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PRINCIPAL INVESTIGATOR: Ira Tabas, M.D., Ph.D.

CONTRACTING ORGANIZATION: Columbia University
New York, NY 10032

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Ira Tabas, M.D., Ph.D.; Wai Hong (Connie) Woo, Ph.D.

E-Mail: iat1@columbia.edu

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**14. ABSTRACT**

There is an epidemic of obesity in the military. Obesity leads to type 2 diabetes, the most dangerous consequence of which is atherothrombotic vascular disease. Over the 4-year grant period, we have made major progress on the Key Tasks. We have gained more in-depth understanding on how CaMKII, NADPH oxidase, and IP3Rs—all AngII targets—trigger apoptosis in ER-stressed macrophages. Moreover, we have made new discoveries related to the AngII receptor adaptor, β-arrestin. Our work with PPARs provided new insight into how these drugs for obese diabetics affect advanced plaque progression. The mechanism of obesity-associated adipokines was advanced by showing that LPS, as a model of adiponectin-LPS complex, can suppress a pro-apoptotic branch of the UPR in vivo by the exact same mechanisms elucidated in vitro. Importantly, we have completed the first comprehensive study of adiponectin effects on atherosclerosis in mice and found that adiponectin by itself does not suppress atherogenesis. Moreover, we found that another obesity-associated adipokine—eNampt—may promote macrophage-associated disease processes in obese subjects. Finally, we have continued our studies on how a specific molecular event that could promote plaque necrosis and likely occurs in obesity—cleavage of the efferocytosis receptor Mertk—occurs in advanced human plaques. In summary, we have made substantial progress in understanding how obesity leads to accelerated heart disease at a molecular-cellular level. Future work spurred by these discoveries is likely to suggest novel therapeutic targets to prevent obesity-associated vascular disease in military personnel and in the general public.

**15. SUBJECT TERMS**
obesity, diabetes, insulin resistance, heart disease, atherosclerosis, macrophages, apoptosis, unfolded protein response, PPAR, adiponectin, angiotensin 2

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## INTRODUCTION:

As described in detail in the original grant application, there is an epidemic of obesity in the military. Obesity leads to insulin resistance syndromes, notably metabolic syndrome and type 2 diabetes. The major cause of death in people with insulin resistance syndromes is atherothrombotic vascular disease, including acute myocardial infarction, sudden death, and stroke. Therefore, when retired military personnel and their families reach middle age, there will be an epidemic of obesity-related vascular disease. This will result in the loss of senior personnel and the expertise they contribute to the military. Moreover, the economic burden of cardiovascular disease in active and retired personnel and their families on the military will be enormous. The impact of this trend is being felt now in the military but will accelerate to a very high level over the next 10-20 years if the current trends are left unchecked. In this context, the overall objective of the proposal is to understand at a cellular and molecular level how obesity/insulin resistance promotes atherothrombotic vascular disease. Accomplishing this goal will suggest new targets for drug therapy, which would greatly benefit both the military and the general population. The emphasis of the work is on a key event in advanced atherosclerosis that leads to acute vascular events, namely, advanced lesional macrophage death. Macrophages are the major cell type in atherosclerotic lesions, and when they die and the cell corpses are not rapidly cleared by neighboring phagocytes ("defective efferocytosis"), necrosis ensues. Plaque necrosis, in turn, promotes plaque disruption and exposure of thrombogenic material. The newly exposed thrombogenic material triggers platelet aggregation (thrombosis), which can acutely obstruct the arterial lumen and cause tissue death (infarction). In the heart, this series of events leads to myocardial infarction and sudden death, and in the brain the consequence is stroke. **Thus, the overall focus of this proposal is advanced lesional macrophage death and defective efferocytosis, and we have excellent progress in the third year of funding in understanding how obesity/insulin resistance can promote these processes.**

## BODY:

### I. Studies related to angiotensin-II (AngII) (Tasks 1-3)

Our work during the 4-year grant period related to AngII has taken us into two directions: AngII targets that affect advanced lesional macrophage apoptosis; and a key adaptor for the AngII receptor, β-arrestin2 (BARR2).

**A. Ang II targets that promote advanced lesional macrophage death—**Our work has focused on three AngII targets: calcium/calmodulin-dependent protein kinase 2 (CaMKII) [1, 2]; NADPH oxidase [3, 4]; and the ER calcium release channel, IP3 receptors (IP3R) [5] (G. Li and I. Tabas, unpublished data). Work during the grant period has revealed a novel and highly important pathway relevant to macrophage apoptosis in advanced atherosclerotic lesions that includes all three molecules. This work has recently been published in *J. Clin. Invest.* and in *J. Cell Biol.* [6, 7] and can be summarized by the scheme in Figure 1. ER stress causes a release of calcium from stores in the ER into the cytosol. This activates CaMKII, which in turn triggers four pathways that are critical for apoptosis: Fas signaling; STAT1 pro-apoptotic signaling; mitochondrial membrane permeabilization; and NADPH oxidase. ER stress also induces the pro-apoptotic protein CHOP, which we know is important in macrophage apoptosis in atherosclerosis from our recent study in *Cell Metabolism* [8]. The mechanism of CHOP is integrated directly into the critical role of cytosolic calcium, because CHOP induces

an oxidase called ERO1α, which in turn activates the ER calcium release channel, IP3R, by promoting the formation of an activating disulfide bond in IP3R. Thus, CHOP amplifies what we refer to as a "calcium engine of death."

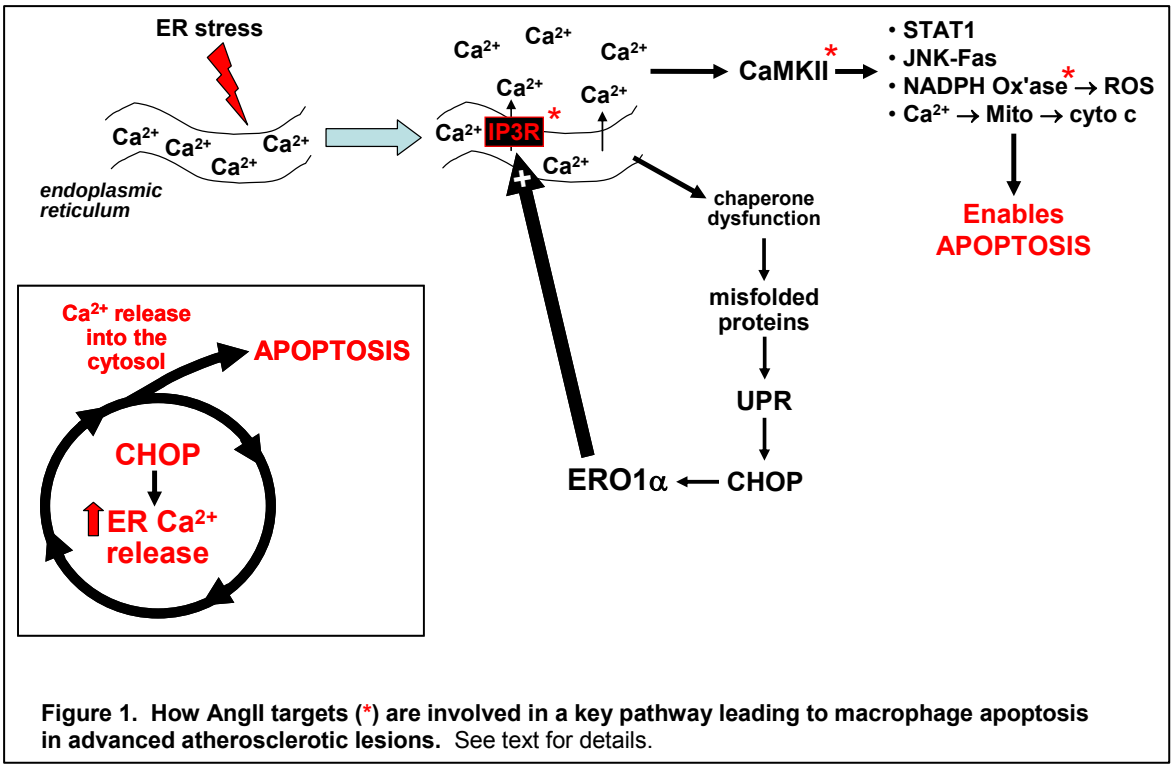


**Figure 1. How AngII targets (*) are involved in a key pathway leading to macrophage apoptosis in advanced atherosclerotic lesions.** See text for details.

**B. BARR2—**The type I AngII receptor can signal through the adaptor β-arrestin (BARR) [9]. Work funded by this grant revealed that BARR2 is induced by ER stress in macrophages and signals a compensatory cell-survival response in the setting of ER stress. The mechanism of cell-survival is through facilitation of a pathway triggered by the epidermal growth factor (EGF) receptor. Thus, AngII pro-apoptotic signaling is counterbalanced by a BARR2 EGF receptor pathway. The data are summarized in a poster recently presented at the Military Health Research Forum (MHRF) in Kansas City (appendix). The working hypothesis is schematized in Figure 2.

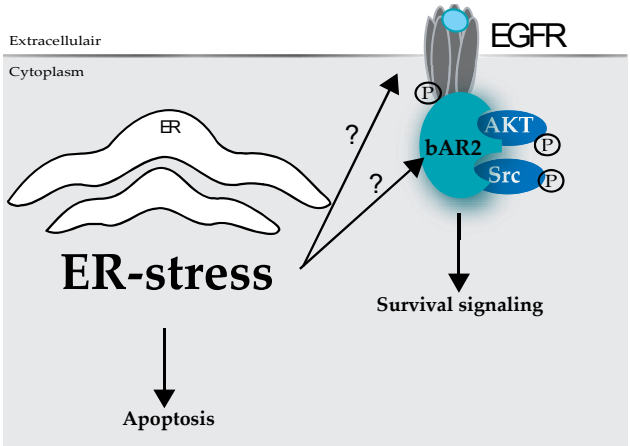


**Figure 2. Model of how β-arrestin2 (bAR2) participates in a comp ensatory ce ll-survival pathway in ER -stressed macrophages through activation of the EGF receptor.** See text and MHRF poster in appendix.

**C. Implications**—It is likely that the high levels of AngII known to exist in obesity contribute to the increased risk of CAD among these subjects. The work funded by this grant will enable an informed and focused examination at a cellular and molecular level as to how AngII promotes plaque progression in vivo. In particularly, each of the components of this pathway depicted in Fig. 1 can be examined to determine its key mechanisms of action *in vivo*. With regard to BARR2, activation of survival signaling by BARR2 and EGFR may prevent accumulation of apoptotic macrophages and

thus advancement of atherosclerosis. This new discovery may pave the way for a novel strategy to prevent advanced lesion progression and thus clinical complications of cardiovascular disease in high-risk obese subjects.

## II. PPAR studies (related to Tasks 3-5)

Our 4 years of work funded by this grant has led to three major discoveries related to PPARs. First, as we published in Circulation [10], we showed that thiazolidinediones (TZDs), which have actions that involve both PPARγ–dependent and –independent effects enhanced macrophage apoptosis by a number of stimuli, including those thought to be important in advanced atherosclerosis. Most importantly, TZD-induced apoptosis was still observed in PPARγ-deficient macrophages, indicating a PPARγ–independent mechanism. To explore the net effect on advanced atherosclerosis *in vivo*, *Ldlr*-/- mice were fed a nondiabetogenic cholesterol-enriched diet to promote midstage lesions. Then, pioglitazone was administered with the diet for an additional 10 weeks. Aortic root lesions from the pioglitazone-treated mice showed a substantial increase in apoptotic cells and plaque necrosis compared with lesions from non–drug-treated mice. Thus, the potential atheroprotective effects of TZDs conferred by insulin sensitization may be partially offset by adverse effects on advanced atherosclerosis. Because the mechanisms of the beneficial and proposed adverse effects may differ, these findings have potentially important implications for drug optimization.

The second discovery is related clearance of apoptotic cells, a process known as efferocytosis. Plaque necrosis in advanced atherosclerotic lesions is caused by the combination of macrophage apoptosis and defective efferocytosis of the apoptotic cells [11]. In a recent study in our laboratory [12], we showed a direct molecular link between a key efferocytosis receptor—Mertk—and both efferocytosis and plaque necrosis in advanced atherosclerotic lesions. It turns out that advanced lesions have several different types of macrophages, often referred to as classically activated inflammatory macrophages and alternatively activated resolution macrophages. In general, the inflammatory macrophages originate from a subset of blood-borne monocytes that express high levels of a marker called Lyc (Lyc-high monocytes), and vice versa for the alternatively activated macrophages (Lyc-low monocytes). PPARs promote the formation of alternatively activated macrophages, while obesity promotes the invasion of inflammatory macrophages [13-15]. Work funded by this grant has allowed us to explore how PPARs and obesity affect monocyte/macrophage subsets and efferocytosis. Our preliminary data show that macrophages derived from Lyc-low macrophages are better able to dispose of apoptotic cells. Most importantly, obesity leads to a dramatic increase in the ratio of Lyc-high:low monocytes (**Figure 3**)—an effect we think is mediated by decreased activation of PPARγ and PPARδ in obesity. In very recent work, we have been able to show that different sub-populations of macrophages in advanced atherosclerotic lesions have different efficiencies of efferocytosis. In future studies stimulated by this grant, we plan to study mouse models of PPAR activation and depletion to test our ideas in a causative manner.

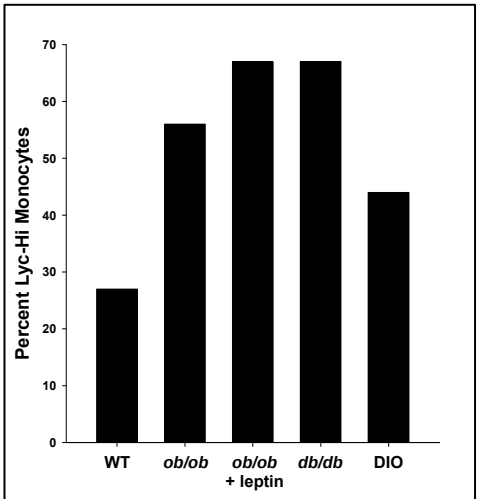


**Figure 3. Obesity is associated with an elevated percentage of Lyc$^{hi}$:Lyc$^{lo}$ monocytes.** Peripheral blood monocytes from thin wild-type (WT) mice and obese ob/ob, db/db, and diet-induced obese mice (DIO) were assayed by FACS for Lyc. Note that addition of leptin to ob/ob mice, which are leptin-deficient, did not reverse the effect, indicating that leptin is not involved.

Third, in a collaborative study tentatively accepted for publication by *Circ. Res*. (appendix), we explored the role of potential upstream activators of PPARs, namely fatty acids, on efferocytosis in obesity. We showed that peritoneal macrophages from ob/ob and ob/ob;ldlr-/- mice showed impaired efferocytosis, reflecting defective PI3K activation during uptake of apoptotic cells. Membrane phospholipids of ob/ob and ob/ob;ldlr-/- macrophages showed an increased content of saturated fatty acids (FAs), and decreased n-3 FAs (Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA)) compared to controls. A similar defect in efferocytosis was induced by treating control macrophages with saturated free FA (FFA)/BSA complexes, while the defect in ob/ob macrophages was reversed by treatment with EPA/BSA or by feeding ob/ob mice a fish oil diet rich in n-3 FAs. There was also defective macrophage efferocytosis in atherosclerotic lesions of ob/ob;Ldlr-/- mice and this was reversed by a fish oil-rich diet. The findings suggest that in obesity and Type 2 diabetes elevated levels of saturated FAs and/or decreased levels of n-3 FAs contribute to decreased macrophage efferocytosis. Beneficial effects of fish oil diets in atherosclerotic cardiovascular disease could involve improvements in macrophage function related to reversal of defective efferocytosis, and could be particularly important in Type 2 diabetes and obesity.

## III. Adiponectin/adipokine studies (Task 6)

Work during the 4-year grant period suggested the possibility that adiponectin, which is influenced by obesity and may protect against heart disease in humans, may be anti-atherogenic particularly in setting of bacteremia, because in that setting it complexes with lipopolysaccharide (LPS) and triggers a pathway that suppresses CHOP. CHOP, as reviewed above, is a major pro-apoptotic effector in advanced lesional macrophages [8]. Even subtle bacteremia may trigger atherogenesis, as suggested by studies linking periodontal disease to CAD [16]. Therefore, the grant has funded two major activities. First, understanding how LPS, a toll-like receptor ligand, suppresses CHOP; and second, understanding the effect of adiponectin on atherosclerosis in vivo in the absence of sepsis, where we predicted a minimal effect.

The first study was recently accepted for publication by Nature Cell Biology [17]. We found that ER stress-induced CHOP expression was suppressed by prior engagement of toll-like receptor (TLR) 3 or 4 through a TRIF-dependent pathway. TLR engagement did not suppress phosphorylation of PERK or eIF-2$\alpha$, which are upstream of CHOP, but phospho-eIF-2$\alpha$ failed to promote translation of the CHOP activator ATF4. In mice subjected to systemic ER stress, pre-treatment with low-dose LPS suppressed CHOP expression and apoptosis in splenic macrophages, renal tubule cells, and hepatocytes, and prevented renal dysfunction and hepatosteatosis. This protective effect of LPS did not occur in Trif-/- mice nor in wild-type mice in which CHOP expression was genetically restored. Thus, TRIF-mediated signals from TLRs selectively attenuate translational activation of ATF4 and its downstream target gene CHOP.

The second study was a collaboration with Dr. Philipp Scherer, UT-Southwestern. The manuscript is currently under revision for *Arterio. Thromb. Vasc. Biol.* (appendix), where it received a favorable review. As mentioned, adiponectin has been implicated in the protection against a cluster of related metabolic disorders including obesity, insulin resistance, and cardiovascular disease. Mice lacking adiponectin display impaired hepatic insulin sensitivity and respond only partially to PPAR$\gamma$ agonists. Adiponectin-mediated metabolic

improvements are associated with alleviation of dyslipidemia and the prevention of ectopic lipid deposition. It has been suggested that adiponectin has anti-atherogenic properties. However, the involvement of adiponectin on the atherogenic process have not been studied systematically in preclinical models of atherosclerosis. We crossed either adiponectin KO (*Adn*$^{-/-}$) or mice with chronically elevated adiponectin levels (*Adn*$^{Tg}$) into the low-density lipoprotein (LDL) receptor (*Ldlr*$^{-/-}$) deficient background. We found that adiponectin levels did not correlate with the development of atherosclerosis under a number of different dietary conditions. We analyzed plaque volume in the aortic root, cholesterol accumulation in the aorta and plaque morphology, none of which were affected by variable circulating adiponectin levels.

In summary, under non-bacteremic conditions, adiponectin does not affect the atherogenic process or plaque progression to necrosis in mouse models of atherosclerosis. However, in the setting of bacteremia, as might occur in a number of subtle conditions in humans such as periodontal disease, adiponectin will likely complex with LPS and facilitate the suppression of CHOP, which would suppress plaque progression based on our recent CHOP mouse study [8]. These are completely novel ideas that change the way we look at the effect of adiponectin, and thus obesity, on heart disease.

As explained last year, we have also studied another adipocytokine—eNampt, which unlike adiponectin increases with obesity. As this year published in *J. Biol. Chem.* [18], we found that eNampt potently blocked macrophage apoptosis induced by a number of ER stressors. The mechanism involved a two-step sequential process: rapid induction of interleukin 6 (IL-6) secretion, followed by IL 6-mediated autocrine/paracrine activation of the pro-survival signal transducer STAT3. The ability of eNampt to trigger this IL 6-/STAT3 cell-survival pathway did not depend on the presence of the Nampt enzymatic substrate nicotinamide in the medium; could not be mimicked by the Nampt enzymatic product nicotinamide mononucleotide (NMN); was not blocked by the Nampt enzyme inhibitor FK866; and showed no correlation with enzyme activity in a series of site-directed mutant Nampt proteins. Thus, eNampt protects macrophages from ER stress-induced apoptosis by activating an IL-6/STAT3 signaling pathway via a non-enzymatic mechanism. These data suggest a novel action and mechanism of eNampt that could affect the balance of macrophage survival and death in the setting of obesity, which in turn could play important roles in obesity-associated diseases. In particular, our working hypothesis is that those obesity-associated conditions in which macrophages are detrimental, including early atherogenesis, white adipose tissue (WAT)-mediated inflammation and insulin resistance, and tumors promoted by tumor-associated macrophages (TAMs), could be exacerbated by the high levels of eNampt in obesity. In support of this idea, we have shown very recently that there is a close correlation between WAT macrophage apoptosis and a fall in eNampt levels when diet-induced obese mice are switched to a low-calorie diet. Stimulated by these findings, we plan to test causation by restoring eNampt and determining whether this will block the loss of WAT macrophages during reversal of obesity. If so, this could have major implications, because WAT macrophages are thought to play an important role in inflammation-induced insulin resistance in the setting of obesity [19].

## IV. Studies in advanced human atherosclerotic lesions (Task 7)

Over the past four years, our major efforts in examining human atheromata have been in the areas of STAT1 and soluble Mer. STAT1 is a pro-apoptotic molecule that is activated by the aforementioned calcium-CaMKII pathway, and we showed in 2008 that it was present and

activated in human atheromata [20]. Over the last year, we have focused on an area related to defective efferocytosis, which, as explained above, promotes plaque necrosis [11]. We recently showed that the efferocytosis receptor Mertk is important in plaque necrosis in atheromata in vivo [12]. We are now interested in the hypothesis that cleavage of Mertk, which is stimulated by inflammation in macrophages and which compromises efferocytosis, can explain at least partially the defective efferocytosis that is known to occur in advanced and inflamed atheromata [21]. As explained last year, we found a remarkably high level of soluble Mer in advanced human lesions but not in uninvolved human arteries. In very recent work, we have verified this finding in ~20 new human specimens, that have various levels of plaque necrosis (not yet analyzed) and various levels of sol Mer (**Figure 4**). We are now attempting to correlate sol Mer level in lesions with plaque vulnerability, and we are well on our way of creating a new mouse model expressing non-cleavable Mer to test causation in plaque progression.

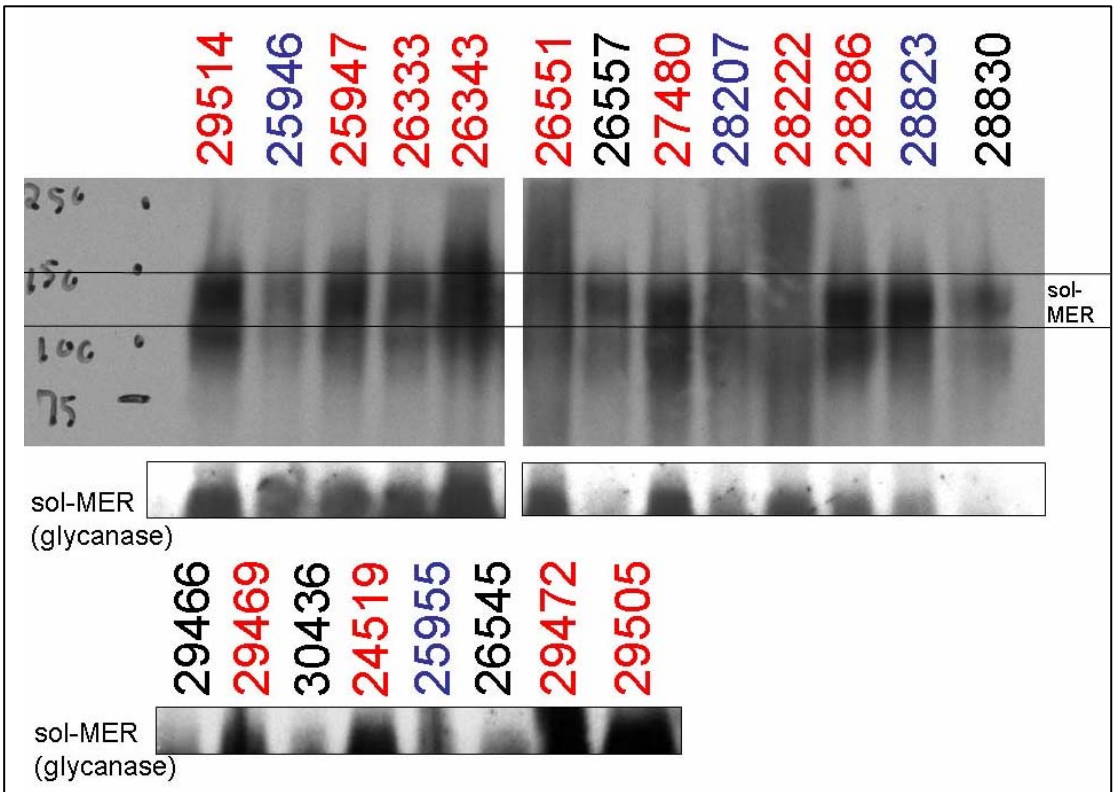


**Figure 4. Soluble Mer i s pres ent i n different l evels i n h uman pl aques.** Carotid a therectomy specimens f rom unidentified subjects (I D3 only ) w ere subjected to Western b lot using a n a nti-human Mertk a ntibody ( upper blot). Soluble Mer runs as a diffuse band due to variations in protein glycosylation. In the blots la beled "(g lycanase)", t he material w as digested with N-glycanase, which cause sol Mer to migrate at a much lower MW in the gel, thus confirming identity of sol Mer. In future studies, we will analyze these specimens for degrees of plaque necrosis to see if that parameter correlates with level of sol Mer.

## KEY RESEARCH ACCOMPLISHMENTS IN YEARS 1-4:

<table>
<tr><th>Task #</th><th>Year 1</th><th>Year 2</th><th>Year 3</th><th>Year 4</th></tr>
<tr><td>1</td><td rowspan="2">• Initiation of key signaling studies that we reasoned would be critical for understanding the role of AngII in Mϕ apoptosis.</td><td rowspan="2">• Elucidation of the key signaling pathways involved in the ER stress-PRR model of apoptosis, with an emphasis on those pathways known to be direct targets of AngII, namely, calcium-CaMKII-NADPH oxidase-ROS. This work resulted in a high-profile manuscript recently accepted by Circulation.</td><td rowspan="2">• AngII target #1: In-depth mechanistic work in on how calcium is released to activate CaMKII<br>• AngII target #2: New insight into how NADPH oxidase promotes apoptosis<br>• New data how the AngII receptor adaptor β-arrestin 2 can influence the balance between apoptosis and cell survival</td><td rowspan="2">• Expanded our understanding of how the AngII targets IP3R, CaMKII, and NADPH oxidase<br>• Mechanism of BARR2 cell-survival pathway elucidated, i.e., through EGF receptor.</td></tr>
<tr><td>2</td></tr>
<tr><td>3</td><td rowspan="3">• Elucidatio n of the role of TZDs on advanced lesional Mϕ death.</td><td rowspan="2">• The TZD study was refined with more in-depth mechanism and completion of in vivo studies, resulting in submission and publication by Circulation.</td><td rowspan="2">• Discovery of how PPARs and obesity can effect monocyte/macrophage subsets<br>• New data on how these monocyte/macrophage subsets affect the efficiency of efferocytosis</td><td rowspan="3">• Major progress was made on how macrophage subsets, which are affected by PPARs, affect efferocytosis in advanced atheromata<br>• Explore role of fatty acids, which can act through PPARs, on efferocytosis in the setting of obesity (in revision for Circ Res.)<br>• In process of setting up study to test the role of PPARδ on this process in atherosclerosis</td></tr>
<tr><td>4</td></tr>
<tr><td>5</td></tr>
<tr><td>6</td><td>• Mech anistic studies on how adiponectin suppresses the UPR.</td><td>• Elucidatio n both in vitro and in vivo that the key UPR-suppressive action of adiponectin is carried out by an adiponectin-LPS complex (holo-adiponectin) through a unique TLR-TRIF/TRAM pathway.</td><td>• Ne w comprehensive data that the adiponectin-LPS UPR suppressive pathway functions in vivo<br>• New study on a 2nd adipokine—eNampt—showing how it promotes macrophage survival and thus may contribute to obesity related macrophage-induced diseases</td><td>• Study on how LPS affects the CHOP pathway published in Nature Cell Biology<br>• Study on how adiponectin affects atherosclerosis in vivo was completed and is now under revision for ATVB<br>• Study on how eNampt affects macrophage survival was published in JBC<br>• New studies underway to test the idea that a decrease in eNampt might affect WAT macrophage apoptosis during weight loss</td></tr>
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| **7** | • Identification of key downstream UPR effectors as candidates to explore in human lesions. | • First demonstration that a key pro-apoptotic signaling molecule downstream of the UPR—Ser-P-STAT1—is expressed in advanced but not early human coronary artery lesions. (Myoishi et al. reported that advanced human lesions express UPR markers, which was correlated with apoptosis and plaque vulnerability.) | • First demonstration that soluble Mer, a marker of defective efferocytosis, is present in advanced human plaques | • Study exploring the presence of sol Mer in human atheroma was expanded |

**REPORTABLE OUTCOMES:**

• Original Publications

Thorp, E., Kuriakose, G., Shah, Y.M., Gonzalez, F.J., Tabas, I. (2007) Pioglitazone increases macrophage apoptosis and plaque necrosis in advanced atherosclerotic lesions of non-diabetic LDL receptor-null mice. Circulation 116:2182-2190.

Lim, W., Timmins, J., Seimon, T.A., Sadler, A., Kolodgie, F., Virmani, R., Schindler, C., and Tabas, I. (2008) Signal transducer and activator of transcription-1 is critical for apoptosis in macrophages subjected to endoplasmic reticulum stress in vitro and in advanced atherosclerotic lesions in vivo. Circulation 117:940-951.

Thorp, E., Cui, D., Kuriakose, G., and Tabas, I. (2008) Mutation of the Mertk receptor promotes apoptotic cell accumulation and plaque necrosis in advanced atherosclerotic lesions of apolipoprotein E-deficient mice. Arterio. Thromb. Vasc. Biol. 28:1421-8.

Senokuchi, T., Liang, C.P., Seimon, T.A., Han, S., Matsumoto, M., J.H., DePinho, R.A., Accili, D., Tabas, I., and Tall, A.R. (2008) FoxOs promote apoptosis of insulin resistant macrophages during cholesterol-induced ER stress. Diabetes 57:2967-76.

Li, Y., Zhang, Y., Dorweiler, B., Cui, D., Wang, T., Woo, C.W., Wolberger, C., Imai, S., Tabas, I. (2008) Extracellular Nampt protects macrophages from ER stress-induced apoptosis via a non-enzymatic interleukin-6/STAT3 signaling mechanism. J. Biol. Chem. 283:34833–34843.

Thorp, E., Li, G., Seimon, T.A., Kuriakose, G., Ron, D., Tabas, I. (2009) Reduced apoptosis and plaque necrosis in advanced atherosclerotic lesions of Apoe-/- and Ldlr-/- mice lacking CHOP. Cell Metabolism 9:474-481.

Timmins, J., Ozcan, L., Seimon, T.A., Li, G., Malagelada, C., Backs, J., Backs, T., Bassel-Duby, R., Olson, E.N., Anderson, M.E., and Tabas, I. (2009) Calcium/calmodulin-dependent protein kinase II links endoplasmic reticulum stress with Fas and mitochondrial apoptosis pathways. J. Clin. Invest. In press.

Li, G., Mongillo, M., Chin, K-T., Harding, H., Ron, D., Marks, A.R., and Tabas, I. (2009) Role of ERO1$\alpha$-mediated stimulation of inositol 1,4,5-triphosphate receptor activity in endoplasmic reticulum stress-induced apoptosis. J. Cell Biol. 186:783-792.

Tabas, I., Seimon, T., Arrelano, J., Li, Y., Forcheron, F., Cui, D., Han, S., Liang, C.P., Tall, A., and Accili, D. (2007) The impact of insulin resistance on macrophage death pathways in advanced atherosclerosis. IN Fatty Acids and Lipotoxicity in Obesity and Diabetes. Novartis Foundation Symposium 286. John Wiley & Sons, Ltd., Chichester, UK, pp. 99-112.

- Submitted manuscripts

Li, S., Sun, Y., Liang, C.P., Thorp, E., Han, S., Jehle, A., Viswanathan, S., Kanter, J., Li, R., Welch, C.L., Hasty, A., Bornfeldt, K., Breslow, J.L., Tabas, I., Tall, A.R. Defective phagocytosis of apoptotic cells by macrophages in atherosclerotic lesions of ob/ob mice and reversal by a fish oil diet. In revision for Circ. Res.

Nawrocki, A.R., Hofmann, S.M., Teupser, D., Basford, J.E., Durand, J.L., Rickes, E.L., Jelicks, L.A., Tanowitz, H.B., Chang, C.H., Hui, D.Y., Woo, C.W., Kuriakose, G., Tabas, I., and Scherer, P.E. (2009) Adiponectin Effects on Atherogenesis in LDL Receptor Null Mice. In revision for Arterio. Thromb. Vasc. Biol.

Liang, C.P., Han, S., Li, G., Senokuchi, T., Tabas, I., Tall, A.R. (2009) Impaired MEK signaling and SERCA expression promotes ER stress and apoptosis in insulin resistant macrophages and is reversed by exenatide treatment. Submitted for publication.

- Reviews

Tabas, I. (2007) Clinical and mechanistic links between diabetes and heart disease. Medscape online: http://www.medscape.com/viewarticle/566297.

Tabas, I., Seimon, T., Timmins, J., Li, G., and Lim, W. (2009) Macrophage apoptosis in advanced atherosclerosis. Annals N.Y. Acad. Sci. 1173:E40–E45

Tabas, I. (2008) Macrophage apoptosis in atherosclerosis: consequences on plaque progression and the role of endoplasmic reticulum stress. Antioxid. Redox Signal. In press/online.

Seimon, T. and Tabas, I. (2009) Mechanisms and consequences of macrophage apoptosis in atherosclerosis. J. Lipid Res., Suppl:S382-7..

Thorp E. and Tabas, I. (2009) Mechanisms and consequences of efferocytosis in advanced atherosclerosis. J. Leukocyte Biol. In press/online.

Thorp, E. and Tabas. I (2009) Differential effects of pioglitazone on advanced atherosclerotic lesions. Correspondence, Am. J. Pathol. 175:1348.

Tabas, I. (2008) Macrophage apoptosis in atherosclerosis: consequences on plaque progression and the role of endoplasmic reticulum stress. *Antioxid. Redox Signal.* In press.

Seimon, T. and Tabas, I. (2008) Mechanisms and consequences of macrophage apoptosis in atherosclerosis. *J. Lipid Res.*, In press.

Tabas, I., Tall, A.R., Accili, D.A. (2009) The impact of macrophage insulin resistance on advanced atherosclerotic plaque progression. Circulation Res. Invited review, submitted.

- Post-doctoral fellows with multiple publications related to this grant

- Oral presentations on the material described in this progress report

- Post-doctoral poster presentation of BARR2 project at 2009 Military Health Research Forum (MHRF) in Kansas City

- Post-doctoral training related to the aforementioned projects in the Tabas laboratory

**CONCLUSIONS:**

Over the last four years, we have gained a wealth of new information related to how obesity—an epidemic in military families and in the general public—can promote obesity's worse consequence, atherothrombotic vascular disease. We have gained new insight into the complexities of AngII, a hormone that is elevated in obesity and known to promote CAD. On the one hand, it affects three major processes known to play a role in advanced lesional macrophage apoptosis. On the other hand, its adaptor BARR2 participates in a cell-survival pathway. How there molecular pathways integrate in advanced atheromata *in vivo* will be a major goal of future studies. We have also gained new insight into the complexity of the PPAR-atherosclerosis connection, which is central to the obesity-CAD epidemic. For example, so-called PPAR activators TZDs have off-target effects that can promote plaque progression, but PPARs themselves can improve efferocytosis in lesions, which will retard plaque progression. These studies provide compelling evidence that new and improved PPAR activators are needed in this setting. In another set of projects, we showed that "pure" adiponectin, which is decreased in obesity, is not anti-atherogenic, as had been previously assumed. Rather, adiponectin can form a complex with LPS, which in turn suppresses pro-apoptotic CHOP. This effect is predicted to suppress lesion progression. Another cytokine that is actually elevated in obesity, eNampt, has macrophage survival effects, which in the setting of WAT, may aggravate insulin resistance and thus atherosclerosis. Finally, studies in two key areas—macrophage apoptosis signaling (STAT1) and efferocytosis (sol Mer)—have been directly related to human atherosclerosis through the study of carotid atherectomy

specimens. In summary, we have made substantial progress in understanding how obesity leads to accelerated heart disease at a molecular-cellular level by the elucidation of novel pathways of macrophage apoptosis and defective efferocytosis in advanced atherosclerosis. Future work in this area, stimulated by the accomplishments during the 4-year grant period, will hopefully suggest new therapeutic approaches to this evolving epidemic in the military and in the general society.

**REFERENCES:**


**(1) Fern RJ, Hahm MS, Lu HK, Liu LP, Gorelick FS, Barrett PQ. Ca2+/calmodulin-dependent protein kinase II activation and regulation of adrenal glomerulosa Ca2+ signaling. Am J Physiol 1995 Dec;269(6 Pt 2):F751-F760.**

**(2) Palomeque J, Valverde CA, Salas MA, Mattiazzi A. CaMKII activation is involved in angiotensin II-mediated cell death. 116 ed. 2007. p. II-114-II-115.**

**(3) Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ Res 1994 Jun;74:1141-8.**

**(4) Lodha S, Dani D, Mehta R, Bhaskaran M, Reddy K, Ding G, et al. Angiotensin II-induced mesangial cell apoptosis: role of oxidative stress. Mol Med 2002 Dec;8(12):830-40.**

**(5) Fuller AJ, Hauschild BC, Gonzalez-Villalobos R, Awayda MS, Imig JD, Inscho EW, et al. Calcium and chloride channel activation by angiotensin II-AT1 receptors in preglomerular vascular smooth muscle cells. Am J Physiol Renal Physiol 2005 Oct;289(4):F760-F767.**

**(6) Timmins JM, Ozcan L, Seimon TA, Li G, Malagelada C, Backs J, et al. Calcium/calmodulin-dependent protein kinase II links endoplasmic reticulum stress with Fas and mitochondrial apoptosis pathways. J Clin Invest. In press 2009.**

**(7) Li G, Mongillo M, Chin KT, Harding H, Ron D, Marks AR, et al. Role of ERO1$\alpha$-mediated stimulation of inositol 1,4,5-triphosphate receptor activity in endoplasmic reticulum stress-induced apoptosis. J Cell Biol 2009;186:783-92.**

**(8) Thorp E, Li G, Seimon TA, Kuriakose G, Ron D, Tabas I. Reduced apoptosis and plaque necrosis in advanced atherosclerotic lesions of *Apoe*-/- and *Ldlr*-/- mice lacking CHOP. Cell Metabolism 2009;9:474-81.**

**(9) Hunyady L, Catt KJ. Pleiotropic AT1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II. Mol Endocrinol 2006 May;20(5):953-70.**

**(10) Thorp E, Kuriakose G, Shah YM, Gonzalez FJ, Tabas I. Pioglitazone increases macrophage apoptosis and plaque necrosis in advanced atherosclerotic lesions of nondiabetic low-density lipoprotein receptor-null mice. Circulation 2007 Nov 6;116(19):2182-90.**

**(11) Tabas I. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. Arterioscler Thromb Vasc Biol 2005 Nov;25(11):2255-64.**

**(12) Thorp E, Cui D, Schrijvers DM, Kuriakose G, Tabas I. Mertk receptor mutation reduces efferocytosis efficiency and promotes apoptotic cell accumulation and plaque necrosis in atherosclerotic lesions of *Apoe*-/- mice. Arterioscler Thromb Vasc Biol 2008 Aug;28(8):1421-8.**

**(13) Odegaard JI, Ricardo-Gonzalez RR, Goforth MH, Morel CR, Subramanian V, Mukundan L, et al. Macrophage-specific PPARγ controls alternative activation and improves insulin resistance. Nature 2007 Jun 28;447(7148):1116-20.**

**(14) Odegaard JI, Ricardo-Gonzalez RR, Red EA, Vats D, Morel CR, Goforth MH, et al. Alternative M2 activation of Kupffer cells by PPARδ ameliorates obesity-induced insulin resistance. Cell Metabolism 2008 Jun;7(6):496-507.**

**(15) Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest 2007 Jan;117(1):175-84.**

**(16) Fardi A, Papadimitriou D. Periodontal and atherosclerosis-induced diseases. Int Angiol 2007 Sep;26(3):197-205.**

**(17) Woo CW, Cui D, Arellano J, Dorweiler B, Harding H, Fitzgerald KA, et al. Adaptive suppression of the ATF4-CHOP branch of the unfolded protein response by toll-like receptor signaling. Nature Cell Biol. In press 2009.**

**(18) Li Y, Zhang Y, Dorweiler B, Cui D, Wang T, Woo CW, et al. Extracellular Nampt promotes macrophage survival via a nonenzymatic interleukin-6/STAT3 signaling mechanism. J Biol Chem 2008 Dec 12;283(50):34833-43.**

**(19) Heilbronn LK, Campbell LV. Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. Curr Pharm Des 2008;14(12):1225-30.**

**(20) Lim WS, Timmins JM, Seimon TA, Sadler A, Kolodgie FD, Virmani R, et al. STAT1 is critical for apoptosis in macrophages subjected to endoplasmic reticulum stress *in vitro* and in advanced atherosclerotic lesions *in vivo*. Circulation 2008;117:940-51.**

**(21) Schrijvers DM, De Meyer GR, Kockx MM, Herman AG, Martinet W. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. Arterioscler Thromb Vasc Biol 2005 Apr 14;25(6):1256-61.**

**APPENDICES:**

- Li, S., Sun, Y., Liang, C.P., Thorp, E., Han, S., Jehle, A., Viswanathan, S., Kanter, J., Li, R., Welch, C.L., Hasty, A., Bornfeldt, K., Breslow, J.L., Tabas, I., Tall, A.R. Defective phagocytosis of apoptotic cells by macrophages in atherosclerotic lesions of ob/ob mice and reversal by a fish oil diet. *Circ. Res.*, accepted pending minor text revision.

- Nawrocki, A.R., Hofmann, S.M., Teupser, D., Basford, J.E., Durand, J.L., Rickes, E.L., Jelicks, L.A., Tanowitz, H.B., Chang, C.H., Hui, D.Y., Woo, C.W., Kuriakose, G., Tabas, I., and Scherer, P.E. (2009) Adiponectin Effects on Atherogenesis in LDL Receptor Null Mice. In revision for *Arterio. Thromb. Vasc. Biol.*

- Tabas, I., Tall, A.R., Accili, D.A. (2009) The impact of macrophage insulin resistance on advanced atherosclerotic plaque progression. Circulation Res. Invited review, submitted.

- Post-doctoral poster presentation of BARR2 project at 2009 Military Health Research Forum (MHRF) in Kansas City

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**Authors:** Suzhao Li, Yu Sun, Chien-Ping Liang, Edward B Thorp, Seongah Han, Andreas W Jehle, Saraswathi Viswanathan, Brian Pridgen, Jenny E kanter, Rong Li, Carrie L Welch, Alyssa H Hasty, Karin E Bornfeldt, Jan L Breslow, Ira Tabas, and Alan R Tall



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

*Running Title: Impaired efferocytosis in ob/ob macrophages*

Suzhao Li[1,2,*], Yu Sun[1], Chien-Ping Liang[1], Edward B. Thorp[1], Seongah Han[1], Andreas W. Jehle[1,7], Saraswathi Viswanathan[4], Brian Pridgen[5], Jenny E. Kanter[6], Rong Li[1], Carrie L.Welch[1], Alyssa H. Hasty[4], Karin E. Bornfeldt[6], Jan L. Breslow[5], Ira Tabas[1,2,3], and Alan R. Tall[1,2]

[1]Departments of Medicine,

[2]Physiology & Cellular Biophysics,

[3]Pathology & Cell Biology, Columbia University, New York, NY 10032, USA

[4]Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN 37232-0615, USA

[5]Laboratory of Biochemical Genetics and Metabolism, Rockefeller University, NY 10065, USA

[6]Department of Pathology, Diabetes and Obesity Center of Excellence, University of Washington, Seattle, WA 98109, USA

[7]Present address: Department of Biomedicine, University Hospital Basel, Hebelstrasse 20, 4031 Basel, Switzerland

*** Correspondence:**

**Division of Molecular Medicine, Department of Medicine, Columbia University, 630 West 168th St., New York, NY 10032.**

**Phone: (212)305-4899; Fax: (212)305-5052; E-mail: sl2181@columbia.edu (S.L.)**

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## ABSTRACT

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. We quantified efferocytosis in peritoneal macrophages and in atherosclerotic lesions of obese ob/ob or ob/ob;Ldlr-/- mice and littermate controls. Peritoneal macrophages from ob/ob and ob/ob;Ldlr-/- mice showed impaired efferocytosis, reflecting defective PI3K activation during uptake of apoptotic cells. Membrane phospholipids of ob/ob and ob/ob;Ldlr-/- macrophages showed an increased content of saturated fatty acids (FAs), and decreased n-3 FAs (Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA)) compared to controls. A similar defect in efferocytosis was induced by treating control macrophages with saturated free FA (FFA)/BSA complexes, while the defect in ob/ob macrophages was reversed by treatment with EPA/BSA or by feeding ob/ob mice a fish oil diet rich in n-3 FAs. There was also defective macrophage efferocytosis in atherosclerotic lesions of ob/ob;Ldlr-/- mice and this was reversed by a fish oil-rich diet. The findings suggest that in obesity and Type 2 diabetes elevated levels of saturated FAs and/or decreased levels of n-3 FAs contribute to decreased macrophage efferocytosis. Beneficial effects of fish oil diets in atherosclerotic cardiovascular disease could involve improvements in macrophage function related to reversal of defective efferocytosis, and could be particularly important in Type 2 diabetes and obesity.


**Key Words:** Efferocytosis Macrophages Atherosclerosis Fatty Acids Mouse

## INTRODUCTION

Patients with Type 2 diabetes experience both accelerated atherogenesis and increased atherosclerotic complications such as plaque rupture and athero-thrombosis [1-3]. While 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 [4] as well as in endothelial cells [5, 6] could also contribute to atherosclerotic plaque formation and complications. In macrophages insulin resistance promotes macrophage apoptosis during the ER stress response, and is associated with increased necrotic core formation in atherosclerotic plaques [4], while in endothelial cells insulin resistance and defective AKT activation lead to impaired NO bioavailability, increased inflammatory gene expression and markedly accelerated atherogenesis [5, 7].

Apoptosis of macrophages and smooth muscle cells in advanced atherosclerotic plaques is thought to lead to increased necrotic core formation, inflammation, plaque disruption and athero-thrombosis [8-10]. Macrophages from ob/ob and Insr-/- mice show increased susceptibility to apoptosis during the ER stress response [11] and decreased inflammatory responses [12]. Increased apoptosis and decreased inflammatory responses of macrophages would not be expected to lead to an enhancement of lesion formation. Efficient phagocytosis of apoptotic cells by macrophages (a process called efferocytosis) is normally anti-inflammatory [13, 14] and should not produce detrimental effects in lesions unless the phagocytic system becomes impaired. In fact, several molecular defects that lead to an enhancement of macrophage apoptosis have been associated with diminished formation of early atherosclerotic lesions [15-17]. In contrast, various induced mutations that cause defective efferocytosis (such as in the mer-TK receptor) lead to increased

necrotic core formation, increased inflammation and accelerated atherogenesis in mouse models [18-20]. Intriguingly, defective macrophage efferocytosis is characteristic of advanced human atherosclerotic lesions [21]. However, very little is known concerning factors that might commonly lead to defective efferocytosis in atherosclerosis.

In the present study we have examined the hypothesis that obesity and Type 2 diabetes might produce a defect in macrophage efferocytosis. We found that peritoneal macrophages from ob/ob mice have impaired efferocytosis, and that atherosclerotic lesions from these mice in the Ldlr-/- background have greatly increased amounts of apoptotic material outside of macrophages indicating a severe defect in efferocytosis. The defect in efferocytosis of ob/ob macrophages did not reflect macrophage insulin resistance, but was reproduced by an exogenous factor i.e. saturated FFA/BSA complexes. Remarkably, a fish oil diet rich in n-3 FAs completely reversed the defect in efferocytosis in ob/ob;Ldlr-/- atherosclerotic lesions.

## MATERIALS AND METHODS

### Animals

About 6-week old obese *ob/ob or db/db* mice together with their lean littermate controls on a C57BL/6J background, as well as TLR4 deletion mutant on C57B6/10ScNJ background [22] and its control mice (C57B6/10ScSnJ) were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). P38 α or ACSL1 deficient macrophages were obtained by crossing the LysM-Cre strain with p38flox/flox or ACSL1-flox mice [23,24]. For study with the AIN76A semi-synthetic diet (0.02% cholesterol), sections of atherosclerotic lesions from diet-fed ob/ob;Ldlr-/- or Ldlr-/- mice for more than 20 weeks were analyzed for lesion area and efferocytosis. The mice used for

*in vitro* peritoneal macrophage efferocytosis or membrane/plasma lipid composition analysis were on the diet for 4 weeks. For olive or fish oil diet study, sections of atherosclerotic lesions from diet-fed ob/ob;Ldlr-/- or Ldlr-/- mice for 6 weeks[25] were analyzed for lesion area and efferocytosis. Ob control and ob/ob mice fed the same diets for 4 weeks were used for *in vitro* macrophage efferocytosis or membrane lipid analysis. Unless otherwise specified, ob control and ob/ob mice were maintained on chow diet for other studies.

**Macrophage isolation and cell culture**

Thioglycollate-elicited peritoneal macrophages were collected from ob/ob or littermate lean control, Ldlr-/- or ob/ob;Ldlr-/- mice 3 days after thioglycollate injection. Cells were pooled from three mice of each genotype and cultured for 1-2 days in DME medium as described [26] . For one day culture, cells were washed 2 hours after plating. For 2-day culture, media were replenished on the second day [26].

***Ex vivo* treatments of macrophages with FFA/BSAs**

Fatty acids, such as lauric (LA)-, myristic (MA)-, palmitic (PA)-, palmitoleic (POA)-, oleic (OA)- or linoleic acid (LOA) were complexed with BSA (Sigma-Aldrich) as described [27]. EPA was prepared as a stock solution in 100% ethanol and then diluted and mixed with essential fatty acid free BSA at a ratio of 5:1 in DMEM/10% FBS before treatment. To study the effects of FFA/BSA on efferocytosis, FFA/BSA complexes were incubated with macrophages for 6-7 h at 0.5mM in DMEM/10% FBS, unless otherwise specified.

***In vitro* efferocytosis**

Analysis of *in vitro* efferocytosis was performed as described [26].

**Assessment of *in vivo* clearance of apoptotic cells by macrophages in the atherosclerotic lesions**

Frozen lesion sections from the proximal aorta from Ldlr-/- or ob/ob;Ldlr-/- mice were analyzed. One separate set of samples was stained with hematoxylin and eosin (Sigma) for quantification of the lesion areas [4]. The necrotic core region was determined as the acellular area of the lesions. For assessment of efferocytosis in the lesions, apoptotic cells in atherosclerotic lesions were detected by the TUNEL (TdT-mediated dUTP nick end labeling) technique using the *in situ* cell death detection kit (Roche)[4], except that the frozen sections of proximal aorta were permeabilized with freshly prepared 0.1% triton and 0.1% 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) [18]. Genomic DNA was stained with Hoechst dye before the slides were mounted with coverslips. Staining was analyzed under an Axiovert 200 fluorescence microscope (Zeiss). Fluorescent images were captured with a CCD camera and analyzed using image analysis software Photoshop (Adobe). Macrophage efferocytosis in the lesion areas was quantified as previously described [18].

**Data analysis**

Results are expressed as mean ± S.E.M. (n is noted in the figure legends or figures), 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 phago-lysosomes [26], we showed that peritoneal macrophages from ob/ob and db/db mice have impaired ability to phagocytose apoptotic cells i.e. impaired efferocytosis [14] (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-mediated phagocytosis (Figure 1C). These findings suggest a generalized defect in the phagocytic activities of ob/ob macrophages.

We next carried out studies to elucidate the mechanism of defective efferocytosis 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 (Fig 1D), indicating a defect in the uptake of apoptotic cells. Prolonged cell culture of ob/ob macrophages for more than two days was associated with a gradual normalization of efferocytotic efficiency (data not shown). To further assess if 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 transplanted 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 PI-3-kinase/AKT activation during efferocytosis in ob/ob macrophages**

An important early event during phagocytosis is the recruitment of PI3K to the phagocytic membrane leading to an increase in generation of PIPs within the phagocytic cup [28, 29]. In control

macrophages, efferocytosis was associated with a rapid increase in PI3K/AKT phosphorylation (Figure 2A) and accumulation of $PIP_3$ 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 $PIP_3$ 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 $PIP_3$ in the phagocytic membrane of control macrophages (Figure 2B and C). Since tyrosine phosphorylation of the regulatory subunit of PI3K (P85) can relieve its inhibitory activity on PI3K [30], 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 in the amount of phosphorylated p85 during efferocytosis in ob 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 if 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 [31]. 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 (Supplementary Figure S1).

**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 efficiency, as previously reported for microbial phagocytosis [32] . However, addition of leptin or various other adipocytokines (visfatin, adiponectin) or lipopolysaccharide did not influence phagocytic efficiency of ob/ob macrophages (Supplementary Figure S2A). Similarly, increases in glucose in the medium had no effect on efferocytosis of ob/ob macrophages (Supplementary Figure S2B). Since ob/ob macrophages show defective insulin signaling [27], we also analyzed efferocytosis in macrophages from Insr-/- mice. However, macrophages from Insr-/- mice showed no significant defect in efferocytosis (Supplementary Figure S2C).

**Exogenous fatty acids alter macrophage efferocytotic efficiency and PI3K activation**

In contrast to non-obese Insr-/- mice [4], 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 [33-35]. We confirmed an increase in plasma FFA levels in ob/ob mice (Figure 3A) and showed that membrane phospholipids of ob/ob macrophages have an increased content of saturated FAs and a decreased content of several unsaturated FAs, including the long chain n-3 FAs, DHA and EPA (Figure 3B). These changes in membrane phospholipids FA composition in ob/ob mice were similar to those observed for plasma lipids, where a decrease in DHA and EPA and an increase in C16:0 and C18:0 fatty acids were also observed (Fig S3A).

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 pre-incubation of WT macrophages with saturated FAs for more than about 5 h induced a defect in efferocytosis (Figure 3C). Since membranes of ob/ob macrophages showed a decreased content of n-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 n-3 fatty acids 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 (Fig 4A) and decreased tyrosine phosphorylation of the regulatory subunit of PI3K during efferocytosis (Fig 4B), resembling the changes seen in ob/ob macrophages (Fig 2A and D). 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 fatty acid composition in ob/ob mice could be responsible for the defect in efferocytosis in ob/ob and ob/ob;Ldlr-/- macrophages.

**Impaired efferocytosis is not reversed by inhibition of TLR4, NF-κB, JNK, p38 or IKK**

Saturated FFAs have recently been shown to induce increased macrophage inflammatory gene expression as a result of enhanced signaling via TLR4 [33]. 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 (Figure 5A and 5B). 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 activities [36-39]. 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 (Figure 5C). To determine if increased signaling via protein kinase Cs 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 (Figure 5D). 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 [40] However, the impairment of efferocytosis by saturated FAs was largely preserved in these cells (Figure 5E). Similarly, incubation of saturated FFA/BSA complexes with macrophages treated with the general ACSL inhibitor triacsin C had no significant effect on efferocytosis (Figure 5E). Together these findings suggested that FFA effects on efferocytosis do not depend on incorporation of fatty acyl CoA derivatives into macrophage lipids, but might be mediated by direct effects of FAs themselves or metabolism of FAs via alternative pathways**.**

**Impaired efferocytosis in atherosclerotic lesions of ob/ob;Ldlr-/- mice**

We next sought to determine if there might be defective macrophage efferocytosis in atherosclerotic lesions of ob/ob mice. Since the ob/ob mutation is not sufficient to produce atherosclerosis, we carried out these studies in ob/ob; Ldlr-/- mice. Mice were fed a high cholesterol, semi-synthetic diet to induce atherosclerosis. Peritoneal macrophages from ob/ob;Ldlr-/- mice fed this diet showed defective efferocytosis compared to Ldlr-/- controls (Fig 6A). On this diet ob/ob;Ldlr-/- mice showed an increase in plasma FFA levels compared to Ldlr-/- controls (Fig 6B). In Ldlr-/- mice fed the atherogenic semi-synthetic diet, levels of n-3 FAs (DHA and EPA) appeared to be reduced ob/ob and ob/control mice fed the chow diet, but the same tendency was observed as noted earlier i.e. there were lower levels of DHA and EPA and C18:2 FAs and increased C16:0 and C18:0 fatty acids in ob/ob;Ldlr-/- compared to Ldlr-/- mice in both plasma lipids and macrophage membrane phospholipids (Figure S3B and S3C). In the ob/ob;Ldlr-/- mice plasma VLDL/LDL and HDL levels were increased compared to Ldlr-/- controls, similar to previous studies[41, 42]. Figure S4A and S4B show data after 4 weeks on diet, Table S1 shows data after more than 20 weeks on diet). We confirmed increased lesion area in ob/ob:Ldlr-/- mice fed the semi-synthetic atherogenic diet for more than 20 weeks compared to Ldlr-/- controls, similar to previous observations on a different diet [42] and also demonstrated increased necrotic core formation in advanced lesions from the former group of mice (Figure 6C). To assess efferocytosis we used a triple staining procedure for apoptotic cells, macrophages and nuclei [18, 21] . This assay is illustrated in Figure 6D. TUNEL positive material that co-localizes 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 non-phagocytosed apoptotic cells. Otherwise, they are scored as associated

with or phagocytosed by macrophages. These assays were scored by a blinded observer. There was a non-significant trend to increased numbers of apoptotic cells within lesions of ob/ob;Ldlr-/- mice, and a significant increase in numbers of non-phagocytosed apoptotic cells, indicating defective efferocytosis in advanced ob/ob atherosclerotic lesions (Figure 6D).

**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, and are rich in n-3 FAs such as EPA and DHA [43]. To determine if 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 about 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 7A). Analysis of membrane phospholipids fatty acids in the mice fed the fish oil diet showed an increased content of n-3 FAs (DHA and EPA) compared to mice receiving the olive oil diet (Figure 7B 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-/- mice**

We next wished to determine if a fish oil rich diet would also improved efferocytosis in atherosclerotic lesions. We carried out an analysis of atherosclerotic lesions from Ldlr-/- and ob/ob:Ldlr-/- 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) [25], there was no overall difference in lesion area after only 6 weeks on olive oil or fish oil diets (Figure 8A). The fish oil diet has a high content of DHA and EPA, allowing us to determine if there might be an in vivo effect on apoptosis and efferocytosis by n-3 FAs [25]. 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 (Figure S5A and S5B). On the olive oil diet the ob/ob:Ldlr-/- mice showed a 2-fold increase in numbers of apoptotic cells and a 3.5-fold increase in the number of unphagocytosed apoptotic cells outside of macrophages, compared to Ldlr-/- controls (Figure 8B). Remarkably, both the increase in apoptosis and the defect in efferocytosis were completely reversed in mice fed the fish oil diet (Figure 8B).

## DISCUSSION

Our studies show that in addition to the known inability of ob/ob macrophages to phagocytose bacteria [32, 44], they show defective efferocytosis and Fc receptor-mediated phagocytosis. Together these findings indicate a generalized defect in the phagocytic activities of ob/ob macrophages. However, unlike the previous reports [32, 44] , we did not find that leptin treatment could reverse defective efferocytosis of ob/ob macrophages. The underlying defect appeared to be related to an altered macrophage membrane phospholipid fatty acid composition with decreased levels of n-3 fatty acids (DHA, EPA) and increased levels of saturated fatty acids (C16:0, C18:0), leading to defective PI3K activation and failure to generate PIP3 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 n-3 FAs, DHA and EPA. Saturated FFA/BSA complexes induced a similar defect in efferocytosis associated with defective PI3K activation in ob control macrophages, while EPA/BSA complexes ameliorated the defect in ob/ob macrophages. Analysis of membrane phospholipids of ob/ob and ob/ob;Ldlr-/- macrophages showed increased saturated FAs and decreased n-3 FAs compared to controls, paralleling changes in the composition of plasma lipid FAs. Thus, exposure of macrophages in vivo to increased saturated FAs and decreased n-3 FAs leads to parallel changes in the composition of membrane phospholipids. Interestingly, feeding a diet high in fish oils appeared to increase membrane n-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. Saturated FFA/BSA complexes still induced a defect in efferocytosis in the presence of triacsin C or ACSL1 deficiency, suggesting that even though membrane phospholipids had altered composition in ob/ob macrophages it could be a direct effect of FFAs or alternative metabolism not requiring fatty acyl CoA formation that produced the defect in efferocytosis. Alteration in membrane fluidity reflecting either increased phospholipid saturated FAs or saturated FFAs, is one potential mechanism to explain the defect in efferocytosis in ob/ob macrophages [45]. However, these studies do not definitely 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)[46, 47 48]. It is possible that increased amounts of saturated fatty acids compete with DHA and EPA for incorporation in membrane phospholipids, and that these n-3 FAs are important for efferocytosis. Products of DHA/EPA such as

lipoxins/resolvins/maresins are known to increase efferocytotic efficiency in macrophages and to promote the resolution of inflammation [49, 50]. DHA and EPA are converted into these anti-inflammatory products by 15-lipoxygenase, probably accounting for the anti-atherogenic activity of this enzyme in certain settings[51]. Thus, reduced levels of plasma and membrane n-3 FAs are likely to be an important factor contributing to defective efferocytosis in ob/ob macrophages, whereas increased levels of n-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 n-3 FAs increase resolvins, protectins in ob/ob mice with beneficial effects on insulin resistance supports this hypothesis [52].

Mouse models of obesity and diabetes, such as ob/ob and db/db mice, show dramatically accelerated atherosclerosis in Apoe-/- and Ldlr-/- backgrounds, but also have pro-atherogenic lipoprotein changes, such as increased VLDL/LDL cholesterol levels[41, 42, 53,]. 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 current study we have analyzed atherosclerotic lesions in ob/ob;Ldlr-/- mice fed two different diets and have found changes suggesting increased apoptosis and defective efferocytosis in lesions of ob/ob;Ldlr-/- mice. In the first study using a semi-synthetic 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 even the chow diet when they were older (6 months old) or in mice fed with the olive oil diet longer (12 weeks) [25,42]. In the latter study, lesion size was decreased by the fish oil diet [25]. The ability of fish oils or n-3 FA supplementation to reduce lesion size has also been noted in several other studies [54, 55] While increased apoptosis in early lesions appears to be associated with an overall decrease in lesion size [56, 57], 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[9, 58].

Apart from the effects of induced or rare mutations in these molecules[18-20], the causes of defective efferocytosis in atherosclerotic lesions are unknown even though this appears to be a common defect in advanced human atherosclerotic lesions [21]. Interestingly, since 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 [59]. The present study suggest that in type 2 diabetes/obesity increased levels of saturated FAs and/or decreased levels of n-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 [59].

Fish oil diets or n-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 [60]. The studies of Serhan and colleagues showing that products of n-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 n-3 FAs in atherosclerotic CVD, i.e. promotion of efferocytosis and resolution of inflammation[49, 50, 51] . 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 n-3 FAs produce defective efferocytosis, and indicate that these defects can be reversed by dietary supplementation with n-3 FAs. Our studies suggest the possibility that fish oil-rich diets currently recommended for treatment of atherosclerotic CVD [60, 61] could be particularly beneficial for CVD in obesity and Type 2 diabetes.

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**REFERENCES**

**1.** Aronson D, Rayfield EJ, Chesebro JH. Mechanisms determining course and outcome of diabetic patients who have had acute myocardial infarction. *Ann Intern Med.* 1997;126(4):296-306.

**2.** Meerarani P, Badimon JJ, Zias E, Fuster V, Moreno PR. Metabolic syndrome and diabetic atherothrombosis: implications in vascular complications. *Curr Mol Med.* 2006;6(5):501-514.

**3.** Silva JA, Escobar A, Collins TJ, Ramee SR, White CJ. Unstable angina. A comparison of angioscopic findings between diabetic and nondiabetic patients. *Circulation.* 1995;92(7):1731-1736.

**4.** Han S, Liang CP, DeVries-Seimon T, Ranalletta M, Welch CL, Collins-Fletcher K, Accili D, Tabas I, Tall AR. Macrophage insulin receptor deficiency increases ER stress-induced apoptosis and necrotic core formation in advanced atherosclerotic lesions. *Cell Metab.* 2006;3(4):257-266.

**5.** Fernandez-Hernando C, Ackah E, Yu J, Suarez Y, Murata T, Iwakiri Y, Prendergast J, Miao RQ, Birnbaum MJ, Sessa WC. Loss of Akt1 leads to severe atherosclerosis and occlusive coronary artery disease. *Cell Metab.* 2007;6(6):446-457.

**6.** Kearney MT, Duncan ER, Kahn M, Wheatcroft SB. Insulin resistance and endothelial cell dysfunction: studies in mammalian models. *Exp Physiol.* 2008;93(1):158-163.

**7.** Kim F, Pham M, Maloney E, Rizzo NO, Morton GJ, Wisse BE, Kirk EA, Chait A, Schwartz MW. Vascular Inflammation, Insulin Resistance, and Reduced Nitric Oxide Production Precede the Onset of Peripheral Insulin Resistance. *Arterioscler Thromb Vasc Biol.* 2008.

**8.** Burke AP, Kolodgie FD, Zieske A, Fowler DR, Weber DK, Varghese PJ, Farb A, Virmani R. Morphologic findings of coronary atherosclerotic plaques in diabetics: a postmortem study. *Arterioscler Thromb Vasc Biol.* 2004;24(7):1266-1271.

**9.** Tabas I. C onsequences and ther apeutic imp lications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficie ncy. *Arterioscler Thromb Vasc Biol.* 2005;25(11):2255-2264.

**10.** Virmani R, Burke AP, Kolodgie F. Morphological characteristics of cor onary atherosclerosis in diabetes mellitus. *Can J Cardiol.* 2006;22 Suppl B:81B-84B.

**11.** Senokuchi T, Liang CP, Seimon TA, Han S, Matsumoto M, Banks AS, Paik JH, Depinho RA, Accili D, Tabas I, Tall AR. FoxOs promote apo ptosis of i nsulin resi stant macrophages during cholesterol-induced ER stress. *Diabetes.* 2008.

**12.** Kjerrulf M, Berke Z, Aspegren A, Umaerus M, Nilsson T, Svensson L, Hurt-Came jo E. Reduced cholesterol a ccumulation by leptin deficient (o b/ob) mouse macrophages. *Inflamm Res.* 2006;55(7):300-309.

**13.** Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apo ptotic cells in vitro inhibit proinflammatory c ytokine production through aut ocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest.* 1998;101(4):890-898.

**14.** Vandivier RW, Henson PM, Douglas IS. Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease. *Chest.* 2006;129(6):1673-1682.

**15.** van Vlijmen BJ, Gerritsen G, Franken AL, Boesten LS, Kockx MM, Gijbels MJ, Vierboom MP, van Eck M, van De Water B, van Berkel TJ, Ha vekes LM. Macrophage p53 deficiency leads to enh anced ather osclerosis in APOE*3-Leiden tran sgenic mice. *Circ Res.* 2001;88(8):780-786.

**16.** Liu J, Thew ke DP, Su YR, Linton MF, Fazio S, Sinensky MS. Reduced macrophage apoptosis is associate d with accelerated atherosclerosis in low-density lipoprotein receptor-null mice. *Arterioscler Thromb Vasc Biol.* 2005;25(1):174-179.

**17.** Arai S, Shelton JM, Chen M, Bradley MN, Castrillo A, Bookout AL, Mak PA, Edwards PA, Mangelsdorf DJ, Tontonoz P, Miyazaki T. A role for the apoptosis inhibitory factor AIM/Spalpha/Api6 in atherosclerosis development. *Cell Metab.* 2005;1(3):201-213.

**18.** Thorp E, Cui D, Schrijvers DM, Kuriakose G, Tabas I. Mertk Receptor Mutation Reduces Efferocytosis Efficiency and Promotes Apoptotic Cell Accumulation and Plaque Necrosis in Atherosclerotic Lesions of Apoe-/- Mice. *Arterioscler Thromb Vasc Biol.* 2008.

**19.** Aprahamian T, Rifkin I, Bonegio R, Hugel B, Freyssinet JM, Sato K, Castellot JJ, Jr., Walsh K. Impaired clearance of apoptotic cells promotes synergy between atherogenesis and autoimmune disease. *J Exp Med.* 2004;199(8):1121-1131.

**20.** Ait-Oufella H, Pouresmail V, Simon T, Blanc-Brude O, Kinugawa K, Merval R, Offenstadt G, Leseche G, Cohen PL, Tedgui A, Mallat Z. Defective mer receptor tyrosine kinase signaling in bone marrow cells promotes apoptotic cell accumulation and accelerates atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2008;28(8):1429-1431.

**21.** Schrijvers DM, De Meyer GR, Kockx MM, Herman AG, Martinet W. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2005;25(6):1256-1261.

**22.** Seimon TA, Obstfeld A, Moore KJ, Golenbock DT, Tabas I. Combinatorial pattern recognition receptor signaling alters the balance of life and death in macrophages. *Proc Natl Acad Sci U S A.* 2006;103(52):19794-19799.

**23.** Devries-Seimon T, Li Y, Yao PM, Stone E, Wang Y, Davis RJ, Flavell R, Tabas I. Cholesterol-induced macrophage apoptosis requires ER stress pathways and engagement of the type A scavenger receptor. *J Cell Biol.* 2005;171(1):61-73.

**24.** Li LO, Ellis JM, Paich HA, Wang S, Gong N, Altshuller GN, Thresher RJ, Kovestr, Watkins SM, Muoio DM, Cline GW, Shulman GI, Colemanr RA. Liver-specific loss of long-chain acyl-CoA synthetase-1 decreases triacylglycerol synthesis and beta-oxidation, and alters phospholipid fatty acid composition. *J Biol Chem.* 2009.

**25.** Saraswathi V, Gao L, Morrow JD, Chait A, Niswender KD, Hasty AH. Fish oil increases cholesterol storage in white adipose tissue with concomitant decreases in inflammation, hepatic steatosis, and atherosclerosis in mice. *J Nutr.* 2007;137(7):1776-1782.

**26.** Jehle AW, Gardai SJ, Li S, Linsel-Nitschke P, Morimoto K, Janssen WJ, Vandivier RW, Wang N, Greenberg S, Dale BM, Qin C, Henson PM, Tall AR. ATP-binding cassette transporter A7 enhances phagocytosis of apoptotic cells and associated ERK signaling in macrophages. *J Cell Biol.* 2006;174(4):547-556.

**27.** Liang CP, Han S, Okamoto H, Carnemolla R, Tabas I, Accili D, Tall AR. Increased CD36 protein as a response to defective insulin signaling in macrophages. *J Clin Invest.* 2004;113(5):764-773.

**28.** Kamen LA, Levinsohn J, Swanson JA. Differential association of phosphatidylinositol 3-kinase, SHIP-1, and PTEN with forming phagosomes. *Mol Biol Cell.* 2007;18(7):2463-2472.

**29.** Marshall JG, Booth JW, Stambolic V, Mak T, Balla T, Schreiber AD, Meyer T, Grinstein S. Restricted accumulation of phosphatidylinositol 3-kinase products in a plasmalemmal subdomain during Fc gamma receptor-mediated phagocytosis. *J Cell Biol.* 2001;153(7):1369-1380.

**30.** Cuevas BD, Lu Y, Mao M, Zhang J, LaPushin R, Siminovitch K, Mills GB. Tyrosine phosphorylation of p85 relieves its inhibitory activity on phosphatidylinositol 3-kinase. *J Biol Chem.* 2001;276(29):27455-27461.

**31.** Senokuchi T, Liang CP, Seimon TA, Han S, Matsumoto M, Banks AS, Paik JH, DePinho RA, Accili D, Tabas I, Tall AR. Forkhead transcription factors (FoxOs) promote apoptosis of insulin-resistant macrophages during cholesterol-induced endoplasmic reticulum stress. *Diabetes.* 2008;57(11):2967-2976.

**32.** Loffreda S, Yang SQ, Lin HZ, Karp CL, Brengman ML, Wang DJ, Klein AS, Bulkley GB, Bao C, Noble PW, Lane MD, Diehl AM. Leptin regulates proinflammatory immune responses. *FASEB J.* 1998;12(1):57-65.

**33.** Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest.* 2006;116(11):3015-3025.

**34.** Boden G, Shulman GI. Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction. *Eur J Clin Invest.* 2002;32 Suppl 3:14-23.

**35.** Savage DB, Petersen KF, Shulman GI. Disordered lipid metabolism and the pathogenesis of insulin resistance. *Physiol Rev.* 2007;87(2):507-520.

**36.** Solinas G, Naugler W, Galimi F, Lee MS, Karin M. Saturated fatty acids inhibit induction of insulin gene transcription by JNK-mediated phosphorylation of insulin-receptor substrates. *Proc Natl Acad Sci U S A.* 2006;103(44):16454-16459.

**37.** Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ, Ye J. Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex. *J Biol Chem.* 2002;277(50):48115-48121.

**38.** Dresner A, Laurent D, Marcucci M, Griffin ME, Dufour S, Cline GW, Slezak LA, Andersen DK, Hundal RS, Rothman DL, Petersen KF, Shulman GI. Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest.* 1999;103(2):253-259.

**39.** Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem.* 2002;277(52):50230-50236.

**40.** Askari B, Kanter JE, Sherrid AM, Golej DL, Bender AT, Liu J, Hsueh WA, Beavo JA, Coleman RA, Bornfeldt KE. Rosiglitazone inhibits acyl-CoA synthetase activity and fatty acid partitioning to diacylglycerol and triacylglycerol via a peroxisome proliferator-activated receptor-gamma-independent mechanism in human arterial smooth muscle cells and macrophages. *Diabetes.* 2007;56(4):1143-1152.

**41.** Hasty AH, Shimano H, Osuga J, Namatame I, Takahashi A, Yahagi N, Perrey S, Iizuka Y, Tamura Y, Amemiya-Kudo M, Yoshikawa T, Okazaki H, Ohashi K, Harada K, Matsuzaka T, Sone H, Gotoda T, Nagai R, Ishibashi S, Yamada N. Severe hypercholesterolemia, hypertriglyceridemia, and atherosclerosis in mice lacking both leptin and the low density lipoprotein receptor. *J Biol Chem.* 2001;276(40):37402-37408.

**42.** Gruen ML, Saraswathi V, Nuotio-Antar AM, Plummer MR, Coenen KR, Hasty AH. Plasma insulin levels predict atherosclerotic lesion burden in obese hyperlipidemic mice. *Atherosclerosis.* 2006;186(1):54-64.

**43.** Connor WE, DeFrancesco CA, Connor SL. N-3 fatty acids from fish oil. Effects on plasma lipoproteins and hypertriglyceridemic patients. *Ann N Y Acad Sci.* 1993;683:16-34.

**44.** Mancuso P, Huffnagle GB, Olszewski MA, Phipps J, Peters-Golden M. Leptin corrects host defense defects after acute starvation in murine pneumococcal pneumonia. *Am J Respir Crit Care Med.* 2006;173(2):212-218.

**45.** Corbett-Nelson EF, Mason D, Marshall JG, Collette Y, Grinstein S. Signaling-dependent immobilization of acylated proteins in the inner monolayer of the plasma membrane. *J Cell Biol.* 2006;174(2):255-265.

**46.** Hall AM, Smith AJ, Bernlohr DA. Characterization of the Acyl-CoA synthetase activity of purified murine fatty acid transport protein 1. *J Biol Chem.* 2003;278(44):43008-43013.

**47.** Hall AM, Wiczer BM, Herrmann T, Stremmel W, Bernlohr DA. Enzymatic properties of purified murine fatty acid transport protein 4 and analysis of acyl- CoA synthetase activities in tissues from FATP4 null mice. *J Biol Chem.* 2005;280(12):11948-11954.

**48.** Jia Z, Moulson CL, Pei Z, Miner JH, Watkins PA. Fatty acid transport protein 4 is the principal very long chain fatty acyl-CoA synthetase in skin fibroblasts. *J Biol Chem.* 2007;282(28):20573-20583.

**49.** Schwab JM, Chiang N, Arita M, Serhan CN. Resolvin E1 and protectin D1 activate inflammation-resolution programmes. *Nature.* 2007;447(7146):869-874.

**50.** Serhan CN, Yang R, Martinod K, Kasuga K, Pillai PS, Porter TF, Oh SF, Spite M. Maresins: novel macrophage mediators with potent antiinflammatory and proresolving actions. *J Exp Med.* 2009;206(1):15-23.

**51.** Merched AJ, Ko K, Gotlinger KH, Serhan CN, Chan L. Atherosclerosis: evidence for impairment of resolution of vascular inflammation governed by specific lipid mediators. *FASEB J.* 2008;22(10):3595-3606.

**52.** Gonzalez-Periz A, Horrillo R, Ferre N, Gronert K, Dong B, Moran-Salvador E, Titos E, Martinez-Clemente M, Lopez-Parra M, Arroyo V, Claria J. Obesity-induced insulin resistance and hepatic steatosis are alleviated by omega-3 fatty acids: a role for resolvins and protectins. *FASEB J.* 2009;23(6):1946-1957.

**53.** Wu KK, Wu TJ, Chin J, Mitnaul LJ, Hernandez M, Cai TQ, Ren N, Waters MG, Wright SD, Cheng K. Increased hypercholesterolemia and atherosclerosis in mice lacking both ApoE and leptin receptor. *Atherosclerosis.* 2005;181(2):251-259.

**54.** Coenen KR, Gruen ML, Lee-Young RS, Puglisi MJ, Wasserman DH, Hasty AH. Impact of macrophage toll-like receptor 4 deficiency on macrophage infiltration into adipose tissue and the artery wall in mice. *Diabetologia.* 2009;52(2):318-328.

**55.** Zampolli A, Bysted A, Leth T, Mortensen A, De Caterina R, Falk E. Contrasting effect of fish oil supplementation on the development of atherosclerosis in murine models. *Atherosclerosis.* 2006;184(1):78-85.

**56.** Seimon T, Tabas I. Mechanisms and consequences of macrophage apoptosis in atherosclerosis. *J Lipid Res.* 2009;50 Suppl:S382-387.

**57.** Gautier EL, Huby T, Witztum JL, Ouzilleau B, Miller ER, Saint-Charles F, Aucouturier P, Chapman MJ, Lesnik P. Macrophage apoptosis exerts divergent effects on atherogenesis as a function of lesion stage. *Circulation.* 2009;119(13):1795-1804.

**58.** Thorp E, Tabas I. Mechanisms and consequences of efferocytosis in advanced atherosclerosis. *J Leukoc Biol.* 2009.

**59.** Bird DA, Gillotte KL, Horkko S, Friedman P, Dennis EA, Witztum JL, Steinberg D. Receptors for oxidized low-density lipoprotein on elicited mouse peritoneal macrophages can recognize both the modified lipid moieties and the modified protein moieties: implications with respect to macrophage recognition of apoptotic cells. *Proc Natl Acad Sci U S A.* 1999;96(11):6347-6352.

**60.** Saito Y, Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Ishikawa Y, Oikawa S, Sasaki J, Hishida H, Itakura H, Kita T, Kitabatake A, Nakaya N, Sakata T, Shimada K, Shirato K. Effects of EPA on coronary artery disease in hypercholesterolemic patients with multiple risk factors: sub-analysis of primary prevention cases from the Japan EPA Lipid Intervention Study (JELIS). *Atherosclerosis.* 2008;200(1):135-140.

**61.** Kris-Etherton PM, Harris WS, Appel LJ. Omega-3 fatty acids and cardiovascular disease: new recommendations from the American Heart Association. *Arterioscler Thromb Vasc Biol.* 2003;23(2):151-152.

## FIGURE LEGENDS

**Figure 1. Defective efferocytosis by macrophages from obese mice.**

**A:** Efferocytosis by obese (ob/ob and db/db) mice and their corresponding littermate lean control mice (ob con and db con). Thioglycollate-elicited mouse peritoneal macrophages were pooled from three mice of each genotype and plated in 24-well plates with 10% FBS for 1- 2 days before phagocytic assay. Macrophages were incubated with CELLTracker red stained apoptotic Jurkat cells for 25 minutes before they were fixed, permeabilized, and stained with an antibody against LAMP1 (Green) for phagocytic index (PI) evaluation. Left, representative images showing the phagocytic assay from ob control and ob/ob peritoneal macrophages. Arrows represent macrophages with internalized apoptotic cells (yellow overlay indicates uptake). Right, quantification results of macrophage efferocytosis of obese and their littermate control mice. **B:** Results of *in vivo* efferocytosis by peritoneal macrophages from ob control and ob/ob mice. **C:** Data of Fc-R mediated phagocytosis by ob control and ob/ob macrophages. **D:** Binding capacities of apoptotic cells between ob control and ob/ob macrophages. Macrophages were incubated with apoptotic cells at 4 °C for 1 hour before binding capacities were measured. **E:** Results of the bone marrow transplantation study with ob control and ob/ob mice. For all experiments, mean and SEM of at least 3 independent experiments, * $P<0.05$, PI: phagocytic index.

**Figure 2. Defective PI-3-kinase/AKT activation in response to phagocytosis of apoptotic cells by ob/ob macrophages.**

**A:** PI3K/AKT signaling during efferocytosis by ob control and ob/ob macrophages. Apoptotic Jurkat T cells were added to the monolayers of macrophages. At the indicated time points after

efferocytosis, unphagocytosed apoptotic cells were removed by rigorous shaking and macrophages were washed with ice cold PBS for three times before lysis. Immunoblotting was performed as indicated above. n=3, *P<0.05. **B:** $PIP_3$ accumulation in phagocytic membranes in response to efferocytosis. Macrophages were incubated with apoptotic cells for 8 minutes before fixation. Left, fluorescent images showing the accumulation of $PIP_3$ in phagocytic membranes (arrows) in ob control, ob/ob and 10μM LY 294002 treated ob control macrophages. Right, Quantification results of the number of areas with $PIP_3$ accumulation in phagocytic membranes per 100 macrophages in response to the stimulus of apoptotic cells. n=3, * P<0.05. **C:** The effects of PI3K inhibitor LY 294002 on efferocytosis of ob control macrophages. Macrophages were pretreated with 10μM LY 294002 1h before efferocytosis were conducted. APC: apoptotic cells. 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, Mean and SEM of 3 independent experiments. P=0.60 for ob control and ob/ob at time 0', P=0.52 at time 5', P=0.14 at time 10', P=0.23 at time 20'.

**Figure 3. Exogenous fatty acids alter macrophage efferocytotic efficiency.**

**A:** Plasma free fatty acids (FFA) level in 5h fasted ob control and ob/ob mice. n=14, *P<0.05. **B:** Composition of saturated fatty acids and unsaturated fatty acids in membrane lipids from ob control and ob/ob macrophages. PA: Palmitic Acids, POA: palmioleic acid, SA: stearic acid, OA: oleic acid, LOA: linoleic acid, AA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid. n=4, *p<0.05. **C:** The time course and effects of saturated FFA/BSA on efferocytosis. Except time course study, cells were treated with 0.5mM FFA for 6-7 hours

before phagocytosis were conducted. n=3, Mean and SEM of 3 independent experiments, * P<0.05. **D:** The effects of FFA/BSA on ob/ob macrophages compared with ob control cells. n=3, Values are expressed as Mean±SEM. Unless specified, * P<0.05 or Not significant (NS) when compared with BSA treated ob/ob macrophages. **E:** The effects of EPA-BSA on efferocytosis by the 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.

**Figure 4. Saturated fatty acid/BSA complexes impair PI3K/AKT activation, resembling the changes seen in ob/ob macrophages.**

**A:** PI3K/AKT activation during efferocytosis in saturated FFA/BSA treated control macrophages. Results are representatives of 3 independent experiments for PI3K/AKT signaling. n=3. **B:** Tyrosine phosphorylation of PI3K regulative subunit P85 during efferocytosis in saturated FFA/BSA treated control macrophages. n=2.

**Figure 5. Impaired efferocytosis is not reversed by inhibition of TLR4, P38, JNK, NF-κB, IKK or PKC signaling or ACSL.**

**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 triacsin 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.

**Figure 6. Defective efferocytosis in late atherosclerotic lesions of ob/ob:Ldlr-/- mice on semi-synthetic diet.**

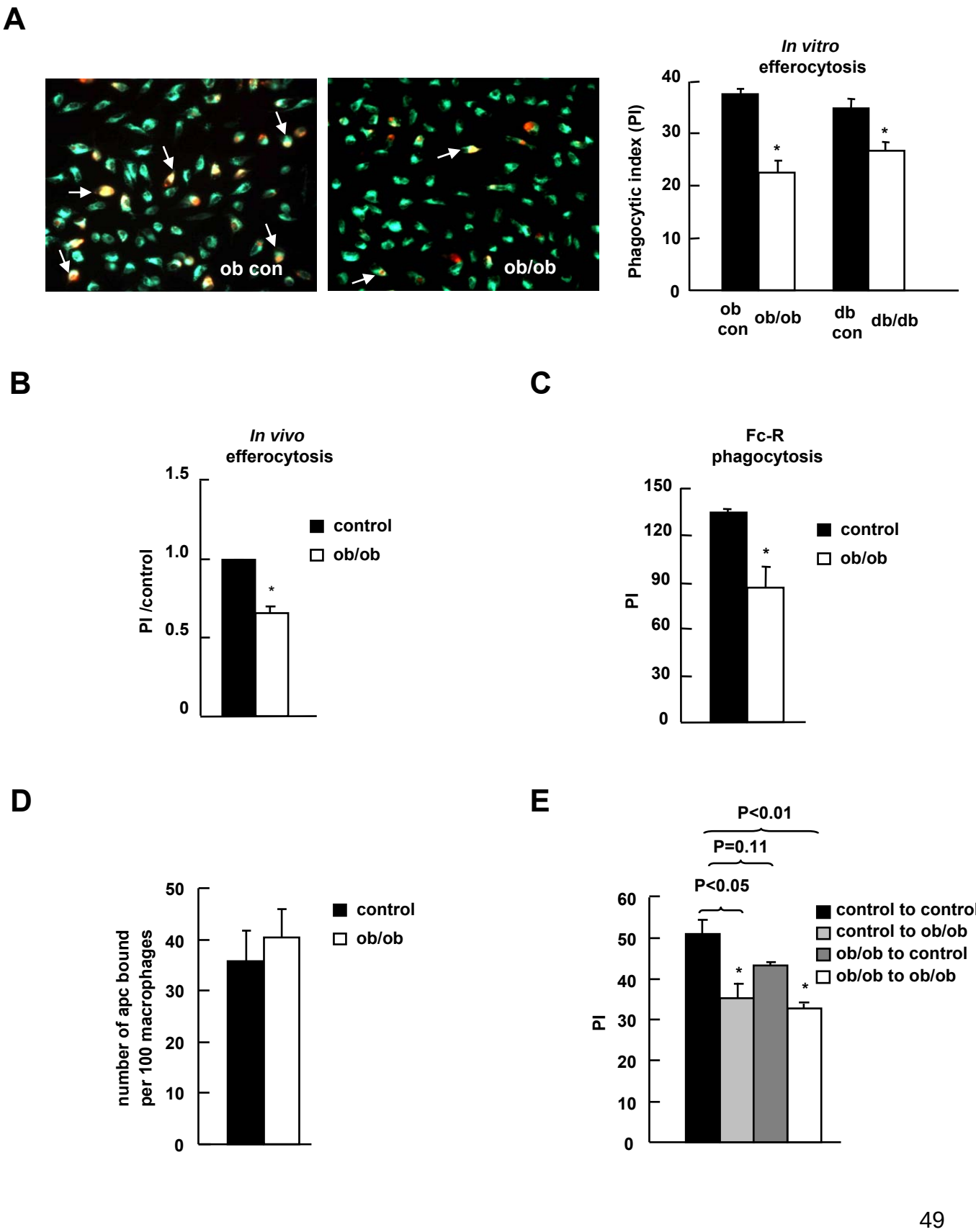
**A:** *In vivo* efferocytosis by peritoneal macrophages from Ldlr-/- control and ob/ob;Ldlr-/- mice. Mice were on AIN76A semi-synthetic diet for 4 weeks before macrophages were collected. Representative data of 2 independent experiments with triplicates. * $P<0.05$. **B:** Plasma free fatty acids (FFA) level in Ldlr-/- or ob/ob;Ldlr-/- mice with the semi-synthetic diet for 4 weeks. n=6, * $P<0.05$. **C:** Proximal aortic root lesion and necrotic core areas per section from Ldlr-/- and ob/ob;Ldlr-/- mice with semi-synthetic diet for more than 20 weeks. n=6 pairs. **D:** Lesional macrophage efferocytosis of the mice described in Figure 6C. Upper, representative images of *in vivo* lesional macrophage efferocytosis from the Ldlr-/- and ob/ob;Ldlr-/- mice. Red: TUNEL positive materials, Blue: Hoechst stained cell nuclei, Green: macrophage specific AIA staining. Lower, quantification results of *in vivo* lesional macrophage efferocytosis (left) and apoptosis (right) from the Ldlr-/- and ob/ob;Ldlr-/- mice. n=6 pairs.

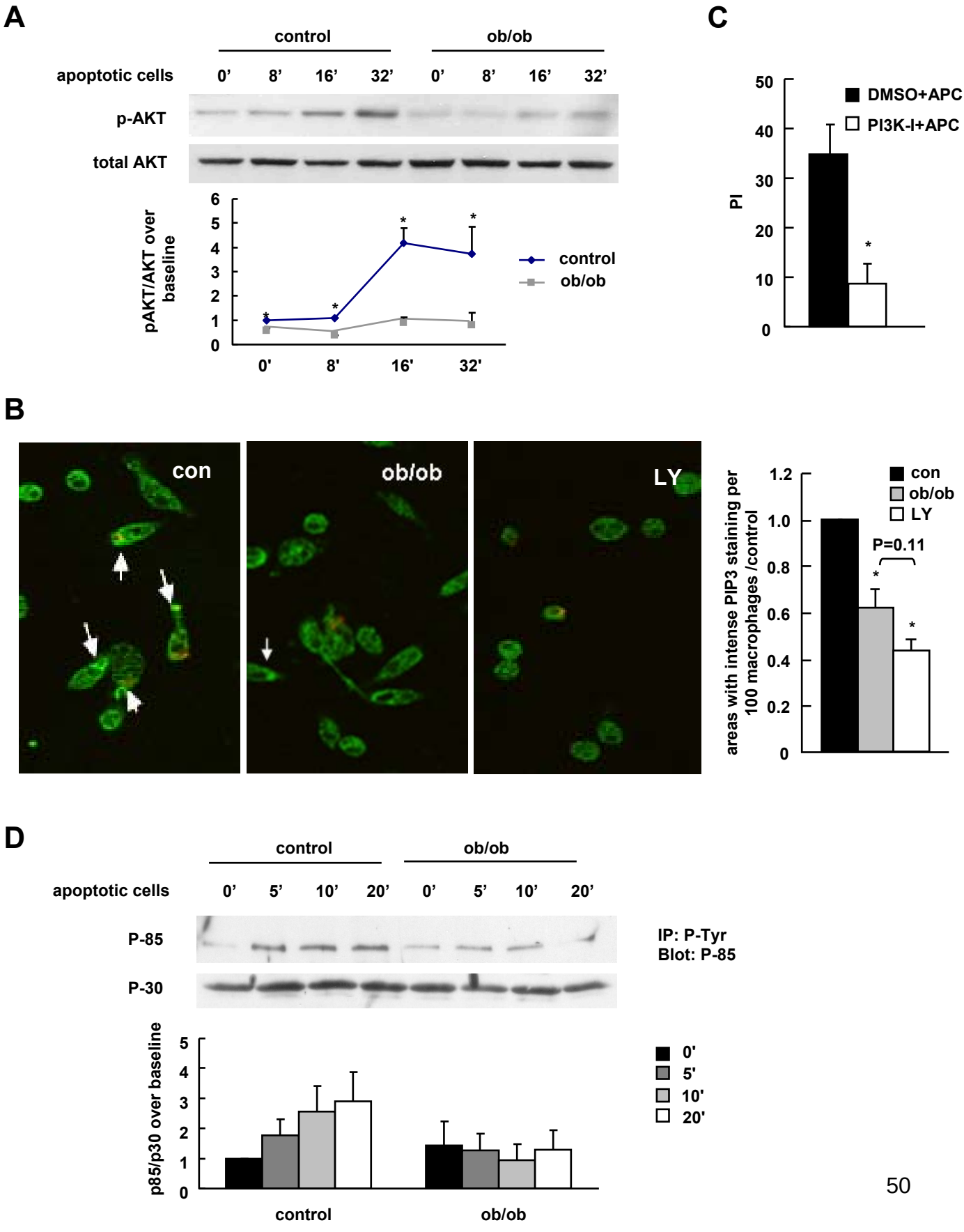
**Figure 7. Fish oil diet reverses the defect in efferocytosis by ob/ob peritoneal macrophages and increases membrane lipid content of n-3 fatty acids.**

**A:** *In vitro* peritoneal macrophage efferocytosis from ob control or ob/ob mice with olive oil or fish oil diets for 4 weeks, n=3. *P<0.05. The inset blot shows the macrophage PI3K/AKT signaling during efferocytosis after treatment with the corresponding diets for 4 weeks. **B:** Composition of saturated fatty acids and unsaturated fatty acids in membrane lipids from control and ob/ob mice on olive oil or fish oil for 4 weeks. PA: Palmitic Acids, POA: palmioleic acid, SA: stearic acid, OA: oleic acid, LOA: linoleic acid, AA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid. n=2, mean value of 2 independent experiments.

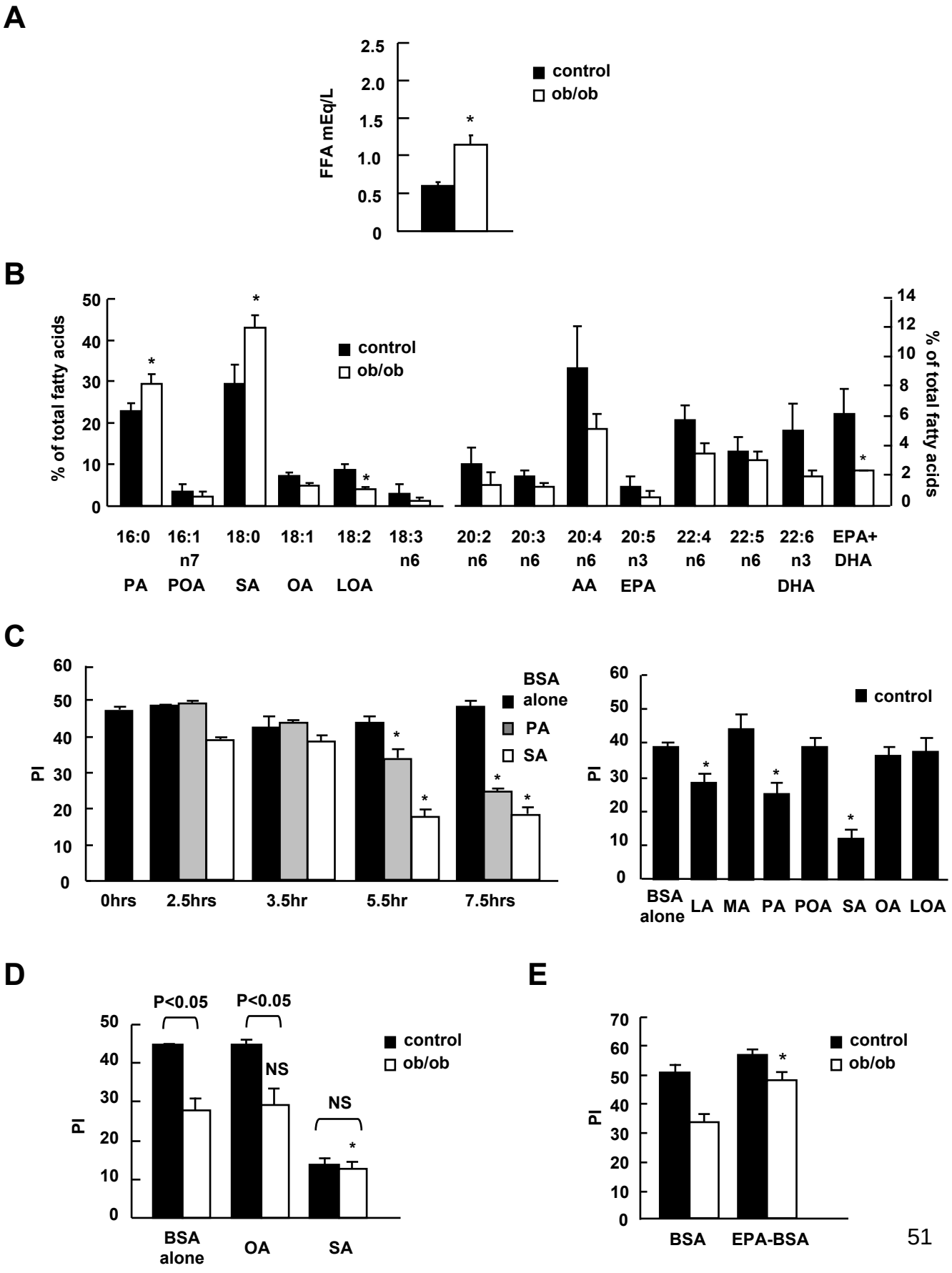
**Figure 8. Increased apoptosis and defective efferocytosis in early atherosclerotic lesions of ob/ob;Ldlr-/- mice: reversal of defects by a fish oil diet.**

**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.

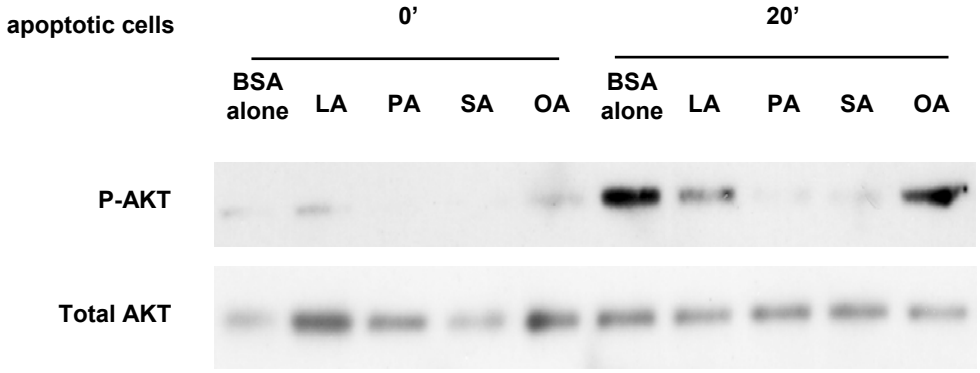

A
ob con
ob/ob
In vitro
efferocytosis
Phagocytic index (PI)
40
30
20
10
0
*
*
ob
con
ob/ob
db
con
db/db
B
In vivo
efferocytosis
PI /control
1.5
1.0
0.5
0
*
control
ob/ob
C
Fc-R
phagocytosis
PI
150
120
90
60
30
0
*
control
ob/ob
D
number of apc bound
per 100 macrophages
50
40
30
20
10
0
control
ob/ob
E
P<0.01
P=0.11
P<0.05
PI
60
50
40
30
20
10
0
*
*
control to control
control to ob/ob
ob/ob to control
ob/ob to ob/ob


A
control
ob/ob
apoptotic cells
0'
8'
16'
32'
0'
8'
16'
32'
p-AKT
total AKT
pAKT/AKT over baseline
control
ob/ob
C
DMSO+APC
PI3K-I+APC
PI
B
con
ob/ob
LY
areas with intense PIP3 staining per 100 macrophages /control
P=0.11
D
control
ob/ob
apoptotic cells
0'
5'
10'
20'
0'
5'
10'
20'
P-85
P-30
IP: P-Tyr
Blot: P-85
p85/p30 over baseline
0'
5'
10'
20'
control
ob/ob

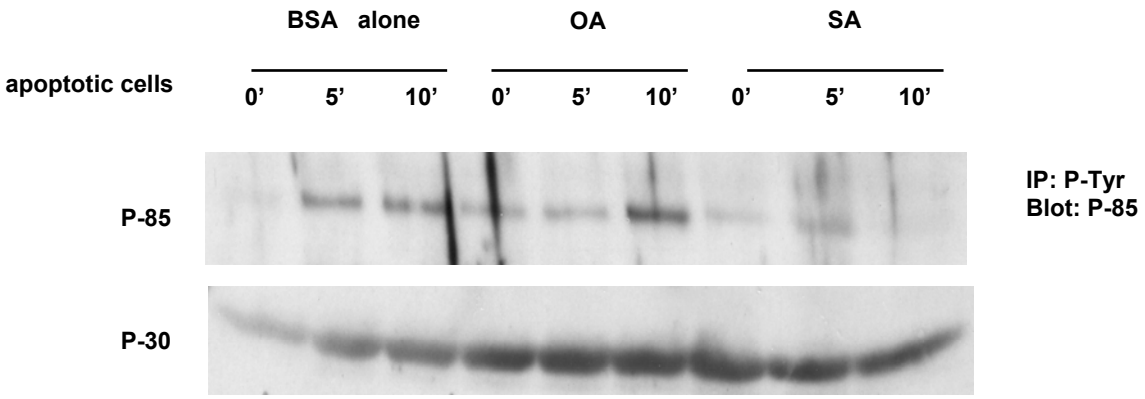
# Figure 3


A
FFA mEq/L
2.5
2.0
1.5
1.0
0.5
0
control
ob/ob
*
B
% of total fatty acids
50
40
30
20
10
0
14
12
10
8
6
4
2
0
control
ob/ob
16:0 PA
16:1 n7 POA
18:0 SA
18:1 OA
18:2 LOA
18:3 n6
20:2 n6
20:3 n6
20:4 n6 AA
20:5 n3 EPA
22:4 n6
22:5 n6
22:6 n3 DHA
EPA+ DHA
C
PI
60
50
40
30
20
10
0
BSA alone
PA
SA
0hrs
2.5hrs
3.5hr
5.5hr
7.5hrs
control
BSA alone
LA
MA
PA
POA
SA
OA
LOA
D
P<0.05
P<0.05
NS
NS
control
ob/ob
BSA alone
OA
SA
E
control
ob/ob
BSA
EPA-BSA

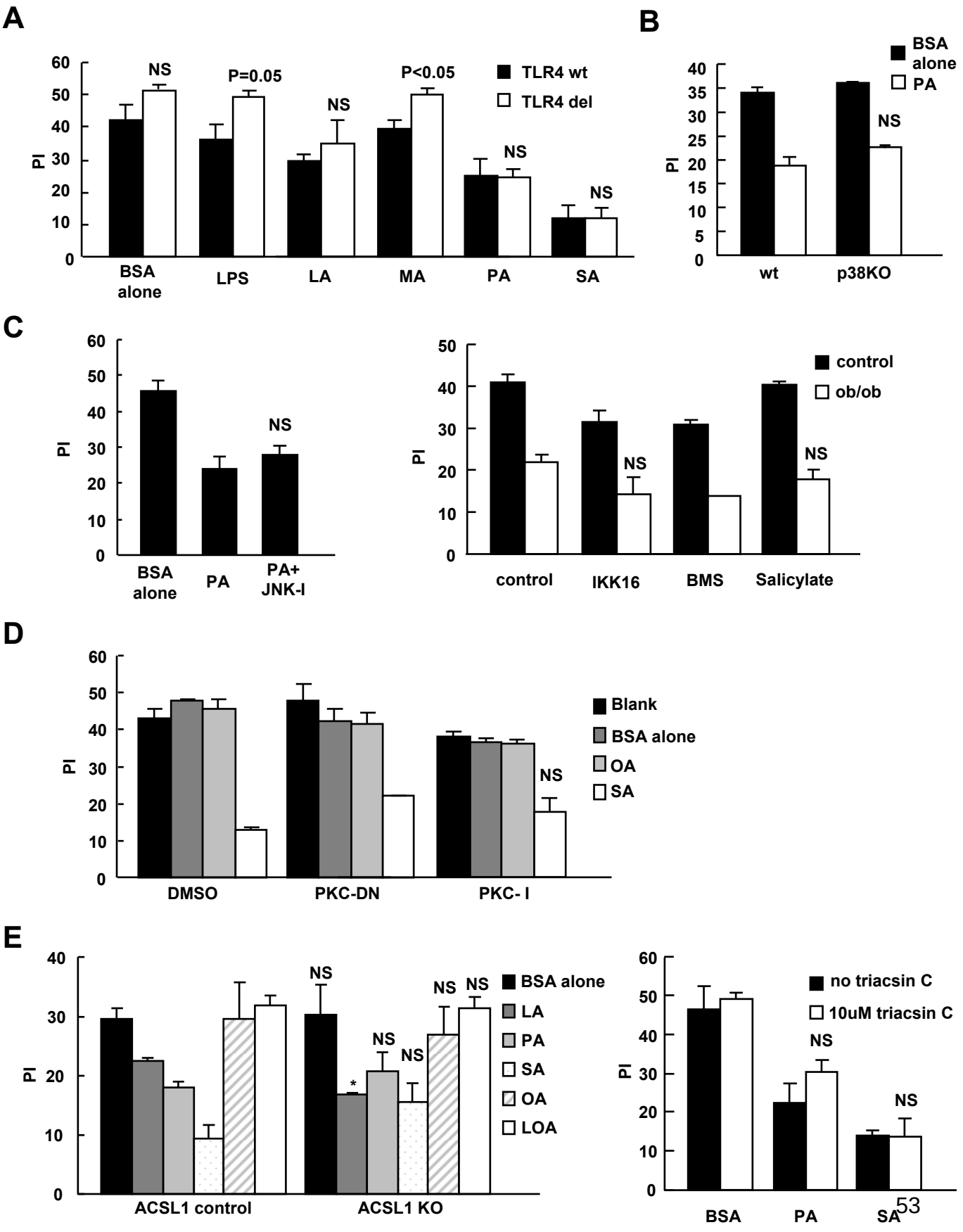
A

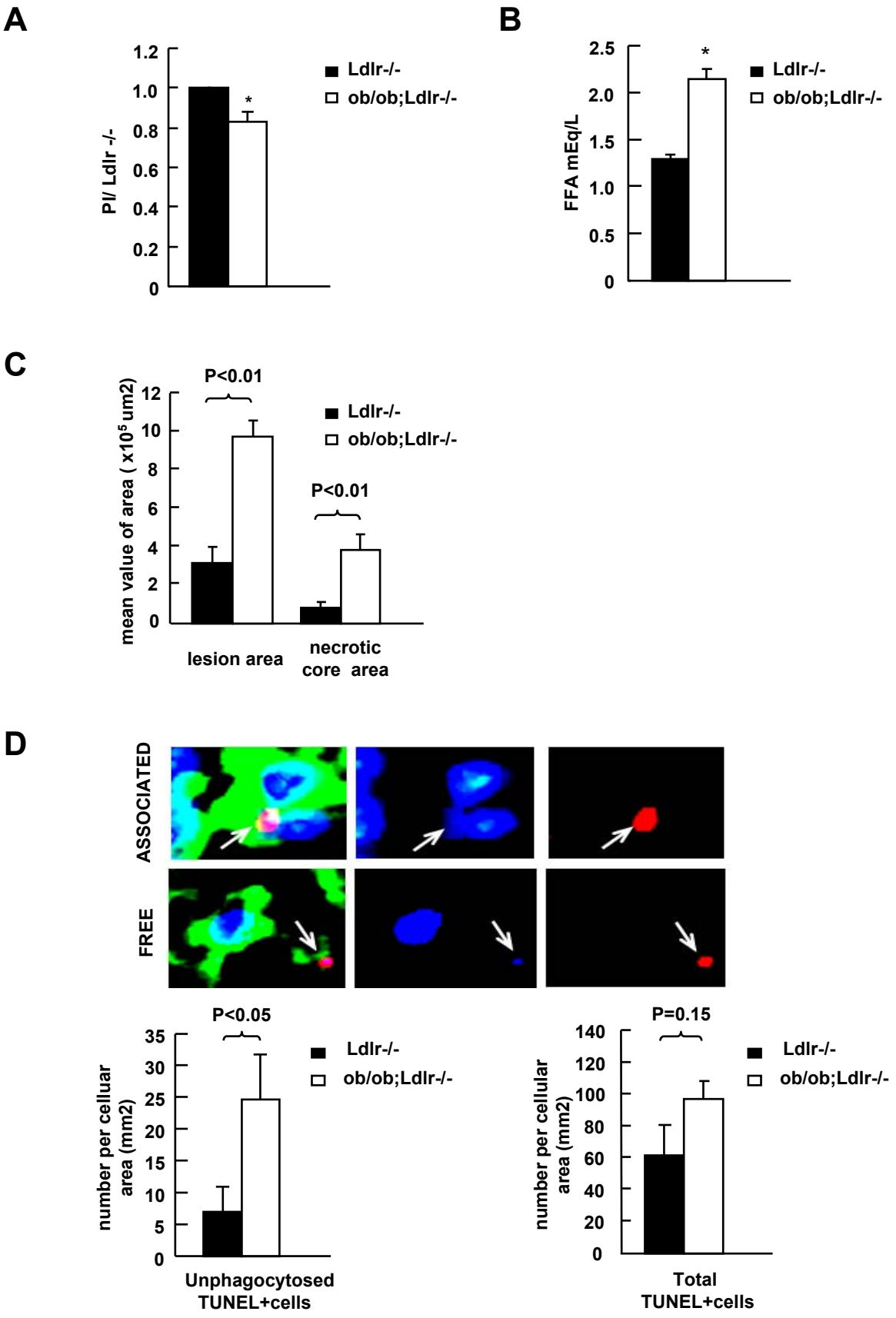

apoptotic cells
0'
20'
BSA alone
LA
PA
SA
OA
BSA alone
LA
PA
SA
OA
P-AKT
Total AKT

B

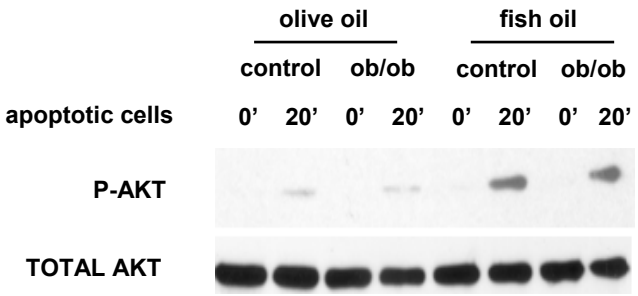

BSA alone
OA
SA
apoptotic cells
0'
5'
10'
0'
5'
10'
0'
5'
10'
P-85
P-30
IP: P-Tyr
Blot: P-85

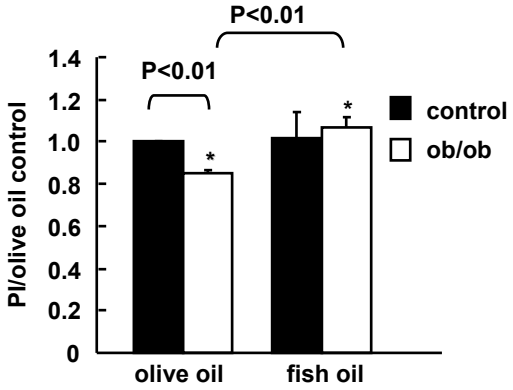
# Figure 5


A
PI
NS
P=0.05
NS
P<0.05
NS
NS
TLR4 wt
TLR4 del
BSA alone
LPS
LA
MA
PA
SA
B
PI
BSA alone
PA
NS
wt
p38KO
C
PI
NS
BSA alone
PA
PA+ JNK-I
control
ob/ob
NS
NS
control
IKK16
BMS
Salicylate
D
PI
Blank
BSA alone
OA
SA
NS
DMSO
PKC-DN
PKC- I
E
PI
NS
NS
NS
NS
*
BSA alone
LA
PA
SA
OA
LOA
ACSL1 control
ACSL1 KO
no triacsin C
10uM triacsin C
NS
NS
BSA
PA
SA

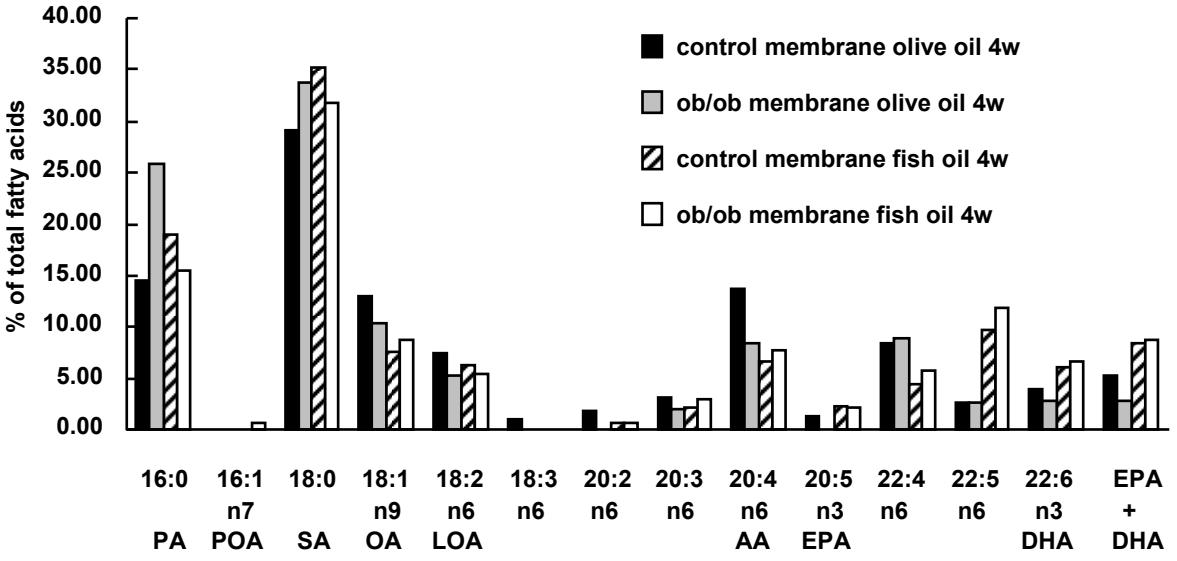

A
PI/ Ldlr -/-
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Ldlr-/-
ob/ob;Ldlr-/-
B
FFA mEq/L
2.5
2.0
1.5
1.0
0.5
0
*
Ldlr-/-
ob/ob;Ldlr-/-
C
mean value of area ( x10^5 um2)
12
10
8
6
4
2
0
P<0.01
P<0.01
Ldlr-/-
ob/ob;Ldlr-/-
lesion area
necrotic core area
D
ASSOCIATED
FREE
P<0.05
number per celluar area (mm2)
35
30
25
20
15
10
5
0
Ldlr-/-
ob/ob;Ldlr-/-
Unphagocytosed TUNEL+cells
P=0.15
number per cellular area (mm2)
140
120
100
80
60
40
20
0
Ldlr-/-
ob/ob;Ldlr-/-
Total TUNEL+cells

A

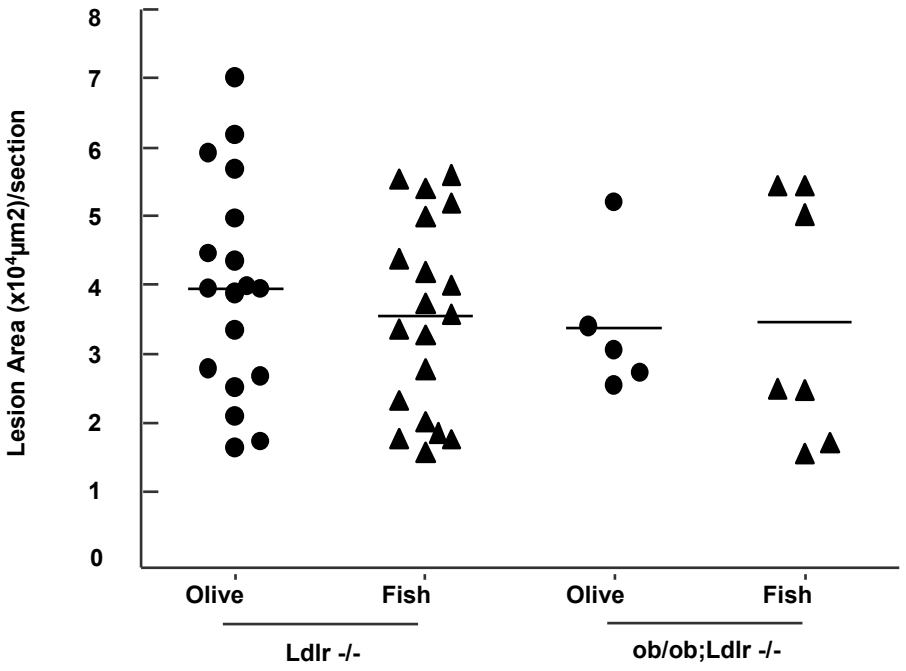

olive oil
fish oil
control
ob/ob
control
ob/ob
apoptotic cells
0'
20'
0'
20'
0'
20'
0'
20'
P-AKT
TOTAL AKT


P<0.01
P<0.01
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
PI/olive oil control
*
*
control
ob/ob
olive oil
fish oil

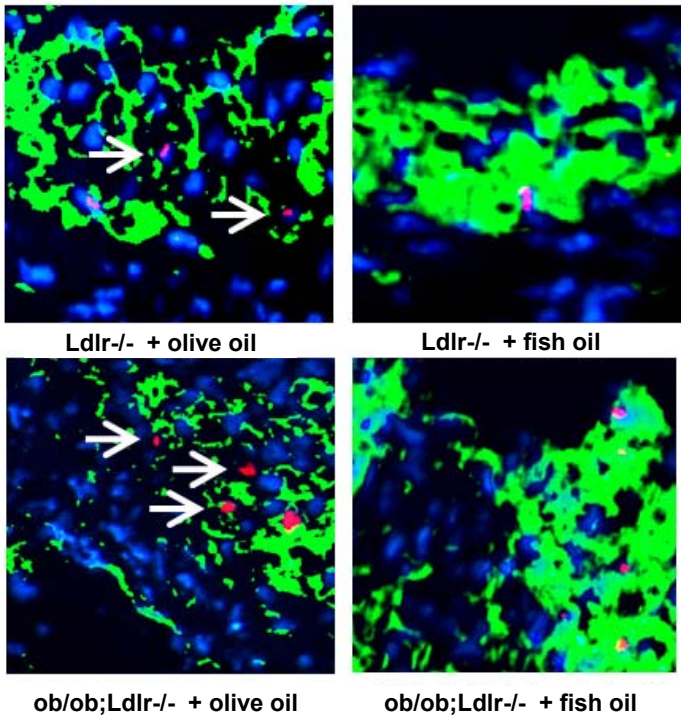
B

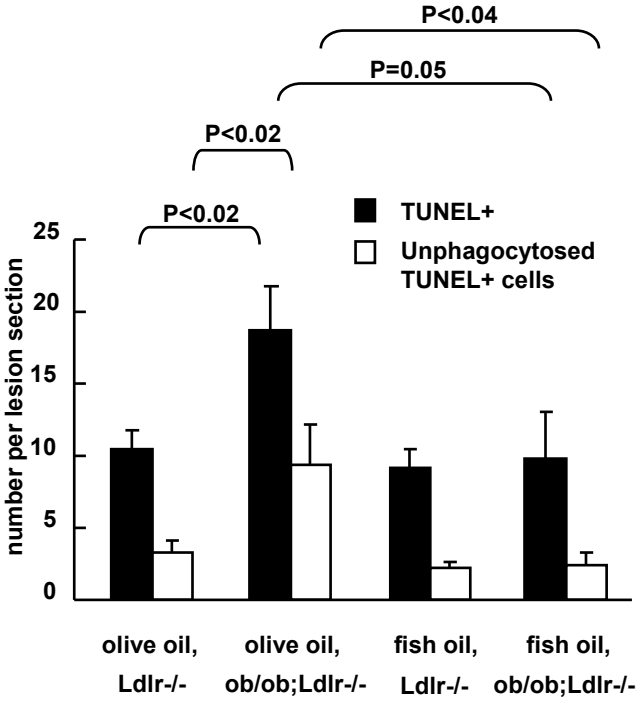

40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
% of total fatty acids
control membrane olive oil 4w
ob/ob membrane olive oil 4w
control membrane fish oil 4w
ob/ob membrane fish oil 4w
16:0 PA
16:1 n7 POA
18:0 SA
18:1 n9 OA
18:2 n6 LOA
18:3 n6
20:2 n6
20:3 n6
20:4 n6 AA
20:5 n3 EPA
22:4 n6
22:5 n6
22:6 n3 DHA
EPA + DHA

A


Lesion Area (x10⁴µm2)/section
8
7
6
5
4
3
2
1
0
Olive
Fish
Olive
Fish
Ldlr -/-
ob/ob;Ldlr -/-

B


Ldlr-/- + olive oil
Ldlr-/- + fish oil
ob/ob;Ldlr-/- + olive oil
ob/ob;Ldlr-/- + fish oil


P<0.04
P=0.05
P<0.02
P<0.02
TUNEL+
Unphagocytosed TUNEL+ cells
number per lesion section
25
20
15
10
5
0
olive oil, Ldlr-/-
olive oil, ob/ob;Ldlr-/-
fish oil, Ldlr-/-
fish oil, ob/ob;Ldlr-/-

**Adiponectin Effects on Atherogenesis in LDL Receptor Null Mice**

Andrea R. Nawrocki[1,*], Susanna M. Hofmann[2], Daniel Teupser[3], Joshua E. Basford[2], Jorge L. Durand[4], Linda A. Jelicks[4], Connie W. Woo[5], George Kuriakose[5], Stephen M. Factor[6], Herbert B. Tanowitz[6], David Y. Hui[2], Ira Tabas[5] and Philipp E. Scherer[7,**]

[1] *Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY, USA*
[2] *Department of Pathology, University of Cincinnati College of Medicine, Cincinnati, OH, USA*
[3] *Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, University Leipzig, Leipzig, Germany*
[4] *Departments of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY, USA*
[5] *Departments of Medicine; Pathology & Cell Biology; and Phsyiology, Columbia University, NY, USA*
[6] *Department of Pathology, Albert Einstein College of Medicine, Bronx, NY, USA*
[7] *Touchstone Diabetes Center, Departments of Internal Medicine and Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX, USA*
** Current address: Department of Obesity, Merck Research Laboratories, Rahway, NJ, USA*

** Corresponding author:
Dr. Philipp E. Scherer, PhD
Touchstone Diabetes Center
The University of Texas Southwestern Medical Center
5323 Harry Hines Blvd.
Dallas, TX 75390-8549
Tel: (214) 648-8715; Fax: (214) 648-8720; E-mail: philipp.scherer@utsouthwestern.edu

Key Words:

Adiponectin, atherosclerosis, LDL receptor knockout mice

**Abstract**

Adiponectin is an adipocyte-derived, secreted protein that has been implicated in the protection against a cluster of related metabolic disorders including obesity, insulin resistance and cardiovascular disease. Mice lacking adiponectin display impaired hepatic insulin sensitivity and respond only partially to PPARγ agonists. Adiponectin-mediated metabolic improvements are associated with alleviation of dyslipidemia and the prevention of ectopic lipid deposition. It has been suggested that adiponectin has anti-atherogenic properties. However, the involvement of adiponectin on the atherogenic process have not been studied systematically in preclinical models of atherosclerosis. We crossed either adiponectin KO ($Adn^{-/-}$) or mice with chronically elevated adiponectin levels ($Adn^{Tg}$) into the low-density lipoprotein (LDL) receptor ($Ldlr^{-/-}$) deficient background. Surprisingly, we found that adiponectin levels did not correlate with the development of atherosclerosis under a number of different dietary conditions. We analyzed plaque volume in the aortic root, cholesterol accumulation in the aorta and plaque morphology, none of which were affected by variable circulating adiponectin levels. In light of the strong clinical correlations reported for adiponectin in cardiovascular disease, the lack of a phenotype in these gain- and loss-of-function studies in mice may suggest lack of causation or alternatively, a role for adiponectin in suppressing an atherothrombotic process, such as plaque rupture or lumenal thrombosis, which is not a component of these murine models. This indicates that the actions of adiponectin on the cardiovascular system are complex and multifaceted, with a minimal direct impact on atherosclerotic plaque formation in the setting of a murine model.

245 words

**Introduction**

Increasing prevalence of obesity and its association with the pathogenesis of cardiovascular disease has evoked a great interest in understanding the impact of adipokines on vascular integrity, systemic inflammation and heart failure (Abel, Litwin et al. 2008). Adipokines are factors secreted from adipose tissue that have been identified as key players in systemic energy management and are dysregulated in obesity (Scherer 2006). These include lipid factors, structural proteins like collagens and hormones implicated in energy homeostasis, lipid metabolism and insulin signaling including leptin, resistin, and retinol binding protein-4. Many pro-inflammatory cytokines (interleukin 1β (IL-1β), IL-6, tumor necrosis factor-α, monocyte chemoattractant protein-1) and pro-thrombotic adipokines (plasminogen activator inhibitor-1) tend to be upregulated in the obese state (Berg and Scherer 2005).

Adiponectin is an adipokine abundantly expressed in differentiated adipocytes and released into the circulation (Shetty, Kusminski et al. 2009). Adiponectin levels are high in the lean state and tend to be lower in obese individuals, provided the obesity is associated with a degree of local cellular dysfunction in adipose tissue that includes an increase in inflammation (Kim, van de Wall et al. 2007). In addition, circulating adiponectin levels correlate inversely with risk factors for cardiovascular disease including body mass index, visceral adiposity, hyperlipidemia and high density lipoprotein cholesterol (HDL-C) levels (Menzaghi, Trischitta et al. 2007). Due to these characteristics, adiponectin has emerged as a useful biomarker for clinical manifestations of a number of disease states associated with metabolic dysfunction. Hotta et al. demonstrated that adiponectin levels of diabetics with coronary artery disease (CAD) are lower than those of diabetics without CAD or of non-diabetics (Hotta, Funahashi et al. 2000). In

contrast, high adiponectin levels are associated with a reduced risk for myocardial infarction in men independently of their glycemic or inflammatory status (Pischon, Girman et al. 2004). Taken together, these epidemiological observations suggest a link between adiponectin and the pathologies of cardiovascular disease, albeit no conclusion can be drawn whether this relationship is correlative or causal.

It has been suggested that adiponectin may also play an anti-atherogenic and anti-inflammatory role (Takemura, Walsh et al. 2007). Increased neointimal proliferation was observed in mice lacking adiponectin and upon viral expression of the globular form in a cuff-injury model in mice (Kubota, Terauchi et al. 2002). Surprisingly, a number of recent studies report an increased risk of all cause and cardiac mortality with elevated adiponectin levels in later and further progressed stages of cardiovascular disease (Cavusoglu, Ruwende et al. 2006). Another recent report concluded that elevated concentrations of adiponectin are independently associated with inducible ischemia in patients with stable coronary heart disease (Zhang, Spies et al. 2009). While adiponectin is measured frequently as marker for cardiovascular disease in the clinical area, to date, no systematic studies have been published that address the direct impact of adiponectin in the progression of atherosclerosis in pre-clinical models. We therefore embarked on a comprehensive analysis of LDL receptor knockout mice that are deficient of adiponectin (adiponectin null mice) or have mildly elevated levels of adiponectin mediated by means of overexpression of an adiponectin transgene (Nawrocki, Rajala et al. 2006) (Combs, Pajvani et al. 2004) and exposed them to various dietary conditions that increase the cholesterol load. Our studies were designed to differentiate between non-obesogenic conditions intended to alleviate effects on adiposity or insulin sensitivity as well as conditions of diet-induced obesity (“Western style diet”). Interestingly, we could not establish a direct role of adiponectin in the

atherosclerotic process. Our observations may suggest lack of direct causation for adiponectin in protecting against atherosclerosis or a role for adiponectin in suppressing an atherothrombotic process, such as plaque rupture or lumenal thrombosis, that is not a component of these murine models (Rosenfeld, Carson et al. 2002).

Our studies also considered the role of peroxisome proliferator-activated receptor γ (PPARγ) gene, which plays a crucial role in adipocyte differentiation. Pharmacologic activation of this nuclear receptor is the single most potent inducer of adiponectin secretion (Pajvani, Hawkins et al. 2004). Thiazolidinediones (TZD), such as rosiglitazone, are selective activators of PPARγ with beneficial effects on hepatic and peripheral insulin sensitivity and on a number of cardiovascular risk factors. However, prolonged TZD treatment has been associated with adverse events such as increased adiposity and edema and more recently substantial concern has been raised about the safety of TZDs on cardiovascular outcomes (Patel, Wyne et al. 2005; Stafylas, Sarafidis et al. 2009). Both pro and anti-atherogenic roles have been attributed to PPARγ. TZD exposure can in fact increase macrophage apoptosis and plaque necrosis in advanced atherosclerotic lesions (Thorp, Kuriakose et al. 2007).

In the present study, we analyzed adiponectin-dependent and independent effects of chronic PPARγ agonism in mice. We examined lesion size as well as effects on cardiac integrity using our genetic models of adiponectin and LDL receptor deficiency. Cardiac remodeling is a well known adaptive mechanism related to obesity (Abel, Litwin et al. 2008). Structural changes in the heart such as left ventricular (LV) hypertrophy precede functional impairment ultimately leading to heart failure. Recently, it has been suggested that adiponectin attenuates cardiac remodeling in models of pressure overload and increases survival of cardiac myocytes when

injected immediately after cardiac ischemia/reperfusion in mice (Shibata, Sato et al. 2005; Ouchi, Shibata et al. 2006). Here we demonstrate that chronic TZD treatment causes LV hypertrophy which is attenuated in adiponectin knockout mice.

## Results

### Deletion of adiponectin does not promote atherosclerosis in lean, LDL receptor deficient mice

To assess a potential atherogenic role of adiponectin, we chose the widely used low density lipoprotein receptor knockout mouse model *(Ldlr-/-)* (Zadelaar, Kleemann et al. 2007). Mice lacking adiponectin (*Adn*$^{-/-}$) (Nawrocki, Rajala et al. 2006) were crossed with *Ldlr*$^{-/-}$ mice to generate double knockout mice (*Ldlr/Adn*$^{-/-}$). Mice were fed a low-fat diet containing 10% kcal from fat and 0.15% cholesterol ("Clinton/Cybulsky diet"), a regimen that causes substantial atherosclerotic lesions within three to four months without associated metabolic complications such as obesity and insulin resistance (Teupser, Persky et al. 2003). Total plasma cholesterol was elevated by the diet to approximately 800 mg/dL in all groups. As shown in Table 1, plasma adiponectin levels were not affected by this type of dietary exposure (**Table 1**, no drug). After 3 months on the diet, mice were lean and had normal glucose levels, thus providing a suitable setting to determine putative, direct effects of adiponectin on lipid metabolism and cardiovascular endpoints without confounding hyperglycemia (**Table 1**; no drug). The lack of adiponectin in *Ldlr/Adn*$^{-/-}$ double knockout mice had no effect on total cholesterol, triglyceride and HDL cholesterol levels and did not alter lipoprotein distribution or particle size as compared to *Ldlr*$^{-/-}$ mice (**Table 1, Suppl. Table 1**). This observation is in line with previous data from *Ldlr*$^{+/+}$*/Adn*$^{-/-}$ single knockout mice (without concomitant disruption of the *LDLR* locus). Lipoprotein particle distribution of *Adn*$^{-/-}$ mice is comparable to wild type C57Bl/6J mice on regular chow (**Suppl. Fig. 1**).

To assess the influence of adiponectin on the vasculature, we determined the progression of atherosclerosis at the aortic root as well as in the brachiocephalic branch of the aorta (BCA). Lesions were observed underneath most of the valve leaflets and near the aortic branches whereas macroscopic lesions were generally absent from the thoracic or abdominal aorta. As shown in Figure 1, the lesion area within the aortic root (**Fig. 1A**) and the BCA (**Fig. 1B**) was not affected by the presence or absence of adiponectin (no drug; *Ldlr*$^{-/-}$ vs. *Ldlr/Adn*$^{-/-}$). This result indicated that adiponectin does not inhibit the atherogenic process, at least not under the dietary conditions chosen. To exclude the possibility that this may be unique to the *Ldlr*$^{-/-}$ mice, we also used apolipoprotein E knockout (*Apoe*$^{-/-}$) mice, but observed a similar results. *Apoe*$^{-/-}$ mice develop widespread lesions throughout the aorta while on regular chow (Getz and Reardon 2006). Similar to the results in the *Ldlr*$^{-/-}$ mice, adiponectin deficiency had no influence on size or distribution of atherosclerotic lesions in *Apoe*$^{-/-}$ mice (data not shown).

**Pharmacological induction of adiponectin by PPARγ agonism does not improve atherosclerosis in lean mice**

PPARγ agonists have been evaluated in various long term human studies for potential cardiovascular benefits with mixed results (Home, Pocock et al. 2007; Nissen and Wolski 2007). Pharmacological activation of PPARγ is the single most potent method to induce the secretion and to elevate plasma adiponectin levels, particularly the high molecular weight form (Pajvani, Hawkins et al. 2003). Selective PPARγ ligands of the thiazolidinedione class (troglitazone and rosiglitazone) have been shown to inhibit the development of atherosclerotic lesions in *Ldlr*$^{-/-}$ under diabetic as well as non diabetic conditions (Li, Brown et al. 2000; Collins, Meehan et al. 2001). To assess whether these effects may be secondary to an up-regulation of adiponectin we

administered a PPARγ selective, non-thiazolidinedione agonist (COOH) at 30 mg/kg body weight per day *in feed*. As expected, COOH treatment increased plasma adiponectin levels approximately four fold over basal (**Table 1, Suppl. Fig. 2**). Furthermore, we confirmed that COOH treatment elicited a robust signature of hepatic PPARγ-responsive genes when analyzed on microarrays (Nawrocki and Scherer, unpublished data).

Quantification of the lesion area revealed no difference within the aortic root while the average area of lesions in the BCA was slightly, but significantly *increased* upon COOH treatment (**Fig 1A and 1B**, *Ldlr*$^{-/-}$; no drug vs. COOH). This effect was not seen in adiponectin null mice (**Fig. 1 A and 1B**, *Ldlr/Adn*$^{-/-}$; COOH) suggesting that neither TZD treatment *per se* nor the associated pharmacologically elevated levels of adiponectin inhibit atherogenesis. Under these conditions (i.e. non-diabetogenic, low fat diet) COOH treatment had no effect on overall plasma glucose levels or body weight. All groups gained similar amounts of body weight (average weight gain 9.9 ± 1.3 grams) over the course of the study. COOH treatment did not affect total and HDL cholesterol levels while triglycerides were slightly elevated at the end of the study (**Table 1**, COOH).

**PPARγ activation causes left ventricular hypertrophy which is attenuated in adiponectin deficient mice**

Activation of PPARγ has been shown to increase heart weight in rodents. This may be a consequence of plasma volume expansion and consequent pressure overload (Arakawa, Ishihara et al. 2004; Nesto, Bell et al. 2004). It also has been proposed that adiponectin plays a protective role in ischemia/reperfusion injury in the heart and may affect cardiac remodeling after pressure overload (Shibata, Ouchi et al. 2004; Shibata, Sato et al. 2005). To elucidate whether adiponectin

is involved in cardiac remodeling, we analyzed the cardiovascular system using non-invasive, high resolution MRI in male *Ldlr*$^{-/-}$ and *Ldlr/Adn*$^{-/-}$ mice after chronic activation of PPARγ. Imaging of the hearts revealed an increase in the left ventricle (LV) volume in response to COOH treatment (**Fig. 2A**). Interestingly, in the absence of adiponectin, the increase in LV volume was attenuated (**Fig. 2B**) suggesting that adiponectin is involved, at least in part, in the PPARγ induced cardiac remodeling. The increase in LV volume coincided with an increase in heart weight (**Fig. 2C**). No alterations were found in the right ventricular volume or the thickness of the ventricle. We also examined sections of the heart with histopathological methods but could not establish a correlation with the degree of fibrosis or the infiltration of inflammatory cells with adiponectin.

**Adiponectin deficiency does not accelerate the development of atherosclerosis concomitant with the metabolic syndrome.**

Based on our initial studies, we concluded that the manifestation of several aspects of the metabolic syndrome, i.e. insulin resistance, increased adiposity and dyslipidemia may be necessary to unravel a putative role of adiponectin in the atherogenic process. This assumption is in line with the fact that genetic ablation of adiponectin causes a very mild phenotype unless mice are metabolically challenged with a high fat diet. In addition, circulating adiponectin levels have been shown to correlate negatively with adiposity (Arita, Kihara et al. 1999). We hypothesized that adiponectin may act indirectly through its overall effects on metabolic and inflammatory parameters. To address this question, we challenged *Ldlr/Adn*$^{-/-}$ mice with a Western style diet (WD) containing high levels of fat, sugar and cholesterol (42% kcal from fat, 42% kcal from carbohydrates and 0.2% cholesterol). In this study we included female mice since

plasma adiponectin levels are several fold higher in female compared to male mice. After 4 months on WD, all mice were obese and hyperglycemic (**Table 2**). Both genders developed severe hypercholesterolemia and hyper-triglyceridemia independent of adiponectin levels and lipoprotein profiles were indistinguishable between *Ldlr/Adn*$^{-/-}$ and *Ldlr*$^{-/-}$ mice (data not shown). Adiponectin levels tended to be lower at the end of the study consistent with an obesity associated suppression of adiponectin secretion (**Table 2**, *Ldlr*$^{-/-}$; week 0 vs. week 16, n.s.).

We assessed the extent of atherosclerosis in the aorta and the aortic root by different analytical methods. First, accumulation of lipids in the aortic wall was quantified by the measurement of the cholesterol content in the aorta excised distal to the aortic root to the right renal artery. This lipid extraction method has been validated previously and shown to correlate well with the assessment of atherosclerotic lesions by en face lipid staining or the measurement of the ratio of intima/media thickness (Sparrow, Burton et al. 2001). The advantage of such measurements is that surface area as well as volume and distribution of lesions are taken into account by including cholesterol depositions that may be undetectable by optical methods. Despite of the additional challenge to the vasculature posed by the increased body weight and elevated basal glucose levels, we did not find a difference in total cholesterol, free cholesterol and cholesterol ester content in aortas from *Ldlr/Adn*$^{-/-}$ mice compared to *LDLR*$^{-/-}$ mice (**Fig. 3A and B**). The relative cholesterol content in the aorta was higher in male than in female mice.

In a separate cohort, we assessed cross-sectional lesion area in the aortic roots as well as necrotic lesion area in both female and male mice (**Fig. 3C**). Note that plaque necrosis is associated with vulnerable plaques and culprit lesions in humans (Virmani, Burke et al. 2006). None of the measurements revealed a correlation between the adiponectin genotype and

morphology of atherosclerotic lesions. We concluded that a) adiponectin does not directly affect the process of atherogenesis, b) adiponectin suppresses an atherothrombotic process, such as plaque rupture or lumenal thrombosis, that is not a component of these murine models (Rosenfeld, Carson et al. 2002) or c) a role of adiponectin may be masked by compensatory mechanisms that could have evolved as a consequence of a germ line deletion of adiponectin (Davis and Scherer 2008; Wong, Krawczyk et al. 2008).

**Elevated adiponectin levels do not prevent atherosclerosis.**

We have shown previously, that overexpression leads to a significant improvement of lipid metabolism (Kim, van de Wall et al. 2007). The transgenic expression of an adiponectin deletion mutant ($Adn^{Tg}$) leads to a moderate increase of steady state adiponectin concentrations in the circulation by approximately 2- to 3-fold over baseline, similar to levels achieved by activation of PPARγ (Combs, Pajvani et al. 2004; Kim, van de Wall et al. 2007). Adiponectin over-expression increases lipid clearance and lipoprotein lipase activity coinciding with an improved suppression of endogenous glucose production. In an *ob/ob* background, adiponectin overexpression caused a complete reversal of the diabetic phenotype along with the correction of dyslipidemia and liver steatosis. This observation is striking in light of the fact that these mice were morbidly obese and displayed significantly higher amounts of adipose tissue than their *ob/ob* littermates (Kim, van de Wall et al. 2007). To study effects of adiponectin overexpression on atherogenesis, we crossed $Adn^{Tg}$ mice into the $Ldlr^{-/-}$ model ($Ldlr/Adn^{Tg}$) and characterized the metabolic profile as well as cardiovascular effects after 4 months on a Western style diet. Body weight, total adiponectin and fasting glucose levels are shown in Table 3. Adiponectin levels had no effect on body weights in this study (**Table 3**). On average, males gained 16.5 ±

0.9 grams and females 11.4 ± 0.8 grams of body mass within 4 months on the Western diet. Adiponectin levels were 3 to 3.5 fold higher in *Ldlr/Adn*$^{Tg}$ compared to *Ldlr*$^{-/-}$ mice. Total circulating adiponectin was slightly higher in females, but not in males, after 4 months of Western diet (**Table 3**).

Fasting glucose was significantly lower in *Ldlr/Adn*$^{Tg}$ mice as compared to *Ldlr*$^{-/-}$ mice at the beginning and at the end of the dietary intervention (**Table 3**). The improved basal glucose levels correlated with an improved response to an oral glucose challenge (oral glucose tolerance test, OGTT) (**Fig. 4A** females, **Fig. 4B** males). Area under the curve for the glucose excursion showed that *Ldlr*$^{-/-}$ were mildly glucose intolerant and that this defect was significantly improved by overexpression of adiponectin.

With respect to the lipid profile, we found that adiponectin overexpression caused a significant reduction of total cholesterol as well as plasma triglyceride in both females and males (**Fig. 5A and 5B**). At the end of the study, plasma from each group was pooled and analyzed by size exclusion chromatography (**Fig. 5C and 5D;** females, **Fig. 5E and 5F;** males). The lipoprotein profile revealed reduced triglyceride (**Fig. 5C and 5E**) and cholesterol (**Fig. 5D and 5F**) levels in all fractions from both female and male *Ldlr/Adn*$^{Tg}$ mice when compared to *Ldlr*$^{-/-}$ mice. As expected, reductions in cholesterol were accounted for mostly by VLDL and IDL/LDL fractions while triglycerides were reduced in VLDL fractions of *Ldlr/Adn*$^{Tg}$ groups.

As above, we evaluated the amount of atherosclerosis by the accumulation of cholesterol and cholesterol esters in the aorta distal to the aortic root to the right renal artery. Once again, elevation of adiponectin levels did not affect the accumulation of cholesterol in the aortic wall (**Fig. 6A** females, **Fig. 6B** males). These results were confirmed by assessment of lesion area

within sections of the aortic root quantified from digital images of hearts (n= 9) per group after staining with Oil red O (**Fig. 6C**). The extent of atherosclerosis within the aortic root was independent of the amount of adiponectin present in both female and male mice (**Fig. 6**, *Ldlr/Adn*$^{Tg}$ vs. *Ldlr*$^{-/-}$). Our data demonstrate that adiponectin has no effect on the formation of lesions despite its beneficial effects on lipid clearance and plasma lipids. In *Ldlr*$^{-/-}$ mice, neither genetic ablation nor chronic elevation of adiponectin correlates with amount or distribution of atherosclerotic lesions.

**Discussion**

The role of adiponectin in cardiovascular disease has been met with considerable interest and examined in a large number of epidemiological studies. Based on these results, a picture has emerged suggesting that high adiponectin levels are highly predictive of a lower incidence of cardiovascular disease in the general population. However, when examined at later stages of cardiovascular disease, high adiponectin levels are indicative of an increased rate of mortality, including mortality from cardiovascular disease (Cavusoglu, Ruwende et al. 2006). This may reflect a compensatory upregulation of a beneficial factor in light of a generally disadvantageous cardiovascular environment. However, the detailed mechanistic connection between late stage cardiovascular disease and upregulation of adiponectin are far from being understood. Underlying reasons for an increase of adiponectin levels at steady state are an increased production rate in adipose tissue, a decreased clearance rate or the combination of the two. We have recently demonstrated that all three possibilities can occur under different physiological conditions (Halberg and Scherer, Diabetes, *in press*).

A number of studies in preclinical models suggest that adiponectin is more than an innocent bystander and convenient marker for cardiovascular integrity. Earlier studies in rodents have suggested an association between low adiponectin and vascular disease particular in acute models of vascular injury such as vascular thickening after cuff-injury (Kubota, Terauchi et al. 2002). Overexpression of a globular form of adiponectin from the liver caused a reduction of atherosclerotic lesion formation (Yamauchi, Kamon et al. 2003). This is consistent with experiments performed *in vitro* using the globular form of adiponectin which suggested a potent anti-inflammatory role. More recently, Libby et al. demonstrated that globular adiponectin

suppressed lipopolysaccharide-induced cytokine release in macrophages and suppressed T-lymphocyte accumulation in atherogenesis in *ApoE*$^{-/-}$ mice (Okamoto, Folco et al. 2008). Prior to this study, the role adiponectin has not been addressed in the less severe *Ldlr*$^{-/-}$ model. Similar to humans carrying a deficient LDL receptor allele due to spontaneous mutations, mice with LDL receptor deficiency have mild hypercholesterolemia and therefore provide an effective small-animal model for the study of atherosclerosis (Ishibashi, Brown et al. 1993). These mice generally manifest an increase in intermediate density lipoproteins (IDL) and LDL without a significant change in HDL levels and plasma triglycerides. Atherosclerosis develops when fed a high fat diet and the rate and extent of disease progression can be manipulated by varying the dietary lipid content. The *Ldlr*$^{-/-}$ rodent model differs from the *ApoE*$^{-/-}$ model which is characterized by spontaneous and rapid formation of lesions even on a standard chow diet. In our studies we used diets that cause substantial hypercholesterolemia but differentially affect the degree of insulin resistance. Surprisingly, while we detected the expected differential susceptibility to metabolic challenges with respect to insulin sensitivity, we failed to detect differences in atherosclerotic read outs in both genetic adiponectin gain- and loss-of-function mice. The discrepancies between our observations in *Ldlr*$^{-/-}$ mice and the data reported from *ApoE*$^{-/-}$ mice are not clear but may be explained by physiological differences between these models.

We have demonstrated previously that adiponectin correlates positively with improved dyslipidemia in mice (Kim, van de Wall et al. 2007). *Adn*$^{Tg}$ mice have dramatically reduced total and VLDL triglycerides and LDL and HDL cholesterol. In contrast, we observed that the lipoprotein profile in the absence of adiponectin in *Adn*$^{-/-}$ mice is unaltered when fed a chow diet.

Here, we demonstrate that even in the presence of severe hypercholesterolemia in the *Ldlr*$^{-/-}$ mouse, overexpression of adiponectin in the *Ldlr/Adn*$^{Tg}$ causes a significant improvement of the dyslipidemia (Figure 5). However, it is important to note that despite of the beneficial effects of adiponectin, plasma lipids levels remain very high and are within a pathophysiological range in both *Ldlr/Adn*$^{Tg}$ and *Ldlr*$^{-/-}$ mice when compared to lean and healthy wild type mice (Supplemental Figure 1). We conclude that adiponectin plays an important role in the regulation of plasma lipids, however the severity of hypercholesterolemia and hypertriglyceridemia in the *Ldlr*$^{-/-}$ model hampers the exploration of a role of adiponectin in the atherogenic process.

By the same token we found that pharmacological activation of PPARγ in consequence chronic elevation of circulating adiponectin levels had no inhibitory effect on atherosclerosis. In fact, we found a small but significant increase in lesion area in the brachioscephalic artery upon treatment with COOH. It is well established that PPARγ agonism has anti-inflammatory effects. Furthermore, deletion of PPARγ specifically in macrophages is sufficient to markedly increase lesion area in LDL receptor knockout mice supporting the notion that activation of this receptor may reduce atherosclerosis. Studies with structurally distinct classes of PPARγ agonists led to somewhat contradicting results, although in most, but not all of these studies, the outcome suggested a beneficial effect (Li, Brown et al. 2000; Collins, Meehan et al. 2001; Thorp, Kuriakose et al. 2007). In most of these studies, reduced lesion area coincided with an improvement of overall metabolic parameters. A meta-analysis of clinical outcome studies in humans indicated that rosiglitazone treatment may be associated with an increased risk of myocardial infarction (Psaty and Furberg 2007). However, a very recent study concluded that while rosiglitazone as a glucose-lowering therapy in people with type 2 diabetes is indeed

increasing the risk of heart failure it does not increase the risk of overall cardiovascular morbidity or mortality compared with standard glucose-lowering drugs (Home, Pocock et al. 2009). Many clinical studies clearly established a negative correlation between adiponectin levels and the incidence of myocardial infarction. Our goal was to minimize indirect effects due improvements of overall metabolic parameters by adiponectin or due to chronic treatment with PPARγ agonists. We chose to study lean, insulin sensitive mice in the hope of dissociating direct actions adiponectin on the vascular wall. To this end, we chose a non-diabetogenic, low fat diet. Notably, we did not observe any signs of inhibition of atherosclerosis, despite a large number of samples, upon treatment with the PPARγ agonist COOH. The variability among rodent data once again underlines the shortcomings of the LDL receptor mouse model in reproducing associations that have been established in humans.

Mice are generally resistant to plaque rupture and myocardial infarction. In our studies, the levels of adiponectin did not correlate with necrotic core size or the acellular area within lesions in the aortic root in our studies. Separate from atherosclerosis *per se*, obesity is also associated with cardiac remodeling. In rodents, adiponectin has been implicated in vasoprotection particularly in models of acute vascular injury, pressure overload and ischemia (Ouchi, Shibata et al. 2006). It has been shown that adiponectin attenuates cardiac hypertrophy and fibrosis in response to a cardiac stressor in angiotensin II treated mice. Here we demonstrate that PPARγ activation causes cardiac hypertrophy, concomitant with an increase in the LV volume and heart to body weight ratio. The TZD-induced hypertrophy is attenuated in adiponectin deficient mice, highlighting a possible direct involvement of adiponectin in this

process as well. Indeed, the positive result in this component of our study serves to further highlight the *lack* of an effect of adiponectin on atherosclerotic lesion size or progression.

## Methods

### Animals and Diets

Animals were maintained in a pathogen free facility and all experimental protocols were approved by the Institutes for Animal Studies of the Albert Einstein College of Medicine and the University of Texas Southwestern Medical Center. Mice were group housed and on alternating 12-hour light and dark cycles under controlled environmental conditions (22-25ºC, 40 -50% humidity) with free access to food and water. Adiponectin knockout mice were generated as described previously (Nawrocki, Rajala et al. 2006) and backcrossed for at least 6 generations onto a C57BL/6J background in all studies with exception of the first study for which the mice were backcrossed 5 times. Adiponectin transgenic mice ($Adn^{Tg}$) were generated as described by Combs et al. (Combs, Pajvani et al. 2004). Both strains were crossbred with C57BL/6J-$Ldlr^{tm1Her}$, stock No. 002207 (Jackson Laboratory, Bar Harbor, Me). Subsequently, double het ($Adn^{+/-}/Ldlr^{+/-}$) offspring were intercrossed to obtain $Ldlr/Adn^{-/-}$ double knockout mice. $Ldlr^{-/-}/Adn^{Tg}$ mice were generated by crossing $Adn^{Tg}$ males containing a single transgene with $Ldlr^{-/-}$ females and subsequently by crossing $Adn^{Tg}/Ldlr^{+/-}$ males with $Ldlr^{-/-}$ males. Mice were genotyped as described before (Nawrocki, Rajala et al. 2006; Kim, van de Wall et al. 2007). All experiments were performed with littermate control groups. Eight week old mice were placed on various diets as described in results: (1) Clinton-Cybulsky diet (Lichtman, Clinton et al. 1999) (Reseach Diets D00110804), (2) Western style diet (Harlan Teklad 88137). The PPARγ agonist, 2-(2-(4-phenoxy-2-propylphenoxy)ethyl)indole-5-acetic acid (COOH) was a kind gift from Merck Research Laboratories (Rahway, NJ).

### Plasma analysis

Plasma glucose and triglyceride determinations were performed from heparinized tail blood using glucose oxidase assay kits (FastBlue B, Sigma-Aldrich). Basal glucose was measured using glucoanalyzer blood glucose strips (MediSense Precision Xtra, Abbott Laboratories, Abbott Park, IL). For oral glucose tolerance tests mice were fasted for 4 hours before given an oral glucose load of 2.5 g glucose/kilogram body weight using a solution of 10% glucose. Blood was drawn at the indicated times and glucose concentrations were measured as above. Access to food was denied during the course of the study. Adiponectin was measured using a rat adiponectin RIA kit (Millipore). Levels of total cholesterol and triglycerides and HDL cholesterol were measured with standard enzymatic assays from tail bleeds (Infinity, Cholesterol and Triglyceride kit; Thermo Scientific, HDL-C kit from Wako Chemicals). Lipoprotein subclass profiles were measured by Liposcience Inc. using nuclear magnetic resonance spectroscopy. For lipoprotein analyses, EDTA plasma aliquots of blood collected by cardiac puncture from anesthetized mice were stored at 4°C until separation. Fifty microliters of EDTA plasma was pooled from 5 mice per group and 200 microliters were separated via FPLC size exclusion chromatography on a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech). The column was equilibrated with PBS and run at a flow rate of 0.2 mL/min and 0.25 ml fractions were collected. Each fraction was assayed for cholesterol and triglycerides.

**Aortic Cholesterol Measurements**

After collection of blood by cardiac puncture, the vasculature was gently perfused through the left ventricle with cold PBS and 3 mmol/L EDTA. Any branches and adipose tissue connected to the aorta was removed carefully and each aorta was excised distal to the aortic root to the right renal artery. The aortas were stored briefly on ice in PBS and then blotted dry, weighed, minced,

and extracted with chloroform/methanol (2:1) according to the method of Folch et al. (Lees, Folch et al. 1959). The lipid extracts were prepared as described by (Sparrow, Burton et al. 2001). Extracts were dried down, resuspended quantitatively in chloroform/methanol (2:1), and stored at -20°C until the time of assay. Total and free cholesterol levels in the aortic extracts were determined with by enzymatic fluorometric assays. Briefly, the solvent was evaporated from aliquots containing 1 to 16 nmol of cholesterol, and the lipid residue was resolubilized in 100 µL of reagent grade ethanol. Aliquots of cholesterol (Sigma-Aldrich) and cholesteryl oleate (Sigma-Aldrich) standard solutions prepared in chloroform/methanol (1:1) were treated similarly. To determine free cholesterol, samples and standards were incubated for 1 hour at 37°C in a total volume of 1.01 mL of 0.1 mol/L potassium phosphate buffer, pH 7.4, containing 0.03% Triton X-100 and 0.9 mmol/L sodium cholate. Cholesterol oxidase (0.18 U; Boehringer Mannheim), peroxidase (2 U; Boehringer Mannheim), and *p*-hydroxyphenylacetic acid (0.5 mg/mL; Aldrich) were added for an additional 1-hour incubation at 37°C. The fluorescent product was measured in a Spex FluoroMax (SPEX Industries, Inc) (excitation 325 nm, emission 415 nm) with acrylic UVT semimicrocuvettes (Evergreen Scientific). For total cholesterol determinations, cholesterol esterase (10 U; Calbiochem) was included in the first incubation step, and cholesteryl oleate was used as a standard. The cholesteryl ester in each sample was calculated by subtracting the value of free cholesterol from that for total cholesterol. Samples for each aorta were run in duplicate at 2 different concentrations. All values are expressed as nmol/mg wet tissue weight.

**Tissue preparation and morphometric analysis of lesions**

Atherosclerosis was determined at the aortic origin as well as in the brachiocephalic branch of the aorta. To quantify lesion area in the aortic root, formalin-fixed hearts were processed as

previously described (Teupser, Persky et al. 2003). Briefly, serial 12 micron sections were cut from the origin of the aortic valve leaflets, throughout the aortic sinus and stained with oil red O and counter-stained with hematoxylin. Mean lesion area was calculated from the analysis of digital images obtained from 5 sections/mouse, using the Image Pro Plus software. To quantify cross-sectional lesion area in the brachiocephalic artery, the Y-shaped piece of brachiocephalic artery was frozen in OCT and sectioned distal to proximal at 10 μm thickness, starting from the subclavian and carotid arteries. Digital images were taken from 6 equidistant (100-μm) oil red O stained sections located 200 to 700 μm from the branching point of the brachiocephalic into the carotid and subclavian arteries. Atherosclerotic lesions lumenal to the internal elastic lamina were quantified on one section per slide. Lesion size was calculated as the mean lesion area of six sections. Plaque necrosis was quantified by measuring the area of hematoxylin and eosin-negative acellular areas in the intima, as described previously (Feng, Zhang et al. 2003). Boundary lines were drawn around these regions, and the area measurements were obtained using Image-Pro-Plus software (version 3.0; Mediacybernetics). Differences between groups were assessed using *t*-test analyses.

**Magnetic Resonance Imaging**

MRI measurements were performed using a Omega 9.4T vertical bore nuclear magnetic resonance spectrometer (GE, Fremont, CA) and a 40 mm inner diameter-60 mm long quadrature birdcage imaging coil (RF Sensors, LLC) (de Souza, Tang et al. 2005).

Mice were anesthetized with isoflurane inhalation anesthesia (2% in medical air) and a set of standard electrocardiographic (ECG) leads was attached to the mouse limbs. The ECG signal was fed to a Gould ECG amplifier linked to the Omega system and a PC. Mice were positioned

head-up in the imaging coil and temperature within the gradient coils was maintained at 25-30$^{o}$C with a NESLAB gradient water cooling system, to prevent hypothermia. Heart rate and ECG were monitored continuously and used as the gating signal triggering the MR spectrometer acquisition, as previously described (Durand, Tang et al. 2006). A routine cardiac gated multi-slice (90° sinc pulse) spin echo sequence was employed. An echo time (TE) of 14 ms and repetition time (TR) of approx. 100-200 ms (depending on heart rate) were used for short axis images of the heart, with a 40 mm field of view (FOV) and a 128 x 256 matrix size (interpolated to 256 x 256 pixels). As previously reported, we used a spin echo sequence (De Souza, Cohen et al. 2005; Durand, Tang et al. 2006), which gives very good contrast between blood and heart wall muscle. Mouse cardiac images were analyzed with MATLAB-based software and heart dimensions were measured by centerline analysis (Durand, Tang et al. 2006). The diastolic LV mass was calculated by using an ellipsoid approximation as described before (Siri, Jelicks et al. 1997).

**Statistical Analyses**

Statistical analysis was performed by using non-parametric t-test (Mann-Whitney test) to determine differences between individual group means. MRI data were compared by using the single factor ANOVA test.

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**References**

Abel, E. D., S. E. Litwin, et al. (2008). "Cardiac remodeling in obesity." Physiol Rev **88**(2): 389-419.

Arakawa, K., T. Ishihara, et al. (2004). "An antidiabetic thiazolidinedione induces eccentric cardiac hypertrophy by cardiac volume overload in rats." Clin Exp Pharmacol Physiol **31**(1-2): 8-13.

Arita, Y., S. Kihara, et al. (1999). "Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity." Biochem Biophys Res Commun **257**(1): 79-83.

Berg, A. H. and P. E. Scherer (2005). "Adipose tissue, inflammation, and cardiovascular disease." Circ Res **96**(9): 939-49.

Cavusoglu, E., C. Ruwende, et al. (2006). "Adiponectin is an independent predictor of all-cause mortality, cardiac mortality, and myocardial infarction in patients presenting with chest pain." Eur Heart J **27**(19): 2300-9.

Collins, A. R., W. P. Meehan, et al. (2001). "Troglitazone inhibits formation of early atherosclerotic lesions in diabetic and nondiabetic low density lipoprotein receptor-deficient mice." Arterioscler Thromb Vasc Biol **21**(3): 365-71.

Combs, T. P., U. B. Pajvani, et al. (2004). "A transgenic mouse with a deletion in the collagenous domain of adiponectin displays elevated circulating adiponectin and improved insulin sensitivity." Endocrinology **145**(1): 367-83.

Davis, K. E. and P. E. Scherer (2008). "Adiponectin: no longer the lone soul in the fight against insulin resistance?" Biochem J **416**(2): e7-9.

De Souza, A. P., A. W. Cohen, et al. (2005). "MR imaging of caveolin gene-specific alterations in right ventricular wall thickness." Magn Reson Imaging **23**(1): 61-8.

de Souza, A. P., B. Tang, et al. (2005). "Magnetic resonance imaging in experimental Chagas disease: a brief review of the utility of the method for monitoring right ventricular chamber dilatation." Parasitol Res **97**(2): 87-90.

Durand, J. L., B. Tang, et al. (2006). "Dyskinesis in Chagasic myocardium: centerline analysis of wall motion using cardiac-gated magnetic resonance images of mice." Magn Reson Imaging **24**(8): 1051-7.

Feng, B., D. Zhang, et al. (2003). "Niemann-Pick C heterozygosity confers resistance to lesional necrosis and macrophage apoptosis in murine atherosclerosis." Proc Natl Acad Sci U S A **100**(18): 10423-8.

Getz, G. S. and C. A. Reardon (2006). "Diet and murine atherosclerosis." Arterioscler Thromb Vasc Biol **26**(2): 242-9.

Home, P. D., S. J. Pocock, et al. (2009). "Rosiglitazone evaluated for cardiovascular outcomes in oral agent combination therapy for type 2 diabetes (RECORD): a multicentre, randomised, open-label trial." Lancet **373**(9681): 2125-35.

Home, P. D., S. J. Pocock, et al. (2007). "Rosiglitazone evaluated for cardiovascular outcomes--an interim analysis." N Engl J Med **357**(1): 28-38.

Hotta, K., T. Funahashi, et al. (2000). "Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients." Arterioscler Thromb Vasc Biol **20**(6): 1595-9.

Ishibashi, S., M. S. Brown, et al. (1993). "Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery." J Clin Invest **92**(2): 883-93.

Kim, J. Y., E. van de Wall, et al. (2007). "Obesity-associated improvements in metabolic profile through expansion of adipose tissue." J Clin Invest **117**(9): 2621-37.

Kubota, N., Y. Terauchi, et al. (2002). "Disruption of adiponectin causes insulin resistance and neointimal formation." J Biol Chem **277**(29): 25863-6.

Kubota, N., Y. Terauchi, et al. (2002). "Disruption of adiponectin causes insulin resistance and neointimal formation." J Biol Chem **277**(29): 25863-25866.

Lees, M., J. Folch, et al. (1959). "A simple procedure for the preparation of brain sulphatides." J Neurochem **4**(1): 9-18.

Li, A. C., K. K. Brown, et al. (2000). "Peroxisome proliferator-activated receptor gamma ligands inhibit development of atherosclerosis in LDL receptor-deficient mice." J Clin Invest **106**(4): 523-31.

Lichtman, A. H., S. K. Clinton, et al. (1999). "Hyperlipidemia and atherosclerotic lesion development in LDL receptor-deficient mice fed defined semipurified diets with and without cholate." Arterioscler Thromb Vasc Biol **19**(8): 1938-44.

Menzaghi, C., V. Trischitta, et al. (2007). "Genetic influences of adiponectin on insulin resistance, type 2 diabetes, and cardiovascular disease." Diabetes **56**(5): 1198-209.

Nawrocki, A. R., M. W. Rajala, et al. (2006). "Mice lacking adiponectin show decreased hepatic insulin sensitivity and reduced responsiveness to peroxisome proliferator-activated receptor gamma agonists." J Biol Chem **281**(5): 2654-60.

Nesto, R. W., D. Bell, et al. (2004). "Thiazolidinedione use, fluid retention, and congestive heart failure: a consensus statement from the American Heart Association and American Diabetes Association." Diabetes Care **27**(1): 256-63.

Nissen, S. E. and K. Wolski (2007). "Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes." N Engl J Med **356**(24): 2457-71.

Okamoto, Y., E. J. Folco, et al. (2008). "Adiponectin inhibits the production of CXC receptor 3 chemokine ligands in macrophages and reduces T-lymphocyte recruitment in atherogenesis." Circ Res **102**(2): 218-25.

Ouchi, N., R. Shibata, et al. (2006). "Cardioprotection by adiponectin." Trends Cardiovasc Med **16**(5): 141-6.

Pajvani, U. B., M. Hawkins, et al. (2003). "Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione -mediated improvement in insulin sensitivity." J Biol Chem.

Pajvani, U. B., M. Hawkins, et al. (2004). "Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione -mediated improvement in insulin sensitivity." J Biol Chem **279**(13): 12152-12162.

Patel, C., K. L. Wyne, et al. (2005). "Thiazolidinediones, peripheral oedema and congestive heart failure: what is the evidence?" Diab Vasc Dis Res **2**(2): 61-6.

Pischon, T., C. J. Girman, et al. (2004). "Plasma adiponectin levels and risk of myocardial infarction in men." Jama **291**(14): 1730-7.

Psaty, B. M. and C. D. Furberg (2007). "Rosiglitazone and cardiovascular risk." N Engl J Med **356**(24): 2522-4.

Rosenfeld, M. E., K. G. Carson, et al. (2002). "Animal models of spontaneous plaque rupture: the holy grail of experimental atherosclerosis research." Curr Atheroscler Rep **4**(3): 238-42.

Scherer, P. E. (2006). "Adipose tissue: from lipid storage compartment to endocrine organ." Diabetes **55**(6): 1537-45.

Shetty, S., C. M. Kusminski, et al. (2009). "Adiponectin in health and disease: evaluation of adiponectin-targeted drug development strategies." Trends Pharmacol Sci **30**(5): 234-9.

Shibata, R., N. Ouchi, et al. (2004). "Adiponectin-mediated modulation of hypertrophic signals in the heart." Nat Med **10**(12): 1384-9.

Shibata, R., K. Sato, et al. (2005). "Adiponectin protects against myocardial ischemia-reperfusion injury through AMPK- and COX-2-dependent mechanisms." Nat Med **11**(10): 1096-103.

Siri, F. M., L. A. Jelicks, et al. (1997). "Gated magnetic resonance imaging of normal and hypertrophied murine hearts." Am J Physiol **272**(5 Pt 2): H2394-402.

Sparrow, C. P., C. A. Burton, et al. (2001). "Simvastatin has anti-inflammatory and antiatherosclerotic activities independent of plasma cholesterol lowering." Arterioscler Thromb Vasc Biol **21**(1): 115-21.

Stafylas, P. C., P. A. Sarafidis, et al. (2009). "The controversial effects of thiazolidinediones on cardiovascular morbidity and mortality." Int J Cardiol **131**(3): 298-304.

Takemura, Y., K. Walsh, et al. (2007). "Adiponectin and cardiovascular inflammatory responses." Curr Atheroscler Rep **9**(3): 238-43.

Teupser, D., A. D. Persky, et al. (2003). "Induction of Atherosclerosis by Low-Fat, Semisynthetic Diets in LDL Receptor-Deficient C57BL/6J and FVB/NJ Mice: Comparison of Lesions of the Aortic Root, Brachiocephalic Artery, and Whole Aorta (En Face Measurement)." Arterioscler Thromb Vasc Biol **23**(10): 1907-1913.

Thorp, E., G. Kuriakose, et al. (2007). "Pioglitazone increases macrophage apoptosis and plaque necrosis in advanced atherosclerotic lesions of nondiabetic low-density lipoprotein receptor-null mice." Circulation **116**(19): 2182-90.

Virmani, R., A. P. Burke, et al. (2006). "Pathology of the vulnerable plaque." J Am Coll Cardiol **47**(8 Suppl): C13-8.

Wong, G. W., S. A. Krawczyk, et al. (2008). "Molecular, biochemical and functional characterizations of C1q/TNF family members: adipose-tissue-selective expression patterns, regulation by PPAR-gamma agonist, cysteine-mediated oligomerizations, combinatorial associations and metabolic functions." Biochem J **416**(2): 161-77.

Yamauchi, T., J. Kamon, et al. (2003). "Globular adiponectin protected ob/ob mice from diabetes and ApoE-deficient mice from atherosclerosis." J Biol Chem **278**(4): 2461-8.

Zadelaar, S., R. Kleemann, et al. (2007). "Mouse models for atherosclerosis and pharmaceutical modifiers." Arterioscler Thromb Vasc Biol **27**(8): 1706-21.

Zhang, M. H., C. Spies, et al. (2009). "Adiponectin and inducible ischemia in patients with stable coronary heart disease: data from the Heart and Soul study." Atherosclerosis **205**(1): 233-8.

**Figure Legends**

**Figure 1**

Adiponectin deficiency has no effect on atherosclerosis in male *Ldlr*$^{-/-}$mice on a non-diabetogenic diet with high levels of cholesterol. Atherosclerosis in *Ldlr*$^{-/-}$ and in *Ldlr/Adn*$^{-/-}$ mice fed a semisynthetic, low fat diet supplemented with 0.15% cholesterol for 3 months. A, Quantitative analysis of lesion area of sections from the aortic root at the level of the valves and B, lesion area within the brachiocephalic artery (BCA). Mice were left untreated (no drug) or were dosed with a selective PPARγ agonist (COOH) *in feed*. PPARγ agonist (COOH) treatment caused a significant increase in the lesion area within the BCA in *Ldlr*$^{-/-}$ mice compared to untreated mice (* $P<0.05$). No other significant differences were observed between groups. Group size n = 12 to 18.

**Figure 2**

*In vivo* cardiac magnetic resonance imaging (MRI) of the heart of anesthetized mice. A, abdominal view of the heart. The left ventricle is indicated by yellow quadrant. MRI was performed on *Ldlr*$^{-/-}$ and in *Ldlr/Adn*$^{-/-}$ with or without treatment with COOH. Data are normalized to average LV volume of no drug treatment group for each genotype and expressed as mean ± SEM. B, fold increase in LV volume in *Ldlr*$^{-/-}$ and *Ldlr/Adn*$^{-/--}$ male mice upon treatment with COOH compared to untreated mice. C, heart weight to body weight ratio. (* $P<0.05$, n = 12 to 18).

**Figure 3**

Adiponectin deficiency has no impact on atherosclerotic lesion area in male or female *Ldlr*$^{-/-}$ mice on a Western style diet. Quantification of atherosclerosis in *Ldlr*$^{-/-}$ and in *Ldlr/Adn*$^{-/-}$ mice fed a diet rich in lipids and carbohydrates for 4 months. Quantification of aortic cholesterol content in A, female and B, male mice. Total cholesterol, free cholesterol and cholesterol esters were measured from lipid extracts from aortas dissected at the right renal artery and proximal to the aortic root. C, Quantitative analysis of lesion area and the necrotic core area of sections from the aortic root at the level of the valves. Data means are shown. No significant differences were found within the groups (n = 11 to 14).

**Figure 4**

Overexpression of adiponectin improves glucose tolerance during oral glucose tolerance test. A, female and B, male *Ldlr/Adn*$^{Tg}$ and *Ldlr*$^{-/-}$ mice were fed a Western style diet for 4 months. Oral glucose tolerance test was performed after 4 hours of fasting. Circulating glucose was measured at times 0, 20, 40, 60, 120 and 180 minutes after an oral glucose challenge. Data are expressed as mean ± SEM at each time point and as area under the curve. (* $P<0.05$, n = 9 to 13)

**Figure 5**

Adiponectin reduces Plasma lipids and improves the plasma lipoprotein profile. A, Total cholesterol was measured in female and male *Ldlr/Adn*$^{Tg}$ and *Ldlr*$^{-/-}$ mice upon feeding of a Western style diet for 4 months. B, plasma triglycerides levels. (* $P<0.05$, n = 6). C – F, Lipoprotein profiles are significantly improved in *Ldlr/Adn*$^{Tg}$ mice compared to *Ldlr*$^{-/-}$mice. Plasma from 5 mice per group was pooled and subfractionated by gel filtration chromatography

and triglyceride and cholesterol was measured in each fraction. Triglyceride and cholesterol content in each fraction is shown from females (C, D) and males (E, F). VLDL, very low density lipoprotein, LDL/IDL, Intermediate and low density lipoprotein, HDL, high density lipoprotein.

**Figure 6**

Overexpression of adiponectin does not inhibit atherosclerosis.Quantification of aortic cholesterol content in A, female and B, male *Ldlr/Adn*$^{Tg}$ and *Ldlr*$^{-/-}$ mice. Total cholesterol, free cholesterol and cholesterol esters were measured from lipid extracts from aortas dissected at the right renal artery and proximal to the aortic root. No significant differences were observed between the groups (n = 7 to 11). C, Quantitative analysis of total lesion area in aortic root from both female and male mice (n = 3).

**Table 1**

| | *Ldlr*$^{-/-}$ *(n = 9)* | *Ldlr/Adn*$^{-/-}$ *(n = 12)* | *Ldlr*$^{-/-}$ *(n = 14)* | *Ldlr/Adn*$^{-/-}$ *(n = 14)* |
|---|---|---|---|---|
| | **No drug** | | **COOH** | |
| Adiponectin (μg/ml) | 8.5 ± 0.4 | | 38.0 ± 5.6* | |
| Weight (g) | 32.7 ± 1.1 | 31.8 ± 0.8 | 33.1 ± 0.7 | 31.8 ± 0.8 |
| Fasting Glucose (mg/dL) | 91.5 ± 7.0 | 83.5 ± 5.2 | 89.6 ± 6.7 | 99.6 ± 5.6 |
| Total Cholesterol (mg/dL) | 885 ± 54 | 898 ± 69 | 802 ± 54 | 812 ± 52 |
| Triglyceride (mg/dL) | 395 ± 29 | 404 ± 37 | 525 ± 31^ | 649 ± 57 |
| HDL-C (mg/dL) | 208 ± 26 | 179 ± 22 | 212 ± 15 | 184 ± 14 |

∗ *Ldlr*$^{-/-}$ *no drug vs. Ldlr*$^{-/-}$ COOH, $p < 0.001$
^ *Ldlr*$^{-/-}$ *no drug vs. Ldlr*$^{-/-}$ COOH, $p < 0.05$

**Table 2**

| | *Ldlr*$^{-/-}$ *(n = 11)* | *Ldlr/Adn*$^{-/-}$ *(n = 13)* | *Ldlr*$^{-/-}$ *(n = 14)* | *Ldlr/Adn*$^{-/-}$ *(n = 12)* |
|---|---|---|---|---|
| | **Males** | | **Females** | |
| Weight (g) | 45.7 ± 2.1 | 43.4 ± 2.5 | 32.2 ± 2.0 | 29.8 ± 1.5 |
| Fasting Glucose (mg/dL) | 200 ± 10 | 187 ± 8 | 192 ± 7 | 188 ± 15 |
| Adiponectin (μg/ml) | | | | |
| Week 0 | 10.7 ± 1.2 | n.d. | 16.4 ± 1.5 | n.d. |
| Week 16 | 7.9 ± 0.3 | n.d. | 13.8 ± 1.0 | n.d. |
| Total Cholesterol (mg/dL) | 1649 ± 177 | 1746 ± 156 | 1193 ± 62 | 1175 ± 114 |
| Triglyceride (mg/dL) | 564 ± 81 | 572 ± 81 | 256 ± 21 | 284 ± 63 |
| HDL-C (mg/dL) | 292 ± 13 | 298 ± 19 | 278 ± 13 | 274 ± 17 |

**Table 3**

| | *Ldlr*$^{-/-}$ | *Ldlr*$^{-/-}$/*Adn*$^{Tg}$ | *Ldlr*$^{-/-}$ | *Ldlr*$^{-/-}$/*Adn*$^{Tg}$ |
|---|---|---|---|---|
| | **Beginning of Study** | | **End of Study** | |
| **Males** *(n =11-12)* | | | | |
| Weight (g) | 23.7 ± 0.7 | 23.6 ± 0.9 | 39.3 ± 1.8# | 41.0 ± 1.8# |
| Fasting Glucose (mg/dL) | 182 ± 6 | 156 ± 6** | 200.5 ± 11.9∗ | 139.4 ± 8.1∗ |
| Adiponectin (μg/ml) | 12.5 ± 0.8 | 44.5 ± 4.6∗ | 13.7 ± 1.3 | 46.0 ± 5.7∗ |
| Total Cholesterol (mg/dL) | 252 ± 17 | 204 ± 6** | 1082 ± 110 | 906 ± 87 |
| Triglyceride (mg/dL) | 182 ± 21 | 91 ± 9** | 415 ± 44 | 346 ± 34 |
| **Females** *(n =15-17)* | | | | |
| Weight (g) | 18.6 ± 0.4 | 18.4 ± 0.4 | 29.5 ± 1.2 | 30.5 ± 1.5 |
| Fasting Glucose (mg/dL) | 148 ± 5 | 127 ± 7 | 173 ± 7∗ | 150 ± 6∗ |
| Adiponectin (μg/ml) | 17.4± 1.6 | 52.9 ± 7.4∗ | 27.2 ± 2.3# | 74.5 ± 8.3∗# |
| Total Cholesterol (mg/dL) | 249 ± 6 | 200 ± 29** | 1248 ± 39 | 1324 ± 83 |
| Triglyceride (mg/dL) | 159 ± 9 | 87 ± 10** | 480 ± 15 | 510 ± 33 |

* *Ldlr*$^{-/-}$ vs. *Ldlr*$^{-/-}$/*Adn*$^{Tg}$, $p < 0.001$
** *Ldlr*$^{-/-}$ vs. *Ldlr*$^{-/-}$/*Adn*$^{Tg}$, $p < 0.05$
# Beginning of Study vs. End of Study, $p < 0.05$

**Supplemental Figure 1**

Lipoprotein profile from male *Adn*$^{-/-}$ and wild type mice on regular chow diet. Plasma was pooled from 5 mice and subfractionated by gel filtration chromatography and triglyceride and cholesterol was measured in each fraction. VLDL, very low density lipoprotein, LDL/IDL, Intermediate and low density lipoprotein, HDL, high density lipoprotein.

**Supplemental Figure 2**

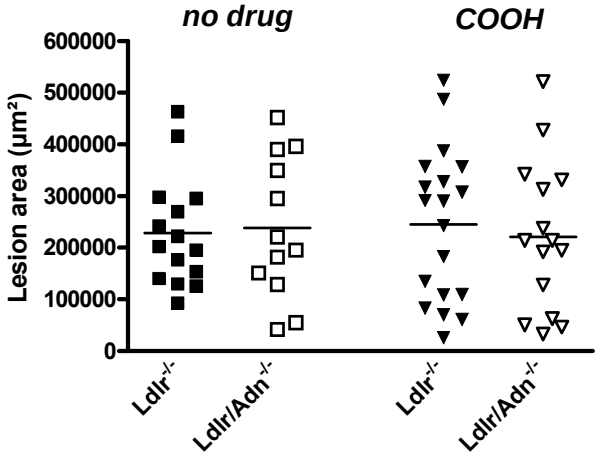
Total plasma adiponectin after administration of PPARγ selective, non-thiazolidinedione agonist (COOH) at 30 mg/kg body weight per day *in feed* at baseline and after 4, 8 and 12 weeks of treatment. COOH caused as significant raise in total circulating adiponectin levels (*$P<0.05$ compared to no drug, n = 12 to 18)

**Supplemental Table 1**

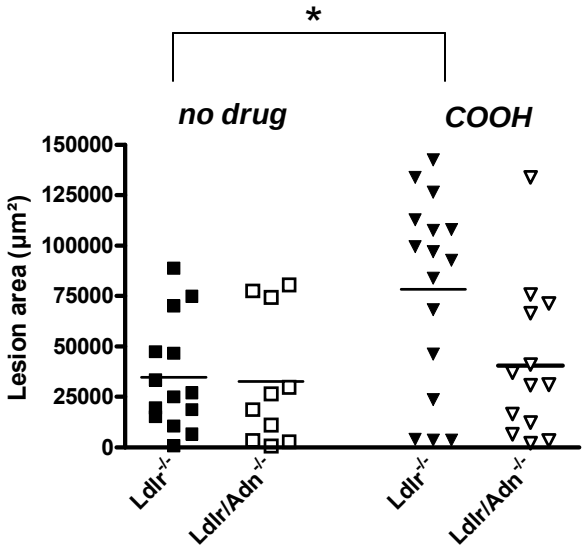
| | *Ldlr*$^{-/-}$ | *Ldlr/Adn*$^{-/-}$ | *Ldlr*$^{-/-}$ | *Ldlr/Adn*$^{-/-}$ |
|---|---|---|---|---|
| | **No drug** | | **COOH** | |
| VLDL particles (nmol) | 797 | 691 | 527 | 623 |
| LDL particles (nmol) | 1125 | 727 | 1181 | 973 |
| HDL particles (nmol) | 19.5 | 23.0 | 6.1 | 8.4 |
| VLDL size (nm) | 57 | 50 | 53 | 54 |
| HDL size (nm) | 9.0 | 9.7 | 9.2 | 9.9 |
| TG total (mg/dL) | 1203 | 952 | 946 | 1190 |
| VLDL-TG (mg/dL) | 1054 | 855 | 817 | 1067 |
| HDL-C (mg/dL) | 45 | 37 | 14* | 12^ |

∗ Ldlr$^{-/-}$ no drug vs. Ldlr$^{-/-}$ COOH, $p < 0.05$
^ Ldlr/Adn$^{-/-}$no drug vs. Ldlr/Adn$^{-/-}$ COOH, $p < 0.05$

1A


no drug
COOH
Lesion area (µm²)
600000
500000
400000
300000
200000
100000
0
Ldlr-/-
Ldlr/Adn-/-
Ldlr-/-
Ldlr/Adn-/-

1B


*
no drug
COOH
Lesion area (µm²)
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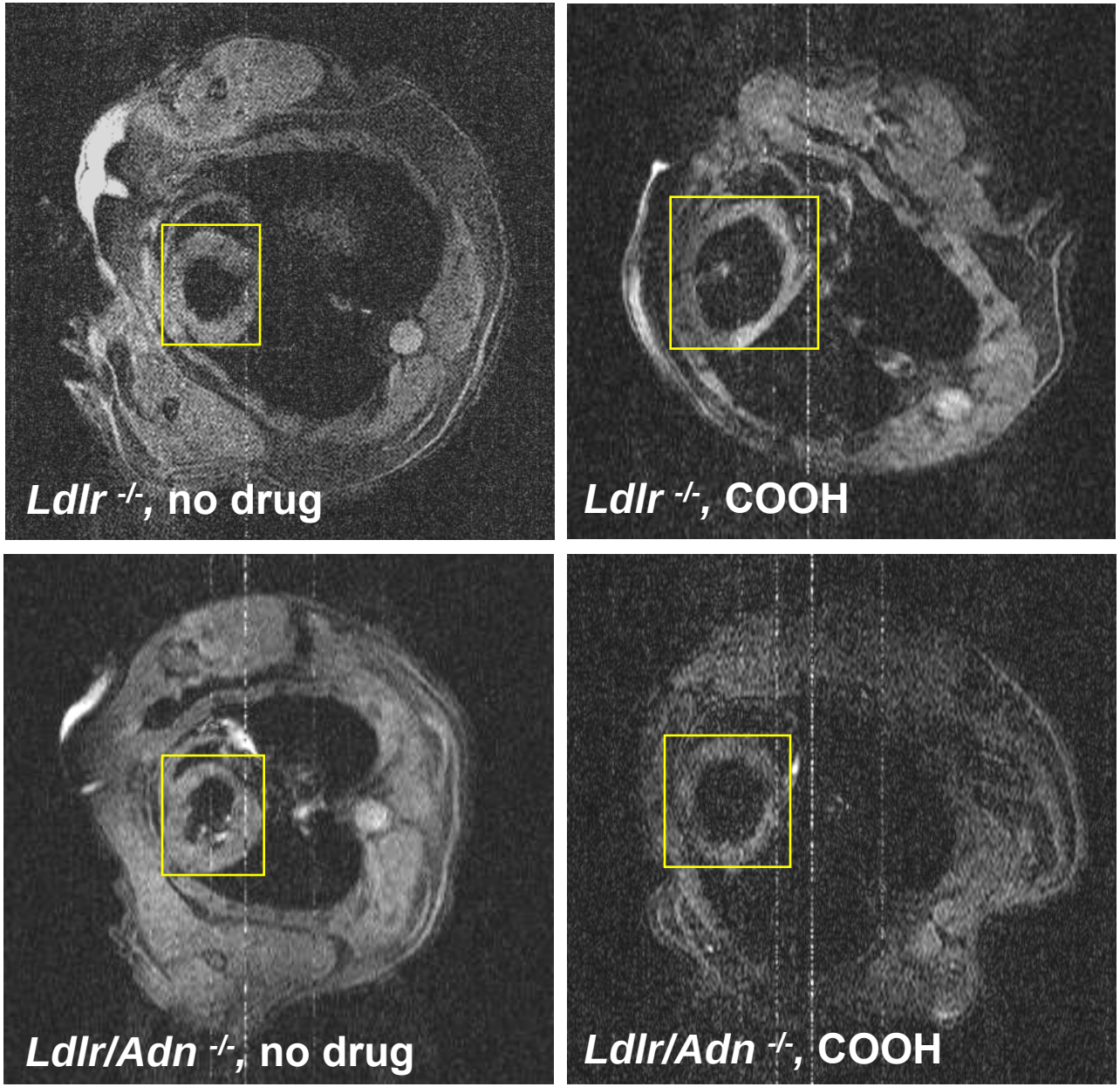
125000
100000
75000
50000
25000
0
Ldlr-/-
Ldlr/Adn-/-
Ldlr-/-
Ldlr/Adn-/-

2A



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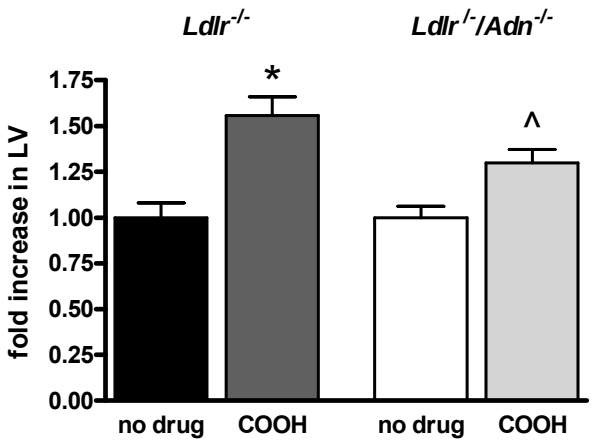
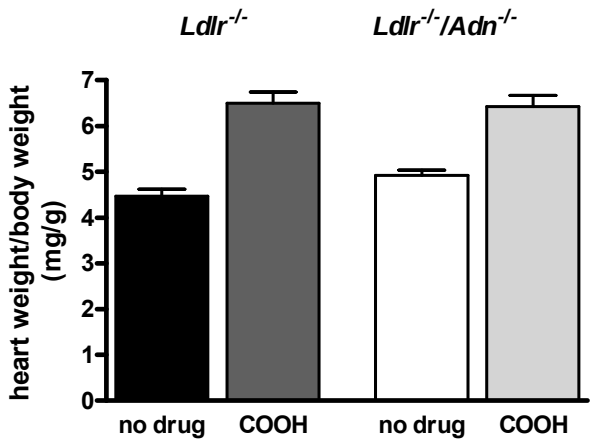

Ldlr-/-
Ldlr-/-/Adn-/-
fold increase in LV
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
^
no drug
COOH
no drug
COOH

2C


Ldlr-/-
Ldlr-/-/Adn-/-
heart weight/body weight (mg/g)
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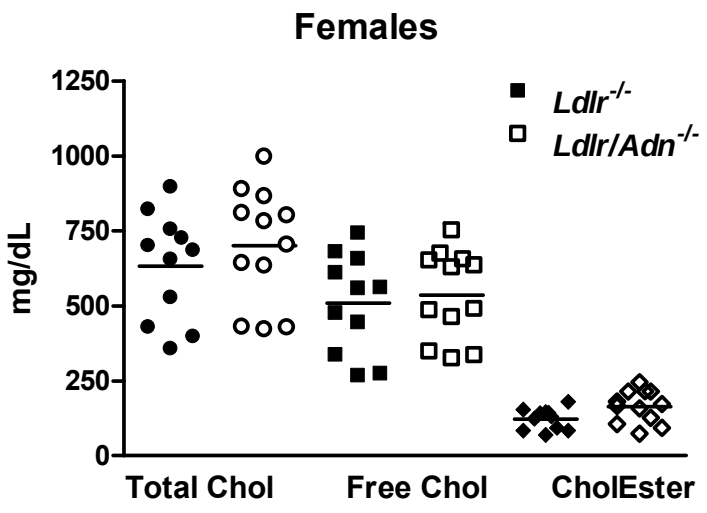
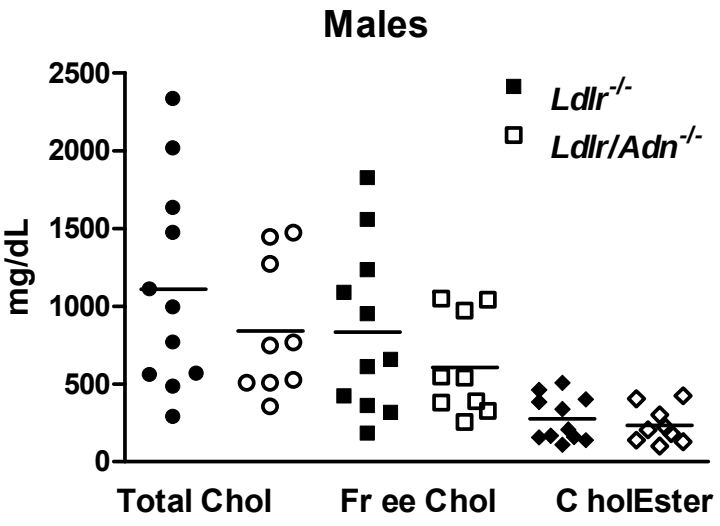
6
5
4
3
2
1
0
no drug
COOH
no drug
COOH

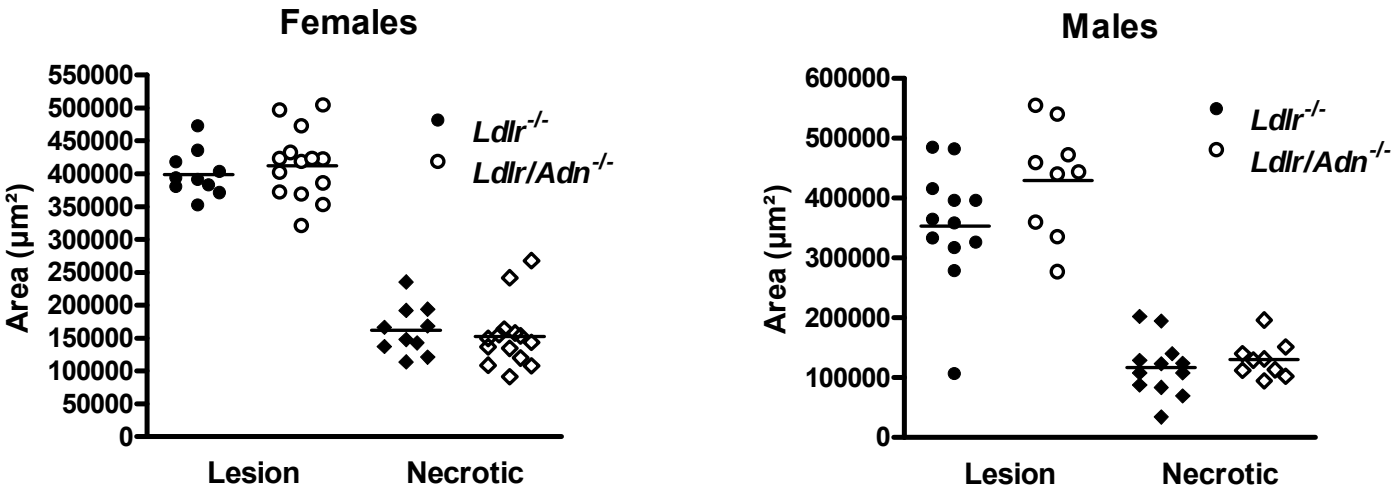
3A

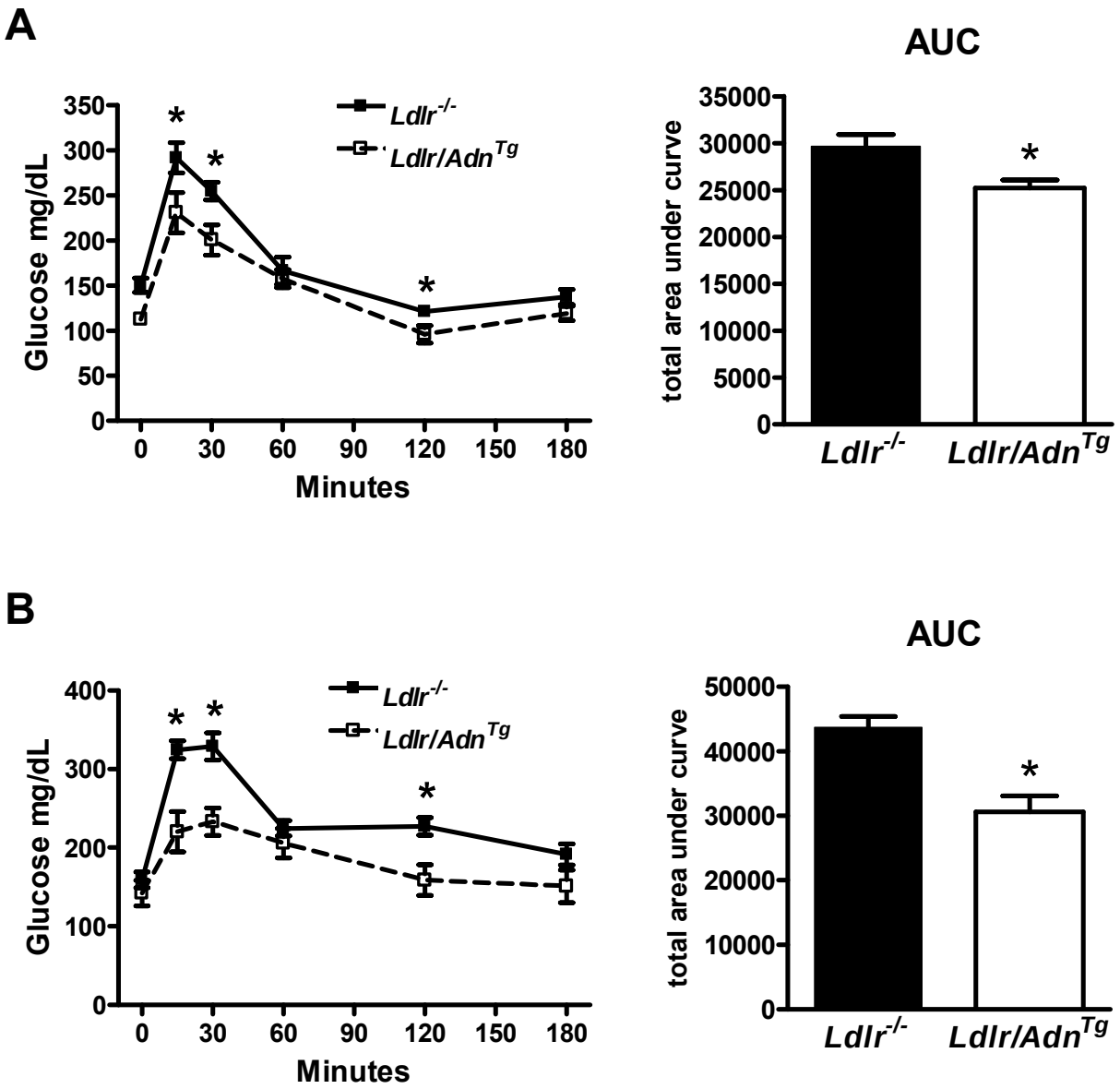

Females
1250
1000
750
500
250
0
mg/dL
Ldlr-/-
Ldlr/Adn-/-
Total Chol
Free Chol
CholEster

3B


Males
2500
2000
1500
1000
500
0
mg/dL
Ldlr-/-
Ldlr/Adn-/-
Total Chol
Fr ee Chol
C holEster

3C


Females
Area (µm²)
550000
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
Lesion
Necrotic
Ldlr-/-
Ldlr/Adn-/-
Males
Area (µm²)
600000
500000
400000
300000
200000
100000
0
Lesion
Necrotic
Ldlr-/-
Ldlr/Adn-/-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Glucose mg/dL
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300
250
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150
100
50
0
0
30
60
90
120
150
180
Minutes
Ldlr-/-
Ldlr/Adn Tg
*
*
*
AUC
total area under curve
35000
30000
25000
20000
15000
10000
5000
0
Ldlr-/-
Ldlr/Adn Tg
*
B
Glucose mg/dL
400
300
200
100
0
0
30
60
90
120
150
180
Minutes
Ldlr-/-
Ldlr/Adn Tg
*
*
*
AUC
total area under curve
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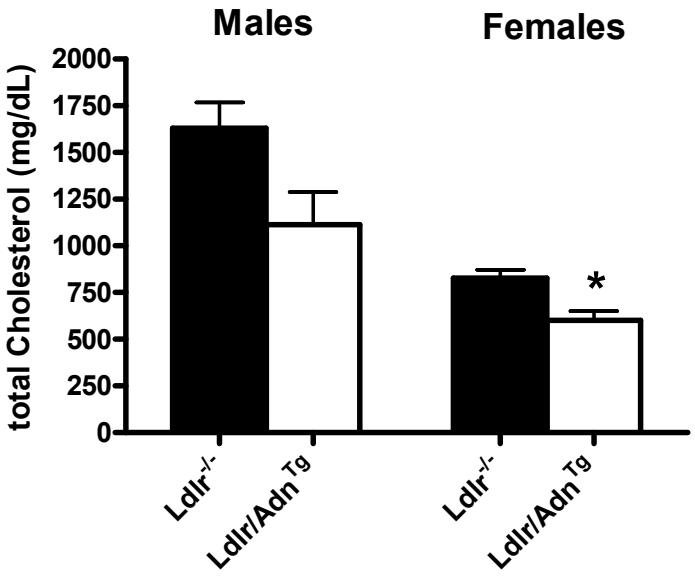
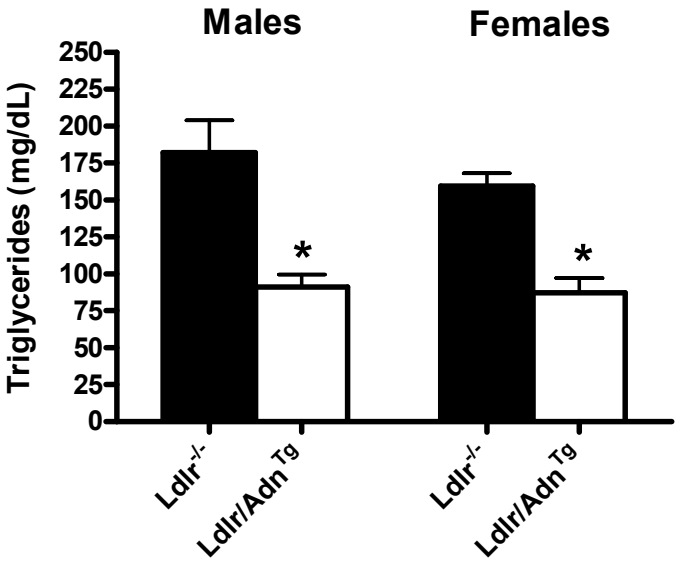
40000
30000
20000
10000
0
Ldlr-/-
Ldlr/Adn Tg
*

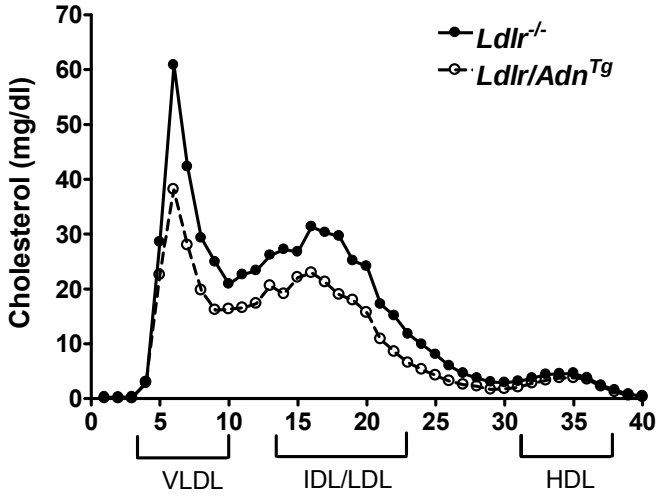
5A


Males
Females
total Cholesterol (mg/dL)
2000
1750
1500
1250
1000
750
500
250
0
*
Ldlr-/-
Ldlr/Adn Tg
Ldlr-/-
Ldlr/Adn Tg

5B


Males
Females
Triglycerides (mg/dL)
250
225
200
175
150
125
100
75
50
25
0
*
*
Ldlr-/-
Ldlr/Adn Tg
Ldlr-/-
Ldlr/Adn Tg

5C


Cholesterol (mg/dl)
70
60
50
40
30
20
10
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20
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30
35
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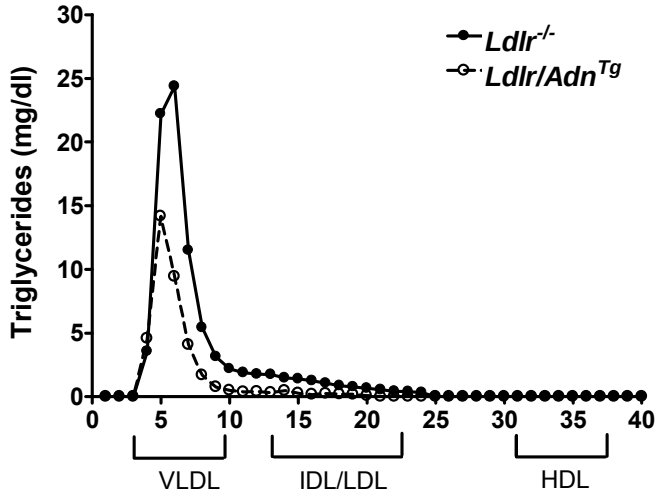
Ldlr/AdnTg
VLDL
IDL/LDL
HDL

5D


Triglycerides (mg/dl)
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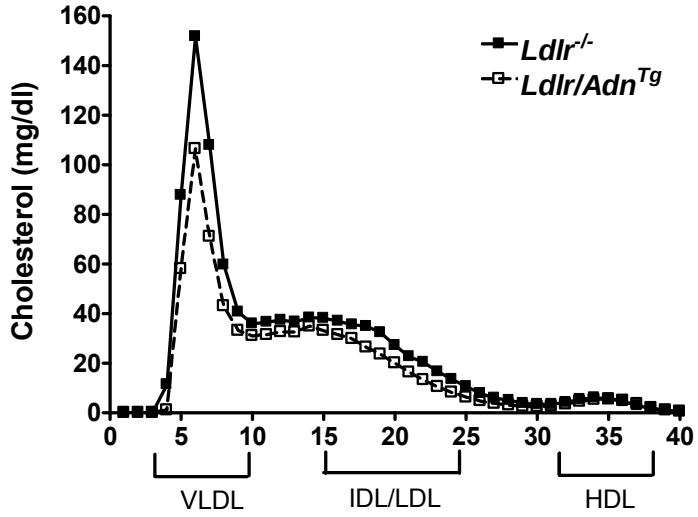
Ldlr/AdnTg
VLDL
IDL/LDL
HDL

5E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140
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100
80
60
40
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0
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15
20
25
30
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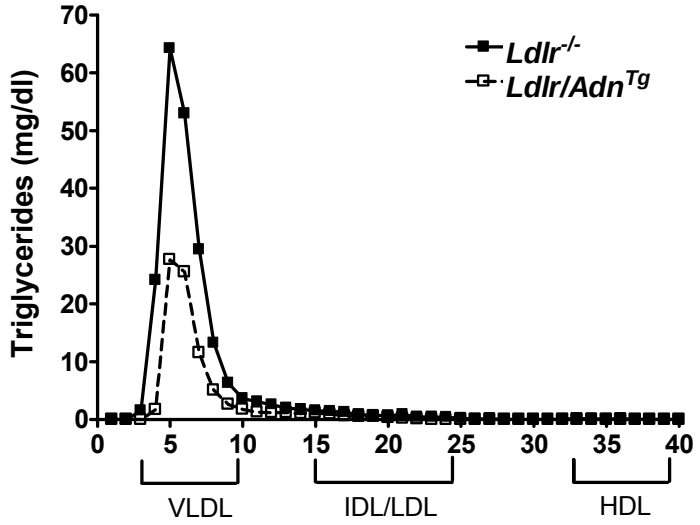
Ldlr/AdnTg
VLDL
IDL/LDL
HDL

5F


Triglycerides (mg/dl)
70
60
50
40
30
20
10
0
0
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20
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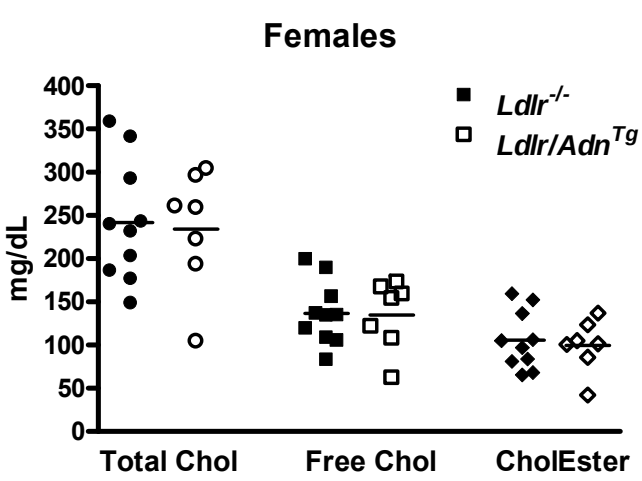
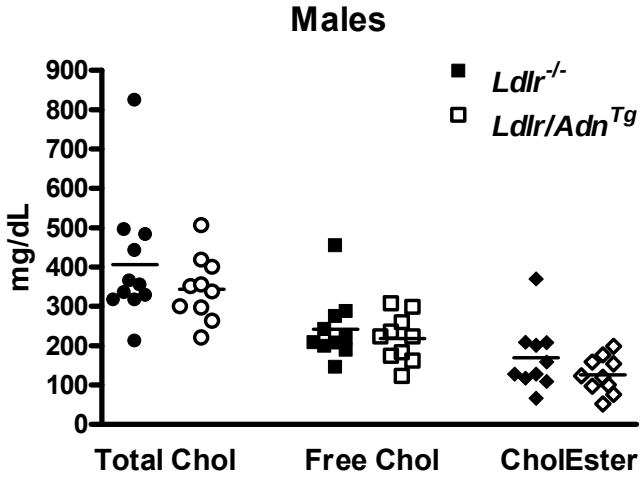
Ldlr/AdnTg
VLDL
IDL/LDL
HDL

6A


Females
mg/dL
400
350
300
250
200
150
100
50
0
Ldlr-/-
Ldlr/Adn Tg
Total Chol
Free Chol
CholEster

6B


Males
mg/dL
900
800
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0
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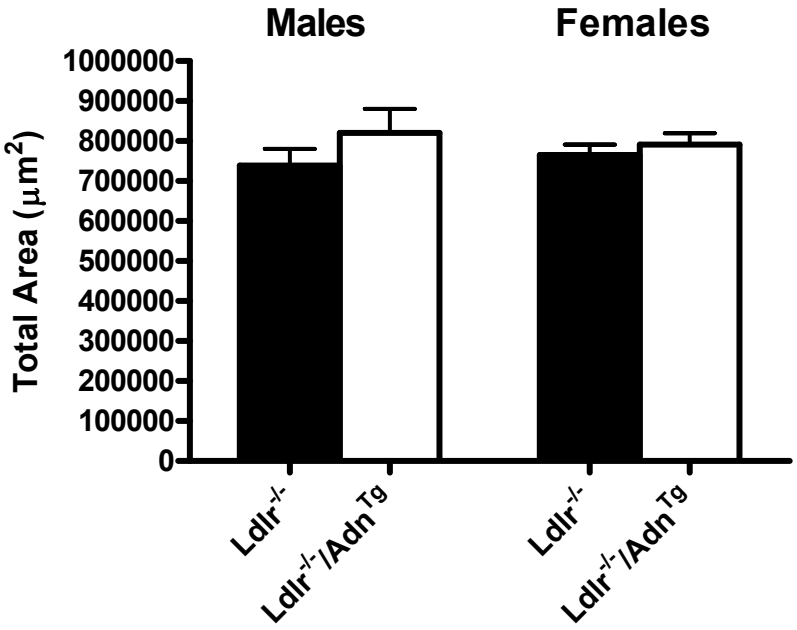
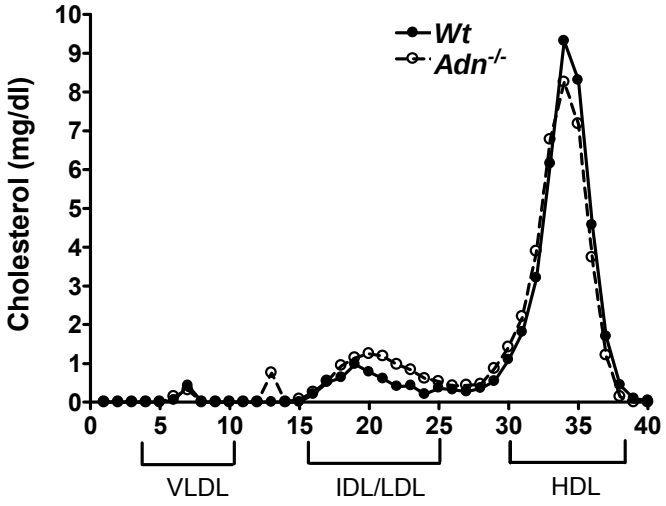
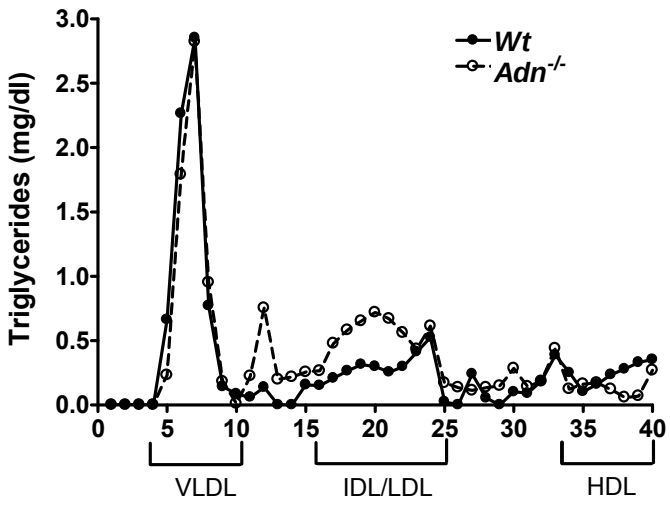
Ldlr/Adn Tg
Total Chol
Free Chol
CholEster

6C

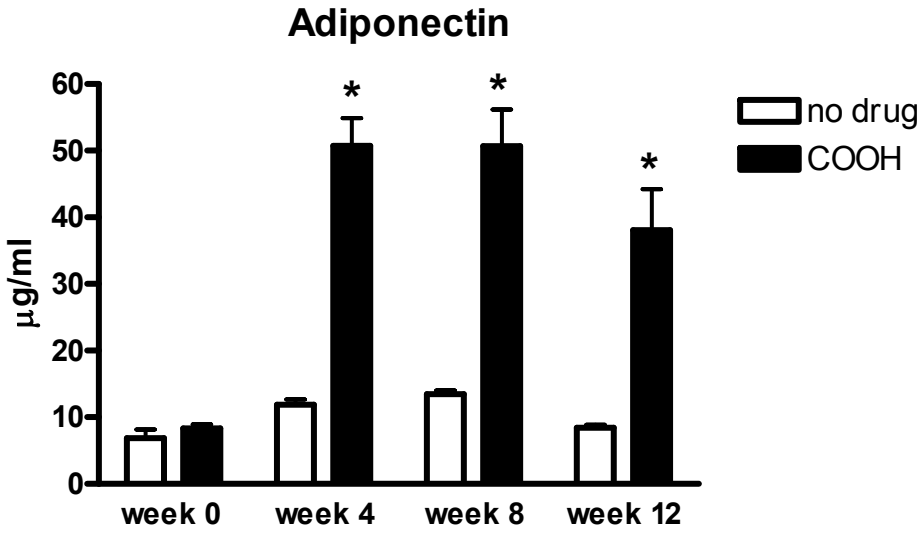

Males
Females
Total Area (μm2)
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
Ldlr-/-
Ldlr-/-/Adn Tg
Ldlr-/-
Ldlr-/-/Adn Tg

Supplemental Figure 1



Supplemental Figure 2



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Invited Review for Cardiovascular Complications of Diabetes and Obesity

# The Impact of Macrophage Insulin Resistance on Advanced Atherosclerotic Plaque Progression

Ira Tabas, Alan Tall, and Domenico Accili

From the Departments of Medicine (I.T., A.R.T., D.A.), Physiology and Cellular Biophysics (I.T., A.R.T), and Pathology and Cell Biology (I.T.), Columbia University, New York, NY

Correspondence to Ira Tabas, MD, PhD, Department of Medicine, Columbia University, 630 West 168th Street, New York, NY 10032. E-mail iat1@columbia.edu
Tel: 212-305-9430
FAX: 212-305-4834

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***Abstract***—Atherothrombotic vascular disease is the major cause of death and disability in obese and diabetic subjects with insulin resistance. Although increased systemic risk factors in the setting of insulin resistance contribute to this problem, it is likely exacerbated by direct effects of insulin resistance on the arterial wall cells that participate in atherosclerosis. A critical process in the progression of atherosclerotic lesions to those that cause clinical disease is necrotic breakdown of plaques. Plaque necrosis, which is particularly prominent in the lesions of diabetics, is caused by the combination of macrophage apoptosis and defective clearance, or efferocytosis, of the apoptotic macrophages. One cause of macrophage apoptosis in advanced plaques is activation of a pro-apoptotic branch of the endoplasmic reticulum stress pathway known as the Unfolded Protein Response (UPR). Macrophages have a functional insulin receptor signal transduction pathway, and down regulation of this pathway in the setting insulin resistance enhances UPR-induced apoptosis. Moreover, other aspects of the obesity/insulin-resistance syndrome may adversely affect efferocytosis. These processes may therefore provide important mechanistic link among insulin resistance, plaque necrosis, and atherothrombotic vascular disease and suggest novel therapeutic approaches to this expanding health problem.

**Key Words:** atherosclerosis ■ insulin resistance ■ diabetes ■ macrophages ■ apoptosis ■ efferocytosis ■ plaque necrosis ■ ER stress ■ Unfolded Protein Response

**Nonstandard Abbreviations and Acronyms:**

UPR: unfolded protein response

VLDL: very low-density lipoprotein

LDL: low-density lipoprotein

HDL: high-density lipoprotein

MMP: matrix metalloproteinase

ER: endoplasmic reticulum

CHOP: CEBP-homologous protein

GADD: growth arrest and DNA damage

PRR: pattern recognition receptor

FC: free cholesterol

TLR: toll-like receptor

SAR: type A scavenger receptor

BAPTA-AM: acetoxymethyl ester of 1,2-bis(O-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid

CaMKII: calcium/calmodulin-dependent protein kinase II

STAT: signal transducer and activator of transcription

IP3: inositol 1,4,5-triphosphate

ERO1$\alpha$: ER oxidase-1$\alpha$

SERCA: sarco/endoplasmic reticulum calcium-dependent ATPase

Mertk: c-mer tyrosine kinase

Apoe: apolipoprotein E

Ldlr: LDL receptor

MFG-E8: milk fat globule epidermal growth factor 8

IVUS: intravascular ultrasound

IRS-2: insulin receptor substrate-2

ERK: extracellular signal-regulated kinases

MEK: MAPK/ERK kinase

Insr: insulin receptor

EPA: eicosapentanoic acid

eNOS: endothelial nitric oxide synthase

VCAM: vascular cells adhesion molecule

PPAR: peroxisome proliferator-activated receptor

The incidence of insulin resistance, metabolic syndrome, and type 2 diabetes is rising rapidly due to the epidemic of obesity in the industrialized world.[1] While a number of disease processes are associated with insulin resistance and type 2 diabetes, the leading cause of morbidity and mortality is cardiovascular disease.[2] An important factor in accelerated heart disease in type 2 diabetes is likely to be insulin resistance and hyperinsulinemia. For example, the risk of cardiovascular disease is increased in metabolic syndrome, which is characterized by insulin resistance without overt hyperglycemia.[3-5] Moreover, rapid weight gain during childhood leads to hyperinsulinemia and increased coronary artery disease risk in adult life.[6] Part of the association between insulin resistance and cardiovascular disease is likely related to associated risk factors, including dyslipidemia (increased VLDL, reduced HDL, and possibly altered LDL), hypertension, and a pro-thrombotic state.[3] However, insulin resistance may have direct pro-atherogenic effects at the level of the arterial wall, and an emerging concept that will be explored in this review is that insulin resistance in lesional macrophages promotes a series of cellular events critical for advanced plaque progression. After a brief review of atherogenesis, we will focus on new findings related to plaque progression and the role of macrophage insulin resistance that have appeared in the literature since the last review of this topic in this journal in 2007.[7]

## Principles of Atherogenesis

### Plaque Initiation and Progression

Atherogenesis begins with the retention of atherogenic lipoproteins in the subendothelium of susceptible areas of the arterial tree.[8] In response to these retained lipoproteins, particularly those that undergo atherogenic modifications such as oxidation and aggregation, a series of biological and maladaptive inflammatory responses ensue: (a) monocytes and other inflammatory cells enter the intima; (b) monocytes differentiate into macrophages, which then ingest retained and modified lipoproteins and become cholesteryl ester-loaded foam cells; (c) macrophages and other inflammatory cells contribute to a state of inflammation that fails to properly resolve; and (d) smooth muscle cells populate the intima, leading to collagen synthesis.[9-12]. At this stage, the plaques are usually asymptomatic due to outward remodeling of the artery to preserve lumenal blood flow and a fibrous cap that protects the lesion from disruption.[13, 14] However, some of these plaques, unrelated to plaque size *per se*, may undergo necrotic breakdown, thinning of the fibrous cap, a heightened state of inflammation, and an accumulation of unesterified cholesterol.[13-19]. Many of the hallmarks of impaired inflammation resolution are evident in these plaques, including continued entry and poor egress of inflammatory cells, defective clearance of apoptotic cells, and a suppressed fibrotic "scarring" response.[12, 20] These so-called "vulnerable plaques" are at risk for plaque disruption through fibrous cap rupture or endothelial erosion, which in turn can trigger acute thrombosis. If the thrombosis is extensive and not quickly resolved, acute vascular occlusion and tissue infarction occurs, leading to acute myocardial infraction, unstable angina, sudden cardiac death, or stroke.

The exact mechanisms of plaque disruption are not known. Cap thinning *per se* may be caused by a combination of protease-mediated digestion of extracellular matrix molecules, particularly by matrix metalloproteinases (MMPs), and decreased collagen synthesis, perhaps exacerbated by death of the collagen-synthesizing cells in the intima.[13] These processes, as well as coagulation and thrombosis, are likely promoted by inflammatory cytokines, many of which are secreted by lesional macrophages.[13] Lesional necrosis of vulnerable plaques, which is caused by the combination of macrophage death and defective clearance, or "efferocytosis," of dead macrophages,[21-23] can promote plaque disruption by a number of mechanisms.[15, 22, 24-27]. For example, although matrix proteases are secreted by living macrophages in lesions, they may also be released by dead and dying macrophages.[28] Moreover, lesional necrosis triggers a heightened state of inflammation, which, as mentioned above, promotes MMP secretion, coagulation, and thrombosis.[29] Finally, the necrotic core is rich in lipids and poor in cells and extracellular matrix, and the structural properties resulting from this composition are thought to contribute to mechanical stresses in the overlying cap, which may contribute to cap rupture.[30] Thus, macrophage death and defective clearance of the dead cells, leading to lesional necrosis, is an important process in the

formation of the vulnerable plaque, and, as described in this review, exacerbations of these processes may help explain accelerated atherothrombotic disease in insulin-resistant stated.

**Mechanisms and Consequences of Macrophage Death and Defective Efferocytosis in Advanced Atheromata**

To understand how insulin resistance may promote advanced plaque progression in general, and plaque necrosis in particular, it is necessary to review our latest understanding of the mechanisms and consequences of macrophage death in advanced atheromata. A number of hypotheses have been conceived to explain advanced lesional macrophage apoptosis, and undoubtedly more than one mechanism is involved. Examples include growth factor deprivation, toxic cytokines, and oxidized lipids or lipoproteins,[31] but there is as yet little proof for these ideas *in vivo*. Recent mechanistic data in cultured cells and correlative and genetic-causation evidence *in vivo* support a role for endoplasmic reticulum (ER) stress in advanced lesional macrophage apoptosis and its major consequence, plaque necrosis. As had been previously demonstrated in other models of ER stress-induced apoptosis, macrophages subjected to ER stress undergo apoptosis in a manner that is partially dependent on the CHOP (GADD153) branch of the ER stress pathway known as the Unfolded Protein Response (UPR).[32, 33] CHOP-mediated apoptosis can be modeled in cultured macrophages by either potent inducers of ER stress or by the combination of more subtle ER stressors and a "second hit." Examples of atherosclerosis-relevant inducers of the single-hit ER stress apoptosis model include 7-ketocholesterol and saturated fatty acids (T. Seimon *et al*., manuscript in preparation).[33] Examples of the two-hit model include the combination of subtle ER stressors, such as very low amounts of oxysterols or fatty acids, plus a second pro-apoptotic hit that involves activation of pattern recognition receptors (PRRs), such as modified lipoproteins or oxidized phospholipids (T. Seimon *et al*., manuscript in preparation).[34, 35] Another example of the two-hit model is incubation of macrophages with atherogenic lipoproteins under conditions of genetic or pharmacologic inhibition of intracellular cholesterol re-esterification.[36, 37] In this model, which is often referred to as the "FC" (free cholesterol) model and is designed to mimic FC-loaded macrophages in advanced atheromata,[38, 39] the ER stress hit is provided by excess accumulation of unesterified cholesterol in the ER membrane, and the second hit is activation of PRRs by the lipoproteins themselves. Thus far, two pairs of PRRs have been identified that can serve as second pro-apoptotic hits in ER-stressed macrophages: toll-like receptor 4 (TLR4) and the type A scavenger receptor (SRA);[34, 35] and TLR2 and CD36 (Seimon *et al*., manuscript in preparation). The contribution of the second hit to apoptosis involves both amplification of pro-apoptotic pathways and suppression of cell-survival pathways that are activated in ER-stressed cells, such as those involving phospho-Akt and NF-κB. The tendency of macrophages to undergo apoptosis when subjected to ER stress in combination with PRR activation may have evolved as a host defense mechanism against intracellular organisms that require living macrophages to survive.

While it has been known that activation of the CHOP pathway of the UPR can cause apoptosis, the molecular mechanisms linking CHOP to death execution pathways is poorly understood. Recent work in our laboratories has provided evidence for a calcium-dependent mechanism in ER stress-induced macrophage apoptosis. ER stress in macrophages leads to the release of calcium from the ER lumen into the cytosol.[32] The cytosolic calcium chelator BAPTA-AM can block ER stress-induced apoptosis in macrophages, and recent work has shown that a key integrator of cytosolic calcium and death execution in these cells is a calcium-responsive kinase called calcium/calmodulin-dependent protein kinase II (CaMKII).[35, 40, 41] Activation of CaMKII leads to multiple death pathways, including induction of the cell-surface death receptor Fas; stimulation of mitochondrial calcium uptake and release of pro-apoptotic cytochrome c from the mitochondria; activation of pro-apoptotic STAT-1; and accumulation of reactive oxygen species through activation of NADPH oxidase.[41] CHOP amplifies this calcium-death pathway by leading to activation of IP3 receptors, which are calcium-release channels in the ER membrane.[42] The mechanism involves oxidative activation of IP3R by the downstream CHOP transcriptional target, ER oxidase-1α (ERO1α). Net calcium release can also be promoted through inhibition of sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA), which pumps calcium back into the ER lumen. SERCA is inhibited by alterations in the ER membrane by certain ER stressors, such as unesterified cholesterol or

saturated fatty acids,[43] and SERCA is down-regulated in the setting of insulin resistance, as will be summarized below.

Macrophage apoptosis by itself would not be expected to be detrimental, because apoptotic cells are normally cleared rapidly by phagocytosis ("efferocytosis") in a manner that prevents post-apoptotic cellular necrosis and that promotes anti-inflammatory processes.[27] Indeed, manipulations that accelerate early lesional macrophage apoptosis decrease lesion cellularity and plaque progression, and *vice versa*,[23, 44] suggesting that efferocytosis is very efficient in the early stages of atherogenesis. This principle has been applied recently to a mouse model diabetes and early atherosclerosis.[45] However, in the later stages of atherosclerosis, macrophage apoptosis is associated with plaque necrosis,[23] and there is evidence in humans that efferocytosis is defective in advanced plaques.[46] The mechanisms of defective efferocytosis in advanced lesions are not known, but several interesting ideas have been advanced based on *in vitro* and *in vivo* observations. For example, oxidized lipids and proteins exist in these plaques, and some of these molecules can competitively inhibit efferocytosis by binding to efferocytosis receptors.[47] In another scenario, the efferocytosis receptor Mertk has been shown to play a role in efferocytosis and plaque necrosis in mouse lesions,[48, 49] and inflammation-induced cleavage of this receptor by membrane sheddases[50] may contribute to defective clearance of apoptotic cells in advanced plaques.

The concept that ER stress-induced macrophage apoptosis in combination with defective efferocytosis in advanced lesions promotes plaque necrosis is supported by a number of genetic-causation studies in mice and by correlative studies in humans. In fat-fed *Apoe*-/- or *Ldlr*-/- mice, ER stress markers are induced as lesions progress.[32, 51-55] Most importantly, genetic targeting of CHOP and STAT-1, the pro-apoptotic signaling transducer activated by the CHOP calcium-CaMKII pathway (above), as well as prevention of cholesterol-induced ER damage, inhibit advanced lesional macrophage apoptosis and plaque necrosis.[33, 41, 51] Moreover, deletion of two PRRs—SRA and CD36—that have been shown to participate as second hits in ER stress-induced apoptosis *in vitro* decreases macrophage apoptosis and plaque necrosis in the lesions of fat-fed *Apoe*-/- mice,[56] and absence of TLR2 decreases apoptosis in macrophage-rich areas in lesions of fat-fed *Apoe*+/- mice.[57] In humans, there are close correlations among markers of ER stress, apoptosis, and plaque vulnerability in coronary arteries.[54] In terms of efferocytosis, studies have shown an increase in plaque necrosis that correlates with a worsening of lesional efferocytosis in several mouse models in which efferocytosis effectors have been targeted, including Mertk, MFG-E8, transglutaminase-2, and complement factor C1q.[48, 58] In summary, *in vitro* and *in vivo* evidence support a model in which macrophage apoptosis in advanced lesions, induced in part by a pro-apoptotic ER stress-calcium pathway, plus defective efferocytosis promote plaque necrosis (Figure 1). Because plaque necrosis is strongly associated with disrupted plaques and acute lumenal thrombosis,[59] and because plaque necrosis is particularly prominent in atherosclerotic lesions from diabetic subjects, as described in the following section, these insights should be useful in our understanding of and therapeutic approaches to accelerated plaque progression in the setting of insulin resistance.

## Macrophage Death and Plaque Progression in Insulin Resistance

### Plaque Necrosis in Diabetic Lesions

It is now well-established that diabetes and insulin resistance are major risk factors for atherothrombotic vascular disease.[3-5] While many theories have arisen to explain this relationship,[60, 61] a common endpoint of plaque progression associated with atherothrombotic vascular disease, as mentioned in the previous section, is plaque necrosis. In this context, a number of independent studies have found that advanced atherosclerotic lesions in diabetic subjects are characterized by particularly large necrotic cores when compared to similarly sized lesions from non-diabetic individuals.[62-67] For example, Burke *et al.*[62] found that necrotic core size in the coronary arteries of subjects who died suddenly was positively correlated with the presence of diabetes independently of other factors. Similar results were found when coronary atherectomy specimens of diabetics and non-diabetics were compared.[63] Nasu *et al.*[66] used virtual histology based on intravascular ultrasound (IVUS) data to assess coronary arterial necrotic cores in non-

diabetic and diabetic patients with stable angina and found an approximate 50% increase in the percent area covered by necrotic cores in the diabetic group. Almost identical findings were reported in similar studies conducted by Hong *et al*.[65] in Korea and Pundziute *et al*.[66] in the Netherlands. A prospective study of subjects with coronary artery disease in which radiofrequency data from IVUS was used to assess necrotic core size in coronary arteries found that only diabetes and age were associated positively with necrotic core size in logistic regression analysis.[67] These collective data raise the issue as to whether the cellular events described in the previous sections, particularly advanced lesional macrophage apoptosis and/or defective efferocytosis, are enhanced in the setting of diabetes, leading to increased plaque necrosis and, ultimately, accelerated atherothrombotic vascular disease.

**The Effect of Insulin Resistance on Macrophage Death Pathways**

In view of the role of insulin resistance in diabetic heart disease and the larger necrotic cores in the coronary arteries of diabetic subjects, we and others have examined how insulin resistance at the level of the macrophage affects mechanisms and consequences of macrophage death *in vitro* and *in vivo*. Macrophages have insulin receptors, and acute exposure of the cells to insulin *in vitro* results in phosphorylation of the insulin receptor, insulin receptor substrate-2 (IRS-2), and Akt, leading, among other responses, to nuclear exclusion and inactivation of FoxO transcription factors.[7, 68] Moreover, pre-treatment of macrophages *in vitro* with high-dose insulin leads to down-regulation of their insulin receptors and suppression of insulin receptor signaling, which is also observed in freshly isolated macrophages from insulin-resistant mice, such as the hyperinsulinemic leptin-deficient *ob*/*ob* mouse.[68] Thus, macrophages show the hallmarks of "insulin resistance" at a cellular level in the setting of high insulin concentrations.

Macrophages rendered insulin resistant through pre-incubation with insulin, genetic deletion of the insulin receptor, or pharmacologic inhibition of insulin signaling, and macrophages freshly isolated from hyperinsulinemic mice, show an increase in the levels of two scavenger receptors, the SRA and CD36.[7, 68] Moreover, when insulin signaling in *ob/ob* mice was restored by administration of a thiazolidinedione, CD36 levels returned to normal in association with restoration of macrophage insulin signaling. As mentioned in the previous section, both SRA and CD36 can serve as "second hits" in ER stress-induced macrophage apoptosis both *in vitro* and in advanced lesional macrophage death and plaque necrosis *in vivo*. In this regard, insulin-resistant macrophages show markedly enhanced apoptosis *in vitro* when exposed to ER stress conditions plus either an SRA-mediated second hit, as is the case with macrophages loaded with lipoprotein-derived unesterified cholesterol, or when exposed to oxidized LDL, a CD36 ligand (T. Seimon *et al*., manuscript in preparation).[55, 69, 70]

ER stress in macrophages triggers compensatory cell-survival pathways, notably those activated by Akt and NF-κB, and apoptosis is temporally correlated with a down-regulation of these pathways and can be accelerated by their inhibition.[35, 71, 72] Moreover, Akt deficiency in *Apoe*-/- mice was shown to enhance lesional macrophage apoptosis and inflammation and plaque progression.[73] In this context, an important observation was that phosphorylation of Akt is suppressed in ER-stressed, insulin-resistant macrophages.[69, 71] Consistent with a decrease in Akt phosphorylation, Senokuchi *et al*.[71] found an increase in nuclear FoxO1 in insulin-resistant, ER-stressed macrophages, which normally translocates to the cytoplasm in response to Akt-dependent phosphorylation.[74] Moreover, macrophages genetically lacking FoxO1, 3 and 4, were resistant to ER stress-induced apoptosis.[71] However, FoxO-overexpression experiments indicated that nuclear localization of these transcription factors was not by itself sufficient for macrophage apoptosis but rather led to an enhancement of apoptosis in the setting of ER stress. The apoptosis-enhancing mechanism of FoxO1 is directly related to the role of another compensatory cell-survival factor in ER-stressed macrophages, namely NF-κB.[71, 72, 75, 76] In ER-stressed macrophages, FoxO1 induces the expression of the NF-κB inhibitor IκBε and thereby enhances apoptosis.[71]

Importantly, insulin resistance potentiates the ER stress response itself.[77] ER stress in macrophages leads to activation of the mitogen-activated protein kinase ERK,[76] and Liang *et al*.[77] found that this response was blunted in insulin-resistant macrophages. Additional studies revealed that the MEK-ERK pathway induces SERCA,[77] which, as explained above, can abrogate ER stress by replenishing ER

lumenal calcium stores and can protect macrophages from ER stress-induced apoptosis by lowering cytosolic calcium levels. Thus, the blunted MEK-ERK-SERCA pathway in insulin-resistant macrophages exacerbates the ER stress response and the calcium-mediated apoptosis pathway described above, and restoration of MEK1 in these cells is protective against both ER stress and apoptosis.[77]

In summary, mechanistic studies using various cell culture models of insulin-sensitive and insulin-resistant macrophages, including primary macrophages freshly harvested from *ob/ob* mice, have revealed an integrated pathway of cell signaling events responsible for the increased apoptotic response to ER stress in the setting of insulin resistance. Key among these events are those related to the compromise of compensatory cell survival pathways and the exacerbation of pro-apoptotic calcium signaling pathways (Figure 2).

**The Effect of Macrophage Insulin Resistance on Murine Atherosclerosis**

To test relevance of enhanced ER stress-induced apoptosis in insulin-resistant macrophages *in vivo*, irradiated *Ldlr*-/- mice were transplanted with bone marrow from *Insr*+/+ or *Insr*-/- mice.[69] After recovery of the graft, the mice were fed a high-fat diet, and lesions were analyzed for overall area and, most importantly, plaque morphology. Consistent with the *in vitro* data, the advanced lesions of the *Insr*-/- → *Ldlr*-/- mice fed the diet for 12 weeks had more apoptotic cells and more plaque necrosis than those of the *Insr*+/+ → *Ldlr*-/- control mice. Overall lesion area, the less important endpoint for the hypothesis being tested, showed no change after 8 weeks of diet and only a modest increase after 12 weeks. Baumgartl *et al.*[78] used the cre-lox system to create *Apoe*-/- with macrophage-targeted deficiency of insulin receptors. After 4 months on a high-fat diet, these mice had a modest decrease in lesion area compared with control *Apoe*-/- mice. Apoptotic cells and necrotic areas were not quantified. Immortalized macrophages derived from these mice had a marked reduction in LPS-induced interleukin-6 (IL-6) secretion. The authors also tested the effects of global and bone marrow-derived IRS-2 deficiency in fat-fed *Apoe*-/- mice. In the holo-knockout model, lesion area was modestly increased, and in the bone marrow transplant model, lesion area was modestly decreased. Plaque morphology was not quantified. The authors interpreted these data as showing that myeloid-derived insulin receptors suppress atherosclerosis by blunting the inflammatory response.[78] Senokuchi *et al.*[71] also observed decreased inflammatory responses during ER stress in insulin-resistant macrophages. In that study, reduced NF-κB responses led to both increased apoptosis, as noted in the previous section, and decreased expression of some inflammatory genes. In summary, a careful comparison of Han *et al.*[69] and Baumgartl *et al.*[78] reveal a common finding of relatively modest effects of macrophage insulin resistance on overall lesion size, with subtle differences between the two studies perhaps arising from differences in genetic background (mixed *vs*. inbred C67Bl/6J), diets used (Western-type diet *vs*. the pro-inflammatory high-cholesterol/bile salt diet), and stage of lesion development. As noted, those specific features of atherosclerotic lesions related to the novel concept that insulin-resistant macrophages are more susceptible to apoptosis, *i.e.*, advanced lesional macrophage apoptosis and plaque necrotic area, were assessed in only one of the two studies, and the data supported that concept.[69]

Hsueh and colleagues[79] developed a model of global insulin resistance and examined its effect on atherosclerosis in the setting of aging. While this model did not focus specifically on macrophage insulin resistance, some interesting observations may give further clues to mechanisms linking macrophage insulin resistance to advanced plaque progression at the level of the arterial wall. The investigators compared 3 mo/old and 12 mo/old *Ldlr*-/- mice fed a high-fat diet for 3 months. The older mice developed worse insulin resistance and worse atherosclerosis, and the lesions in these mice appeared to be associated with a marked increase in plaque necrosis. The insulin-resistant older mice had a blunted anti-oxidant response that might be caused by a defective DJ-1—Nrf2 anti-oxidant pathway,[80] and a higher lesional expression of the NADPH oxidase subunit, p47. Atherosclerosis and plaque morphology were improved by treating the mice with the NADPH oxidase inhibitor and anti-oxidant, apocynin. One implication of these findings is that aging, a major risk factor for cardiovascular disease in humans,[81] may interact with insulin resistance to promote plaque necrosis, and in this regard it is interesting to note that aging is associated with both enhanced ER stress and defective efferocytosis.[82, 83] Second, a critical

downstream pro-apoptotic effector of ER stress and ER calcium release is activation of NADPH oxidase, and, given the pathways described in Figure 2, this response may be further enhanced in the setting of insulin resistance. Although vitamin E has not been shown to be effective in decreasing cardiovascular risk in humans,[84] more targeted anti-oxidants, such as NADPH oxidase inhibitors, in the specific setting of insulin resistance and possibly aging, may be more mechanistically justified and have more promise.

**How Insulin Resistance Might Affect Efferocytosis**

The increase in plaque necrosis in diabetic lesions raises the important issue as to whether efferocytosis is defective in these lesions and, if so, how this is mechanistically linked to insulin resistance. For example, defective phosphatidylinositol 3-kinase signaling in the setting of insulin resistance could, in theory, lead to a defect in efferocytosis in general and a specific defect in Mertk-mediated efferocytosis in particular.[85, 86] Using an *in situ* assay that quantifies the percentage of apoptotic cells that have been engulfed by phagocytic macrophages *vs*. not associated with phagocytes,[48] Li *et al*.[87] found that the aortic root lesions of Western diet-fed *ob/ob*;*Ldlr*-/- mice had evidence of defective efferocytosis compared with lesions of Western diet-fed *Ldlr*-/- mice. *In vitro* studies showed that primary macrophages isolated from *ob/ob* mice have a defect in efferocytosis that was associated with defective PI3 kinase activity, but those from *Insr*-/- mice do not. Further studies revealed that the key defect in *ob/ob* macrophages was an increase in the saturated fatty acid:unsaturated fatty acid ratio in the macrophage membranes, perhaps through "stiffening" the plasma membrane to the point where phagocytosis is compromised.[88] The efferocytosis defect on *ob/ob* macrophage could be corrected by treating the cells with the omega-3 polyunsaturated fatty acid eicosapentanoic acid (EPA), and similar results were found when macrophages were harvested from EPA-fed *ob/ob* mice. Most importantly, lesional efferocytosis was improved in *ob/ob*;*Ldlr*-/- mice by EPA feeding, which interestingly has also been associated with protection from heart disease in humans.[89] The precise mechanism of how saturated fatty acid impair efferocytosis and how EPA improves it is still under investigation, as are other possible links between insulin resistance and clearance of apoptotic cells. Nonetheless, we can begin to imagine an integrated model in which direct effects of insulin resistance on advanced lesional macrophage apoptosis, combined with defective efferocytosis caused by systemic fatty acid defects in the setting of insulin resistance, can at least partially explain the large neurotic cores and accelerated thrombotic vascular disease in diabetics (Figure 2).

## Conclusions and Future Directions

This review focused on one key feature of diabetes, insulin resistance; one type of lesional cell, the macrophage; and one overall context of atherosclerosis, advanced plaque progression. Even within this focused area of research, more work is needed to further define mechanisms whereby insulin resistance affects specific signaling pathways involved in the panoply of atherosclerosis-relevant macrophage activities, including, interaction with lipoproteins and intracellular metabolism of lipoprotein-derived lipids; inflammation and the resolution thereof; stress responses, including oxidative, heat shock, and ER stress; secretion of proteases, pro-coagulant molecules, and other factors involved in plaque progression; phagocytosis, efferocytosis, and antigen presentation; apoptosis-cell survival balance; and interaction with other cells and extracellular matrix. Moreover, it is likely that insulin resistance affects these processes differently in different subsets of macrophages and in other types of myeloid cells, notably dendritic cells, mast cells, and neutrophils. A limitation of our *in vivo* studies has been the lack of a mouse model that fully recapitulates features of human plaque disruption and athero-thrombosis,[90] and so further developments to improve mouse models of diabetic atherothrombotic vascular disease is an important goal. Nonetheless, it is becoming clear that key morphologic features of such plaques are worsened by ER stress[33] and insulin resistance in macrophages.[69]

Beyond the specific areas of plaque macrophages, insulin resistance, and advanced plaque progression, other areas of focus may offer additional clues as to why heart disease is enhanced in diabetes.[61] For example, decreased insulin signaling in endothelial cells, through impaired Akt signaling, is also likely to

have important pro-atherogenic consequences through decreased eNOS activity and increased expression of inflammatory genes and VCAM-1.[73] In the liver, hyperinsulinemia and insulin signaling may increased VLDL secretion while having the opposite effects on LDL receptor expression.[91] The other major feature of type 2 diabetes, hyperglycemia, may promote plaque instability by enhancing the inflammatory response in macrophages through effects on plasma triglyceride-rich lipoproteins and free fatty acids.[92] Hyperglycemia may also cause endothelial cell abnormalities, including oxidative stress and RAGE-induced inflammation, that promote the earlier stages of atherogenesis.[93, 94] Interestingly, there are recent data suggesting that hyperglycemia may exert some its pro-atherogenic effects in endothelial cells through FoxO1 and also through the induction of ER stress.[95, 96] These hyperglycemia-endothelial cell studies, together with the insulin resistance-macrophage studies described in this review, raise the interesting possibility that hyperglycemia may affect mostly the earlier stages of atherogenesis, while insulin resistance has its greatest effect on promoting advanced plaque progression. In this context, a recent analysis of the Veterans Affairs Diabetes Trial found that intensive glucose lowering reduced cardiovascular events in diabetics with a coronary artery calcium store < 100 (multivariable hazard ratio [HR] = 0.08, p=0.03), but not in those with a calcium score >100 (HR = 0.74, p=0.21).[97] Smooth muscle cells, a key cell type in the generation of the "protective" fibrous cap in advanced lesions, and platelets, the final effector of acute vascular occlusion, may be affected by insulin resistance, hyperglycemia, or fatty acid abnormalities, which provide additional opportunities for investigation.[61] Continued progress in these areas will provide a more complete understanding of how multiple features of diabetes promotes heart disease.

The ultimate goal of these studies is to complement our current efforts at identifying and treating systemic risk factors that promote cardiovascular disease in diabetics. Despite the relative success of this strategy, risk is still very high,[98], and the tremendous scale of this epidemic is such that overall risk will still be high even if compliance is improved and the experimental modalities prove useful. Further understanding of the specific mechanisms of increased vascular disease in diabetics, particularly at the molecular level in arterial wall cells, may be a promising approach for further eradication in the future—and one that should be additive or even synergistic with reduction of lipid and other systemic risk factors. One approach is to increase insulin sensitivity in diabetic macrophages, such has been demonstrated recently using a PPARγ activator *in vivo*[68] and 1,25$(OH)_2$ vitamin D *in vitro*.[99] Another approach is to develop agents to prevent ER stress or downstream pro-apoptotic processes in macrophages by pharmacologic means, *e.g.*, through the use of chemical chaperones[100] or inhibitors of the calcium-mediated pro-apoptotic pathway.[101, 102] Moreover, in view of the importance of defective efferocytosis in the generation of plaque necrosis and the *ob/ob* efferocytosis study described above, experimental therapeutic modalities designed to enhance efferocytosis[58, 103] may be particularly useful in diabetics. Delivery of such drugs to plaques might be facilitated by specific vehicles targeted to plaques,[104] while clinical assessment in phase 2 and phase 3 studies could be assisted by imaging techniques such as carotid MRI, that have the capacity to measure important plaque features such as necrotic core area and cap thickness.[105] Studies in these areas occurring in parallel with ongoing efforts at systemic risk reduction offer the best chance to curb the growing epidemic of diabetes-associated atherothrombotic vascular disease.

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## Disclosures

None.

## References


1. Yach D, Stuckler D, Brownell KD. Epidemiologic and economic consequences of the global epidemics of obesity and diabetes. *Nat Med.* 2006;12:62-66.

2. Fox CS, Coady S, Sorlie PD et al. Increasing cardiovascular disease burden due to diabetes mellitus: the Framingham Heart Study. *Circulation.* 2007;115:1544-1550.

3. Grundy SM. Obesity, metabolic syndrome, and cardiovascular disease. *J Clin Endocrinol Metab.* 2004;89:2595-2600.

4. Haffner SM, D'Agostino R, Jr., Mykkanen L et al. Insulin sensitivity in subjects with type 2 diabetes. Relationship to cardiovascular risk factors: the Insulin Resistance Atherosclerosis Study. *Diabetes Care.* 1999;22:562-568.

5. Gami AS, Witt BJ, Howard DE et al. Metabolic syndrome and risk of incident cardiovascular events and death: a systematic review and meta-analysis of longitudinal studies. *J Am Coll Cardiol.* 2007;49:403-414.

6. Barker DJ, Osmond C, Forsen TJ, Kajantie E, Eriksson JG. Trajectories of growth among children who have coronary events as adults. *N Engl J Med.* 2005;353:1802-1809.

7. Liang CP, Han S, Senokuchi T, Tall AR. The macrophage at the crossroads of insulin resistance and atherosclerosis. *Circ Res.* 2007;100:1546-1555.

8. Tabas I, Williams KJ, Boren J. Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation.* 2007;116:1832-1844.

9. Ross R. Cell biology of atherosclerosis. *Annu Rev Physiol.* 1995;57:791-804.

10. Glass CK, Witztum JL. Atherosclerosis. the road ahead. *Cell.* 2001;104:503-516.

11. Lusis AJ. Atherosclerosis. *Nature.* 2000;407:233-241.

12. Tabas I. Macrophage death and defective inflammation resolution in atherosclerosis. *Nature Rev Immunol.* 2010.

13. Aikawa M, Libby P. The vulnerable atherosclerotic plaque: pathogenesis and therapeutic approach. *Cardiovasc Pathol.* 2004;13:125-138.

14. Kolodgie FD, Virmani R, Burke AP et al. Pathologic assessment of the vulnerable human coronary plaque. *Heart.* 2004;90:1385-1391.

15. Burke AP, Virmani R, Galis Z, Haudenschild CC, Muller JE. 34th Bethesda Conference: Task force #2--What is the pathologic basis for new atherosclerosis imaging techniques? *J Am Coll Cardiol.* 2003;41:1874-1886.

16. Kruth HS. Localization of unesterified cholesterol in human atherosclerotic lesions. Demonstration of filipin-positive, oil-red-O-negative particles. *Am J Pathol.* 1984;114:201-208.

17. Guyton JR, Klemp KF. Development of the atherosclerotic core region. Chemical and ultrastructural analysis of microdissected atherosclerotic lesions from human aorta. *Arterioscler Thromb.* 1994;14:1305-1314.

18. Small DM. George Lyman Duff memorial lecture. Progression and regression of atherosclerotic lesions. Insights from lipid physical biochemistry. *Arteriosclerosis.* 1988;8:103-129.

19. Lundberg B. Chemical composition and physical state of lipid deposits in atherosclerosis. *Atherosclerosis.* 1985;56:93-110.

20. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol.* 2008;8:349-361.

21. Schaefer HE. The role of macrophages in atherosclerosis. *Hamatol Bluttransfus.* 1981;27:137-142.

22. Ball RY, Stowers EC, Burton JH, Cary NR, Skepper JN, Mitchinson MJ. Evidence that the death of macrophage foam cells contributes to the lipid core of atheroma. *Atherosclerosis.* 1995;114:45-54.

23. Tabas I. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. *Arterioscler Thromb Vasc Biol.* 2005;25:2255-2264.

24. Libby P, Clinton SK. The role of macrophages in atherogenesis. *Curr Opin Lipidol.* 1993;4:355-363.

25. Hegyi L, Skepper JN, Cary NRB, Mitchinson MJ. Foam cell apoptosis and the development of the lipid core of human atherosclerosis. *J Pathol.* 1996;180:423-429.

26. Naghavi M, Libby P, Falk E et al. From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part I. *Circulation.* 2003;108:1664-1672.

27. Fadok VA, Bratton DL, Henson PM. Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. *J Clin Invest.* 2001;108:957-962.

28. Fadok VA, Bratton DL, Guthrie L, Henson PM. Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases. *J Immunol.* 2001;166:6847-6854.

29. Young JL, Libby P, Schonbeck U. Cytokines in the pathogenesis of atherosclerosis. *Thromb Haemost.* 2002;88:554-567.

30. Ohayon J, Finet G, Gharib AM et al. Necrotic core thickness and positive arterial remodeling index: emergent biomechanical factors for evaluating the risk of plaque rupture. *Am J Physiol Heart Circ Physiol.* 2008;295:H717-H727.

31. Tabas I. Apoptosis and plaque destabilization: the role of macrophage apoptosis induced by cholesterol. *Cell Death & Differentiation.* 2004;11:S12-S16.

32. Feng B, Yao PM, Li Y et al. The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages. *Nat Cell Biol.* 2003;5:781-792.

33. Thorp E, Li G, Seimon TA, Kuriakose G, Ron D, Tabas I. Reduced apoptosis and plaque necrosis in advanced atherosclerotic lesions of *Apoe*-/- and *Ldlr*-/- mice lacking CHOP. *Cell Metabolism.* 2009;9:474-481.

34. DeVries-Seimon T, Li Y, Yao PM et al. Cholesterol-induced macrophage apoptosis requires ER stress pathways and engagement of the type A scavenger receptor. *J Cell Biol.* 2005;171:61-73.

35. Seimon TA, Obstfeld A, Moore KJ, Golenbock DT, Tabas I. Combinatorial pattern recognition receptor signaling alters the balance of life and death in macrophages. *Proc Natl Acad Sci U S A.* 2006;103:19794-19799.

36. Yao PM, Tabas I. Free cholesterol loading of macrophages induces apoptosis involving the fas pathway. *J Biol Chem.* 2000;275:23807-23813.

37. Yao PM, Tabas I. Free cholesterol loading of macrophages is associated with widespread mitochondrial dysfunction and activation of the mitochondrial apoptosis pathway. *J Biol Chem.* 2001;276:42468-42476.

38. Shio H, Haley NJ, Fowler S. Characterization of lipid-laden aortic cells from cholesterol-fed rabbits. III. Intracellular localization of cholesterol and cholesteryl ester. *Lab Invest.* 1979;41:160-167.

39. Katz SS, Shipley GG, Small DM. Physical chemistry of the lipids of human atherosclerotic lesions. Demonstration of a lesion intermediate between fatty streaks and advanced plaques. *J Clin Invest.* 1976;58:200-211.

40. Lim WS, Timmins JM, Seimon TA et al. STAT1 is critical for apoptosis in macrophages subjected to endoplasmic reticulum stress *in vitro* and in advanced atherosclerotic lesions *in vivo*. *Circulation.* 2008;117:940-951.

41. Timmins JM, Ozcan L, Seimon TA et al. Calcium/calmodulin-dependent protein kinase II links endoplasmic reticulum stress with Fas and mitochondrial apoptosis pathways. *J Clin Invest.* 2009. In press.

42. Li G, Mongillo M, Chin KT et al. Role of ERO1α-mediated stimulation of inositol 1,4,5-triphosphate receptor activity in endoplasmic reticulum stress-induced apoptosis. *J Cell Biol.* 2009. In press.

43. Li Y, Ge M, Ciani L et al. Enrichment of endoplasmic reticulum with cholesterol inhibits SERCA2b activity in parallel with increased order of membrane lipids. Implications for depletion of ER calcium stores and apoptosis in cholesterol-loaded macrophages. *J Biol Chem.* 2004;279:37030-37039.

44. Gautier EL, Huby T, Witztum JL et al. Macrophage apoptosis exerts divergent effects on atherogenesis as a function of lesion stage. *Circulation.* 2009.

45. Secchiero P, Candido R, Corallini F et al. Systemic tumor necrosis factor-related apoptosis-inducing ligand delivery shows antiatherosclerotic activity in apolipoprotein E-null diabetic mice. *Circulation.* 2006;114:1522-1530.

46. Schrijvers DM, De Meyer GR, Kockx MM, Herman AG, Martinet W. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2005;25:1256-1261.

47. Chang M-K, Bergmark C, Laurila A et al. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: Evidence that oxidation-specific epitopes mediate macrophage recognition. *Proc Natl Acad Sci USA.* 1999;96:6353-6358.

48. Thorp E, Cui D, Schrijvers DM, Kuriakose G, Tabas I. Mertk receptor mutation reduces efferocytosis efficiency and promotes apoptotic cell accumulation and plaque necrosis in atherosclerotic lesions of *Apoe*-/- mice. *Arterioscler Thromb Vasc Biol.* 2008;28:1421-1428.

49. it-Oufella H, Pouresmail V, Simon T et al. Defective mer receptor tyrosine kinase signaling in bone marrow cells promotes apoptotic cell accumulation and accelerates atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2008;28:1429-1431.

50. Sather S, Kenyon KD, Lefkowitz JB et al. A soluble form of the Mer receptor tyrosine kinase inhibits macrophage clearance of apoptotic cells and platelet aggregation. *Blood.* 2007;109:1026-1033.

51. Feng B, Zhang D, Kuriakose G, Devlin CM, Kockx M, Tabas I. Niemann-Pick C heterozygosity confers resistance to lesional necrosis and macrophage apoptosis in murine atherosclerosis. *Proc Natl Acad Sci U S A.* 2003;100:10423-10428.

52. Zhou J, Lhotak S, Hilditch BA, Austin RC. Activation of the unfolded protein response occurs at all stages of atherosclerotic lesion development in apolipoprotein E-deficient mice. *Circulation.* 2005;111:1814-1821.

53. Gargalovic PS, Gharavi NM, Clark MJ et al. The unfolded protein response is an important regulator of inflammatory genes in endothelial cells. *Arterioscler Thromb Vasc Biol.* 2006;26:2490-2496.

54. Myoishi M, Hao H, Minamino T et al. Increased endoplasmic reticulum stress in atherosclerotic plaques associated with acute coronary syndrome. *Circulation.* 2007;116:1226-1233.

55. Sanson M, Auge N, Vindis C et al. Oxidized low-density lipoproteins trigger endoplasmic reticulum stress in vascular cells: prevention by oxygen-regulated protein 150 expression. *Circ Res.* 2009;104:328-336.

56. Manning-Tobin JJ, Moore KJ, Seimon TA et al. Loss of SR-A and CD36 activity reduces atherosclerotic lesion complexity without abrogating foam cell formation in hyperlipidemic mice. *Arterioscler Thromb Vasc Biol.* 2009;29:19-26.

57. Madan M, Amar S. Toll-like receptor-2 mediates diet and/or pathogen associated atherosclerosis: proteomic findings. *PLoS ONE.* 2008;3:e3204.

58. Thorp E, Tabas I. Mechanisms and consequences of efferocytosis in advanced atherosclerosis. *J Leukocyte Biol.* 2009.

59. Virmani R, Burke AP, Kolodgie FD, Farb A. Vulnerable plaque: the pathology of unstable coronary lesions. *J Interv Cardiol.* 2002;15:439-446.

60. Plutzky J, Viberti G, Haffner S. Atherosclerosis in type 2 diabetes mellitus and insulin resistance: mechanistic links and therapeutic targets. *J Diabetes Complications.* 2002;16:401-415.

61. D'Souza A, Hussain M, Howarth FC, Woods NM, Bidasee K, Singh J. Pathogenesis and pathophysiology of accelerated atherosclerosis in the diabetic heart. *Mol Cell Biochem.* 2009.

62. Burke AP, Kolodgie FD, Zieske A et al. Morphologic findings of coronary atherosclerotic plaques in diabetics: a postmortem study. *Arterioscler Thromb Vasc Biol.* 2004;24:1266-1271.

63. Moreno PR, Murcia AM, Palacios IF et al. Coronary composition and macrophage infiltration in atherectomy specimens from patients with diabetes mellitus. *Circulation.* 2000;102:2180-2184.

64. Nasu K, Tsuchikane E, Katoh O et al. Plaque characterisation by Virtual Histology intravascular ultrasound analysis in patients with type 2 diabetes. *Heart.* 2008;94:429-433.

65. Hong YJ, Jeong MH, Choi YH et al. Plaque characteristics in culprit lesions and inflammatory status in diabetic acute coronary syndrome patients. *JACC Cardiovasc Imaging.* 2009;2:339-349.

66. Pundziute G, Schuijf JD, Jukema JW et al. Type 2 diabetes is associated with more advanced coronary atherosclerosis on multislice computed tomography and virtual histology intravascular ultrasound. *J Nucl Cardiol.* 2009;16:376-383.

67. Garcia-Garcia HM, Serruys PW, Mintz GS et al. Synergistic effect of cardiovascular risk factors on necrotic core in coronary arteries: a report from the global intravascular radiofrequency data analysis registry. *JACC Cardiovasc Imaging.* 2009;2:629-636.

68. Liang CP, Han S, Okamoto H et al. Increased CD36 protein as a response to defective insulin signaling in macrophages. *J Clin Invest.* 2004;113:764-773.

69. Han S, Liang CP, DeVries-Seimon T et al. Macrophage insulin receptor deficiency increases ER stress-induced apoptosis and necrotic core formation in advanced atherosclerotic lesions. *Cell Metabolism.* 2006;3:257-266.

70. Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidized low density lipoprotein. *J Biol Chem.* 1993;268:11811-11816.

71. Senokuchi T, Liang CP, Seimon TA et al. Forkhead transcription factors (FoxOs) promote apoptosis of insulin-resistant macrophages during cholesterol-induced endoplasmic reticulum stress. *Diabetes.* 2008;57:2967-2976.

72. Thorp E, Kuriakose G, Shah YM, Gonzalez FJ, Tabas I. Pioglitazone increases macrophage apoptosis and plaque necrosis in advanced atherosclerotic lesions of nondiabetic low-density lipoprotein receptor-null mice. *Circulation.* 2007;116:2182-2190.

73. Fernandez-Hernando C, Ackah E, Yu J et al. Loss of akt1 leads to severe atherosclerosis and occlusive coronary artery disease. *Cell Metab.* 2007;6:446-457.

74. Nakae J, Park BC, Accili D. Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a Wortmannin-sensitive pathway. *J Biol Chem.* 1999;274:15982-15985.

75. Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science.* 1996;274:782-784.

76. Li Y, Schwabe RF, DeVries-Seimon T et al. Free cholesterol-loaded macrophages are an abundant source of tumor necrosis factor-alpha and interleukin-6: model of NF-kappaB- and map kinase-dependent inflammation in advanced atherosclerosis. *J Biol Chem.* 2005;280:21763-21772.

77. Liang CP, Han S, Li G, Senokuchi T, Tabas I, Tall AR. Defective MEK signaling and SERCA activity contribute to increased apoptosis in insulin resistant macrophages. *Diabetes.* 2008;57:A198.

78. Baumgartl J, Baudler S, Scherner M et al. Myeloid lineage cell-restricted insulin resistance protects apolipoproteinE-deficient mice against atherosclerosis. *Cell Metab.* 2006;3:247-256.

79. Collins AR, Lyon CJ, Xia X et al. Age-accelerated atherosclerosis correlates with failure to upregulate antioxidant genes. *Circ Res.* 2009;104:e42-e54.

80. Chen XL, Kunsch C. Induction of cytoprotective genes through Nrf2/antioxidant response element pathway: a new therapeutic approach for the treatment of inflammatory diseases. *Curr Pharm Des.* 2004;10:879-891.

81. Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. *Circulation.* 1998;97:1837-1847.

82. Naidoo N. The endoplasmic reticulum stress response and aging. *Rev Neurosci.* 2009;20:23-37.

83. Aprahamian T, Takemura Y, Goukassian D, Walsh K. Ageing is associated with diminished apoptotic cell clearance in vivo. *Clin Exp Immunol.* 2008;152:448-455.

84. Williams KJ, Fisher EA. Oxidation, lipoproteins, and atherosclerosis: which is wrong, the antioxidants or the theory? *Curr Opin Clin Nutr Metab Care.* 2005;8:139-146.

85. Hu B, Punturieri A, Todt J, Sonstein J, Polak T, Curtis JL. Recognition and phagocytosis of apoptotic T cells by resident murine tissue macrophages require multiple signal transduction events. *J Leukoc Biol.* 2002;71:881-889.

86. Hall MO, Agnew BJ, Abrams TA, Burgess BL. The phagocytosis of os is mediated by the PI3-kinase linked tyrosine kinase receptor, mer, and is stimulated by GAS6. *Adv Exp Med Biol.* 2003;533:331-336.

87. Li S, Sun Y, Thorp E et al. Defective phagocytosis of apoptotic cells by macrophages in atherosclerotic lesions of ob/ob mice and its reversal by a fish oil diet. 2009.

88. Calder PC, Bond JA, Harvey DJ, Gordon S, Newsholme EA. Uptake and incorporation of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis. *Biochem J.* 1990;269:807-814.

89. Leaf A. Historical overview of n-3 fatty acids and coronary heart disease. *Am J Clin Nutr.* 2008;87:1978S-1980S.

90. Rosenfeld ME, Carson KG, Johnson JL, Williams H, Jackson CL, Schwartz SM. Animal models of spontaneous plaque rupture: the holy grail of experimental atherosclerosis research. *Curr Atheroscler Rep.* 2002;4:238-242.

91. Han S, Liang CP, Westerterp M et al. Hepatic insulin signaling regulates VLDL secretion and atherogenesis in mice. *J Clin Invest.* 2009;119:1029-1041.

92. Johansson F, Kramer F, Barnhart S et al. Type 1 diabetes promotes disruption of advanced atherosclerotic lesions in LDL receptor-deficient mice. *Proc Natl Acad Sci U S A.* 2008;105:2082-2087.

93. Nishikawa T, Edelstein D, Du XL et al. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature.* 2000;404:787-790.

94. Harja E, Bu DX, Hudson BI et al. Vascular and inflammatory stresses mediate atherosclerosis via RAGE and its ligands in apoE-/- mice. *J Clin Invest.* 2008;118:183-194.

95. Tanaka J, Li Q, Banks AS et al. Foxo1 links hyperglycemia to LDL oxidation and eNOS dysfunction in vascular endothelial cells. *Diabetes.* 2009.

96. Khan MI, Pichna BA, Shi Y, Bowes AJ, Werstuck GH. Evidence Supporting a Role for Endoplasmic Reticulum Stress in the Development of Atherosclerosis in a Hyperglycaemic Mouse Model. *Antioxid Redox Signal.* 2009.

97. Reaven PD, Moritz TE, Schwenke DC et al. Intensive Glucose Lowering Therapy Reduces Cardiovascular Disease Events in VADT Participants with Lower Calcified Coronary Atherosclerosis. *Diabetes.* 2009.

98. Preis SR, Pencina MJ, Hwang SJ et al. Trends in cardiovascular disease risk factors in individuals with and without diabetes mellitus in the framingham heart study. *Circulation.* 2009;120:212-220.

99. Oh J, Weng S, Felton SK et al. 1,25(OH)2 vitamin d inhibits foam cell formation and suppresses macrophage cholesterol uptake in patients with type 2 diabetes mellitus. *Circulation.* 2009;120:687-698.

100. Balch WE, Morimoto RI, Dillin A, Kelly JW. Adapting proteostasis for disease intervention. *Science.* 2008;319:916-919.

101. Zhang R, Khoo MS, Wu Y et al. Calmodulin kinase II inhibition protects against structural heart disease. *Nat Med.* 2005;11:409-417.

102. Yu J, Weiwer M, Linhardt RJ, Dordick JS. The role of the methoxyphenol apocynin, a vascular NADPH oxidase inhibitor, as a chemopreventative agent in the potential treatment of cardiovascular diseases. *Curr Vasc Pharmacol.* 2008;6:204-217.

103. Mitchell S, Thomas G, Harvey K et al. Lipoxins, aspirin-triggered epi-lipoxins, lipoxin stable analogues, and the resolution of inflammation: stimulation of macrophage phagocytosis of apoptotic neutrophils in vivo. *J Am Soc Nephrol.* 2002;13:2497-2507.

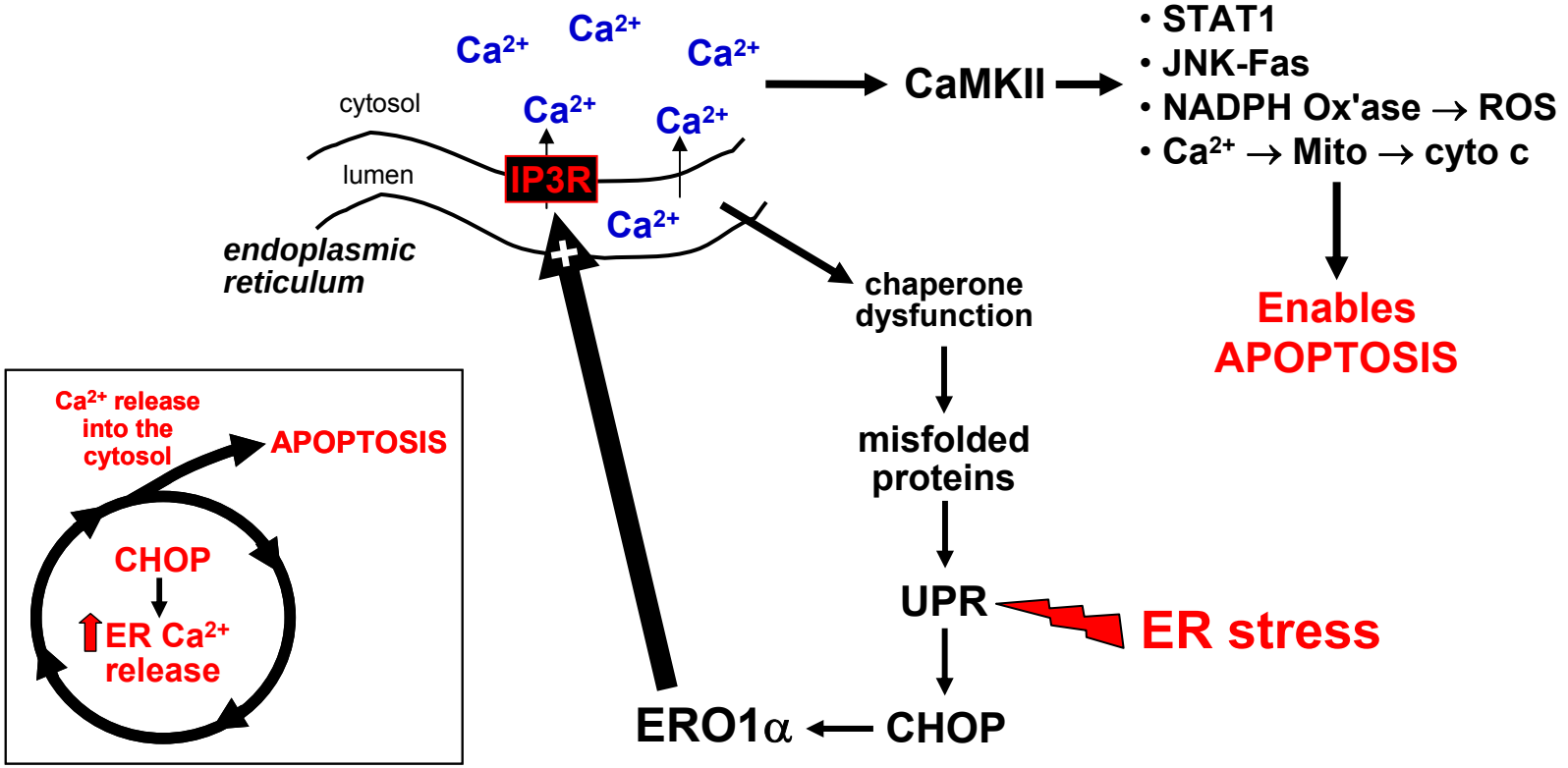
104. Wickline SA, Neubauer AM, Winter PM, Caruthers SD, Lanza GM. Molecular imaging and therapy of atherosclerosis with targeted nanoparticles. *J Magn Reson Imaging.* 2007;25:667-680.

105. Silvera SS, Aidi HE, Rudd JH et al. Multimodality imaging of atherosclerotic plaque activity and composition using FDG-PET/CT and MRI in carotid and femoral arteries. *Atherosclerosis.* 2009.

## Figure Legends

**Figure 1.** A CHOP-calcium pathway of ER stress-induced apoptosis in macrophages. A diverse array of ER stress-provoking events, many of which exist in advanced atheromata, trigger the UPR and lead to induction of the downstream effector CHOP. CHOP induces ERO1$\alpha$, which in turn oxidatively activates IP3R calcium release channels in the ER. IP3R-mediated calcium release begins a pro-apoptotic cascade involving activation of CaMKII by cytosolic calcium and subsequent downstream apoptotic processes, as listed in the figure and as described in the text. In addition, the resulting low level of calcium in the ER lumen likely causes dysfunction of calcium-dependent protein chaperones, which amplifies UPR activation. The central concept is that pro-apoptotic CHOP functions, at least in part, by promoting calcium-induced death as part of a positive feedback cycle (see *inset*).

**Figure 2.** Cellular-molecular mechanisms by which macrophage insulin resistance promotes ER stress-induced macrophage apoptosis and advanced plaque progression. At least three pro-apoptotic processes are enhanced in ER stressed macrophages: (1) ER stress normally activates a compensatory MEK-ERK-SERCA pathway to lower cytoplasmic calcium and replenish ER lumenal stores. This pathway is blocked in the setting of insulin resistance, leading to enhanced activation of calcium-mediated apoptotic pathways (increased cytosolic calcium) and further UPR-CHOP activation (decreased ER lumenal calcium). (2) CD36 and SRA, two PRRs that, when activated, are synergistic with ER stress in inducing apoptosis, are up-regulated in insulin-resistant macrophages. (3) Increased nuclear FoxO in insulin-resistant macrophages induces I$\kappa$B$\varepsilon$, thereby suppressing a compensatory NF-$\kappa$B cell-survival pathway. In addition to these pro-apoptotic processes, increased levels of saturated fatty acids in the setting obesity compromise the ability of macrophages to engulf apoptotic cells. Apoptotic cells that are not efficiently cleared become secondarily necrotic and, over time, accumulate into necrotic cores in advanced plaques. These necrotic cores, which are particularly large in diabetic atheromata, are thought to contribute to plaque disruption. See text for details.

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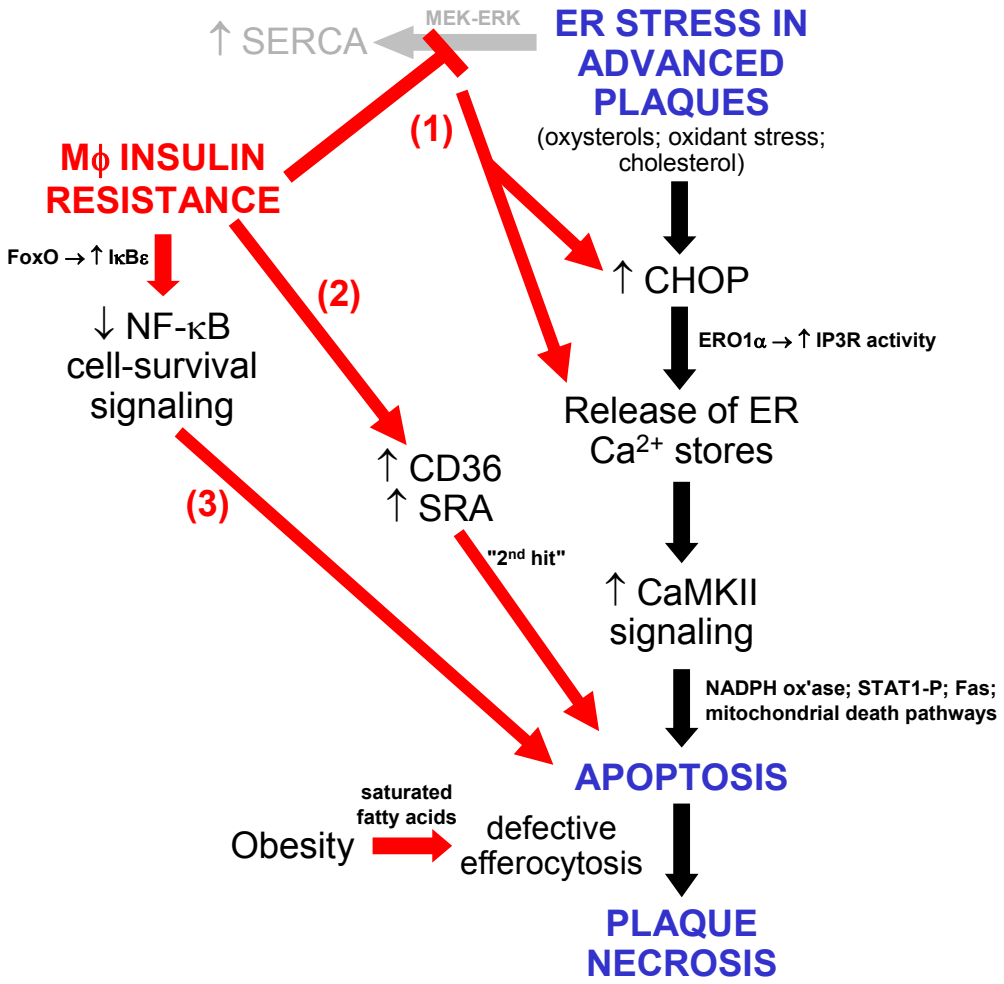


**Figure 2 .** Cellular-molecular mechanisms by wh ich ma crophage in sulin re sistance promotes ER stress-induced macrophage apoptosis and advanced plaque progression. At leas t th ree pro-apoptotic processes are enhanced in ER s tressed mac rophages: (1) ER stress normally activates a compensatory MEK-ERK-SERCA pathway to lower cytoplasmic calcium and replenish ER lumenal stores. This pathway is blocked in the setting of insulin resistance, leading to enhanced activation of calcium-mediated apoptotic pathways (increased cytosolic calcium) and further UPR-CHOP activation (decreased ER lumenal calcium). (2 ) CD36 and SRA, two PRRs that, when activated, are synergistic with ER s tress in inducing apoptosis, are up-regulated in insulin-resistant macrophages. (3) Increased nuclear FoxO in insulin-resistant macrophages induces IkBε, thereby suppressing a compensatory NF-κB cell-survival pathway. In addition to these pro-apoptotic processes, increased levels of saturated fatty acids in the setting obesity compromise the ability of macrophages to engulf apoptotic cells. Apoptotic cells that are not efficiently cleared become secondarily necrotic and, o ver time , accu mulate into necrotic cores in advanced p laques. T hese nec rotic cores, which a re pa rticularly large in diabetic atheromata, are thought to contribute to plaque disruption. See text for details.

P24-7

# Beta-Arrestin 2: a critical integrator of survival signaling in macrophage apoptosis

Judith C Sluimer[1], Robert J Lefkowitz[2], Ira A Tabas[1]

[1] Columbia University, Dept. Medicine, New York, NY, [2] Duke University Medical Center, HHMI, Durham,NC

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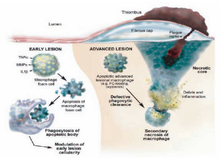
## Introduction

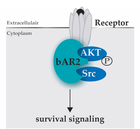
There is an epidemic of obesity worldwide, including among military personnel and their families. The most important detrimental consequence of obesity is atherothrombotic vascular disease, the cause of unstable angina, myocardial infarction, sudden cardiac death, and stroke. These clinical complications of atherosclerosis are mainly caused by thrombus formation, which results from rupture of an atherosclerotic plaque.




Dead macrophages accumulate in advanced atherosclerosis as macrophage cell death (apoptosis) is increased, and the clearance of dead macrophages is decreased compared to early atherosclerosis. Dead cell accumulation results in increased necrotic core size and inflammation, causing plaque rupture.

In previous work, we have shown that in the early phases of apoptosis a pro-survival pathway is triggered that aims to rescue macrophages from apoptosis. This signal transduction pathway is headed by the activation of the signaling protein AKT through its phosphorylation.

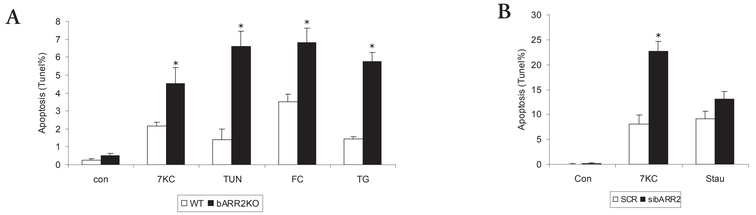
Beta-arrestin 2 (BARR2) is a protein that is often essential for AKT phosphorylation. BARR2 functions as a scaffold bringing AKT and its activator SRC kinase together, and is thus essential for induction of survival signaling. Deficiency of BARR2 and concomittant reduction of pro-survival signaling has been shown to enhance apoptosis in several cell types, but its function in plaque macrophages is unknown.




## Hypothesis

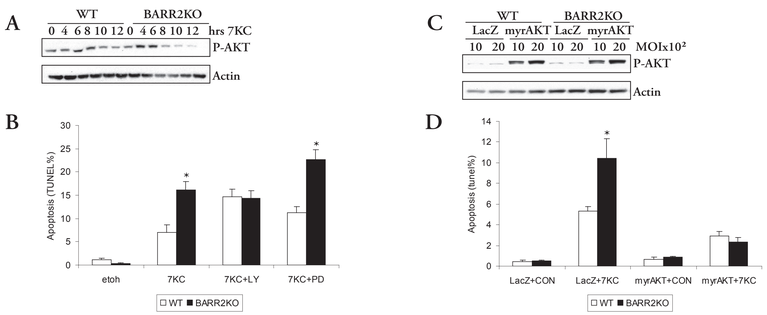
BARR2 deficiency in advanced plaque macrophages leads to reduced survival signaling and thus to increased macrophage apoptosis.

## Results



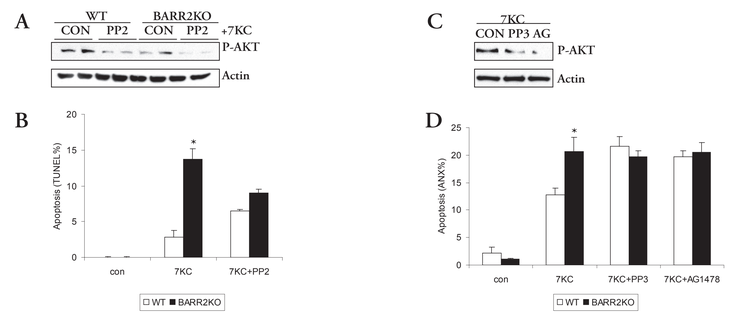

Complete and partial knock-down of BARR2 increased macrophage apoptosis *in vitro*

A. Peritoneal macrophages of methyl-BSA immunized C57Bl/6 and BARR2 KO mice were treated with apoptotic stimuli for 18hrs. These stimuli mimic endoplasmatic reticulum (ER-)stress, which is observed in advanced atherosclerosis: 7ketocholesterol (7KC, 35uM), tunicamycin (5ug/ml), free cholesterol loading (FC; acLDL(50ug/ml)+ACAT inhibitor (58035, 10ug/ml) and thapsigargin (TG, 2.5uM). The percentage of TUNEL+ cells were quantified. B. Apoptosis induced by 7KC is also enhanced when BARR2 expression is silenced using siRNA treatment. Apoptosis is not enhanced in BARR2 macrophages after staurosporin treatment (100nM), which does not induce ER-stress. * P<0.05



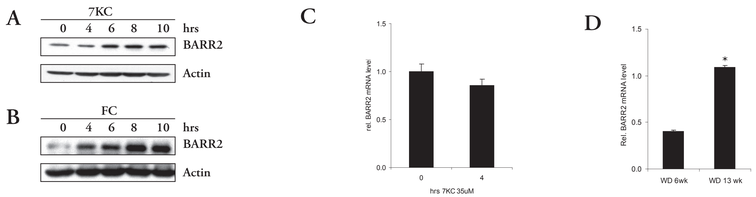

BARR2-dependent survival involves AKT activation

A. WT and BARR2KO macrophages were incubated with 7KC for the indicated times and P-AKT was detected using western blot. P-AKT signaling was induced by 7KC in WT macrophages, while BARR2KO showed less sustained activity. Actin was used as loading control. B. Chemical inhibition of AKT activity by LY294002 (LY, 10uM) enhanced WT apoptosis, induced by 7KC for 18hr, to the same level as BARR2KO. In contrast, the EKR inhibitor PD98059 (PD, 10uM) further enhanced 7KC-induced apoptosis in both WT and KO macrophages. This indicates that AKT activity is essential for BARR2 dependent survival signaling, whereas ERK is not. C. WT and BARR2KO macrophages were transfected with control and constitutively active AKT (myrAKT) adenoviral DNA (MOI 1000-2000) and after 48hrs expression was confirmed using western blot. D. Myr-AKT, but not control adenovirus (MOI 1000) rescued the enhanced apoptosis in BARR2KO macrophages, also confirming that AKT activity is essential for bARR2-dependent survival in macrophages. * P<0.05




BARR2 and AKT-dependent survival requires SRC and EGFR activation

A. WT and BARR2KO macrophages were pre-treated with SRC kinase inhibitor PP2 (10uM), incubated with 7KC for 9hrs and P-AKT was detected using western blot. P-AKT signaling was reduced in WT and BARR2KO macrophages. Actin was used as loading control. B. WT and BARR2KO macrophages were pre-treated with PP2 followed by 7KC for 18hrs to induce apoptosis. SRC inhibition enhanced WT apoptosis to the same level as BARR2KO, suggesting that SRC initiates AKT activity, which is essential for BARR2-dependent survival in macrophages. C. Macrophages were pre-treated with inhibitors of the epidermal growth factor receptor, EGFR (PP3, 10uM, Ag1478 5uM), and 7KC for 9hrs and P-AKT was detected using western blot. D. WT and BARR2KO macrophages were pre-treated with EGFR inhbitors followed by 7KC for 18hrs to induce apoptosis. EGFR inhibition enhanced WT apoptosis to the same level as BARR2KO, suggesting that the EGFR is also essential for AKT and BARR2-dependent survival in macrophages. * P<0.05




BARR2 expression is enhanced in ER-stress conditions and in advanced atherosclerotic plaques

A. Macrophages were incubated with 7KC or FC (B) for the indicated timepoints and bAR2 protein was detected using western blot. ER-stress stimuli induced BARR2 protein. Actin was used as loading control. C. BARR2 mRNA, assessed by real-time RT-PCR, was unchanged in macrophages incubated with 7KC. D. Atherogenic low density lipoprotein receptor (LDLR) KO mice were fed a western diet for 6 or 13 wks to induce atherosclerosis. Plaque tissue was isolated from frozen aortic root sections using laser-capture microdissection and BARR2 mRNA was assessed by real-time RT-PCR. * P<0.05

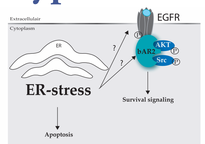
## Conclusions

* BARR2 knockdown (siRNA and genetic) enhanced macrophage apoptosis
* Survival signaling by BARR2 requires SRC kinase and activation of AKT
* BARR2 signaling depends on the epidermal growth factor receptor
* Expression of BARR2 is increased by ER-stress and with plaque progression

## Future experiments

* Study effect of macrophage-specific BARR2 knockdown on plaque size+apoptosis
* Silence EGFR using siRNA and assess survival in WT vs. BARR2KO macrophages
* Study mechanism of the BARR2 translational increase in ER-stress
* Investigate mechanisms of EGFR activation and its effect on atherosclerosis

## Hypothesis




## Impact statement

Activation of survival signaling by BARR2 and EGFR may prevent accumulation of apoptotic macrophages and thus advancement of atherosclerosis. This new discovery may pave the way for a novel strategy to prevent advanced lesion progression and thus clinical complications of cardiovascular disease in high-risk obese subjects.

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