Defective Phagocytosis of Apoptotic Cells by Macrophages in Atherosclerotic Lesions of ob/ob Mice and Reversal by a Fish Oil Diet

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Rationale: The complications of atherosclerosis are a major cause of death and disability in type 2 diabetes. Defective clearance of apoptotic cells by macrophages (efferocytosis) is thought to lead to increased necrotic core formation and inflammation in atherosclerotic lesions.

Objective: To determine whether there is defective efferocytosis in a mouse model of obesity and atherosclerosis.

Methods and Results: We quantified efferocytosis in peritoneal macrophages and in atherosclerotic lesions of obese ob/ob or ob/ob;Ldlr<sup>−/−</sup> mice and littermate controls. Peritoneal macrophages from ob/ob and ob/ob;Ldlr<sup>−/−</sup> mice showed impaired efferocytosis, reflecting defective phosphatidylinositol 3-kinase activation during uptake of apoptotic cells. Membrane lipid composition of ob/ob and ob/ob;Ldlr<sup>−/−</sup> macrophages showed an increased content of saturated fatty acids (FAs) and decreased ω-3 FAs (eicosapentaenoic acid and docosahexaenoic acid) compared to controls. A similar defect in efferocytosis was induced by treating control macrophages with saturated free FA/BSA complexes, whereas the defect in ob/ob macrophages was reversed by treatment with eicosapentaenoic acid/BSA or by feeding ob/ob mice a fish oil diet rich in ω-3 FAs. There was also defective macrophage efferocytosis in atherosclerotic lesions of ob/ob;Ldlr<sup>−/−</sup> mice and this was reversed by a fish oil–rich diet.

Conclusions: The findings suggest that in obesity and type 2 diabetes elevated levels of saturated FAs and/or decreased ω-3 FAs contribute to decreased macrophage efferocytosis. Beneficial effects of fish oil diets in atherosclerotic cardiovascular disease may involve improvements in macrophage function related to reversal of defective efferocytosis and could be particularly important in type 2 diabetes and obesity. (Circ Res. 2009;105:1072-1082.)

Key Words: efferocytosis ▪ macrophages ▪ atherosclerosis ▪ fatty acids ▪ mouse

Patients with type 2 diabetes experience both accelerated atherogenesis and increased atherosclerotic complications such as plaque rupture and atherothrombosis. Although diabetic dyslipidemia is a major factor underlying accelerated atherogenesis, mechanisms acting at the level of the vessel wall may also be involved in plaque formation and complications. Recent studies have suggested that insulin resistance in macrophage foam cells, as well as in endothelial cells, could also contribute to atherosclerotic plaque formation and complications. In macrophages insulin resistance promotes macrophage apoptosis during the endoplasmic reticulum stress response, and is associated with increased necrotic core formation in atherosclerotic plaques, whereas in endothelial cells, insulin resistance and defective protein kinase B (AKT/PKB) activation lead to impaired nitric oxide (NO) bioavailability, increased inflammatory gene expression and markedly accelerated atherogenesis.

Apoptosis of macrophages and smooth muscle cells in advanced atherosclerotic plaques is thought to lead to increased necrotic core formation, inflammation, plaque disruption and atherothrombosis. Macrophages from ob/ob and insulin receptor knockout (Insr<sup>−/−</sup>) mice show increased susceptibility to apoptosis during the endoplasmic reticulum stress response and decreased inflammatory responses. Increased apoptosis and decreased inflammatory responses of macrophages would not be expected to lead to an enhancement of lesion formation, because efficient efferocytosis is normally antiinflammatory and should not produce detrimental effects unless the phagocytic system becomes im-

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1072
paired. In fact, several molecular defects that lead to an enhancement of macrophage apoptosis have been associated with diminished formation of early atherosclerotic lesions. In contrast, various induced mutations that cause defective efferocytosis (such as in the Mer-tyrosine kinase receptor) lead to increased necrotic core formation, increased inflammation, and accelerated atherogenesis in mouse models. In the present study, we show defective efferocytosis in macrophages of ob/ob mice both in peritoneal macrophages and within atherosclerotic lesions and relate these changes to alterations in membrane fatty acid (FA) composition.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Animals

Animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University. Obese ob/ob or db/db mice (~6 weeks old), together with their lean littermate controls, low-density lipoprotein receptor knockout (Ldlr−/−) and ob/ob;Ldlr−/− on C57BL/6J background were obtained from The Jackson Laboratory (Bar Harbor, Me). For study with the AIN76A semisynthetic diet (0.02% cholesterol), sections of animals were used for in vitro peritoneal macrophage efferocytosis or membrane/plasma lipid composition analysis. For olive or fish oil diet study, sections of atherosclerotic lesions, apoptotic cells were detected as before, except that the frozen sections were permeabilized with 0.1% triton and sodium citrate on ice for 2 minutes. After TUNEL staining, samples were blocked in 10% goat serum and stained with macrophage-specific rabbit anti-mouse antibody AIA (Accurate Chemical and Scientific). Genomic DNA was stained with Hoechst dye before the slides were mounted with coverslips. Fluorescent images were captured and macrophage efferocytosis in the lesion areas was quantified as previously described.

Data Analysis

Results are expressed as means±SEM (n is noted in the figure legends or figure), and statistical significance of differences was evaluated with Student’s t test.

Results

Macrophages of ob/ob Mice Have Impaired Ability to Phagocytose Apoptotic Cells

Using a fluorescence assay that identifies apoptotic cells within macrophage phagosomes, we showed that peritoneal macrophages from ob/ob and db/db mice have impaired efferocytosis (Figure 1A). An in vivo defect in efferocytosis was shown by instilling apoptotic cells into the peritoneal cavity of mice during macrophage elicitation (Figure 1B, in vivo). In addition, ob/ob macrophages showed a defect in Fc receptor (Fc-R)–mediated phagocytosis (Figure 1C). These findings suggest a generalized defect in the phagocytic activities of ob/ob macrophages. The decrease in efferocytosis was not associated with diminished binding of apoptotic cells to the cell surface of ob/ob macrophages (Figure 1D), indicating a defect in the uptake of apoptotic cells. Prolonged cell culture of ob/ob macrophages for more than 2 days was associated with a gradual normalization of efferocytotic efficiency (data not shown). To further assess whether defective efferocytosis of ob/ob macrophages might be cell autonomous, we carried out a bone marrow transplantation experiment. When ob control bone marrow was transplanted into lethally irradiated ob/ob mice, the ob control peritoneal macrophages acquired a similar defect in efferocytosis, as seen in ob/ob mice transplanted with ob/ob bone marrow. In contrast, when ob/ob bone marrow was trans-
planted into ob control mice, efferocytotic efficiency of peritoneal macrophages was not significantly different from ob control to ob control transplanted mice (Figure 1E). These findings indicate that the defect in efferocytosis of ob/ob macrophages is reversible and most likely arises from factors exogenous to the macrophage.

**Decreased Phosphatidylinositol 3-Kinase/AKT Activation During Efferocytosis in ob/ob Macrophages**

An important early event during phagocytosis is the recruitment of phosphatidylinositol 3-kinase (PI3K) to the phagocytic membrane, leading to an increase in generation of phosphatidylinositol phosphates (PIPs) within the phagocytic cup. In control macrophages, efferocytosis was associated with a rapid increase in PI3K/AKT phosphorylation (Figure 2A) and accumulation of PIP₃ in the phagocytic cup (seen as bright localized staining as indicated by arrows in Figure 2B). ob/ob macrophages had markedly defective PI3K/AKT signaling during efferocytosis and impaired accumulation of PIP₃ in phagocytic membranes (quantification showed ~40% decrease in signal in phagocytic cups in ob/ob macrophages compared to control). Similarly, the synthetic PI3K inhibitor LY 294002 produced a severe defect in efferocytosis associated with decreased accumulation of PIP₃ in the phagocytic membrane of control macrophages (Figure 2B and 2C). Because tyrosine phosphorylation of the regulatory subunit of PI3K (P85) can relieve its inhibitory activity on PI3K,21 we carried out immunoprecipitation with a phosphotyrosine antibody and then SDS-PAGE and Western blotting with an antibody against the PI3K regulatory subunit P85. There was a rapid increase in the amount of phosphorylated p85 during
efferocytosis in control macrophages but not in ob/ob macrophages. This suggests that p85 phosphorylation is impaired in ob/ob macrophages during efferocytosis, leading to decreased PI3K/AKT activation (Figure 2D). To determine whether decreased AKT activation might be directly responsible for defective efferocytosis of ob/ob macrophages, we transfected macrophages with adenovirus expressing a constitutively active form of AKT, myr-AKT.6 However, this did not restore the defect in efferocytosis in ob/ob macrophages, suggesting that defective PIP₃ generation resulting from decreased PI3K activation is responsible for defective efferocytosis, rather than decreased AKT signaling (Online Figure I).

**Defective Efferocytosis Is Not Reversed by Adipocytokines**

The bone marrow transplantation experiments showed that the defect in efferocytosis of ob/ob macrophages arose from interaction of the macrophages with exogenous factors. In an attempt to identify the relevant exogenous factors, we first considered whether there might be a direct effect of leptin on phagocytic

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**Figure 2.** Defective PI3K/AKT activation during efferocytosis by ob/ob macrophages. A, PI3K/AKT signaling during efferocytosis by ob control and ob/ob macrophages. *P*<0.05 (n=3). B, PIP₃ accumulation in phagocytic membranes. Macrophages were incubated with apoptotic cells for 8 minutes before fixation. Left, Fluorescent images showing the accumulation of PIP₃ in phagocytic membranes (arrows) of ob control, ob/ob, and 10 μmol/L LY 294002–treated control macrophages. Right, Quantification results of the number of areas with PIP₃ accumulation in phagocytic membranes. *P*<0.05 (n=3). C, PI3K inhibitor LY 294002 on efferocytosis of ob control macrophages. Macrophages were pretreated with 10 μmol/L inhibitor 1 hour before efferocytosis (n=3). *P*<0.05. D, Tyrosine phosphorylation of PI3K regulative subunit P85 during efferocytosis by ob control and ob/ob macrophages. For Western blot, representative of 3 independent experiments. For quantification results, means and SEM of 3 independent experiments. *P*=0.60 for ob control and ob/ob at 0 minute; *P*=0.52 at 5 minutes; *P*=0.14 at 10 minutes; *P*=0.23 at 20 minutes.
efficiency, as previously reported for microbial phagocytosis. However, addition of leptin or various other adipocytokines (visfatin, adiponectin) or lipopolysaccharide did not influence phagocytic efficiency of ob/ob macrophages (Online Figure II, A). Similarly, increases in glucose in the medium had no effect on efferocytosis of ob/ob macrophages (Online Figure II, B).

Because ob/ob macrophages show defective insulin signaling, we also analyzed efferocytosis in macrophages from Insr<sup>−/−</sup> mice. However, macrophages from Insr<sup>−/−</sup> mice showed no significant defect in efferocytosis (Online Figure II, C).

**Figure 3.** Exogenous FAs alter macrophage efferocytotic efficiency. A, Plasma FFA level in 5-hour fasted ob control and ob/ob mice (n=14). *P<0.05. B, Composition of saturated FAs and unsaturated FAs in total membrane lipids (both esterified and nonesterified FAs from whole cell membranes) from ob control and ob/ob macrophages. The whole cell membrane includes both plasma membrane and subcellular membranes. AA indicates arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LOA, linoleic acid; OA, oleic acid; PA, palmitic acids; POA, palmitoleic acid; SA, stearic acid. *P<0.05 (n=4). C, Time course and effects of saturated FFA/BSA on efferocytosis. Except for time course study, cells were treated with 0.5 mmol/L FFA for 6 to 7 hours before efferocytosis (n=3). *P<0.05. D, The effects of FFA/BSA on ob/ob macrophages compared with ob control cells (n=3). Unless specified, *P<0.05 or NS when compared with BSA-treated ob/ob macrophages. E, Effects of EPA/BSA on efferocytosis by ob control and ob/ob macrophages. Representative data of 2 independent experiments with triplicates. *P<0.05 when compared with BSA-treated ob/ob macrophages.

**Exogenous FAs Alter Macrophage Efferocytotic Efficiency and PI3K Activation**

In contrast to nonobese Insr<sup>−/−</sup> mice, ob/ob mice show marked elevations in plasma FFA levels, and elevated levels of FFAs especially saturated FAs, are thought to have an
important role in inducing insulin resistance and inflammatory responses in these mice. We confirmed an increase in plasma FFA levels in ob/ob mice (Figure 3A) and showed that total membrane lipids of ob/ob macrophages have an increased content of saturated FAs and a decreased content of several unsaturated FAs, including the long chain ω-3 FAs docosahexaenoic acid (DHA) and EPA (Figure 3B). These changes in membrane FA composition in ob/ob mice were similar to those observed for total plasma lipids, where a decrease in DHA and EPA and an increase in C16:0 and C18:0 FAs were also observed (Online Figure III, A).

The changes in plasma and membrane FAs in ob/ob mice led us to examine the effects of adding exogenous FFA/BSA complexes on efferocytosis. Strikingly, incubation of wild-type macrophages with saturated FFA/BSA complexes but not unsaturated FFA/BSA complexes induced a marked defect in efferocytosis comparable to that observed in ob/ob macrophages. A time course study showed that preincubation of WT macrophages with saturated FAs for more than ~5 hours induced a defect in efferocytosis (Figure 3C). Because membranes of ob/ob macrophages showed a decreased content of ω-3 FAs, we determined effects of incubating ob control and ob/ob macrophages with EPA/BSA complexes. Whereas incubation of ob/ob macrophages with saturated FFA/BSA complexes worsened the defect in efferocytosis (Figure 3D), incubation with EPA/BSA complexes eliminated the defect in efferocytosis in ob/ob macrophages (Figure 3E). Thus, either a decrease in plasma and macrophage membrane ω-3 FAs and/or an increase in plasma and membrane saturated FAs could be responsible for the defect in efferocytosis in ob/ob macrophages.

Interestingly, the incubation of wild-type macrophages with saturated FFA/BSA complexes led to defective PI3K/AKT signaling (Figure 4A) and decreased tyrosine phosphorylation of the regulatory subunit of PI3K during efferocytosis (Figure 4B), resembling the changes seen in ob/ob macrophages (Figure 2A and 2D). This indicates a similar underlying defect in PI3K activation in ob/ob and saturated FA-treated control macrophages and suggests that changes in plasma and macrophage membrane FA composition in ob/ob mice could be responsible for the defect in efferocytosis in ob/ob and ob/ob;Ldlr<sup>−/−</sup> mice. We also carried out experiments that ruled out a role of increased signaling via Toll-like receptor 4 or effects mediated via 1xB kinase, c-Jun N-terminal kinase, or protein kinase C in saturated FA–treated macrophages efferocytosis (Online Figure IV).

**Impaired Efferocytosis in Atherosclerotic Lesions of ob/ob;Ldlr<sup>−/−</sup> Mice**

We next sought to determine whether there might be defective macrophage efferocytosis in atherosclerotic lesions of ob/ob mice. Because the ob/ob mutation is not sufficient to produce atherosclerosis, we carried out these studies in ob/ob;Ldlr<sup>−/−</sup> mice. Mice were fed a high cholesterol, semisynthetic diet to induce atherosclerosis. Peritoneal macrophages from ob/ob;Ldlr<sup>−/−</sup> mice fed this diet showed an increase in plasma very-low-density lipoprotein/low-density lipoprotein (VLDL/LDL) and high-density lipoprotein (HDL) levels compared to Ldlr<sup>−/−</sup> controls, similar to those observed for total plasma lipids. After 4 weeks on diet, ob/ob;Ldlr<sup>−/−</sup> mice showed an increase in plasma FFA levels compared to Ldlr<sup>−/−</sup> controls (Figure 5B). On this diet, ob/ob;Ldlr<sup>−/−</sup> mice showed an increase in plasma FFA levels compared to Ldlr<sup>−/−</sup> controls (Figure 5B). In Ldlr<sup>−/−</sup> mice fed the atherogenic semisynthetic diet, levels of ω-3 FAs (DHA and EPA) appeared to be reduced than ob/ob and ob/control mice fed the Chow diet, but the same tendency was observed as noted earlier, ie, there were lower levels of DHA and EPA and C18:2 FAs and increased C16:0 and C18:0 FAs in ob/ob;Ldlr<sup>−/−</sup> compared to Ldlr<sup>−/−</sup> mice in both plasma and macrophage membrane lipid composition (Online Figure III, B and C). In the ob/ob;Ldlr<sup>−/−</sup> mice, plasma very-low-density lipoprotein/low-density lipoprotein (VLDL/LDL) and high-density lipoprotein (HDL) levels were increased compared to Ldlr<sup>−/−</sup> controls, similar to previous studies. (Online Figure V, A and B, shows data after 4 weeks on diet; Online Table I shows data after more than 20 weeks on diet.) We confirmed increased lesion area in ob/ob;Ldlr<sup>−/−</sup> mice fed the semisynthetic atherogenic diet for...
more than 20 weeks compared to Ldlr<sup>−/−</sup> controls, similar to previous observations on a different diet<sup>26</sup> and also demonstrated increased necrotic core formation in advanced lesions from the former group of mice (Figure 5C). To assess efferocytosis, we used a triple staining procedure for apoptotic cells, macrophages, and nuclei.<sup>13,27</sup> This assay is illustrated in Figure 5D. TUNEL-positive material that colocalizes with nuclei (Hoechst-positive) signifies apoptotic cells. If apoptotic cells are not within macrophages (defined as AIA staining positive material in close proximity to another nucleus), they are scored as nonphagocytosed apoptotic cells. Otherwise, they are scored as associated with or phagocytosed by macrophages. These assays were scored by a blinded observer. There was a nonsignificant trend to increased numbers of apoptotic cells within lesions of ob/ob;Ldlr<sup>−/−</sup> mice and a significant increase in numbers of nonphagocytosed apoptotic cells, indicating defective efferocytosis in advanced ob/ob atherosclerotic lesions (Figure 5D).

**A Diet Rich in Fish Oils Reverses the Defect in Efferocytosis in ob/ob Mice**

As noted above, EPA/albumin complexes produced an improvement in efferocytosis in ob/ob macrophages (Figure 3E). Fish oil–rich diets are beneficial for atherosclerotic cardiovascular disease (CVD), and are rich in ω-3 FAs such as EPA and DHA.<sup>28</sup> To determine whether fish oils could enhance efferocytosis in vivo, we fed ob control and ob/ob mice either olive oil– or fish oil–enriched diets (containing 40% fat) for ∼4 weeks and then isolated peritoneal macrophages from these mice. Efficiency of efferocytosis was significantly decreased in olive oil–treated ob/ob mice compared to ob control mice and this defect was reversed in fish oil–treated ob/ob mice relative to ob control mice, paralleling marked improvements in AKT phosphorylation on the fish oil diet (Figure 6A). Analysis of membrane lipid composition in the mice fed the fish oil diet showed an increased content of ω-3 FAs (DHA and EPA) compared to mice

**Figure 5.** Defective efferocytosis in late atherosclerotic lesions of ob/ob;Ldlr<sup>−/−</sup> mice on AIN76A semisynthetic diet. A, In vitro efferocytosis by peritoneal macrophages from Ldlr<sup>−/−</sup> and ob/ob;Ldlr<sup>−/−</sup> mice fed semisynthetic diet for 4 weeks. Representative data of 2 independent experiments with triplicates. *P<0.05. B, Plasma FFA level of the diet-fed Ldlr<sup>−/−</sup> or ob/ob;Ldlr<sup>−/−</sup> mice (n=6). *P<0.05. C, Proximal aortic root lesion and necrotic core areas per section from Ldlr<sup>−/−</sup> and ob/ob;Ldlr<sup>−/−</sup> mice with semisynthetic diet for more than 20 weeks (n=6 pairs). D, Lesional macrophage efferocytosis of the mice described in C. Top, Representative images of in vivo lesional macrophage efferocytosis. Red indicates TUNEL-positive materials; blue, Hoechst-stained cell nuclei; green, macrophage-specific AIA staining. Bottom, Quantification results of in vivo lesional macrophage efferocytosis (left) and apoptosis (right) from the Ldlr<sup>−/−</sup> and ob/ob;Ldlr<sup>−/−</sup> mice (n=6 pairs).
receiving the olive oil diet (Figure 6B shows mean results of two separate analyses). However, there was no consistent change in membrane saturated FAs in mice fed the fish oil diet.

A Fish Oil–Enriched Diet Reverses Defective Efferocytosis in ob/ob;Ldlr<sup>−/−</sup> Mice

We next wished to determine whether a fish oil–rich diet would also improve efferocytosis in atherosclerotic lesions. We carried out an analysis of atherosclerotic lesions from Ldlr<sup>−/−</sup> and ob/ob;Ldlr<sup>−/−</sup> mice fed olive oil or fish oil diets. Studies were performed at an early time point in fatty streak lesions. In contrast to later time points when mice treated with olive oil displayed larger atherosclerotic lesions than those receiving fish oil (12 weeks),<sup>16</sup> there was no overall difference in lesion area after only 6 weeks on olive oil or fish oil diets (Figure 7A). The fish oil diet has a high content of DHA and EPA, allowing us to determine whether there might be an in vivo effect on apoptosis and efferocytosis by ω-3 FAs.<sup>16</sup> Analysis of plasma lipid FA composition showed a marked increase in DHA and EPA, with decreases in C18:1 and C20:4 FAs in mice on the fish oil diet compared to the olive oil diet (Online Figure VI, A and B). On the olive oil diet, the ob/ob;Ldlr<sup>−/−</sup> mice showed a 2-fold increase in numbers of apoptotic cells and a 3.5-fold increase in the number of nonphagocytosed apoptotic cells outside of macrophages, compared to Ldlr<sup>−/−</sup> controls (Figure 7B). Remarkably, both the increase in apoptosis and the defect in efferocytosis were completely reversed in mice fed the fish oil diet (Figure 7B).

Discussion

Our studies show that in addition to the known inability of ob/ob macrophages to phagocytose bacteria,<sup>22,29</sup> they show defective efferocytosis and Fc-R–mediated phagocytosis. Unlike the previous reports,<sup>22,29</sup> we did not find that leptin treatment could reverse these defects. The underlying defect appeared to be related to an altered macrophage membrane lipid composition with decreased levels of ω-3 FAs (DHA, EPA) and increased levels of saturated FAs (C16:0, C18:0), leading to defective PI3K activation and failure to generate PIP<sub>3</sub> in the macrophage phagocytic membrane.

Our data strongly suggest that the defect in efferocytosis of ob/ob macrophages was related to increased concentrations of saturated FAs and/or decreased concentrations of the ω-3 FAs DHA and EPA. Saturated FFA/BSA complexes induced a similar defect in efferocytosis associated with defective PI3K activation in control macrophages, whereas EPA/BSA complexes ameliorated the defect in ob/ob macrophages. Analysis of membrane lipid composition of ob/ob and ob/ob;Ldlr<sup>−/−</sup> macrophages showed increased saturated FAs and decreased ω-3 FAs compared to controls, paralleling changes in the composition of plasma lipid FAs. Thus, exposure of macro-
phages in vivo to increased saturated FAs and decreased ω-3 FAs leads to parallel changes in the composition of membrane lipids. Interestingly, feeding a diet high in fish oils appeared to increase membrane ω-3 FAs without appreciable changes in content of saturated FAs and led to a reversal of the defect in ob/ob efferocytosis in peritoneal and lesional macrophages. Products of DHA/EPA such as lipoxins/resolvins/maresins are known to increase efferocytotic efficiency in macrophages and to promote the resolution of inflammation.30,31 DHA and EPA are converted into these antiinflammatory products by 15-lipoxygenase, probably accounting for the antiatherogenic activity of this enzyme in certain settings.32 Thus, reduced levels of plasma and membrane ω-3 FAs are likely to be an important factor contributing to defective efferocytosis in ob/ob macrophages, whereas increased levels of ω-3 FAs and products such as lipoxins/resolvins/maresins are likely to explain beneficial effects of fish oil diets on efferocytosis. A recent report showing that ω-3 FAs increase resolvins and protectins in ob/ob mice with beneficial effects on insulin resistance supports this hypothesis.33

Mouse models of obesity and diabetes, such as ob/ob and db/db mice, show dramatically accelerated atherosclerosis in apolipoprotein E knockout and Ldlr−/− backgrounds but also have proatherogenic lipoprotein changes, such as increased VLDL/LDL cholesterol levels.25,26,34 Thus, the potential contribution of factors acting at the level of the vessel wall may be obscured by increased hyperlipidemia on the ob/ob background. In the present study, we have analyzed atherosclerotic lesions in ob/ob;Ldlr−/− mice fed 2 different diets. In the first study using a semisynthetic diet (more than 20 weeks), we found that in advanced lesions of ob/ob;Ldlr−/− mice there was increased lesion area, increased necrotic core, and defective efferocytosis compared to Ldlr−/− controls. In the second study, we analyzed lesions from ob/ob;Ldlr−/− mice or Ldlr−/− controls that had been fed olive oil or fish oil diets for a short period (6 weeks). In these early lesions, we documented increased apoptosis and defective efferocytosis in the ob/ob;Ldlr−/− mice compared to Ldlr−/− controls. It is important to note that, even though at this stage overall lesion size was similar in the different groups, it was larger in the ob/ob;Ldlr−/− mice fed the chow diet when they were older (6 months old) or in mice fed with the olive oil diet longer (12 weeks).16,26 In the latter study, lesion size was decreased by the fish oil diet.16 The ability of fish oils or ω-3 FA supplementation to reduce lesion size has also been noted in several other studies.35,36 Although increased apoptosis in early lesions appears to be associated with an overall decrease in lesion size,37,38 our observations indicate defective efferocytosis in both early and late lesions of ob/ob;Ldlr−/− mice. Defective efferocytosis likely contributes to defective resolution of inflammation and leads to increased lesion size and features of instability such as necrotic core formation when lesions become advanced.39,40

Figure 7. Increased apoptosis and defective efferocytosis in early atherosclerotic lesions of ob/ob;Ldlr−/− mice: reversal of defects by a fish oil diet. A, Proximal aortic root lesion areas per section from Ldlr−/− and ob/ob;Ldlr−/− mice with either olive oil or fish oil diet for 6 weeks. B, Representative images (left) and quantification results (right) of in vivo macrophage efferocytosis in the atherosclerotic lesions from 6-week olive oil– or fish oil–treated Ldlr−/− and ob/ob;Ldlr−/− mice (n=9 for olive oil or fish oil treated Ldlr−/− mice; n=5 for olive oil or fish oil treated ob/ob;Ldlr−/− mice).
Apart from the effects of induced or rare mutations,13–15 the causes of defective efferocytosis in atherosclerotic lesions are unknown even though this appears to be a common defect in advanced human atherosclerotic lesions.27 Interestingly, because similar clearance mechanisms are involved, it has been speculated that oxidized phospholipids in oxidized LDL or derived from apoptotic cells may compete with new apoptotic cells for clearance by macrophages.41 The present study suggests that in type 2 diabetes/obesity, increased levels of saturated FAs and/or decreased levels of ω-3 FAs may give rise to a defect in efferocytosis. Once defective efferocytosis has been initiated by such a mechanism, accumulation of oxidized lipids in lesions may worsen the defect by the proposed competitive mechanism.41

Fish oil diets or ω-3 long chain FA supplementation ameliorate atherosclerotic CVD in humans, but these effects are not correlated with improvements in traditional risk factors such as plasma LDL or HDL levels.42 The studies of Serhan and colleagues showing that products of ω-3 FAs in macrophages help to resolve inflammation and promote efferocytosis have suggested novel mechanisms to explain the beneficial effects of diets enriched in fish oils or supplemented with ω-3 FAs in atherosclerotic CVD, ie, promotion of efferocytosis and resolution of inflammation.30–32 Our studies in the ob/ob mouse model extend these important observations by suggesting that in obesity and diabetes increases in the levels of saturated FAs and decreases in ω-3 FAs produce defective efferocytosis and indicate that these defects can be reversed by dietary supplementation with ω-3 FAs. Our studies suggest the possibility that fish oil–rich diets currently recommended for treatment of atherosclerotic CVD42,43 could be particularly beneficial for CVD in obesity and type 2 diabetes.

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Disclosures
None.

References


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METHODS

Animals
Toll-like-receptor 4 (TLR4) deletion mutant on C57B6/10ScNJ background and its control mice (C57B6/10ScSnJ) were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). P38α or long-chain acyl-CoA synthetase (ACSL)-1 deficient macrophages were obtained by crossing the LysM-Cre strain with p38flox/flox or ACSL1-flox mice.

Reagents and antibodies
A rat anti–mouse monoclonal antibody (clone 1D4B) against LAMP1 was obtained from BD Biosciences. All fluorescent second antibodies and CellTracker Red were obtained from Invitrogen-Molecular Probes, except for those used in studies Fc-R phagocytosis (Jackson Immunoresearch Labs). IKK16 was from Torcis Bioscience. JNK inhibitor SP600125, myristoylated protein kinase C (PKC) peptide inhibitor and PKC inhibitor GF109203X, Lipopolysaccharide (LPS), BMS-345541 and salicylate were from Sigma. NEFA measurement kit from Wako Pure Chemical Industries Ltd. was used to measure plasma FFAs. Triacsin C was from Fermentek Biotechnology.

Immunoblot analysis and immunoprecipitation
Immunoblot analysis using total lysates (25 µg) from macrophages was performed as described except that primary antibodies against phosphor-AKT (Ser473) and total AKT were used (Cell Signaling). For immunoprecipitation (IP) experiments with phosphor-tyrosine beads, after adding apoptotic cells at different time points, cells were washed and collected in lysis buffer with 1% Triton X-100. 150µg total cell lysates was used with 25μl p-tyrosine agrose beads (Santa Cruz Biotechnology) for overnight IP. IP products were washed 3 times with lysis buffer or PBS (at least once with lysis buffer). Antibodies against phosphor-tyrosine or P85-PI3K (Upstate Cell Signaling Solutions) were used for blotting.

In vitro Fc-R mediated phagocytosis
Macrophages were washed 3 times with DME blank medium and then incubated with IgG covered sheep red blood cells (SRBCs) for 25 minutes. Macrophages were washed with ice-cold PBS 3 times and stained with a rabbit anti-SRBC antibody on ice for 10 minutes with 5% donkey serum. A FITC-conjugated anti-rabbit antibody was used as second antibody to identify the SRBCs that were bound but not internalized. Cells were then fixed, permeabilized and blocked before being stained again with rabbit anti-SRBC to recognize the phagocytosed cells. A rhodamine Red-conjugated anti-rabbit antibody was used as secondary antibody.

Immunofluorescent microscopy
For macrophage efferocytosis, after washing with PBS, cells were fixed, permeabilized, blocked and stained with primary antibody (anti-mouse lamp1) and secondary antibody (Alexa 488 conjugated goat anti–rat). For PIP staining, cells were permeabilized with 0.5% saponin. Mouse anti-PIP was used at 1:50 as primary antibody (Echelon). Goat anti-mouse IgM Alexa 488 was used as second antibody (Jackson ImmunoResearch).
Confocal and fluorescent pictures were taken as previously 4. Brightness was adjusted with Photoshop 6.0 (Adobe).

Bone marrow transplantation (BMT) study
BMT was performed as previously described in the lab with a well-established method 6. Briefly, recipient female ob control or ob/ob mice (5-6 weeks) were given acidified water (pH 4.5) containing 100 mg/liter neomycin (Sigma) and 10 mg/liter polymyxin B sulfate (Sigma) 1 week before and 2 weeks after BM transplantation. They were lethally irradiated before transplantation. Bone marrow was collected from femurs and tibias of 6-week-old male donor mice (ob control or ob/ob). Each recipient mouse was injected with about 4–5 × 10^6 bone marrow cells through the tail vein. Six weeks after BM transplantation, peripheral blood DNA was collected with Ultraclean DNA spin Kit (MO BIO) for PCR screening of Y chromosome gene to check donor bone marrow reconstitution. The primers used have been previously described 7. Thioglycollate-elicited peritoneal macrophages were collected from the recipient mice about 6 weeks after BMT and in vitro efferocytosis were evaluated as above.

Phagocytosis of apoptotic cells by peritoneal macrophages in vivo
Analysis of in vivo peritoneal macrophage efferocytosis was conducted similarly to those reported with minor adjustment 8, 9. In brief, macrophages were recruited into the peritoneum by injection of thioglycollate. Three days after injection, CellTracker red labeled apoptotic Jurkat T cells (0.5 to 1.5 × 10^7 cells) were injected into the abdomen of mice. After 1 h, peritoneal cells were collected from the abdominal cavity. To remove erythrocytes and unphagocytosed apoptotic bodies, cells were incubated on polystyrene dishes for 20 minutes and washed 3 times gently. Cells were then fixed, permeabilized and stained with anti-lamp1 antibody to assess efferocytosis as above. Since the number of macrophages collected from ob/ob mice is usually much smaller than that collected from ob control mice (2 to 3 times fewer cells), the phagocytic indices were quantified from those pairs of ob control and ob/ob mice for which the ratios between the number of injected apoptotic cells and the number of collected peritoneal macrophages were similar.

Membrane fraction collection and membrane fatty acid extraction for GC analysis of lipid composition
Peritoneal macrophages were allowed to adhere for 2 h in DMEM/10%FBS after collection. Cells were washed 3 times with ice cold PBS to get rid of any unbound non-macrophage cells. Macrophages were then homogenized in homogenized buffer (20mM HEPES with 1mM CaCl\textsubscript{2} and MgCl\textsubscript{2}, 1mM DTT and protease inhibitors at pH 7.4). After that, one-fourth volume of 30% sucrose was added before homogenization. The homogenized mixture was then centrifuged at 1,500xg for 10 minutes at 4 °C. The supernatant was further spun at 150,000xg for 1 hr at 4 °C. The whole membrane pellet including both plasma membrane and subcellular membranes was homogenized and resuspended in a buffer with 20mM HEPES, 0.25M sucrose, 1mM DTT and protease inhibitors at pH 7.4. The total membrane fatty acids were extracted according to the method described by Lepage 10 before GC analysis. Briefly, whole membrane samples were dissolved in hexane together with standards and then methylated with acetyl chloride and methanol at 100°C for 1 h. After that, samples were precipitated with
potassium carbonate 6% and centrifuged at 2000 rpm for 10 minutes. Supernatants were dried down under nitrogen gas and resuspended in hexane before GC analysis.

**Adenovirus transfection**
Either ob control or ob/ob macrophages were transfected with adenovirus encoding control vector (LacZ) or constitutively active form of AKT, Myr-AKT (1) for 24-48 hours before efferocytosis were conducted.

**Ex vivo treatments of macrophages with various adipocytokines and glucose conditions**
Peritoneal macrophages were treated at 37°C in DME culture medium with 100ng/ml leptin (R&D), LPS 100ng/ml (Sigma), 50µg/ml adiponectin, or 100ng/ml visfatin for 24 h before efferocytosis were assessed. Mouse adiponectin and visfatin were generated in the laboratory of Dr. Ira Tabas. For studies on the potential effects of glucose concentrations, cells were incubated with either 25mM or 5mM D-glucose in DMEM/10% FBS for 1–2 days before analysis of efferocytosis.

**Plasma lipids extraction**
Plasma were collected from mice fasted for 5 hours and lipids were extracted as above and methylated for GC analysis.

**SUPPORTING INFORMATION**
**Impaired efferocytosis is not reversed by inhibition of TLR4, NF-κB, JNK, p38, IKK, or ACSL1**
Saturated FFAs have recently been shown to induce increased macrophage inflammatory gene expression as a result of enhanced signaling via TLR4 11. However, LPS treatment did not induce a defect in efferocytosis and macrophages with mutant TLR4 or macrophages deficient in MyD88 (not shown) or p38α map kinase still showed a major defect in efferocytosis when treated with saturated FFA/BSA complexes (Online Figure VI-A and VI-B). Impaired signaling via PI3K/AKT can arise as result of decreased tyrosine or increased serine phosphorylation of IRS proteins due to increased IKK, NF-κB or JNK activities12-14. By analogy there could be similar effects on signaling proteins that become tyrosine phosphorylated during phagocytosis. However, IKK, NF-κB and JNK inhibitors did not affect the decrease in efferocytosis induced by saturated FAs or the defect in ob/ob macrophages (Online Figure VI-C). To determine if increased signaling via protein kinase Cs (PKCs) could be involved, we treated macrophages with myristoylated -PKC-DN (dominant negative) or with a potent PKC inhibitor GF 109203X but this did not lead to an improvement in the inhibition of efferocytosis by saturated FAs (Online Figure VI-D). In order to determine if effects of FFAs were mediated via fatty acyl CoA formation, we incubated saturated FFA/BSA complexes with macrophages containing a specific KO of long-chain acyl-CoA synthetase (ACSL)-1, the principal ACSL isoform in these cells 15. However, the impairment of efferocytosis by saturated FAs was largely preserved in these cells (Online Figure VI-E). Similarly, incubation of saturated FFA/BSA complexes with macrophages treated with the general ACSL inhibitor triacsin C had no significant effect on efferocytosis (Online Figure VI-E).
Together these findings suggested that FFA effects on efferocytosis do not depend on incorporation of fatty acyl CoA derivatives into macrophage lipids via ACSL-1 or other ACSLs that are inhibited by triacsin C. However, not all members of the fatty acyl CoA synthase family are inhibited by triacsin C\(^{16, 17, 18}\), so these findings do not exclude the possibility that other ACSLs are involved in mediating effects of saturated fatty acids.

**Discussion**

Saturated FFA/BSA complexes still induced a defect in efferocytosis in the presence of triacsin C or ACSL1 deficiency. Alteration in membrane fluidity reflecting either increased membrane lipid composition of saturated FAs or saturated FFAs, is one potential mechanism to explain the defect in efferocytosis in ob/ob macrophages\(^ {19}\). However, these studies do not rule out the importance of Acyl-CoA synthesis in modulation of efferocytosis. Enzymes with acyl-CoA synthetase activity (such as fatty acid transport proteins Fatp1 and Fatp4) that prefer these n-3 unsaturated FAs are not inhibited by triacsin C (or ACSL1-deficiency)\(^ {16, 17, 18}\). It is possible that increased amounts of saturated fatty acids compete with DHA and EPA for incorporation into membrane lipids, and that these n-3 FAs are important for efferocytosis.

**REFERENCES**

7. Ranalletta M, Wang N, Han S, Yvan-Charvet L, Welch C, Tall AR. Decreased atherosclerosis in low-density lipoprotein receptor knockout mice transplanted
with Abcg1-/- bone marrow. *Arterioscler Thromb Vasc Biol.* 2006;26:2308-2315.


FIGURE LEGENDS

Online Figure I. AKT over expression does not correct the defective efferocytosis of ob/ob macrophages. Ob control and ob/ob macrophages were transfected with adenovirus expressing constitutively active form of AKT, myr-AKT for 24-48 hours before efferocytosis was conducted. Data are shown as average of 2 independent experiments.

Online Figure II. Defective efferocytosis is not reversed by adipocytokines or changing glucose condition and not found in insulin receptor knockout macrophages (Insr-/-).  
A: The effects of different adipocytokines on efferocytosis by ob/ob macrophages. Cells were treated at least overnight with various adipocytokines before phagocytosis were conducted.  
B: Changes in glucose concentration in the medium did not reverse the defect.  
C: Efferocytosis between Insr wildtype and Insr-/- macrophages. n=3, values are expressed as Mean±SEM.

Online Figure III. Increased content of saturated fatty acids and decreased content of n-3 fatty acids in lipids from plasma or macrophage membranes of ob/ob or ob/ob;Ldlr-/- mice.  
A: Composition of saturated fatty acids and unsaturated fatty acids in plasma lipids from ob control and ob/ob mice. n=1.  
B: Composition of saturated fatty acids and unsaturated fatty acids in membrane lipids from Ldlr-/- and ob/ob;Ldlr-/- macrophages. n=2.  
C: Composition of saturated fatty acids and unsaturated fatty acids in plasma lipids from Ldlr-/- control and ob/ob; Ldlr-/- mice. n=2. For all experiments, PA: Palmitic Acids, POA: pal mioleic acid, SA: stearic acid, OA: oleic acid, LOA: linoleic acid, AA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid.

Online Figure IV. Impaired efferocytosis is not reversed by inhibition of TLR4, P38, JNK, NF-κB, IKK or PKC signaling or ACSL1.  
A: The effects of saturated FFA/BSA on efferocytosis in macrophages with mutant TLR4.  
B: The effects of saturated FFA/BSA on efferocytosis in p38α knockout macrophages. Not significant (NS) when compared to PA/BSA treated WT cells.  
C: The effects of JNK, IKK or NF-κB inhibitors on efferocytosis in saturated FFA/BSA treated control macrophages (left) or in ob/ob macrophages (right). Not significant when compared to PA/BSA treated control cells (left). Not significant when compared to ob/ob macrophages under control condition (right).  
D: The effects of PKC inhibitors on efferocytosis in saturated FFA/BSA treated ob control macrophages. Not significant when compared to SA/BSA treated control cells with DMSO.  
E: The effects of saturated FFA/BSA on efferocytosis in ACSL1 knockout bone marrow derived macrophages or tria cesin C treated ob control macrophages. Not significant when compared to FFA/BSA treated ACS1 control cells. For all experiments, the inhibitors were added together with saturated FFA/BSAs. LA: lauric acid, MA: myristic acid, PA: palmitic acid, SA: stearic acid, OA: oleic acid, LOA: linoleic acid. Except for BMS and PKC-DN where n=2, values are expressed as Mean±SEM for at least 3 experiments. NS: Not significant.
Online Figure V. Plasma lipoprotein and lipid profiles of ob control and ob/ob mice on chow diet, Ldlr-/- and ob/ob;Ldlr-/- mice on 4-week semi-synthetic diet.

A: Plasma total cholesterol, triglyceride (TG), HDL-Cholesterol (HDL-C) of ob control and ob/ob mice on chow diet, Ldlr-/- and ob/ob;Ldlr-/- mice on 4-week semi-synthetic diet. n=4 for ob control and ob/ob mice; n=6 for Ldlr-/- and ob/ob;Ldlr-/- mice. * P<0.05 when compared to ob control mice; # P<0.05 when compared to ob/ob mice; $ P<0.05 when compared to Ldlr-/- mice.

B: FPLC cholesterol profile of ob control and ob/ob mice on chow diet, Ldlr-/- and ob/ob;Ldlr-/- mice on 4-week semi-synthetic diet. Average value of 2 independent experiments.

Online Figure VI. Fish oil diet increases the contents of DHA and EPA in plasma lipids from Ldlr-/- or ob/ob;Ldlr-/- mice.

A: Composition of saturated fatty acids and unsaturated fatty acids in plasma lipids from Ldlr-/- control and ob/ob; Ldlr-/- mice on olive oil or fish oil for 6 weeks. n=2. B: Composition of saturated fatty acids and unsaturated fatty acids in plasma lipids from Ldlr-/- mice on olive oil or fish oil for 12 weeks. n=2. PA: Palmitic Acids, POA: palmitoleic acid, SA: stearic acid, OA: oleic acid, LOA: linoleic acid, AA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid.

Online Table I. Plasma lipid profile of the Ldlr-/- and ob/ob;Ldlr-/- mice on semi-synthetic diet for more than 20 weeks. Plasma were collected from Ldlr-/- or ob/ob;Ldlr-/- mice on semi-synthetic diet at week 20 or 32. Total cholesterol, VLDL, LDL, HDL and Triglyceride (Tg) were measured.
Online Figure I

A

![Bar graph showing PI (Protein Intensity) levels for LacZ and Myr-AKT in control and ob/ob conditions. The graph compares the protein intensity for control (solid black bars) and ob/ob (open bars) across the two conditions.](image-url)
Online Figure III

A

% of total fatty acids

PA  POA  SA  OA  LOA  n7  n9  n6  n6  n6  n3  n6  n6  n6  n3  EPA  DHA

control plasma
ob/ob plasma

B

% of total fatty acids

PA  POA  SA  OA  LOA  n7  n9  n6  n6  n6  n3  n6  n6  n6  n3  EPA  DHA

Ldlr-/- membrane
semisynthetic diet
ob/ob;Ldlr-/- membrane
semisynthetic diet

C

% of total fatty acids

PA  POA  SA  OA  LOA  n7  n9  n6  n6  n6  n3  n6  n6  n6  n3  EPA  DHA

Ldlr-/- plasma
semisynthetic diet
ob/ob;Ldlr-/- plasma
semisynthetic diet
Online Figure IV

A

B

C

D

E
Online Figure V

A

B
Online Figure VI

A

B
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<td></td>
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<td>153 ± 83</td>
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