formation, we visualized the LRs using Alexa Fluor 594 conjugated cholera toxin subunit β (CTB), which selectively binds to LRs in the plasma membrane [35]. Exposure of adipocytes to palmitate increased CTB-stained microdomains of LRs compared with controls, while pre-exposure to either apoA-I or HDL reduced these microdomains (Figure 2*A*). Finally we investigated whether apoA-I and HDL blocked NOX4 translocation into LRs isolated by ultracentrifugation. Non-LRs sediment in the lower fractions while LRs float towards the top of the centrifuge tube. The presence of LRs was judged by detection of caveolin-1 (CAV1). In the absence of palmitate exposure, all NOX4 protein was present in non-LR fractions. After exposure to palmitate, NOX4 proteins were translocated into LRs (Figure 2*B*). However, pre-exposure of cells to either apoA-I or HDL inhibited palmitate-induced translocation of NOX4 into LRs (Figure 2*B*). These results imply that apoA-I and HDL disrupt the formation of LRs, causing disturbance of palmitate-induced NOX4 translocation, which in turn inhibits chemotactic factor expression.

The anti-inflammatory effect of apoA-I and HDL on palmitate-induced chemotactic factor production is dependent on ABC transporters and scavenger receptor B1

Since reverse cholesterol transport by apoA-I and HDL is mediated by transporters in the plasma membrane (ABCA-1, ABCG-1 and SRB-1), we first tested which of these transporters is important for apoA-I or HDL to exert their effects on palmitate-induced chemotactic factor gene expression. ABCA-1, ABCG-1 and SRB-1 silencing in 3T3-L1 adipocytes by their specific siRNAs was confirmed by demonstrating significant reduction of their respective expression levels by RT-PCR (data not shown) and Western blotting (Supplemental figure III). When ABCA-1 was silenced, the effect of apoA-I on inhibiting *Saa3* and *Mcp-1* gene expression induced by palmitate was reversed, while HDL still inhibited palmitate-induced expression of these chemotactic factors (Figure 3 *A* and *B*). Conversely, the ability of HDL to inhibit palmitate-induced chemotactic factor gene expression was reversed by silencing ABCG-1 or SRB-1, while apoA-I still inhibited these events (Figure 3 *C–F*).

ApoA-I and HDL regulate palmitate-induced formation of LRs, translocation of NOX4 and ROS generation via ABCA-1, ABCG-1 and SRB-1

To investigate the roles of ABCA-1, ABCG-1 and SRB-1 in LR formation induced by palmitate, we evaluated CTB-stained microdomains in the plasma membrane of adipocytes in which these transporters had been silenced using siRNA. In control cells, palmitate resulted in increased CTB-stained microdomains. In ABCA-1 silenced cells, apoA-I did not decrease the CTB-stained LRs comparing while HDL did (Figure 4). Conversely, in ABCG-1 or SRB-1 silenced cells, HDL failed to reduce the CTB-stained LRs while apoA-I did (Figure 4).

Next, we investigated whether ABCA-1, ABCG-1 and SRB-1 regulate NOX4 translocation into LRs, since we had previously shown that exposure of adipocytes to palmitate lead to translocation of NOX4 to LRs [29]. Consistent with the findings with CTB staining, in ABCA-1 silenced cells, palmitate-induced NOX4 translocation into LRs was not blocked by apoA-I while HDL inhibited NOX4 translocation (Figure 5). Conversely, in ABCG-1 or SRB-1 silenced cells, palmitate-induced NOX4 translocation was not blocked by HDL while apoA-I inhibited NOX4 translocation (Figure 5).

Finally, we evaluated how these transporters affect NOX4 derived ROS generation. Similar to the findings with CTB staining and NOX4 translocation, silencing ABCA-1 reduced the ability of apoA-I to suppress palmitate-induced ROS generation, while HDL still reduced palmitate-induced ROS (Figure 6). Silencing of ABCG-1 or SRB-1 reduced the ability of HDL to suppress palmitate-induced ROS generation, while apoA-I still reduced palmitate-induced ROS (Figure 6).

Since silencing these transporters could potentially affect palmitate uptake, thereby changing palmitate concentration in cells, we measured the uptake of ^{14}C-labeled palmitate in adipocytes in which these transporters had been silenced. None of silenced cells, either in the presence or absence of HDL or apoA-I showed alterations in palmitate uptake (data not shown). These findings all indicate that apoA-I exerts its anti-inflammatory effect on adipocytes via ABCA-1, and HDL does so via ABCG-1 and SRB-1.

Adipose tissue inflammation is reduced in human apoA-I transgenic mice

To extend our in vitro findings in 3T3-L1 adipocytes to an in vivo model, we used male apoA-I transgenic mice fed a high-fat, high-sucrose, cholesterol containing diet (HFHSC), which previously has been shown to result in obesity, insulin resistance, and atherosclerosis [32]. ApoA-I and HDL cholesterol levels are increased about two fold in mice overexpressing the human apoA-I transgene [36]. ApoA-I transgenic mice and their littermates showed the same weight gain with either chow or the HFHSC diet (data not shown). As expected, lipoprotein distribution profiles of apoA-I transgenic mice fed chow or HFHSC diet revealed increased HDL particles (supplemental figure IV *A* and *E*). Plasma triglyceride and cholesterol levels were increased in apoA-I$^{tg/tg}$ mice fed the HFHSC diet (supplemental figure IV *B* and *C*). However, there were no changes in plasma triglyceride and cholesterol profiles between LDLR$^{-/-}$ and LDLR$^{-/-}$ apoA-I$^{tg/tg}$ mice fed the HFHSC diet (supplemental figure IV *F* and *G*). Levels of SAA (a circulating inflammatory marker) showed a trend towards reduction in the apoA-I transgenic mice fed the HFHSC diet, but the changes did not reach statistical significance (supplemental figure IV *D* and *H*). Next, we investigated inflammation in adipose tissue. First, in response to consumption of the HFHSC diet expression of chemotactic factor gene, *Saa3*, was decreased in intra-abdominal adipose tissue of apoA-I$^{tg/tg}$ (Figure 7*A*) and LDLR$^{-/-}$ apoA-I$^{tg/tg}$ mice (Supplemental figure V) versus controls (C57BL/6 or LDLR$^{-/-}$), whereas another chemotactic factor gene, *Mcp-1*, was only decreased in intra-abdominal adipose tissue of LDLR$^{-/-}$ apoA-I$^{tg/tg}$ mice (Figure 7*A* and Supplemental figure V). Second, mRNA expression of macrophage markers was decreased in intra-abdominal adipose tissue of apoA-I$^{tg/tg}$ (Figure 7*B*) and LDLR$^{-/-}$ apoA-I$^{tg/tg}$ mice (Supplemental figure V) fed the HFHSC diet. Immunohistochemical staining also showed a decrease of macrophages (detected with Mac2) in intra-abdominal adipose tissue of apoA-I$^{tg/tg}$ mice compared to C57BL/6 mice fed the HFHSC diet (Figure 7*C*). Third, mRNA expression level of pro-inflammatory cytokines (*Tnf*α, *Il1*β and *Il6*) was decreased in intra-abdominal adipose tissue of apoA-I$^{tg/tg}$ (Figure 7*D*) and LDLR$^{-/-}$ apoA-I$^{tg/tg}$ mice (Supplemental figure V) fed the HFHSC diet. Interestingly, *Nox4* mRNA expression level was decreased in apoA-I$^{tg/tg}$ (Figure 7*E*) as well as LDLR$^{-/-}$ apoA-I$^{tg/tg}$ mice (Supplemental figure V) fed the HFHSC diet. These findings imply that apoA-I and HDL also exert an anti-inflammatory effect on adipose tissue in vivo.

DISCUSSION

We previously have shown that both excess glucose [9] and saturated fatty acids such as palmitate [10] increase the expression of the monocyte chemotactic factors, SAA3 and MCP-1, which induce monocyte chemotaxis. In this study we show that apoA-I and HDL have anti-inflammatory properties in cultured adipocytes in addition to their well-described effects on vascular cells such as endothelial cells and macrophages [16, 18–20, 37]. Addition of either normal human HDL or its major apolipoprotein, apoA-I, both reduced palmitate-induced expression of *Saa3* and *Mcp-1* in a dose dependent and additive fashion. These effects are likely related to the cholesterol content of adipocyte membranes, since they could be mimicked by removal of cholesterol from cells by exposure to MβCD and restored by adding back cyclodextrin to which cholesterol had been added. Moreover, the anti-inflammatory effect of apoA-I appeared to be ABCA-1 dependent, since it could be blocked by silencing ABCA-1. The effects of intact HDL appeared to depend on ABCG-1 and SRB-1, since they could be reversed by silencing either of these genes. All of these proteins are present in 3T3-L1 adipocytes in vitro. We also were able to show that *Saa3* and *Mcp-1* expression and macrophage accumulation in adipose tissue were reduced in vivo in mice with high HDL levels as a result of apoA-I over-expression. Finally, we had previously shown that palmitate induction of SAA3 and MCP-1 in adipocytes was dependent on the generation of reactive oxygen species by NOX4 after translocation to lipid rafts [29]. In this study we extend those findings by demonstrating that NOX4 translocation to lipid rafts is inhibited by both HDL and apoA-I. Several mechanisms have been proposed by which HDL might be able to exert an anti-inflammatory effect on cells, including induction of scavenger receptor class B [38] and hydrolysis of some HDL components by endothelial lipase [39], both PPAR-α dependent processes. In addition, effects of apoA-I and intact HDL on ABAC1 and ABCG-1-mediated cholesterol efflux from macrophages have been invoked to play a role in the anti-inflammatory properties of HDL in macrophages [19, 20]. A similar mechanism appears to be operative in adipocytes, since palmitate increased the cholesterol content of rafts, as assessed by cholera toxin fluorescence, an affect that was reduced by pre-treatment of the cells with either HDL or apoA-I. Additional evidence that the cholesterol content of the cell membrane plays an important role in determining the ability of adipocytes to respond to palmitate is the observation that disruption of cell membrane rafts by the addition of cyclodextrin [40] had the same anti-inflammatory effect as exposure of cells to either HDL or apoA-I, and that the pro-inflammatory effect could be restored by exposing the cells to cyclodextrin that had been pre-loaded with cholesterol.

ABCA-1 transports cholesterol from cell membranes to lipid-poor apoA-I [41], whereas whole HDL accepts cholesterol from both ABCG-1 [42, 43] and SRB-1 [44]. Although ABCG-1 is reported to reside in the endoplasmic reticulum, it could mediate transfer of cholesterol to the plasma membrane from where it could be desorbed to exogenous lipid acceptors such as HDL [45]. Our results indicate that silencing ABCA-1 reversed the anti-inflammatory effect of apoA-I but had no influence on the effect of intact HDL. Conversely, silencing ABCG-1 or SRB-1 reversed the inhibitory effect of HDL but not apoA-I on palmitate induction of chemotactic factors. Because of the known functions of these proteins in transferring cholesterol to either apoA-I or HDL, these findings are consistent with the cholesterol content of plasma membranes playing a major role in the ability of adipocytes to respond to the pro-inflammatory effects of palmitate.

Although some controversy exists regarding the definition or even the existence of lipid rafts [46], proteins involved in cell signaling clearly cluster in certain domains of the plasma membrane. Accumulation of free cholesterol of the plasma membrane is associated with increased signaling via TLR4 [19, 20], which reside in these domains – the presence of apoA-I or HDL reduces the cholesterol content of these rafts and TLR4 signaling in other cell

types [20]. The presence of ABCG-1 and HDL also protect against endothelial cell dysfunction in mice fed a western-type diet, consistent with an inhibition of activation of TRL4 by SFA in endothelial cells [47, 48]. We recently showed that ROS generation by NOX4, a member of the NADPH family of oxidases, plays a critical role in palmitate-induction of chemotactic factor production in adipocytes, and is the major reason for generation of ROS in these cells[29]. NOX4 is translocated to the plasma membrane after exposure of adipocytes to palmitate, and disruption of adipocytes membranes by exposure to cyclodextrin blocked both NOX4 translocation and stimulation of chemotactic factor expression induced by palmitate [29]. In the present study, we show that pre-exposure of cells to either HDL or apoA-I markedly inhibited the translocation of NOX4 to "lipid raft" fractions of the plasma membrane and ROS generation. Moreover, silencing ABCA-1 reversed the effect of apoA-I but not HDL, whereas silencing ABCG-1 or SRB-1 reversed the effect of HDL but not apoA-I on palmitate-mediated NOX4 translocation to the plasma membrane. In view of the effects of apoA-I and HDL and the silencing of these transporter proteins on chemotactic factor gene expression, these findings are consistent with apoA-I and HDL effects on NOX4 translocation playing an important role in their ability to influence gene expression.

To test whether similar changes occur in vivo, we chose to emulate saturated fatty acid induced adipose tissue inflammation in control mice and in mice expressing the human apoA-I transgene. We previously have shown that a diet rich in saturated fat and sucrose with a moderate amount of added cholesterol, the so-called HFHSC diet, led to profound pro-inflammatory effects on intra-abdominal adipose tissue [27]. To test the effect of apoA-I and HDL in vivo, we chose to study apoA-I transgenic mice, which have increased levels of human HDL [36, 49]. These mice show a reduction of atherosclerosis when present on an atherosclerosis prone background [49], suggesting that vascular inflammation is reduced in these mice. Our findings indicate that high levels of apoA-I that result from expression of the human apoA-I transgene also inhibit the expression of monocyte chemotactic factors, and the accumulation of macrophages in adipose tissue in mice. Moreover, expression of inflammatory genes such as *Tnf*α *and Il6* were reduced in adipose tissue from these mice, consistent with our in vitro findings. The reason why MCP-1 expression was not reduced in adipose tissue from the apoA-I transgenic mice on the C57BL6 background whereas it was in the transgenic mice on the LDLR deficient background is not clear. However, macrophage and inflammatory genes were reduced in both apoA-I transgenic strains of mice.

Potential shortcomings of our study include lack of comparison of gene expression in the adipocyte versus stromal vascular fractions, and of knowledge of the cellular distribution of NOX4 in adipose tissue. These should be determined in future studies of this nature.

Accumulation of macrophages in adipose tissue of both mice and humans is a hallmark of obesity[3, 4]. Adipose tissue inflammation and obesity are features of the metabolic syndrome in humans [50] and in a mouse model that has many of the features of the metabolic syndrome [27]. Moreover, obesity-associated inflammation predisposes to the development of both type 2 diabetes and cardiovascular disease [50]. Therefore, strategies to reduce adipose tissue inflammation have potentially important therapeutic implications. Although simply raising HDL cholesterol levels has not been shown to reduce atherosclerosis [51] nor do some genetic polymorphs that raise HDL levels protect against cardiovascular disease, it is clear from human epidemiological studies that HDL has a strong inverse relationship with cardiovascular disease [52]. In addition, experiments in mice that either overexpress or are lacking in apoA-I clearly show a very strong relationship with atherosclerosis[49, 53]. HDL also clearly has anti-inflammatory [12–14] and other [14] potentially atheroprotective properties on cells of the artery wall. The findings presented in this study, which demonstrate anti-

inflammatory effects of HDL and apoA-I on adipose tissue inflammation, offer additional possibilities for the prevention of diabetes and atherosclerosis associated with adipose tissue inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

SOURCES OF FUNDING

NIH grants HL094352, HL092969 and DK-035816

Nonstandard Abbreviations

MCP-1	monocyte chemoattractant protein-1
SAA	serum amyloid A
CAV1	caveolin-1
FFA	free fatty acids
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IBX	3-isobutyl-1-methylxanthine
ROS	reactive oxygen species
LRs	Lipid rafts
ApoA-I	apolipoprotein A-I
SFAs	saturated fatty acids
siRNA	small interfering RNA
MβCD	Methyl-β-cyclodextrin
ABCA-1	ATP-binding cassette (ABC) A-1
ABCG-1	ATP-binding cassette (ABC) G-1
SRB-1	scavenger receptor B-1 (SRB-1)
CTB	cholera toxin subunit β
HFHSC	high fat, high sucrose, cholesterol-containing diet
NOX4	NADPH oxidase 4

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Novelty and Significance

What Is Known?

- High density lipoproteins (HDL) and apolipoprotein A-I (apoA-I) have atheroprotective effects, which may be in part due to their anti-inflammatory properties.
- The mechanism by which HDL and apoA-I exert their anti-inflammatory effect in macrophages and endothelial cells appears to be via cholesterol depletion of lipid rafts on cell membranes.

What New Information Does This Article Contribute?

- Cholesterol depletion of lipid rafts in adipocytes by HDL, apoA-I or methyl-β-cyclodextrin inhibited palmitate-mediated chemotactic factor expression.
- HDL and apoA-I have anti-inflammatory effects on adipocytes similar to other cell types such as macrophages and endothelial cells.
- Similar findings were observed in adipose tissue in vivo as a result of apoA-I overexpression.

HDL and apoA-I exert anti-inflammatory effects on macrophages and endothelial cells by depleting cholesterol in lipid rafts. However, it unclear whether HDL and apoA-I have similar effects on adipocytes. We found that both HDL and apoA-I inhibit palmitate-mediated induction of monocyte chemotactic factor gene expression in cultured adipocytes by a mechanism consistent with the disruption and the removal of cholesterol from lipid rafts. Moreover, overexpression of apoA-I in vivo reduced chemotactic factor expression and macrophage accumulation in adipose tissue in mice fed a high-fat diet. These findings indicate that HDL and apoA-I have anti-inflammatory effects on adipocytes and adipose tissue, similar to their better-known effects on vascular cells such as macrophages and endothelial cells. Because macrophage accumulation in adipose tissue is an important harbinger of insulin resistance and cardiovascular disease (CVD), these results have important translational implications for the prevention and the management of insulin resistance and obesity-associated CVD by controlling adipose tissue inflammation and its downstream consequences.

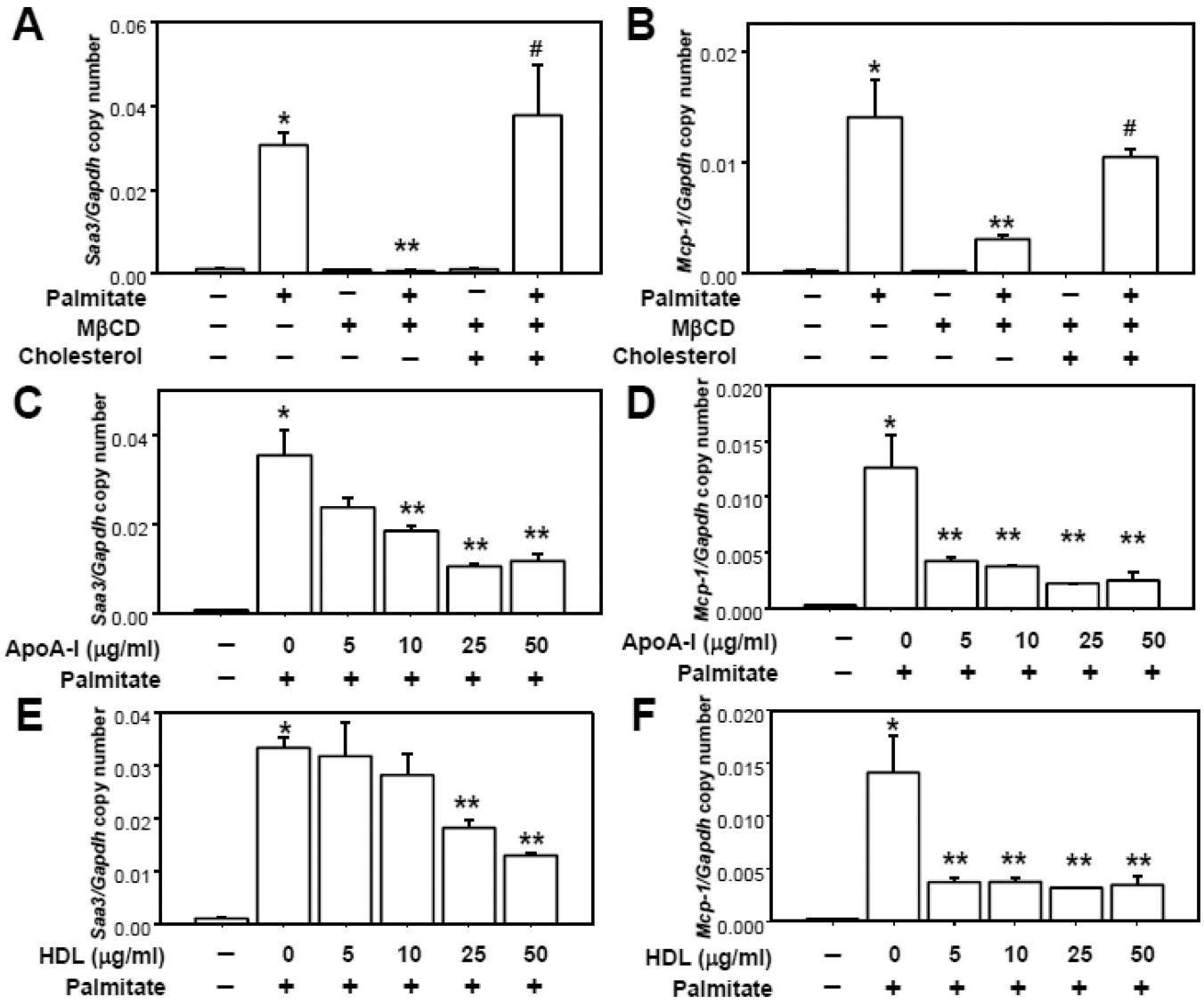
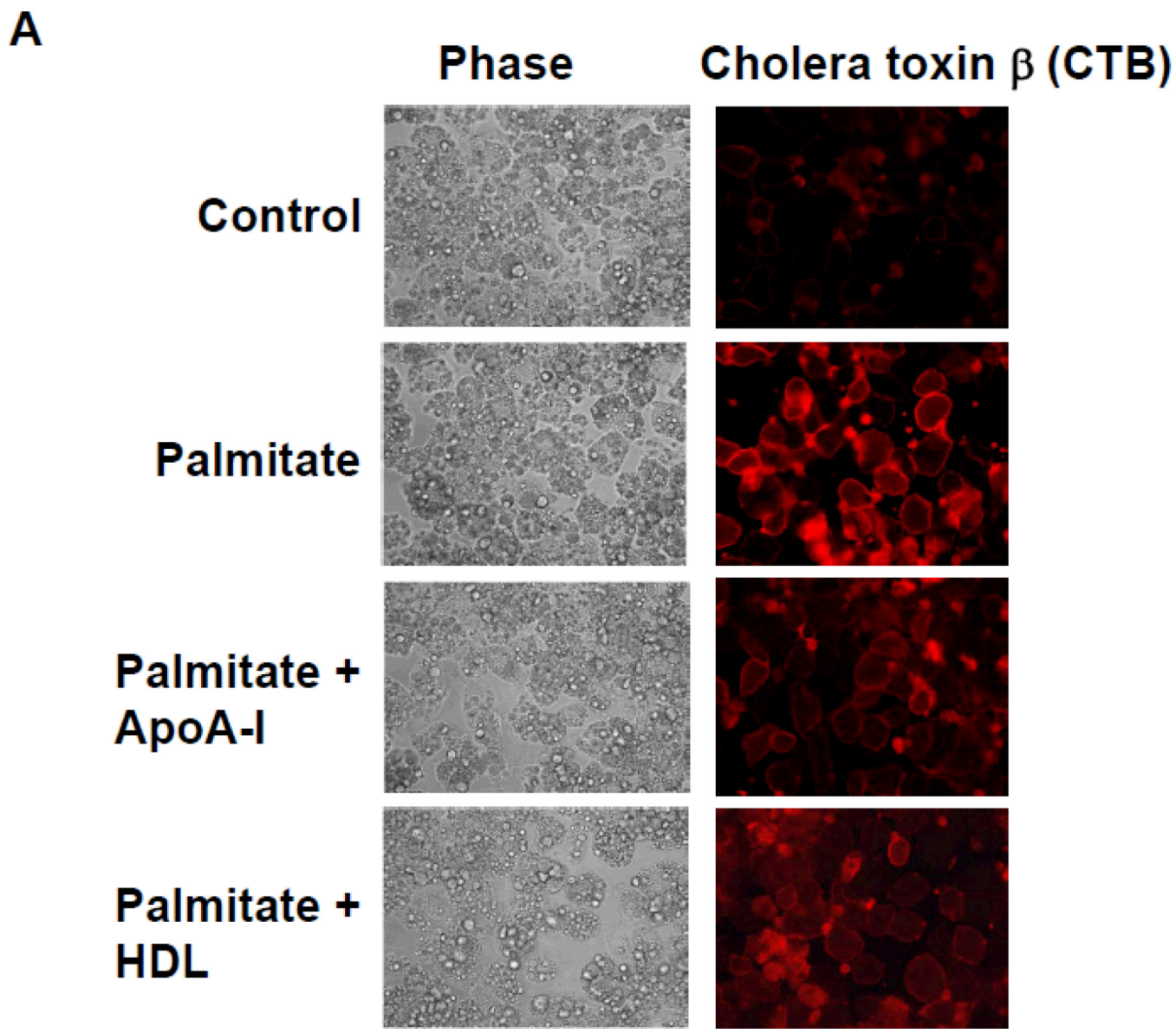


Figure 1. MβCD, apoA-I and HDL inhibits chemotactic factor expression

3T3-L1 adipocytes were pre-exposed to MβCD (10μmol/ml, *A* and *B*), cholesterol loaded MβCD (*A* and *B*), apoA-I and/or HDL (at the indicated concentrations in μg protein/ml), *C–F*) for 6h. After that, adipocytes were incubated with or without 250 μmol/L palmitate for 24h. *Saa3* and *Mcp-1* gene expression was analyzed by multiplex real-time RT-PCR, normalized to GAPDH (*A–F*). **P* < 0.001 vs. control media, ***P* < 0.001 vs. palmitate, #*P* < 0.001 vs. palmitate plus MβCD.



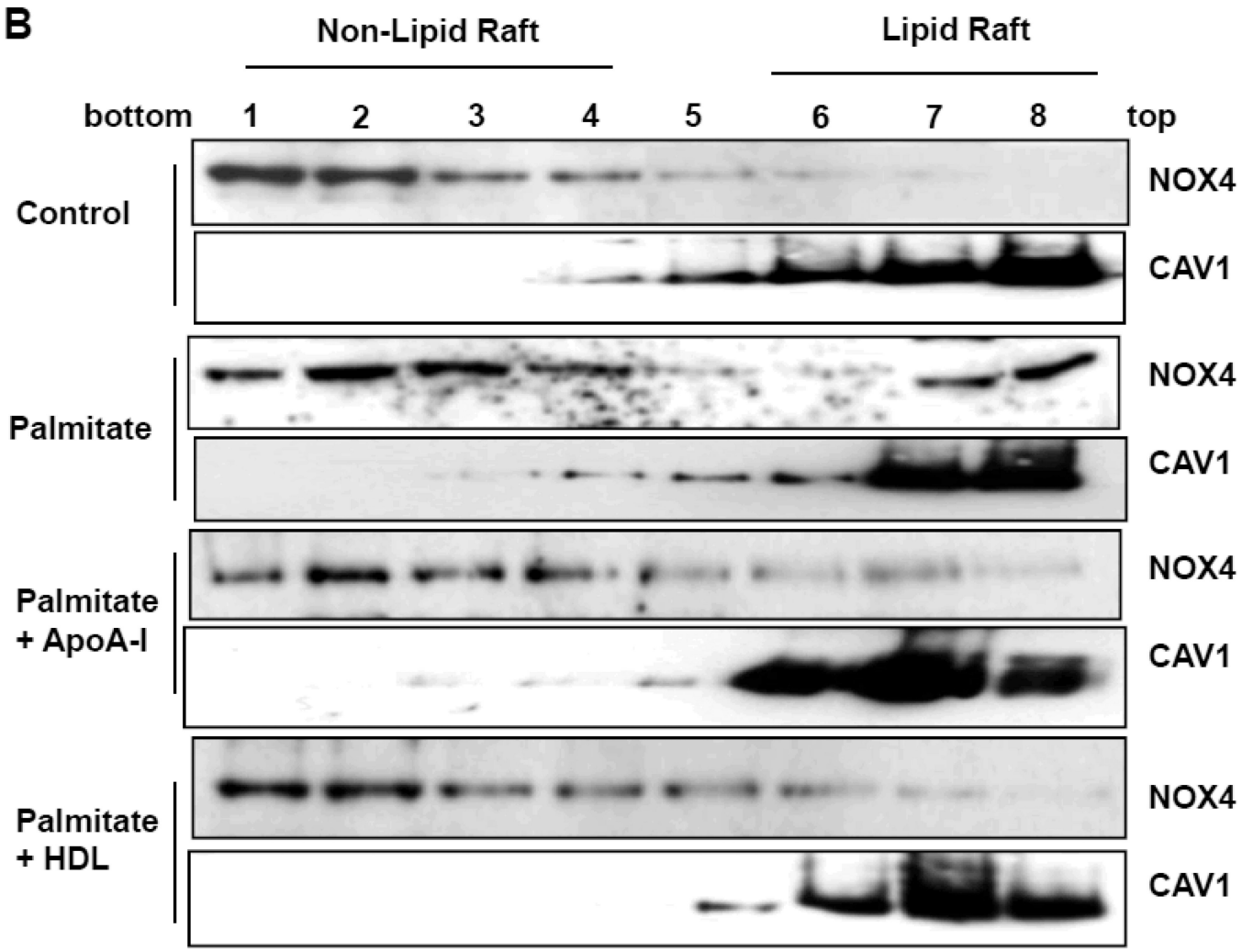


Figure 2. ApoA-I and HDL disrupt palmitate-induced LR formation and NOX4 translocation into LRs

3T3-L1 adipocytes were pre-exposed to apoA-I (50μg protein/ml) or HDL (50μg protein/ml) for 6h. After that, adipocytes were incubated with or without 250μmol/L palmitate for 24h. *A*. LRs were stained by Alexa Fluor 594 conjugated cholera toxin subunit β (CTB) and photographed by fluorescent microscopy (Nikon Eclipse 80i, original magnification ×400). *B*. LRs were isolated and fractionated by ultracentrifugation using a detergent-free fractionation method. Proteins from OptiPre-gradient fractions were immunoblotted with anti-NOX4 antibody and anti-caveolin-1 (CAV1) antibody (*B*). Fractions 6 to 8 contain LRs and fractions 1 to 4 are non-LR containing fractions.

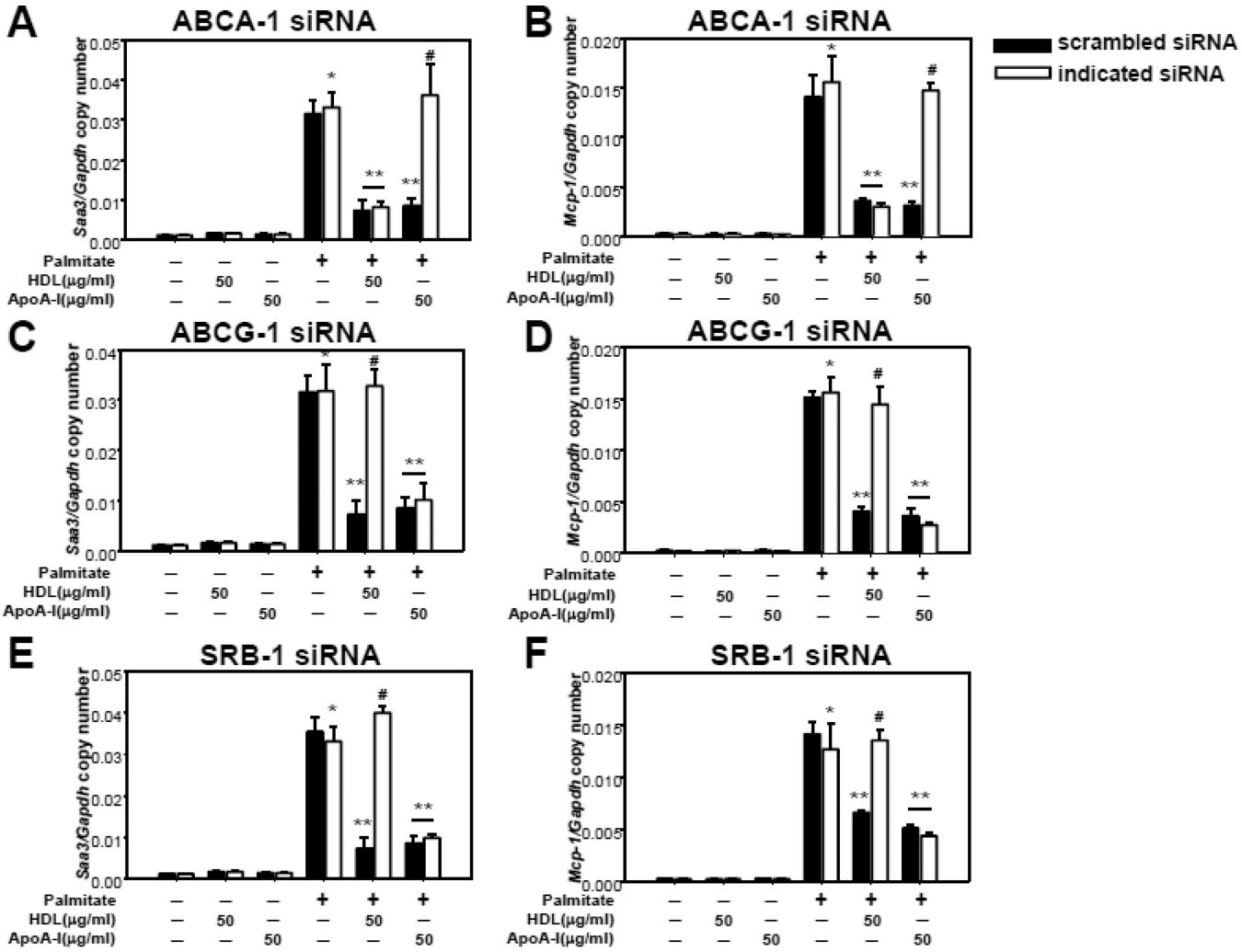
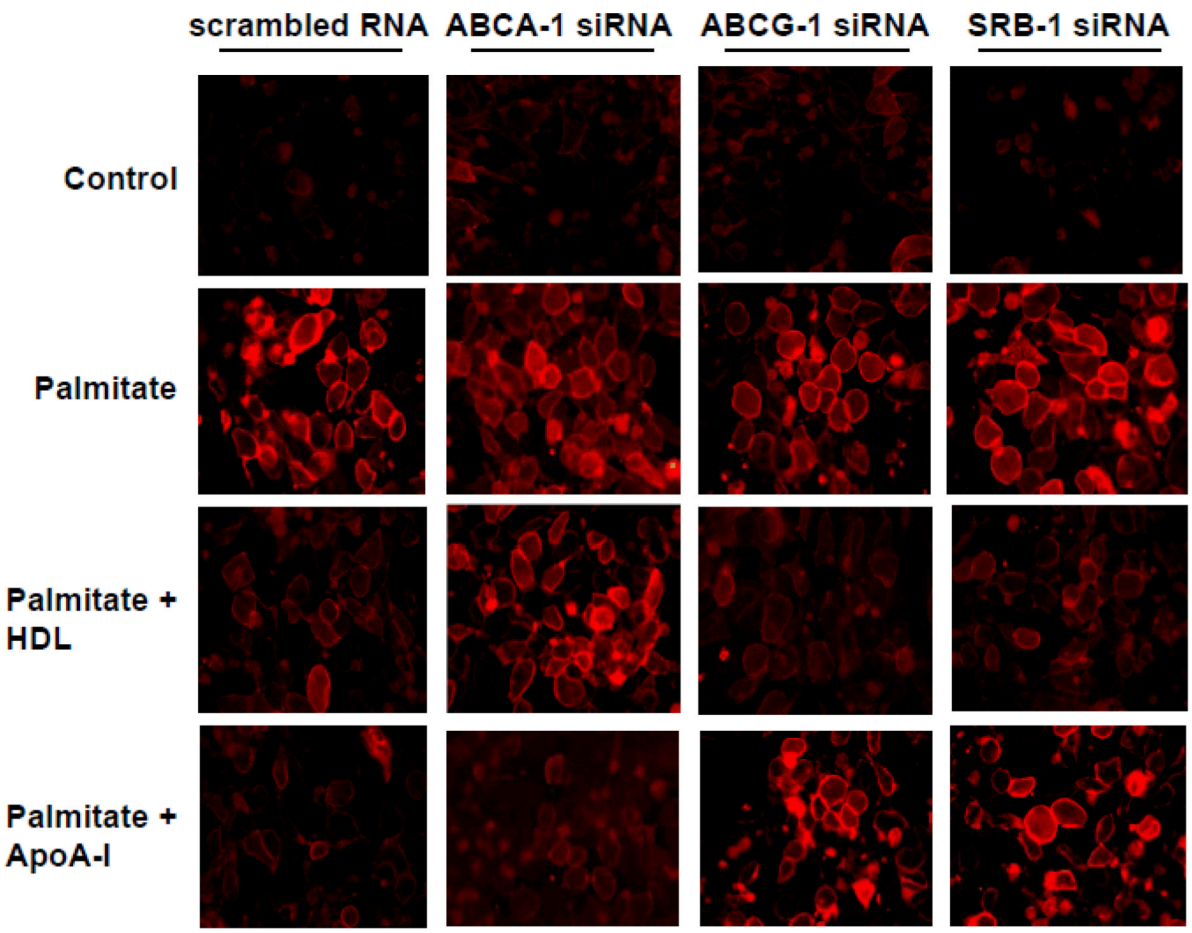


Figure 3. The effects of apoA-I and HDL on palmitate-induced chemotactic factor gene expression are mediated by ABCA-1, ABCG-1 and SRB-1

3T3-L1 adipocytes were transfected with a siRNA specific for ABCA-1 (*A* and *B*), ABCG-1 (*C* and *D*), SRB-1 (*E* and *F*) or a scrambled siRNA (negative control) as indicated. 24h later the siRNA was removed and the cells were cultured for a further 3 days. After that, the cells were pre-exposed to apoA-I (50μg/ml) or HDL (50μg protein/ml) for 6h, and then incubated with or without added palmitate (250μmol/L) for 24h. Total RNA was isolated and analyzed by multiplex real-time RT-PCR using *Saa3*-specific (*A, C* and *E*) or *Mcp-1*-specific (*B, D* and *F*) primers and normalized to *Gapdh*. *$P < 0.001$ vs. control media, **$P < 0.001$ vs. palmitate, #$P < 0.001$ vs. palmitate plus HDL or apoA-I.



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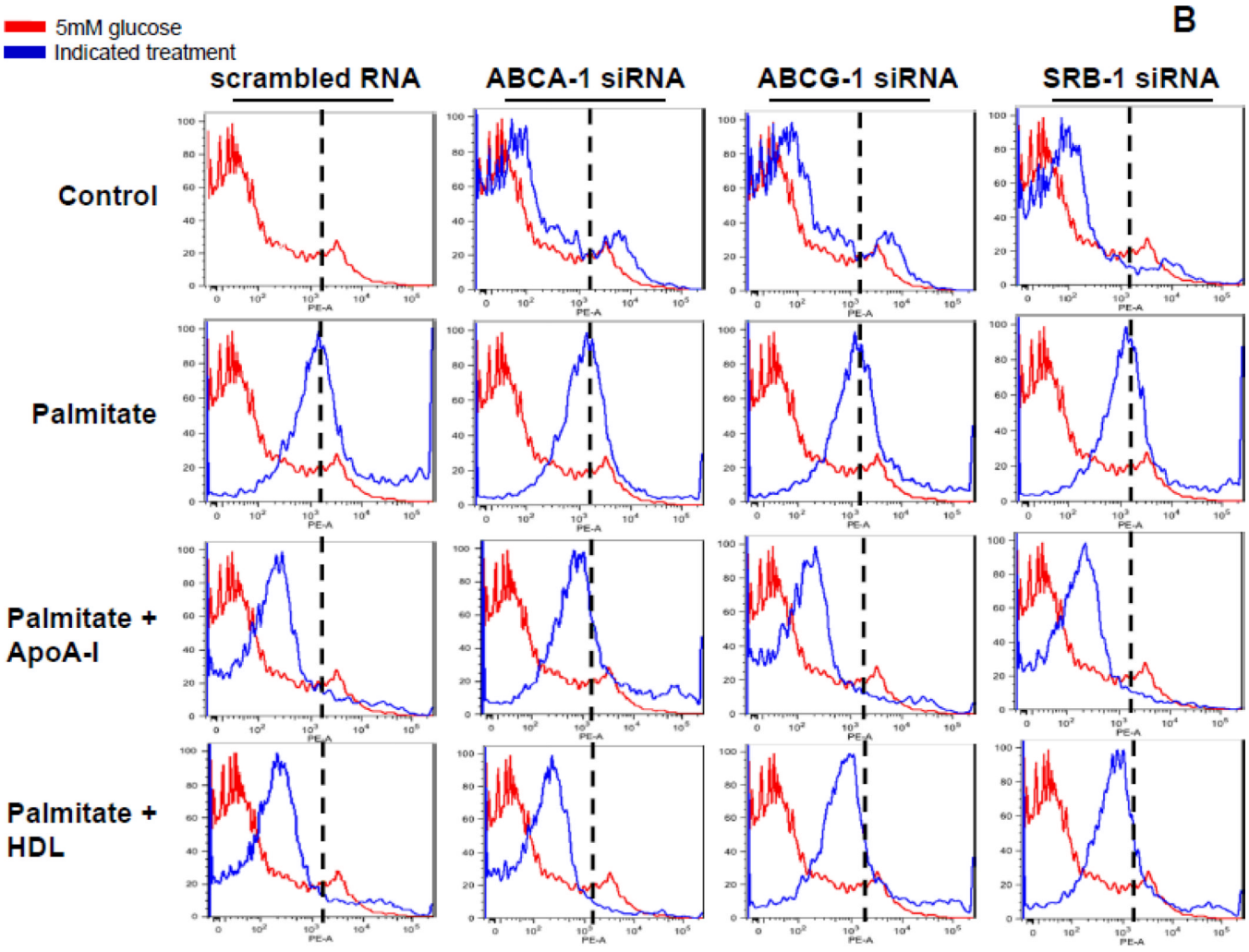


Figure 4. CTB-stained LRs are modulated by HDL and apoA-I via ABCA-1, ABCG-1 and SRB-1

3T3-L1 adipocytes were transfected with a siRNA specific for ABCA-1, ABCG-1, SRB-1 or a scrambled siRNA (negative control) as indicated. 24h later the siRNA was removed and the cells were cultured for a further 3 days. After that, the cells were pre-exposed to apoA-I (50μg/ml) or HDL (50μg protein/ml) for 6h, and then incubated with or without added palmitate (250 μmol/L) for 24h. *A*. LRs were stained by Alexa Fluor 594 conjugated cholera toxin subunit β (CTB) and photographed by fluorescent microscopy (Nikon Eclipse 80i, original magnification ×400). *B*. Cells in which LRs were stained by CTB were subjected to FACS analysis to quantify LR formation. Cells exposed to control media are shown in red and the peak value for cells exposed to 250μmol/L palmitate are indicated by the dashed lines. Cells exposed to the indicated treatments are shown in blue.

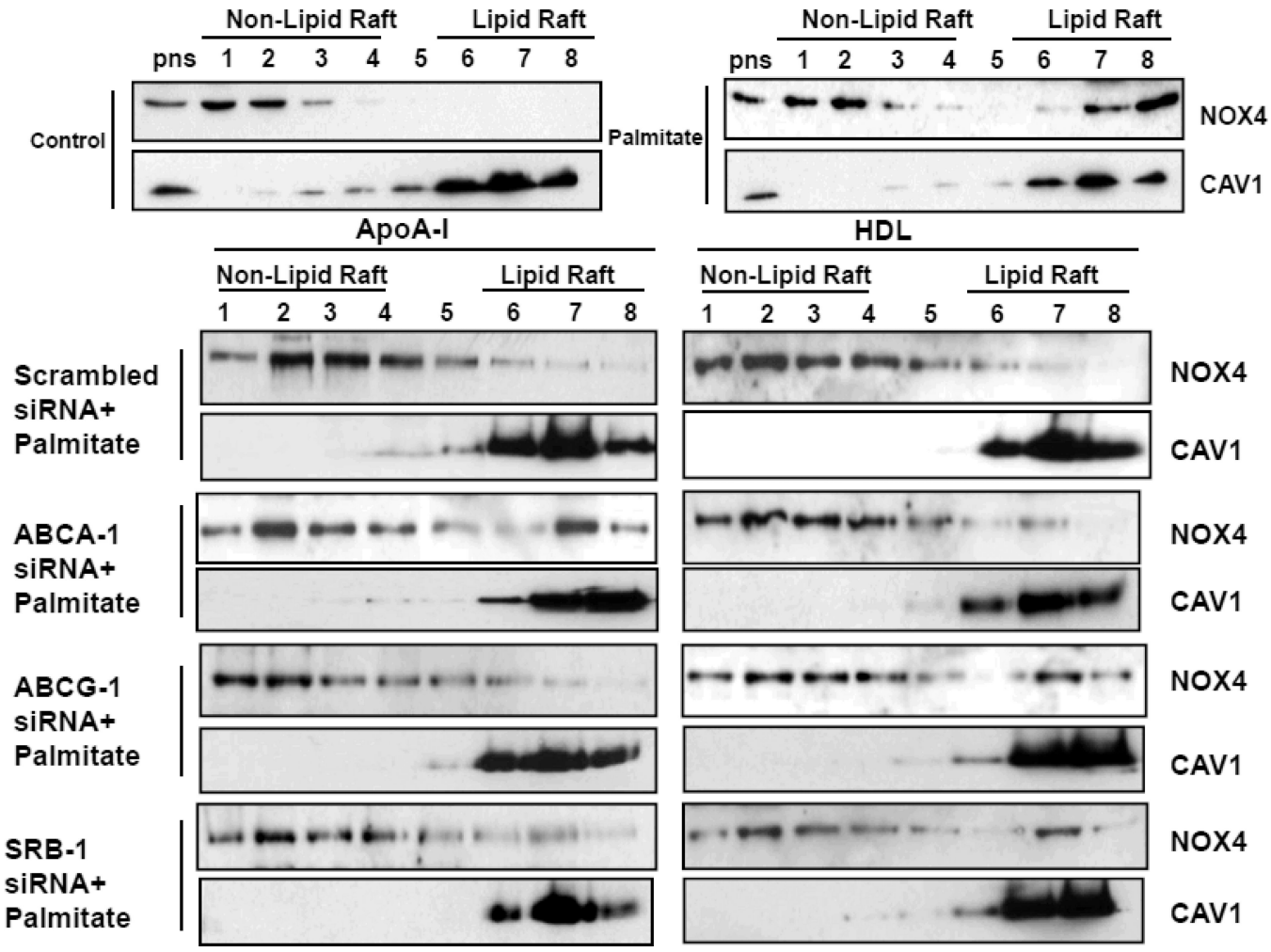


Figure 5. HDL and apoA-I block the palmitate-induced NOX4 translocation via ABCA-1, ABCG-1 and SRB-1

3T3-L1 adipocytes were transfected with a siRNA specific for ABCA-1, ABCG-1, SRB-1 or a scrambled siRNA (negative control) as indicated. 24h later siRNA was removed and the cells were cultured for a further 3 days. After that, the cells were pre-exposed to apoA-I (50μg/ml) or HDL (50μg protein/ml) for 6h, and then incubated with or without added palmitate (250μmol/L) for 24h. LRs were isolated and fractionated by ultracentrifugation using a detergent-free fractionation method. Proteins from OptiPre-gradient fractions were immunoblotted with anti-NOX4 and anti-caveolin-1 (CAV1) antibodis. PNS: the post-nuclear supernatant fraction.

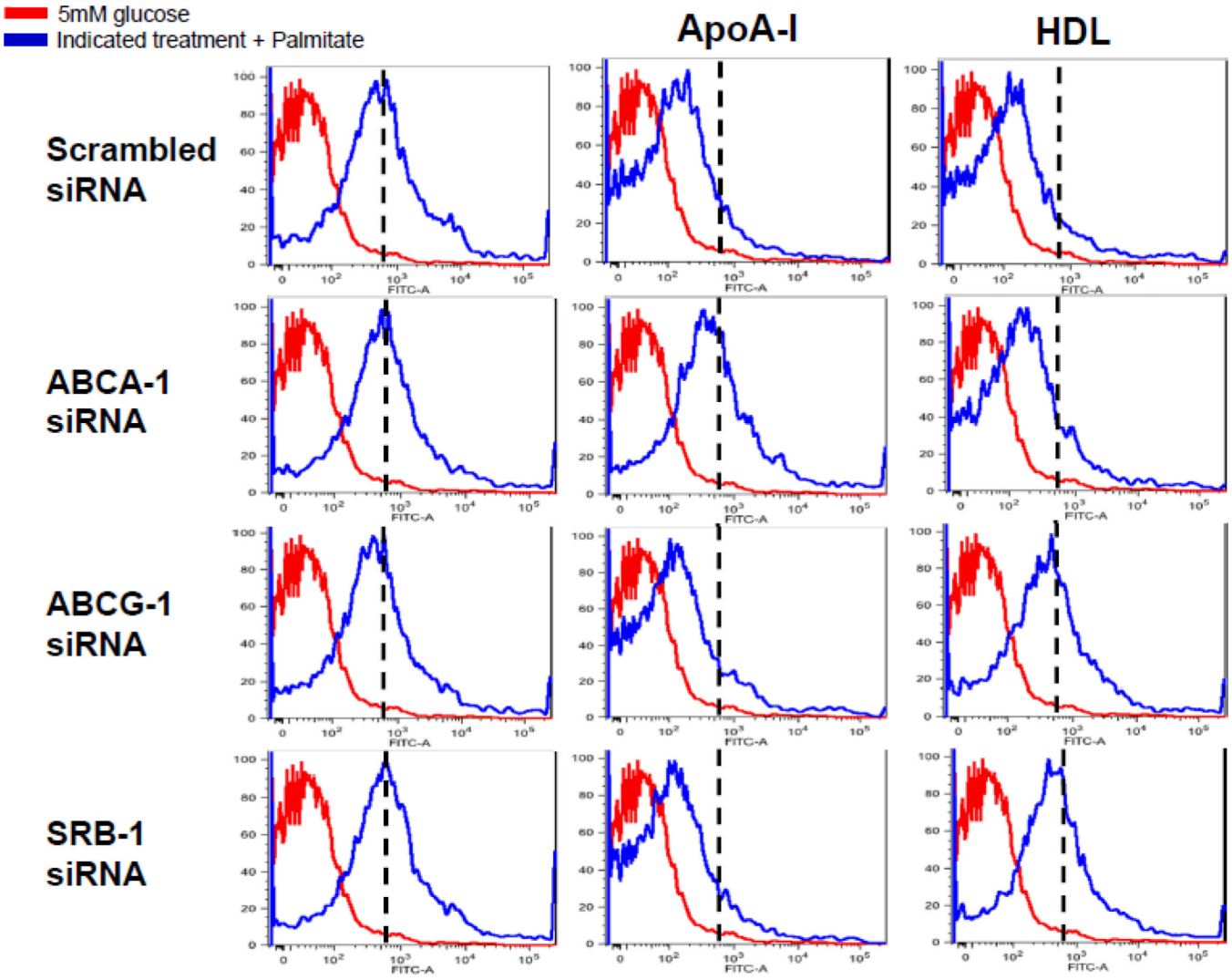
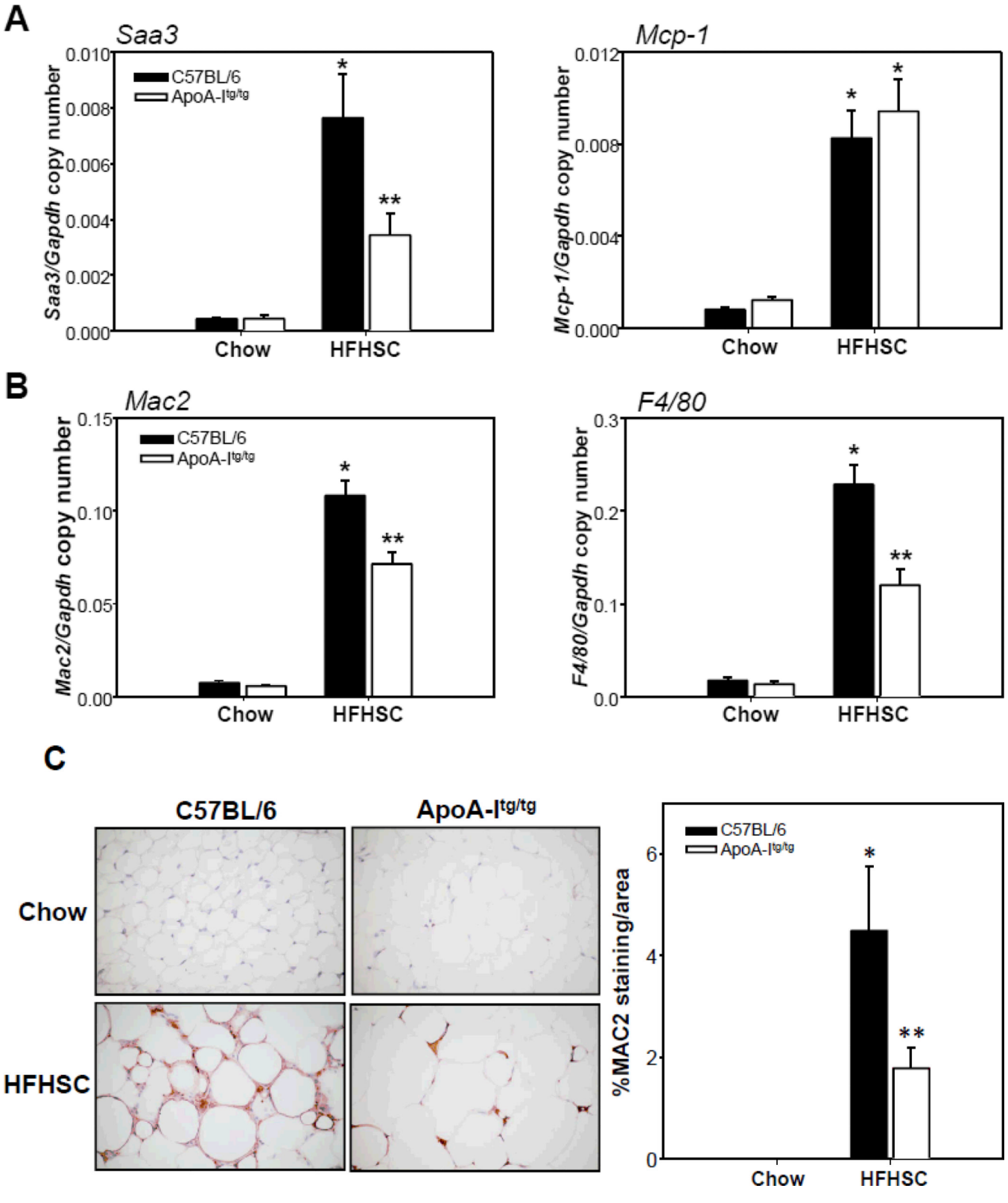


Figure 6. ApoA-I and HDL affect palmitate-induced NOX4-derived ROS generation via ABCA-1, ABCG-1 and SRB-1

3T3-L1 adipocytes were transfected with a siRNA specific for ABCA-1, ABCG-1, SRB-1 or a scrambled siRNA (negative control) as indicated. 24h later the siRNA was removed and the cells were cultured for a further 3 days. After that, the cells were exposed to apoA-I (50μg/ml) or HDL (50μg protein/ml) for 6h, and then incubated with or without added palmitate (250μmol/L) for 24h. Cells were subjected to FACS analysis using CM-H_2DCFDA. Results are plotted as counts (number of cells) on the vertical axis, versus DCF fluorescence intensity on the horizontal axis. Cells exposed to control media are shown in red and the peak value for cells exposed to 250μmol/L palmitate are indicated by the dashed lines. Cells exposed to the indicated treatments are shown in blue.



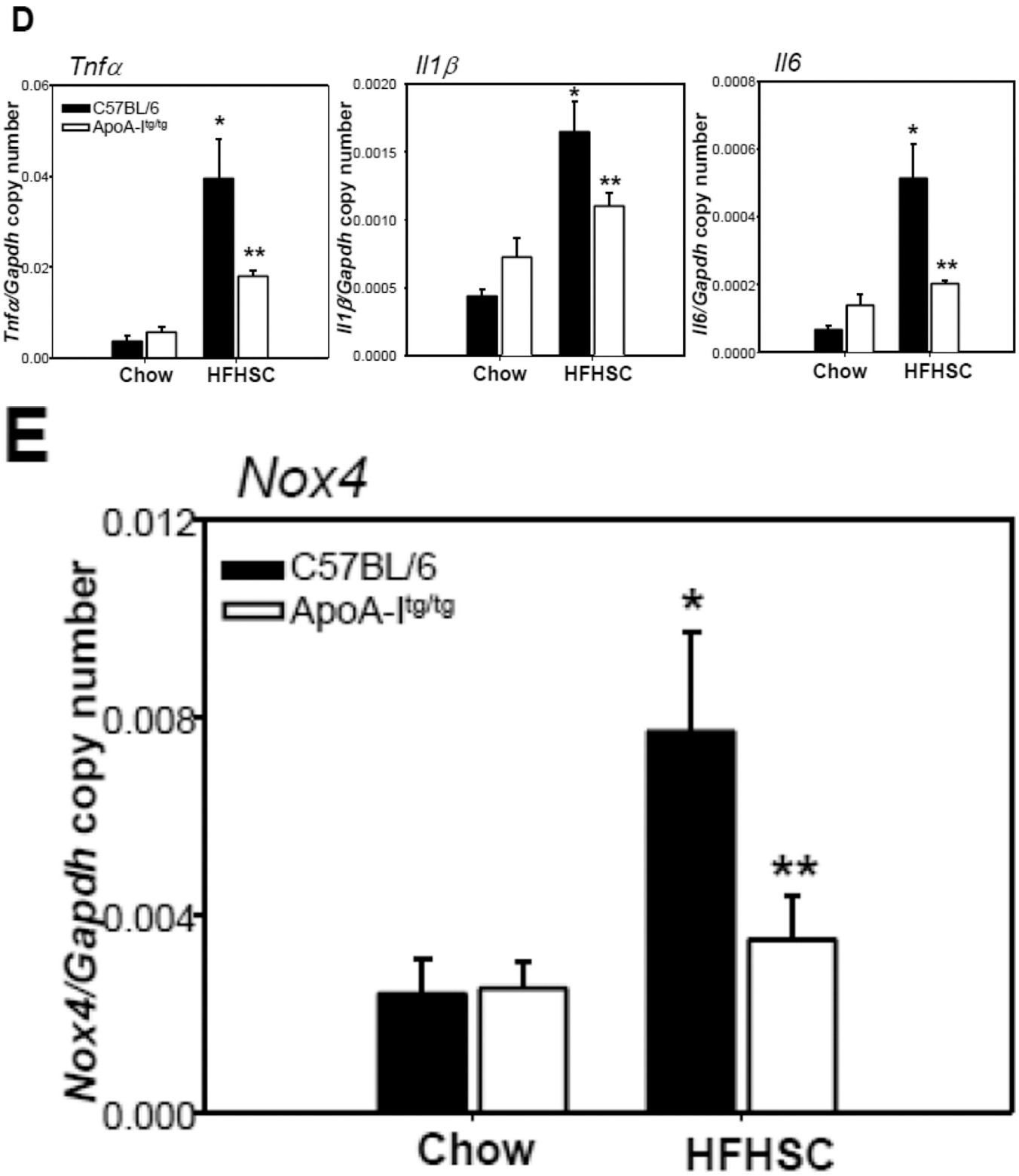


Figure 7. Overexpression of human apoA-I inhibits chemotactic factor expression and macrophage accumulation in adipose tissue of mice

ApoA-I$^{tg/tg}$ and C57BL/6 control mice were fed chow or a high-fat, high-sucrose, cholesterol-containing (HFHSC) diet for 24 weeks (*A*–*E*; n=9). Epididymal fat was isolated and analyzed by real-time RT-PCR using *Saa3, Mcp-1* (*A*), *Mac2, F4/80* (*B*), *Tnf*α, *Il1*β, *Il6* (*D*) *and Nox4* (*E*)-specific primers and probes and normalized to *Gapdh*. Epididymal fat was isolated and analyzed by immunohistochemistry using a Mac-2 antibody (*C*), which detects murine macrophages. Tissues were photographed using microscopy (original magnification ×60. **P* < 0.005 vs. chow, ***P* < 0.005 vs. C57BL/6 or LDLR$^{-/-}$ in HFHSC.