Deficiency of Adipose Differentiation-Related Protein Impairs Foam Cell Formation and Protects Against Atherosclerosis

Antoni Paul, Benny Hung-Junn Chang, Lan Li, Vijay K. Yechoor, Lawrence Chan

Abstract—Foam cells are a hallmark of atherosclerosis. However, it is unclear whether foam cell formation per se protects against atherosclerosis or fuels it. In this study, we investigated the role of adipose differentiation-related protein (ADFP), a major lipid droplet protein (LDP), in the regulation of foam cell formation and atherosclerosis. We show that ADFP expression facilitates foam cell formation induced by modified lipoproteins in mouse macrophages in vitro. We show further that Adfp gene inactivation in apolipoprotein E–deficient (ApoE−/−) mice reduces the number of lipid droplets in foam cells in atherosclerotic lesions and protects the mice against atherosclerosis. Moreover, transplantation of ADFP-null bone marrow–derived cells effectively attenuated atherosclerosis in ApoE−/− mice. Deficiency of ADFP did not cause a detectable compensatory increase in the other PAT domain proteins in macrophages in vitro or in vivo. Mechanistically, ADFP enables the macrophage to maintain its lipid content by hindering lipid efflux. We detected no significant difference in lesion composition or in multiple parameters of inflammation in macrophages or in their phagocytic activity between mice with and without ADFP. In conclusion, Adfp inactivation in ApoE−/− background protects against atherosclerosis and appears to be a relatively pure model of impaired foam cell formation. (Circ Res. 2008;102:1492-1501.)

Key Words: ADFP ■ atherosclerosis ■ foam cells ■ lipid droplets

The lipid-laden macrophage or “foam cell” is a hallmark of atherosclerosis.1–3 Foam cell formation is generally thought of as a protective mechanism whereby the vessel wall rids itself of potentially harmful lipids. Furthermore, macrophages, whether engorged with lipids or not, play a key role in the mediation and modulation of inflammation, and much atherosclerosis research has targeted the role of macrophages in the inflammation pathways that underlie atherogenesis.3,4 Nonetheless, atherosclerosis is more than inflammation of the artery (or arteritis5,6); despite some overlap in pathology, there are differences between atherosclerotic and nonatherosclerotic arteritis.7 A fundamental question in the field remains: How crucial is unfettered lipid accumulation in macrophages in protecting against or fueling atherosclerosis progression or, alternatively, does an inherent impairment in the ability of the macrophages to accumulate lipid in an animal alter its susceptibility to atherosclerosis?

Lipids do not occur free in the cytoplasm of foam cells but are sequestered inside special “bags” called lipid droplets (LDs) or fat bodies. These structures are stabilized and circumscribed by LD proteins (LDPs).8,9 A large number of proteins are associated with LDs, and the most abundant and unique LDPs that occur in lipid-laden cells of the body are the PAT domain proteins, named after perilipin (PLIN).10,11 adipose differentiation-related protein (ADFP), and tail-interacting protein of 47 kDa (TIP47).12,13 More recently, another LDP, S3-12,14 has been classified in the same group.8,9 It is reasonable to hypothesize that the PAT domain proteins play an enabling role in lipid accumulation in macrophages.

In this communication, we show that ADFP plays a key role in foam cell formation, and its absence severely restricts the ability of the macrophages to become foam cells in vitro. Furthermore, we found that ADFP is the most upregulated PAT domain protein in atheromas. We then used the atherosclerosis-prone apolipoprotein E–null (ApoE−/−) mouse to show that genetic ablation of ADFP expression greatly restricts foam cell formation in vivo, and this “defect” alone is sufficient to protect these mice against atherosclerosis development. The study demonstrates that foam cell formation per se, without perturbation in the inflammatory balance, is a crucial pathogenic event in atherosclerosis development.

Materials and Methods

Mice

Adfp−/− mice in C57BL/6J background (F8)15 were crossed 2 times with ApoE−/− mice (B6.129P2-Apoem1Jae; The Jackson Laboratory) to generate ApoE−/−/Adfp−/− mice. These mice were used as breeding
pairs to generate the ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> and ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> littersmates used in the study. Atherosclerosis development was analyzed in male and female mice of both genotypes at 20 weeks of age. For the bone marrow (BM) transfer study, twenty-two 8-week-old ApoE<sup>−/−</sup> females were distributed into 2 groups with similar cholesterol levels and subjected to 10-Gy total body irradiation, and the BM was reconstituted with cells from ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> or ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> mice. Atherosclerosis development was analyzed 14 weeks after BM transfer. Plasma cholesterol and triglyceride levels were measured using kits from Thermo Electron. Plasma lipoproteins were fractionated by fast-performance liquid chromatography gel filtration using Superose 6B columns (Pharmacia LKB Biotechnologie). Mice had free access to regular chow and water during the study. All animal experiments were conducted following protocols approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine.

### Analysis of Atherosclerotic Lesions

We measured atherosclerosis development in 6 sections of the aortic sinus spanning the region from the very proximal aorta to the point that contains 3 complete leaflets based on the method of Paigen et al<sup>16</sup> as previously described. We performed immunohistochemistry using primary antibodies specific for ADFP (Progen), PLIN (Progen), TIP47 (a gift from Dr C. Sztalryd, University of Maryland, Baltimore), S3-12 (a gift from Dr P.E. Bickel, University of Texas, Houston), and Macrophages (Mac-3, Santa Cruz Biotechnology), as previously described. TUNEL staining was performed using kits from Promega. Stainings with hematoxylin/eosin, Masson’s trichrome, Pearls’ iron, and Van Kossa (calcium) were performed using standard protocols.

### Electron Microscopy Analysis of Atherosclerotic Lesions

Aortic sinuses were processed in the Integrated Microscopy Core at Baylor College of Medicine. Images of foam cells in random sections of 3 pairs of Adfp<sup>−/−</sup>/ApoE<sup>−/−</sup> and Adfp<sup>+/−</sup>/ApoE<sup>−/−</sup> littersmates were taken at ×4000 with a Hitachi H-7500 microscope. The total area of the sections and the number and the area of the LDs were analyzed in trilipate using the AxiosView image analysis system (Carl Zeiss Vision).

### Experiments in Cultured Macrophages

RAW 264.7 macrophages were cultured following standard procedures. Thioctylcylate-elicited peritoneal macrophages were harvested as previously described. In some experiments, 50 µg/mL acetylated (ac)LDL or oxidized (ox)LDL (Intracel Resources) were added to the culture media. To visualize LDs, oil red O and Nile red stainings were performed following standard procedures. Binding and uptake of Dil-acLDL were analyzed as previously described. For [H]cholesterol labeling, the cells were incubated in DMEM 0.2% BSA containing acLDL (50 µg/mL) labeled with [1α,2α(N)−3H]cholesterol (Amersham; specific activity 142470 cpm/µg acLDL). To study the efflux of [H]cholesterol, after labeling, washing, and equilibrating, the cells were incubated with DMEM 0.2% BSA containing human apoA-I (10 µg/mL; Intracel Resources). Aliquots of the media were collected at different time points and immediately centrifuged to remove cell debris. At the final time point, the cells were lysed, and the radioactivity in the cells and in the supernatants was determined by scintillation counting. To estimate the rate of intracellular cholesterol ester (CE) accumulation, peritoneal macrophages were exposed to 50 µg/mL cold acLDL for 20 hours, washed, and pulsed with [9,10 (n)−3H]oleic acid complexed with BSA (molar ratio oleic acid to BSA 1:3; specific activity of oleic acid 7034 cpm/nmol) for 4 and 24 hours. Lipid fractions were separated by thin-layer chromatography, and the amount of [9,10 (n)−3H]oleic acid incorporated into CEs was determined by scintillation counting. Micromolar acyl-coenzyme A:cholesterol acyltransferase (ACAT) activity was determined by measuring the rate of incorporation of [14C]oleoyl-coenzyme A into the CE fraction as previously described. To determine CE hydrolyase activity, cells were loaded with cold acLDL (50 µg/mL) (overnight), washed and pulsed with [9,10 (n)−3H]oleic acid for 24 hours. Cholesterol esterification was blocked with the ACAT inhibitor CP113818 (10 µmol/L, a gift from Dr T.Y. Chang, Dartmouth Medical School, Hanover, NH), apoA-I (10 µg/mL) was added to the culture media, and the rate of disappearance of intracellular [3H]-labeled CE was analyzed by thin-layer chromatography at 3, 6, 9, and 23 hours.

Cellular lipids were extracted with hexane:isopropanol (3:2, vol/vol) and protein pellets were dissolved in 0.2 N NaOH, neutralized with HCl, and measured with enzymatic kits (Bio-Rad Laboratories). For thin-layer chromatography, the total lipids were evaporated to dryness, redissolved in chloroform, spotted on silica gel plates, and separated using hexane-diethyl ether:ether:acetic acid (75:35:1) as previously described.

### Western Blot and Real-Time Quantitative PCR Analysis

Western blotting and quantitative (q)PCRs were performed as described. Primer sequences are available in the online data supplement at http://circres.ahajournals.org. Relative gene expression levels were determined from threshold cycle (Ct) values normalized to GAPDH.

### Statistical Analysis

Data were analyzed using SPSS 11.0 for Windows. Statistical analyses were carried out using Mann–Whitney U or t tests. Differences were considered significant when P values were <0.05.

### Results

ADFP Is the Major LDP Stimulated by Lipid Accumulation in Macrophages In Vitro and in Atherosclerotic Lesions of ApoE<sup>−/−</sup> Mice

To tease out the proteins that empower macrophages to transform into foam cells, we first examined the mRNA level of different PAT domain proteins in RAW 264.7 cells, a mouse macrophage cell line, cultured in the absence or presence of acLDL (50 µg/mL for 24 hours). Under basal conditions, we could readily detect Adfp and Tip47 transcripts. The addition of acLDL-stimulated Adfp mRNA to ≅180% of basal but had no effect on the level of Tip47 mRNA (Figure 1a and 1b). The basal level of Plin mRNA was barely detectable and substantially lower than that of Adfp and Tip47 and remained unchanged when acLDL was added (Figure 1a and 1b). We could not detect any S3-12 mRNA whether or not acLDL was included in the incubation medium (Figure 1a and 1b). By immunoblot analysis (Figure 1c), ADFP and TIP47 were readily detectable in RAW264.7 cellular extracts. acLDL treatment significantly increased the intensity of the ADFP band (to ≅180%) but was without effect on the intensity of the TIP47 band (Figure 1d). We were unable to detect immunoreactive PLIN or S3-12, either in the absence or presence of acLDL, indicating that the low level Plin mRNA present was not translated into a detectable amount of protein.

Next, we screened for the expression of mRNA for these proteins in atherosclerotic arteries in C57BL/6 ApoE<sup>−/−</sup> mice, comparing their levels with those of nonatherosclerotic C57BL/6 ApoE<sup>−/−</sup> mice. We harvested the aortas at 24 weeks of age, when the aortic sinuses of ApoE<sup>−/−</sup> mice were studded with atherosclerotic lesions, whereas those of wild-type ApoE<sup>−/−</sup> mice were free of atherosclerotic involvement. In contrast to the transcripts for Plin, Tip47, and S3-12, which
showed no difference between ApoE−/− and wild-type mice, the level of Adfp mRNA increased to ~350% in the aortic sinuses of ApoE−/− mice (Figure 1e). Therefore, among the major LDPs, only the accumulation of ADFP correlates with LD accumulation in macrophages in vitro, and only Adfp expression is stimulated in the aortic sinuses in vivo in mice. These findings agree with previous reports showing that ADFP is highly upregulated in human atherosclerotic lesions.22–24 We note that previous reports did not simultaneously examine all the major LDPs as we did.

Absence of ADFP Restricts Atherosclerosis Development in ApoE−/− Mice
We next examined whether the absence of ADFP would affect atherosclerosis development in male and female ApoE−/−/Adfp−/− and ApoE−/−/Adfp+/− littermates. There was no difference in body weight, plasma cholesterol, and triglyceride levels throughout the study (Table I in the online data supplement). The mice also displayed identical plasma lipoprotein profiles whether or not they produced ADFP (supplemental Figure 1a and b). We euthanized the mice at 20 weeks of age and compared the atherosclerotic lesion size in the aortic sinus areas (Figure 2a through 2d). As previously observed in ApoE−/− mice, female animals developed larger lesions than males in both genotypes. In males, the lack of ADFP was associated with a 58% reduction in lesion size (from 137 ± 98 × 10^3 to 57 ± 40 × 10^3 μm^2). In females, there was similarly a significant reduction of 40% in lesion size in mice lacking ADFP (from 575 ± 213 × 10^3 to 345 ± 153 × 10^3 μm^2). Therefore, the absence of ADFP protects against atherosclerosis development in both male and female ApoE−/− mice.

ADFP protein and mRNA are present in diverse cell types when they accumulate lipid.25,26 In support of an important role for ADFP in fat accumulation is the fact that absence of ADFP decreases lipid accumulation in the liver when mice are fed a high-fat diet.15 To exclude the possibility that absence of ADFP in other tissues accounts for the protection against atherosclerosis, we transferred BM cells from ApoE−/−/Adfp−/− or ApoE−/−/Adfp+/− female mice to 8-week-old ApoE−/−/Adfp+/− recipient female mice, and compared the extent of aortic atherosclerosis in the recipients 14 weeks after BM transfer (Figure 2e). We found that mice that received BM from ADFP-deficient donors had smaller lesions compared with those that received BM cells from ApoE−/−/Adfp+/− donors (size
reduced from $425 \pm 69 \times 10^3 \ \mu m^2$ to $322 \pm 71 \times 10^3 \ \mu m^2$). Therefore, absence of Adfp in BM-derived cells alone is sufficient to protect against atherosclerosis in ApoE<sup>−/−</sup> mice.

No Compensatory Upregulation of Other Major PAT Domain Proteins in the Absence of ADFP

To examine whether other PAT domain proteins undergo compensatory upregulation when ADFP is missing, we isolated peritoneal macrophages from ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> and ApoE<sup>−/−</sup>/Adfp<sup>+/+</sup> mice. S3-12 and TIP47 mRNA could be detected, but not PLIN mRNA. However, we found no difference in any of these transcripts between cells
with and without ADFP expression whether or not the macrophages were exposed to oxLDL, and exposure to oxLDL did not increase the expression of any of these genes (Figure 3a and 3b). Therefore, Adfp<sup>−/−</sup> macrophages did not overexpress TIP47, as was observed in fibroblastic cell lines isolated from Adfp<sup>−/−</sup> embryos<sup>27</sup>; they were much more like Adfp<sup>−/−</sup> liver cells,<sup>15</sup> which also did not upregulate the production of TIP47 or other PAT domain proteins.

Because PAT domain proteins have been shown to exhibit substantial posttranslational regulation,<sup>28</sup> we next analyzed protein expression by immunoblotting (Figure 3c). We found TIP47, but not PLIN or S3-12, in extracts of macrophages isolated from ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> and ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> mice. As expected, ADFP was detected only in ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> animals. The relative concentration of immunoreactive TIP47 was the same whether or not the macrophages expressed

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Figure 3. a and b, RT-PCR (ADFP and TIP47 23 PCR cycles; S3-12 and PLIN 35 cycles) (a) and qPCR analysis (n = 3, *P < 0.05) (b) of the mRNA expression of the main PAT family proteins in peritoneal macrophages isolated from ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> and ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> mice cultured with or without 50 μg/mL oxLDL. WAT indicates white adipose tissue. c, Western blot analysis of peritoneal macrophages cultured with or without 50 μg/mL oxLDL. FL indicates fatty liver. d, qPCR analysis of TIP47 mRNA expression in aortic sinuses of ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> and ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> mice (n = 4). e through h, Immunoreactive ADFP (brown color) (e), TIP47 (brown color) (f), PLIN (g), and S3-12 (h) in atherosclerotic lesions of female ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> (left images) and ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> (right images) mice. Scale bar=50 μm. i, Quantification of percentage of area positively stained for TIP47 in lesions of ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> and ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> mice (n = 7).
ADFP. Furthermore, in contrast to ADFP, the expression of TIP47 protein was not affected by the addition of oxLDL. This oxLDL-regulated Adfp expression and absence of an effect on Tip47 expression is consistent with that observed in acLDL-regulated gene expression in RAW 264.7 cells (Figure 1). To determine whether the lack of compensation of Tip47 extends to the in vivo situation, we quantified the amount of Tip47 mRNA in the RNA isolated from the aortic sinus of ApoE<sup>−/−</sup> mice and found that the concentration of Tip47 mRNA did not differ between mice that expressed ADFP and those that did not (Figure 3d). Next, we analyzed sections of aortic sinus lesions of ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> and ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> mice by immunohistochemistry. We readily detected ADFP and TIP47 (Figure 3e and 3f) but not PLIN or S3-12 in the lesions (Figure 3g and 3h). In addition, the relative distribution and density of immunoreactive TIP47 in the aortic sinus lesions was similar in ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> versus ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> mice (Figure 3i).

Absence of ADFP Restricts Foam Cell Formation
The rationale for the experiments on atherosclerosis development in the 2 types of ApoE<sup>−/−</sup> mice is based on the hypothesis that ADFP modulates foam cell formation in these animals. To assess whether this is true, we first used peritoneal macrophages isolated from ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> and ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> mice as an in vitro model. As shown in Figure 4a through 4c, the lack of ADFP reduced significantly the accumulation of LDs after overnight incubation with oxLDL. Similar experiments performed in macrophages isolated from C57BL/6jApoE<sup>−/−</sup> mice yielded comparable results (supplemental Figure II). Next, we examined the aortic sinus lesions by transmission electron microscopy. Atherosclerotic plaques from both ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> and ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> mice contained lipid-laden foam cells (Figure 4d and 4e). However, the number of LDs per unit area was reduced by >50% in lesions of mice lacking ADFP (Figure 4f). Interestingly, size distribution analysis revealed that the decrease in the number of intracellular LDs affected droplets of all sizes in lesions of ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> mice (Figure 4g). Thus, the effect of ADFP on foam cell formation in vivo is reflected in atherosclerotic lesions in vivo.

Histopathologic Analysis of Atherosclerotic Lesions and Atherosclerosis-Related Macrophage Functions
As expected, small lesions, commonly observed especially in male mice, consisted mainly of fatty streaks with macrophage infiltration, whereas the larger lesions, found mainly in females, contained much more abundant collagen deposition and necrotic cores (supplemental Figure III). Quantitative analysis of lesions of female mice of both genotypes showed no significant differences in the density of apoptotic cells, total cell number content and macrophage content, collagen-positive or calcium-staining area, or necrotic core areas (supplemental Figure IVa through IVg). Furthermore, the lack of ADFP did not affect the appearance of iron deposition, a marker of intraplaque hemorrhage, which was very scarce in mice of either genotype (supplemental Figure IVh).

Corroborating the findings in vivo, we observed a similar rate of apoptosis when peritoneal macrophages from ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> and ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> mice were cultured in the presence of acLDL (50 µg/mL) for 24 hours (supplemental Figure V). We also found that the expression of cytokines (tumor necrosis factor-α, IL [interleukin]-1α, IL-1β, CXCL1, CXCL2, IL-10, IL-6, JE, and macrophage colony-stimulating factor), cytokine receptors (CCR1, CCR2, CCR3, and CCR5) and inducible NO synthase, under basal conditions and after exposure to 50 µg/mL oxLDL for 24 hours, was similar in the 2 genotypes (supplemental Figure VI). Furthermore, the expression of these genes was also similar in aortic sinuses of ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> and ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> mice (supplemental Figure VII). Finally, we detected no difference in NO production and phagocytic activity in macrophages from mice with and without ADFP expression (supplemental Figures VIII and IX).

In sum, we found no difference in macrophage functions that have been associated with protection or propensity to develop atherosclerosis in mice that express or do not express ADFP. The only difference thus seems to be their capacity to accumulate lipid. We next examined how the lack of ADFP affects this property.

Mechanism of Reduced Lipid Accumulation in Macrophages Without ADFP
We used peritoneal macrophages to assess whether the lack of ADFP affects cholesterol trafficking. First, we used Dil-acLDL, a fluorescence-labeled acLDL, to assess whether the absence of ADFP affects lipoprotein binding or uptake and found no differences in either process between macrophages isolated from ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> and ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> mice (Figure 5a). To more accurately quantify cholesterol uptake, we cultured peritoneal macrophages with [1α,2α(N)-H]cholesterol-labeled acLDL in the absence of extracellular acceptors and measured the intracellular [1α,2α(N)-H]cholesterol at 4 and 16 hours by scintillation counting. Again, there was no difference between the 2 genotypes (Figure 5b). After lipoproteins are taken up by macrophages, CEs are hydrolyzed in the endocytic compartment by the lysosomal acid lipase and free cholesterol is exported to other cellular sites, such as the plasma membrane, where it can remain as a structural component or be effluxed to extracellular acceptors, or the endoplasmic reticulum, where it can be reesterified by ACAT-1 and stored in cytoplasmic LDs. We used [1α,2α(N)-H]cholesterol-labeled macrophages to assess whether the absence of ADFP influences cholesterol efflux to a plasma acceptor, apoA-I. As shown in Figure 5c, the amount of cholesterol effluxed increased over time in both ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> and ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> cells; however, at the 16-hour time point, the cholesterol effluxed was 57.5% higher in ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> cells (13.7 ± 2.1% versus 8.7 ± 3.5% in ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> cells, Figure 5c). Next, we measured the incorporation of [9,10 (m) H]oleic acid to the CE pool to assess the accumulation of newly synthesized CE. In this case, the macrophages isolated from ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> mice presented a significant (93%) increase in newly synthesized CE accumulation (504 ± 185 nmol/mg protein in ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> cells versus 261 ± 119 nmol/mg protein in ApoE<sup>−/−</sup>/Adfp<sup>+/−</sup> cells, Figure 5d). To determine whether the higher CE accumulation is the result of increased microsomal ACAT

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activity, we performed subcellular fractionation of macrophages and measured ACAT activity in the microsomal fraction. As shown in Figure 5e, the ACAT activity was similar in microsomes isolated from ApoE−/−/Adfp−/− and ApoE−/−/Adfp+/+ mice. This suggests that the higher CE accumulation in macrophages from ApoE−/−/Adfp−/− mice is caused by the action of ADFP in facilitating CE storage in LDs or in preventing CE from hydrolysis. To differentiate between these 2 possibilities, we determined whether the absence of ADFP affects the rate of hydrolysis of the stored CE. We found no difference in the rate of CE hydrolysis between macrophages that express and those that do not express ADFP (Figure 5f), suggesting a main role of ADFP in facilitating CE storage in LDs.

Finally, we examined by qPCR the expression of other key molecules involved in intracellular cholesterol homeostasis, including (1) the principal receptors involved in modified LDL uptake: SR-A1 and CD36; (2) major molecules involved in CE esterification: ACAT1 and ACAT2; (3) molecules involved in CE transport: ATP-binding cassette A1 (ABCA1) and ATP-binding cassette G1 (ABCG1); and (4) molecules involved in CE degradation: lecithin:cholesterol acyltransferase (LCAT) and cholesterol ester hydrolase (CEH). We found that the expression of these molecules was not significantly different between macrophages from ApoE−/−/Adfp+/- and ApoE−/−/Adfp−/− mice.
Figure 5. a, Dil-acLDL binding (left) and uptake (right) in peritoneal macrophages isolated from ApoE−/−/Adfp−/− (n=3) and ApoE−/−/ Adfp−/− (n=4) mice. b, Time course of uptake of [3H]cholesterol-labeled acLDL in peritoneal macrophages isolated from ApoE−/−/Adfp−/− and ApoE−/−/Adfp−/− mice (n=4). c, Time course of cholesterol efflux to apoA-I in peritoneal macrophages from ApoE−/−/Adfp−/− and ApoE−/−/Adfp−/− mice (n=9, **P<0.01). The efflux is expressed as a percentage of cholesterol effluxed with respect to the total cholesterol (intracellular + extracellular). d, Time course of the rate of intracellular cholesterol esterification (n=5, *P<0.05). e, ACAT activity in isolated microsomes (n=4). f, Time course or CE hydrolysis in [3H]cholesterol-labeled macrophages in which cholesterol reesterification was blocked with the ACAT inhibitor CP 113818 (10 μmol/L) (n=4). g, qPCR of analysis of proteins involved in intracellular lipid homeostasis in macrophages. Note that there were no changes between cells that express or do not express Adfp (*P<0.05 of cells cultured with 50 μg/mL oxLDL vs untreated cells; n=3). ABC indicates ATP-binding cassette; HSL, hormone-sensitive lipase; SR, scavenger receptor.
in reverse cholesterol transport: ATP-binding cassette A1, ATP-binding cassette G1, and scavenger receptor BI; and (3) other key molecules involved in intracellular lipid metabolism, including NPC1 (Nieman–Pick type C1 protein), ACAT-1, fatty acid-binding protein aP2, and hormone-sensitive lipase. As shown in Figure 5g (with primer sequences in supplemental Table II), there were no differences in the expression level of any of these molecules when we compared macrophages of the 2 genotypes. Therefore, the data suggest that ADFP directly facilitates lipid storage in LDs, and its absence results in a detour of intracellular cholesterol toward efflux pathways.

Discussion

In this study, we have addressed whether the process of foam cell formation per se is a key determinant in atherosclerosis progression. We have selected ADFP because of previous studies suggesting its possible involvement in foam cell formation. ADFP expression increases following lipid loading (with oleic acid, oxLDL, or acLDL) of human monocytes or macrophage/monocyctic cell lines. Adfp mRNA is detected in macrophage/lipid-rich areas of endarterectomy specimens, and the level is 3.5-fold higher in atherosclerotic lesions than in healthy areas of the same artery. Adfp gene transfer stimulates lipid accumulation in mouse fibroblasts and in human macrophages incubated with acLDL. Moreover, siRNA knockdown of Adfp expression in THP-1 macrophages reduces triglycerides and total and esterified cholesterol in these cells.

To determine whether other PAT domain proteins increase their expression to compensate for the absence of ADFP, we measured their expression level in macrophages and found no difference between mice with or without Adfp expression. This contrasts with a collaborative study between us and Drs C. Sztalryd and C. Londos, in which we screened the same PAT domain proteins and found that clonal embryonic fibroblastic cells isolated from the same Adfp−/− mice display increased Tip47 expression. On the other hand, Adfp−/− macrophages behave like Adfp−/− liver cells in that absence of ADFP does not change the level of expression of Tip47 or other PAT domain protein genes. This lack of compensation suggests that lipid accumulation in macrophages may be limited by the total PAT domain protein concentration, which stays consistently below wild-type levels in Adfp−/− mice. We note that many other proteins, which are present in lower concentrations than the PAT domain proteins, have been isolated from LDs, and we cannot exclude the possibility that some of them are upregulated when ADFP is missing. Nonetheless, the data indicate that the impaired ability of Adfp−/− macrophages to form LDs is clearly not restored to normal by compensatory overexpression of other LDPs, if indeed it has taken place.

Lipid-laden foam cells form because they take up excess lipids, mainly from cholesterol-rich LDL, or they are too slow in letting go of their lipids, having to store the excess cholesterol in LDs. Another possible way to inhibit foam cell formation is by inactivating ACAT-1, because cholesterol must be esterified in the endoplasmic reticulum before it can be stored in LDs. However, ACAT inactivation or inhibition in macrophages appears to be toxic to the cell, possibly because of excess free cholesterol trafficking to the endoplasmic reticulum membranes, giving rise to downstream effects, including increased synthesis of cytokines, macrophage apoptosis, and accelerated atherosclerosis with grossly necrotic lesions. In contrast, after close examination of lesions and cultured macrophages, we did not find any evidence of toxicity in Adfp−/− cells. Interestingly, a recent report by Zhao et al showed that the transgenic expression of a neutral CE hydrolase, an enzyme that hydrolyzes the CE stored in LDs, enhances cholesterol efflux, and reduces atherosclerosis in mice. Taken together, these data suggest that the LD may be a direct drug target for antiatherosclerosis therapy.

Our findings indicate that Adfp−/− mice appear to be a relatively pure model of inhibition of foam cell formation. Macrophages isolated from ApoE−/−/Adfp−/− mice exhibit an impaired ability to accumulate intracellular LDs, but they do not differ in other properties that are known to affect atherosclerosis susceptibility, such as the ability to produce inflammatory cytokines or in their phagocytic activity. For many years, foam cells have been considered a hallmark of atherosclerosis. Herein, we have presented direct evidence that a relative failure of foam cell formation per se protects against atherosclerosis development.

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Disclosures

None.

References

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Online Figure Legends

**Online Figure I.** CHOL in FPLC fractions of male (a) and female (b) $ApoE^{-/-}/Adfp^{-/-}$ and $ApoE^{-/-}/Adfp^{+/+}$ mice.

**Online Figure II.** (a) Representative Oil Red O staining of peritoneal macrophages isolated from $Adfp^{-/-}$ and $Adfp^{+/+}$ mice in C57BL6/J $ApoE^{+/+}$ background that were exposed to acLDL (50 μg/ml) for 24h; (b) Nile Red staining (green fluorescence) and (c) computer assisted quantification of LDs in peritoneal macrophages from $Adfp^{-/-}$ and $Adfp^{+/+}$ in C57BL6/J $ApoE^{+/+}$ mice treated with acLDL (50μg/ml) for 24 h (n=3, *p<0.03).

**Online Figure III.** Immunostaining of macrophages (brown color, upper panels) and Mason’s trichrome (which stains collagen blue, smooth muscle cells red and nuclei black, lower panels) showing different stages of atherosclerosis development in lesions of male and female mice.

**Online Figure IV.** (a) Representative sections and (b) quantification of the rate of apoptosis in atherosclerotic lesions (n=3). (c) Total cell number in lesions (n=7); (d) % of necrotic cores related to the total lesion area (n=7); (e) % of lesion area that stained positive for macrophages; (f) collagen content (n=7); (g) calcium content (n= 8); and (h) iron deposition in lesions of $ApoE^{-/-}/Adfp^{-/-}$ and $ApoE^{-/-}/Adfp^{+/+}$ mice. No significant differences were observed in any of the measured parameters.
Online Figure V. (a) Representative fields and (b) measurement of apoptosis, assessed by TUNEL, in cultured macrophages pre-treated with acLDL for 24 hours (n= 4).

Online Figure VI. qPCR analysis of expression of inflammatory markers in peritoneal macrophages isolated from ApoE<sup>−/−</sup>/Adfp<sup>+/+</sup> (WT) or ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> (KO) mice cultured O/N in the absence (-ox) or in the presence (+ox) of oxLDL (50 µg/ml) (*p<0.05 of cells cultured with 50 µg/ml of oxLDL vs. untreated cells; n=3). Note that treatment with oxLDL increased the expression of IL-6 and CCR1, but decreased the expression of CXCL1, CCR2, CCR5 and iNOS. However, compared to macrophages expressing ADFP, the lack of ADFP did not change the expression of any of the genes under any of the experimental conditions.

Online Figure VII. qPCR analysis of expression of inflammatory markers in aortic sinuses isolated from ApoE<sup>−/−</sup>/Adfp<sup>+/+</sup> (ADFP+/+) or ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> (ADFP−/−) normalized to the scavenger receptor CD-36 (n=4). No significant differences were observed between mice of both genotypes.

Online Figure VIII. NO<sub>2</sub>– production in peritoneal macrophages. Peritoneal macrophages isolated from ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> and ApoE<sup>−/−</sup>/Adfp<sup>+/+</sup> mice were cultured in the presence or in the absence of 50 µg/mL oxLDL for 24 h. Supernatants were assayed for NO<sub>2</sub>– by the Griess reagent. Data are expressed as micromoles of NO<sub>2</sub>– per mg of protein.

Online Figure IX. Representative fields and quantification of phagocytic activity in peritoneal macrophages isolated from ApoE<sup>−/−</sup>/Adfp<sup>−/−</sup> and ApoE<sup>−/−</sup>/Adfp<sup>+/+</sup> mice. Peritoneal macrophages
isolated from 4 ApoE\(^{-/-}\)/Adfp\(^{-/-}\) and 4 ApoE\(^{-/-}\)/Adfp\(^{+/+}\) mice were loaded O/N with acLDL (50 µg/mL) and incubated for 150 min with apoptotic thymocytes labeled with CellTracker Green (1,500,000/mL). Cells were counterstained with DAPI. The activity for each mouse was obtained by counting the number of apoptotic cells per macrophage in 5 different fields.
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<th>Females</th>
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<td>ADFP&lt;sup&gt;+&lt;/sup&gt;apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>8 weeks</td>
<td>19.8 ± 2.0</td>
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<td>15.8 ± 0.9</td>
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<tr>
<td>15 weeks</td>
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**Online Table I.** BWs and plasma lipids.
### Online Table II. Sequences of the primers used for qPCR.

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Online Figure I/R2
Online Figure III/R2

MALES

ADFP+/+

ADFP−/−

FEMALES

ADFP+/+

ADFP−/−
Online Figure IV/R2
Online Figure V/R2
Inflammatory markers in peritoneal macrophages from ADFP^{+/+} and ADFP^{−/−} mice

* = p<0.05 vs. non treated with oxLDL

Online Figure VI/R2
Inflammatory markers in aortic sinuses of ADFP^+/+apoE^−/− and ADFP^−/−apoE^−/− mice

Online Figure VII/R2
NO2-Production

µM/mg protein

ADFP^{+/+}  ADFP^{--}  ADFP^{+/+}  ADFP^{--}

BASELINE  24h oxLDL

Online Figure VIII/R2
Online Figure IX/R2