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Myeloid *atg5* deletion impairs n-3 PUFA-mediated atheroprotection

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Abstract

Background and aims: Dietary long-chain (≥ 20 carbons) n-3 polyunsaturated fatty acids (PUFAs) reduce atherosclerosis and enhance macrophage autophagy activation. How macrophage autophagy impacts atherosclerotic progression, particularly when comparing dietary n-3 PUFA supplementation vs. saturated fat feeding, is unknown.

Methods: We generated myeloid-specific autophagy-deficient and control mice in the *Ldlr*^{-/-} background by transplanting bone marrow from myeloid-specific autophagy-related (*atg*) 5 knockout mice and wild type controls into irradiated *Ldlr*^{-/-} recipients. After 7 weeks for recovery from radiation, mice were fed an atherogenic diet containing 0.2% cholesterol and 20% calories as palm oil (PO diet), or 10% calories as PO plus 10% calories as fish oil (FO diet) for 16 weeks.

Results: Compared to PO, FO significantly reduced plasma cholesterol, triglyceride, hepatic neutral lipid, and aortic caspase-1 cleavage, but increased aortic efferocytosis, leading to attenuated atherosclerosis in *Ldlr*^{-/-} mice receiving wild type bone marrow. Myeloid *atg5* deletion had little impact on plasma lipid concentrations and hepatic neutral lipid content, regardless of diet. Myeloid *atg5* deletion increased aortic caspase-1 cleavage, decreased aortic efferocytosis and worsened atherosclerosis only in the FO-fed *Ldlr*^{-/-} mice.

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Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Conclusions: Deficient myeloid autophagy significantly attenuated FO-induced atheroprotection, suggesting that dietary n-3 PUFAs reduce atherosclerosis, in part, by activation of macrophage autophagy.

Graphical Abstract

Keywords

atherosclerosis; fish oil; inflammation; macrophage; apoptosis; autophagy

Introduction

Cardiovascular disease, mainly caused by atherosclerosis, is the leading cause of death worldwide (1). Atherosclerosis is a lipid disorder and a chronic inflammatory disease driven by the accumulation of cholesterol-loaded macrophage foam cells in the artery wall (2–5). Autophagy is an intracellular homeostatic process by which cells deliver protein aggregates, damaged organelles, and invading pathogens to lysosomes for degradation. Autophagy has been detected in almost all major cell types (i.e., macrophages, endothelial cells, and smooth muscle cells) of human atherosclerotic plaques using transmission electron microscopy (6). Additionally, the classical markers of autophagy, including light chain (LC)3-II and p62/SQSTM1, are found in both human and mouse atherosclerotic plaques. For example, elevated levels of LC3-II were observed in advanced human plaques via immunoblotting and immunohistochemical analyses (7). LC3-II expression was 5-fold lower in symptomatic (unstable) plaques than in asymptomatic (stable) plaques (8), suggesting that autophagy declines as atherosclerosis progresses. Furthermore, p62/SQSTM1 and polyubiquitinated proteins, colocalizing with plaque macrophages (9–11), exist in human atherosclerotic plaques and *apoE*^{-/-} mouse atheroma lesions, suggesting that atherosclerosis progression is associated with defective autophagy.

Studies using genetic autophagy-defective mice support the concept that dysregulation of autophagy contributes to a variety of pathologic conditions, including aging, type 2 diabetes, and cardiovascular diseases (12–14). Mechanistically, macrophage autophagy can be

activated by atherogenic lipids such as oxidized LDL (oxLDL) and 7-ketocholesterol, one of the primary oxysterols in oxLDL (15). Activated autophagy enhances macrophage efferocytosis, lowers oxidative stress, and reduces apoptosis, hence, promoting macrophage survival (15). Enhanced autophagy activation is also necessary for delivering lipid droplets to lysosomes for hydrolysis to generate free cholesterol for ABCA1-dependent cholesterol efflux in macrophage foam cells, thus reducing macrophage foam cell formation (16). Additionally, autophagy is anti-inflammatory, as evidenced by enhancing macrophage alternative (M2) and decreasing classic (M1) activation (17). Moreover, autophagy negatively regulates the nucleotide-binding oligomerization domain leucine-rich repeat containing receptor (NLRP3) inflammasome in macrophages by promoting degradation of pro-IL-1 β (18) and the NLRP3 inflammasome complex (19), and decreasing production and release of mitochondrial reactive oxygen species and oxidized mitochondrial DNA through clearing dysfunctional mitochondria (20, 21).

Fish oil (FO), which is enriched in long-chain (> 20 carbons) n-3 polyunsaturated fatty acids (PUFAs; i.e., 20:5 n-3 (eicosapentaenoic acid, EPA) and 22:6 n-3 (docosahexaenoic acid, DHA)), is atheroprotective in mice, nonhuman primates, and humans (22–31). The anti-atherosclerotic effect of the FO is primarily due to its lipid-lowering and anti-inflammatory properties. Recently, we (32) reported that compared to saturated fat (palm oil, PO)-enriched diet, dietary supplementation of n-3 and n-6 PUFAs markedly inhibited NLRP3 inflammasome activation. We also showed that PUFA-enriched diets enhance autophagic activation and that autophagy is required for dietary PUFA-mediated NLRP3 inflammasome repression. However, how macrophage autophagy impacts atherosclerotic progression, particularly when comparing dietary n-3 PUFA supplementation vs. saturated fat feeding, is unknown.

In this study, we examined the role of autophagy in atherosclerosis progression in the context of dietary n-3 PUFAs vs. saturated fat feeding. Our results showed that myeloid *atg5* deletion worsens atherosclerosis in the FO-fed *Ldlr*^{-/-} mice, suggesting that macrophage autophagy is required for dietary n-3 PUFA-induced atheroprotection.

Materials and Methods

Dietary oils

The fruit oil of the palm, *Elaeis guineensis* Jacq (a member of the Arecaceae family) was purchased from Shay and Company (Portland, OR, USA). A certificate of analysis is on file for reference and retention samples are deposited at the Wake Forest School of Medicine. The FO (20:5 n-3 and 22:6 n-3 enriched) source was *Brevoortia tyrannis* Latrobe, a member of the Clupeidae family, was manufactured and provided by Omega Protein (Houston, TX, USA) with a report of analysis on file for reference.

Animals, bone marrow transplantation (BMT), and atherogenic diet feeding

Animals: *Ldlr*^{-/-} (stock 002207) and *LysMcre* (stock 004781) mice were purchased from Jackson Laboratories. *Atg5*^{flox/flox} mice were purchased from Riken BioResource Center (stock RBRC02975). *Atg5*^{flox/flox} mice and *LysMcre* mice were intercrossed to generate

heterozygous *LysMcre⁺atg5^{flox/+}* mice. Intercrossing *LysMcre⁺atg5^{flox/+}* mice resulted in the production of *LysMcre⁺atg5^{flox/flox}* (atg5 myeloid-specific KO, atg5MSKO) mice. *Atg5^{flox/flox}* mice were used as the wild type (WT) controls for atg5MSKO mice. Mice were housed in a specific pathogen-free facility on a 12 h light/dark cycle.

BMT: Female *Ldlr^{-/-}* recipient mice (Jackson Laboratories) at 7 wks of age were fasted overnight and received a sublethal dose of radiation (900 rads) 4 h prior to bone marrow injection. Bone marrow (BM) cells were harvested from cleaned femurs and tibias of male donor (*atg5^{flox/flox}* and atg5MSKO) mice and resuspended in serum-free RPMI 1640 medium. 7×10^6 BM cells per mouse were injected into the retro-orbital venous plexus of anesthetized irradiated female *Ldlr^{-/-}* recipient mice. Recipient mice received autoclaved acidified (pH 2.7) water supplemented with 100 mg/L neomycin and 10 mg/L polymyxin B sulfate 3 days before and 2 wks after the transplantation. Mice were then given autoclaved acidified water until the end of the study. Repopulation of blood leukocytes after BMT was evaluated after 6 wks of recovery and 16 wks of atherogenic diet feeding by determining the percentage expression of the Y-chromosome-associated sex-determining region Y gene (Sry) in genomic DNA obtained from white blood cells, as described previously (31). Additionally, *atg5* mRNA expression was measured in resident peritoneal macrophages isolated from BMT mice after 16 wks of atherogenic diet feeding to confirm the success of BMT.

Atherogenic diet feeding: After ~7 wks for recovery from radiation, *Ldlr^{-/-}* recipient mice were randomly assigned to two groups consuming two atherogenic diets (n=8–10/diet group): 1) PO diet which contains 20% calories as PO and 0.2% cholesterol; 2) FO diet which contains 10% calories as PO plus 10% calories as FO and 0.2% cholesterol, for an additional 16 wks to induce atherosclerosis. Diets were prepared by the diet kitchen in the Department of Pathology at Wake Forest School of Medicine; composition and quality control data for similar atherogenic diets have been published (29, 30, 32).

All animal experimental protocols were approved by the Wake Forest University Animal Care and Use Committee.

Plasma and hepatic lipid analysis

Blood samples were taken from tail veins following a 4 h fast during the light cycle before, and at 4 wks intervals after atherogenic diet feeding began. Plasma cholesterol (Wako) and triglyceride (TG) (Roche) were determined by enzymatic analysis according to the manufacturer's instructions. Liver lipids were extracted with chloroform: methanol (2:1) and the extract was used for enzymatic assays (cholesterol, Wako; TG, Roche). Data were normalized to liver protein mass, measured by the Lowry protein assay.

Peritoneal macrophage culture

Resident peritoneal macrophages were harvested from mice by flushing the peritoneal cavity with cold PBS. The peritoneal cells were plated in RPMI-1640 media containing 100 U/ml penicillin and 100 µg/ml streptomycin. After 2 h incubation, floating cells were removed by

washing with PBS and adherent macrophages were lysed using Trizol (Invitrogen) for RNA extraction.

Bioplex assay and Real-time PCR

Concentrations of cytokines/chemokines in plasma were measured using Bioplex assay according to the manufacturer's instructions. Total RNA in aorta, liver, and peritoneal macrophages was extracted using Trizol (Invitrogen) according to the manufacturer's protocols. cDNA preparation and real-time PCR were conducted, as described previously (32).

Blood cell flow cytometry

Peripheral blood was collected by submandibular vein puncture into heparin-containing tubes. Red blood cells were removed from flow cytometry preparations by treatment with ACK lysing buffer (Gibco, cat# A10492). The remaining white blood cells were incubated with Ly6C/G-PerCP-Cy5.5 (BD Pharmingen, cat# 552093), CD115-APC (eBioscience, cat# 17-1152-82) and V450-CD45 (BD Horizon, cat# 560501) (32). Monocytes were identified as CD45⁺CD115⁺ and further separated into Ly6C^{high} and Ly6C^{low} subsets, and neutrophils were identified as CD45⁺CD115⁻Ly6G⁺. Data were acquired on a BD FACS Canto II instrument (BD Biosciences, Becton, Dickinson and Company, Franklin Lakes, NJ) and analyzed using FACSDiva software v6.1.3 (BD Biosciences).

Quantification of aortic root lesion area

Aortic root atherosclerosis was assessed as described by Daugherty et al. (33). Aortic roots were embedded in Optimal Cutting Temperature (Tissue Tek) media in a plastic mold, frozen, and cut at 8 μ m intervals. Sections were collected from the aortic root moving toward the apex of the heart, fixed in 10% buffered formalin, stained in 0.5% Oil Red O for 25 min, and counterstained with hematoxylin. Stained sections were photographed with a Nikon Digital Sight DSFi1 camera and quantified using NIH ImageJ software. We defined the intimal area as the lesion area between the internal elastic lamina and the luminal surface of the aorta. Aortic root sections were quantified from 7–8 mice per group. To measure necrosis, boundary lines were drawn using NIH ImageJ software around regions that were free of staining. We used a 3000 μ m² threshold in order to avoid counting regions that may not represent substantial areas of necrosis, as described before (34). Results were expressed as cross-sectional plaque area (oil red O) or plaque necrosis (necrosis core), or percent of total plaque area or necrosis core.

Immunohistochemistry staining

Macrophages, T cells or IL-18 in aortic root were stained by incubating aortic root sections with the primary antibodies to CD68 (Bio-Rad), CD3 (Abcam), or IL-18 (Abcam), followed by the biotinylated secondary antibody. The staining was visualized using ABC reagent (ABC vector kit; Vector) and DAB substrate chromogen (Dako). For active caspase-1 staining, a fluorescent peptide-based probe for active caspase-1 (FAM-YVAD-fmk; FLICA Caspase-1 Kit 98, ImmunoChemistry Technologies) was used and FLICA staining was performed as reported before (35) with minor modification. The sections were then stained

with the primary antibodies to CD68 (Bio-Rad), followed by the Texas Red anti-rat secondary antibody (Vector). FLICA fluorescent signal was quantified as relative integrated fluorescence intensity normalized to total plaque as reported (35) by using NIH ImageJ software. Apoptotic cells in atherosclerotic lesions were stained using the Click-iT Plus TUNEL Assay kit (Thermo Fisher) according to the manufacturer's protocol. The sections were then stained with CD68 antibody (Bio-Rad) and nuclei were stained with DAPI. Only TUNEL-positive cells that co-localized with DAPI-positive nuclei were counted as apoptotic cells. Efferocytosis was determined by counting the number of macrophage-associated versus free apoptotic cells, following established methods published before (15, 34, 36, 37). Results were expressed as stained cross-sectional area (CD68, IL-18), or cells per unit area (CD3).

Statistical analysis

Data are presented as the mean $\pm$ SEM unless indicated otherwise. Differences were compared with two-way ANOVA using GraphPad Prism software with post hoc Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant.

Results

Myeloid autophagic deficiency does not affect plasma or liver lipid content

Myeloid autophagy-defective mice are reported to have accelerated progression of atherosclerosis (9, 15). Our previous study demonstrated that dietary PUFA supplementation enhances macrophage autophagy (32). To examine the role of macrophage autophagy in n-3 PUFA-mediated atheroprotection, we transplanted BM from male WT or atg5MSKO mice into irradiated female *Ldlr*^{-/-} recipient mice. Atherosclerosis extent was measured after 16 weeks of an atherogenic diet: 1) PO diet which contains 0.2% cholesterol and 20% calories as PO; or 2) FO diet which contains 0.2% cholesterol, 10% calories as PO and 10% calories as FO. The 16 wks of diet feeding time was chosen based on multiple studies from our group using the same atherogenic diets (29–31, 38). Fatty acid composition in PO and FO atherogenic diets (Supplemental Table 1) were similar to those published previously (30, 32), showing that FO vs. PO diet is enriched in EPA (7 vs. 0.4% FA) and DHA (5 vs. 0.3 % FA) and PO vs. FO diet is enriched in palmitic acid (44.2 vs. 32.6% FA) and oleic acid (35.6 vs. 22.9% FA), respectively. Transplantation efficiency was ~ 90% based on the male *Syr* gene expression in blood leukocytes isolated from recipient mice examined at 6 wks post-radiation (Supplemental Figure 1A and B) or after 16 wks of atherogenic diet feeding (Supplemental Figure 1C and D). Moreover, *atg5* transcript was significantly lower (~80% reduction) in *Ldlr*^{-/-} mice receiving bone marrow from atg5MSKO mice regardless of genotype, indicating the success of BMT (Supplemental Figure 1E).

PO- and FO-fed mice gained similar amounts of weight over 16 wks of diet feeding regardless of myeloid genotype (Figure 1A). Consistent with previous reports (29–31), FO reduced plasma total cholesterol (TC) and plasma triglyceride (TG), relative to PO. Myeloid autophagy deficiency did not change plasma lipid concentrations (Figure 1B–C). Consistent with the plasma lowering effects, FO also reduced hepatic neutral lipid, including cholesteryl ester (CE) and TG, in recipient mice receiving WT or atg5MSKO BM relative to

PO-fed counterparts (Figure 1D). However, no genotypic difference was observed within each diet group, indicating that reduced plasma and liver lipid concentrations in FO- vs. PO-fed mice were independent of macrophage autophagy activation. As FO and autophagy both regulate inflammation, we also assessed liver inflammation by measuring mRNA expression of macrophage markers (CD68 and F4/80) and pro-inflammatory mediators (such as IL-1 β , NLRP3, IL-6, and TNF- α) by qPCR. We found that FO significantly lowered CD68, F4/80, and NLRP3 transcript expression, relative to PO, in WT mice, suggesting that FO decreases hepatic macrophage infiltration induced by atherogenic diet (Supplemental Figure 2). However, myeloid autophagy deficiency did not significantly alter macrophage markers or inflammatory mediator expression (Supplemental Figure 2). Overall, our results suggest that myeloid atg5 deficiency has a minimal effect on plasma and liver lipid and hepatic inflammation.

Myeloid autophagic deficiency promotes atherosclerosis in a diet-specific manner

Myeloid autophagic deficiency accelerates atherosclerosis progression in *Ldlr*^{-/-} and *apoE*^{-/-} atherogenic mice fed a Western-type diet, likely by enhancing macrophage NLRP3 inflammasome activation (9) or impairing efferocytosis and promoting plaque necrosis (15). However, whether macrophage autophagy differentially impacts atherosclerotic progression in the context of dietary n-3 PUFA supplementation vs. saturated fat feeding is unknown. As expected given the 50% reduction of plasma TC and TG with FO supplementation, relative to PO (Figure 1), FO significantly reduced atherosclerosis in recipient mice receiving WT and atg5MSKO BM, as indicated by smaller aortic root lesion size (Oil-Red-O staining) (Figure 2A and B), less necrotic core area (Figure 2C), reduced macrophage (CD68⁺) staining (Figure 2D and E), and reduced T cell (CD3⁺) staining (Figure 2F and G). Unexpectedly, however, distinct roles for myeloid autophagy were noted in the PO vs. FO setting. Whereas atherosclerosis was not higher in PO diet-fed recipient mice receiving BM from atg5MSKO vs. WT mice, atherosclerosis was significantly increased in FO-fed recipient mice receiving atg5MSKO vs. WT BM, as shown by significantly increased aortic root lesion, necrotic core area, a trend toward increased macrophage staining, and significantly increased T cell staining, suggesting that FO-mediated atheroprotection is, in part, mediated by myeloid autophagy activation. Note that myeloid autophagy-mediated atheroprotection in FO-fed mice was independent of lipid-lowering effects because myeloid autophagy deficiency did not alter plasma or liver lipid concentrations (Figure 1).

Myeloid autophagic deficiency promotes aortic root caspase-1 cleavage in a diet-specific manner

Autophagy negatively regulates the NLRP3 inflammasome activation (9, 18–21). Activation of NLRP3 inflammasome activates caspase-1, which then cleaves pro-IL-1 β and pro-IL-18 into biologically active fragments (39–41). The NLRP3 inflammasome was reported to promote atherosclerosis (42, 43). To investigate whether the increased atherosclerosis in recipient mice receiving atg5MSKO vs. WT BM in the setting of FO feeding results from enhanced inflammasome activation, we measured caspase-1 activity or cleavage by staining aortic root sections with FLICA probes. Additionally, in order to specially define macrophage caspase-1 activity, we co-stained aortic root sections with FLICA probes and CD68 antibodies. As shown in Figure 3A and B (left panel), active caspase-1 staining was

reduced in aortic root intimal plaques of FO- vs. PO-fed recipient mice receiving WT BM, suggesting that FO reduces caspase-1 activity in atherosclerotic plaques. By contrast, FO did not reduce caspase-1 activity in recipient mice receiving atg5MSKO BM, indicating that myeloid autophagy is required for the suppressive effect of FO on aortic root caspase-1 cleavage. In PO-fed mice, myeloid autophagy deficiency did not increase FLICA staining in aortic root intimal plaques (Figure 3B, left panel), suggesting a diet-dependent effect of autophagy on aortic root caspase-1 activity. A similar pattern of FLICA staining was observed in CD68+ cells among groups (Figure 3B, right panel). Consistent with the reduced caspase-1 activity, FO also lowered IL-18, an end product of the caspase-1 activity, in aortic plaques of mice receiving WT BM, as shown by decreased IL-18 staining, when compared to PO-fed counterparts (Figure 3C and 3D). However, we did not observe a significant increase in IL-18 staining in FO-fed recipient mice receiving atg5MSKO vs. WT BM. In addition to cleaved caspase-1 staining, we also measured mRNA expression of aortic inflammatory genes, including NLRP3 inflammasome components (IL-1 β , IL-18, NLRP3, and caspase-1), M1 (IL-6 and TNF- α), and M2 macrophage markers (arginase-1, YM-1 and others); however, no differences were observed among groups (Supplemental Figure 3).

Myeloid autophagic deficiency worsens aortic root efferocytosis in a diet-specific manner

One of the mechanisms by which autophagy protects against atherosclerosis is that autophagy activation enhances macrophage efferocytosis, lowers oxidative stress, and reduces apoptosis, hence, promoting macrophage survival (15). Next, we conducted an *in situ* efferocytosis assay in the aortic root to quantify the ratio of TUNEL-labeled apoptotic cells in lesions that are associated with CD68-positive macrophages (i.e., internalized or macrophage surface-attached cell corpses) vs. those un-associated with CD68-positive macrophages (i.e., free cell corpses) (34, 36). We found that compared to PO, FO significantly increased efferocytosis in the aortic root intimal area in recipient mice receiving WT but not atg5MSKO BM, suggesting that FO-mediated efferocytosis is dependent on autophagy (Figure 4). The percentage of apoptotic cells in the aortic root intima did not significantly differ among groups (data not shown).

Myeloid autophagic deficiency does not significantly impact systemic inflammation

We then investigated whether myeloid autophagy deficiency impacts systemic inflammation and if so, whether the effects exhibit a diet-specific pattern. To do this, we first examined whether myeloid autophagy deficiency alters blood monocyte/neutrophil composition in FO vs. PO-fed mice. To do this, we measured the percentage of Ly6C^{high} and Ly6C^{low} blood monocytes (identified as CD115⁺Ly6G⁻) and neutrophils (CD115⁻Ly6G⁺) from 9-wk and 14-wk diet-fed mice as we described before (32). Myeloid autophagy deficiency did not significantly change blood monocyte and neutrophil composition among groups of 9 or 14-wks diet-fed mice (Supplemental Figure 4A and B).

Next, we measured cytokine and chemokine concentrations in plasma from recipient mice receiving BM from WT and atg5MSKO mice after 16-wk diet feeding using Bioplex cytokine arrays. As expected, FO vs. PO significantly lowered plasma cytokines (IL-6 and IL-12p40) and chemokines (KC) regardless of genotype (Figure 5A). Myeloid autophagy defect significantly increased plasma levels of the caspase-1 product IL-18, but not IL-1 β ,

regardless of diet (Figure 5B). Myeloid autophagy defect did not significantly impact other cytokine concentrations in FO- or PO-fed mice regardless of genotype. Taken together, myeloid autophagic deficiency has a minimal effect on systemic inflammation.

Discussion

The anti-atherosclerotic effect of FO is largely due to its lipid-lowering and anti-inflammatory properties. To date, no studies have shown whether and to what extent macrophage autophagy activation contributes to the atheroprotective effects of n-3 PUFAs. In the current study, we demonstrate a diet-specific atherosclerosis enhancement in myeloid autophagy-deficient *Ldlr*^{-/-} mice fed FO, but not PO. Our conclusion is founded in the following key observations: 1) FO atheroprotection is attenuated in myeloid autophagy-defective mice; and 2) FO-mediated reduction of aortic root caspase-1 activation and augmentation of aortic root efferocytosis is dependent upon myeloid cell autophagy.

Progression of atherosclerosis leads to deficiency in macrophage autophagy. Mice with macrophage-specific autophagy defects have impaired in vivo reverse cholesterol transport (16) and increased atherosclerotic plaque progression (9, 15). Moreover, intraperitoneal administration of trehalose (a natural sugar) as an inducer of macrophage autophagy-lysosomal biogenesis, caused atherosclerosis regression in *apoE*^{-/-} mice without affecting plasma cholesterol (10). Together, these studies suggest that macrophage autophagy attenuates atherosclerosis progression and promotes its regression. Mechanistically, macrophage autophagic activation protects against atherosclerosis by promoting cholesterol efflux (16), inactivating the NLRP3 inflammasome (9), and enhancing efferocytosis (15). As our previous reports show that dietary n-3 PUFA supplementation reduces atherosclerosis progression in *Ldlr*^{-/-} mice, in the present study, we investigated whether n-3 PUFAs are atheroprotective, at least in part, by activating macrophage autophagy. Indeed, we observed that myeloid autophagy-deficiency modestly, but significantly dampens FO-induced atheroprotection, as shown by increased neutral lipid accumulation (oil red O staining), necrotic core formation, and macrophage (CD68+ cells) and T cell (CD3+ cells) staining. Elevated atherosclerosis in FO-fed myeloid autophagy-deficient mice was associated with increased aortic intimal caspase-1 activation, and decreased efferocytosis, suggesting that myeloid autophagic activation is part of atheroprotective actions of FO.

Exaggerated atherosclerosis caused by myeloid *atg5* deletion was only observed in FO- but not in PO-fed *Ldlr*^{-/-} recipient mice, a result different from that reported by Liao et al. (15), in which they observed increased atherosclerosis in 12-wk or 16-wk Western diet-fed *atg5MSKOLdlr*^{-/-} mice. Several possibilities may explain the discrepant outcome. First, our *atg5MSKOLdlr*^{-/-} mice were generated by transplanting bone marrow from WT and *atg5MKSO* mice into *Ldlr*^{-/-} mice. Despite the 90% transplantation efficiency as evidenced by *Syr* gene expression in blood leukocytes, peritoneal macrophages from some *Ldlr*^{-/-} recipient mice receiving bone marrow from *atg5MSKO* mice contained a considerable amount of *atg5* transcript (Supplemental Figure 1), suggesting that either cre-induced *atg5* knock-out in myeloid cells, or the BMT (or both) was not efficient in all mice, which is a limitation of the current study. Second, we used a 20% PO-enriched diet (~44% of FA content is palmitate) as our atherogenic diet, different from the Western diet (TD88137,

Harlan Teklad; 28.9% of FA content is palmitate) used by Liao et al. (15). Third, we examined advanced atherosclerosis after 16 wks of FO- or PO-enriched diet feeding (38). As autophagy is gradually impaired during atherosclerosis progression of (9–11) and palmitate inhibits autophagy by promoting atg5 protein degradation (44) and by inhibiting AMP-activated protein kinase (45), we speculate that our PO-enriched diet vs. TD88137 diet (44% vs. 29% FAs as palmitate) likely accelerates autophagy deficiency in *Ldlr*^{-/-} mice during the 16-wk diet feeding. In support of this concept, we observed almost no LC3-II expression in PO-fed *Ldlr*^{-/-} mouse aorta, compared to FO-fed mice (32) (see Figure 5A in ref (32)). Given this, it is possible that we may have missed the optimal time window for observation of increased atherosclerosis in our PO-fed *Ldlr*^{-/-} mice receiving atg5MSKO BM.

Dysregulation of autophagy is associated with non-alcoholic fatty liver disease (NAFLD). Deletion of atg7 (46) or atg14 (47) in hepatocytes exacerbated NAFLD in high-fat diet-fed mice, whereas overexpression of atg7 (48) or atg14 (47) improves hepatic steatosis. The beneficial effect of autophagy on hepatic steatosis primarily results from its ability to mobilize and break down intracellular lipids in hepatocytes. Myeloid *atg5* deletion promotes hepatic proinflammatory macrophage polarization in mice fed a high-fat diet and treated with low-dose LPS (17), suggesting that defects in macrophage autophagy lead to hepatic inflammation. However, myeloid *atg5* deletion does not significantly impact plasma lipids and lipoproteins in *Ldlr*^{-/-} or *apoE*^{-/-} mice fed a western diet (15). In our study, we observed a similar level of plasma and liver lipids within each diet group between WT and myeloid *atg5* deficient mice. Additionally, we found that myeloid autophagy deficiency had a minimal effect on hepatic inflammation in *Ldlr*^{-/-} mice, at least under the condition of atherogenic diet feeding. However, because Kupffer cells are not efficiently deleted from the liver during the time course of the BMT study (49), we cannot rule out the possibility that residual *atg5* in Kupffer cells in our *atg5MSKO Ldlr*^{-/-} mice may attenuate the effect of myeloid *atg5* deletion on hepatic lipid and inflammatory cytokine production.

Taken together, our current study highlights a diet-specific role of autophagy in atheroprotection and supports the concept that during atherosclerosis progression, in saturated fatty acid-enriched macrophages (i.e., PO-fed mouse macrophages), autophagy was rapidly delayed and ultimately impaired. As a consequence, saturated fatty acid-fed mice developed more atherosclerosis with defective autophagy, increased lipid accumulation, apoptosis, and NLRP3 inflammasome activation. In contrast, n-3 PUFA supplementation promotes efficient autophagy activation in macrophages, thereby facilitating macrophage cholesterol efflux, enhancing efferocytosis, lowering apoptosis and NLRP3 inflammasome activation, and attenuating atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

Atg	autophagy related
BM	bone marrow
BMT	bone marrow transplantation
CE	cholesteryl ester
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FA	fatty acid
FO	fish oil
GPR	G protein-coupled receptor
LC	light chain
LPS	lipopolysaccharide
MSKO	myeloid specific knockout
NLRP3	nucleotide-binding oligomerization domain leucine-rich repeat containing receptor protein
PO	palm oil

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Highlights

1. Myeloid atg5 deletion worsens atherosclerosis in the n-3 PUFA-fed mice.
2. Myeloid atg5 deletion increases aortic caspase-1 cleavage in the n-3 PUFA-fed mice.
3. Myeloid atg5 deletion decreases aortic efferocytosis in the n-3 PUFA-fed mice.

Figure 1. Myeloid *atg5* deletion does not affect plasma and liver lipids.

Irradiated *Ldlr*^{-/-} mice receiving bone marrow from WT or *atg5* myeloid-specific knockout (MSKO) mice were fed atherogenic diets for 16 weeks. Mouse body weight (A), fasting (4 h) plasma total cholesterol (TC) (B), and triglyceride (TG) (C) concentrations were measured by enzymatic assays and were integrated over time (0–16 weeks) and expressed as area under the curve (AUC). Livers were harvested from 16-week diet-fed mice and lipid extracted to measure TC, free cholesterol (FC), cholesteryl ester (CE) and TG by enzymatic assays (D). CE mass was calculated as (TC-FC)×1.67 and normalized to protein mass. Each symbol represents an individual mouse. Data are expressed as mean ± SEM; n = 7–8 per group. *, *p*<0.05; **, *p*<0.01; ***, *p*<0.001; ns: not significant.

Figure 2. Myeloid autophagic deficiency promotes atherosclerosis in a diet-specific manner. Irradiated *Ldlr*^{-/-} mice transplanted with WT or atg5MSKO bone marrow were fed atherogenic diets for 16 weeks. (A and B) Quantification of aortic root Oil-Red-O-positive intimal area (plaque area and % plaque area). (C) Quantification of the aortic necrotic intimal area (total necrotic area and % necrotic area). (D-E) Quantification of CD68⁺ cells (macrophages) in the aortic root intimal area. (F-G) Quantification of CD3⁺ cells (T cells) in aortic root intimal area (number of CD3 positive cells in lesion). Scale bars in A and D = 100 μm . Each symbol represents an individual mouse. Data are expressed as mean $\pm$ SEM. n = 7–8 per group. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

Figure 3. Reduction of aortic root caspase-1 activation by FO supplementation is dependent upon myeloid autophagy.

Irradiated *Ldlr*^{-/-} mice transplanted with WT or atg5MSKO bone marrow were fed atherogenic diets for 16 weeks. (A and B) Quantification of FLICA⁺ (cleaved caspase-1) cells in aortic root intimal area (B, left panel) and co-localized with CD68⁺ cells (B, right panel). DAPI (blue; nuclei), FLICA (green), and CD68 (red, macrophages). The white dash line in the FO-fed WT aortic root image indicates the boundary between lesion and aorta intima. (C and D) Quantification of IL-18⁺ cells in aortic root intimal area. Scale bar=50 μm . Each symbol represents an individual mouse. Data are expressed as mean $\pm$ SEM. n = 7–8 per group. * $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$; ns: not significant.

Figure 4. Increase of aortic root efferocytosis by FO supplementation is dependent upon myeloid autophagy.

Irradiated *Ldlr*^{-/-} mice transplanted with WT or atg5MSKO bone marrow were fed atherogenic diets for 16 weeks. (A and B) Quantification of aortic root intimal area efferocytosis (the ratio of macrophage-associated: free apoptotic cells). The free and macrophage-associated apoptotic cells were indicated by white and yellow arrows, respectively. DAPI (blue; nuclei), TUNEL (red), and CD68 (green; macrophages) were labeled fluorescently. Scale bar=50 μm. Each symbol represents an individual mouse. Data are expressed as mean ± SEM. n = 7–8 per group. *, $p < 0.05$; ns: not significant.

Figure 5. Differential effects of fish oil and myeloid autophagy deficiency on plasma cytokine/chemokines.

Irradiated *Ldlr*^{-/-} mice transplanted with WT or atg5MSKO bone marrow were fed atherogenic diets for 16 weeks. Plasma cytokine and chemokine concentrations were quantified by multiplex assay. Each symbol represents an individual mouse. Data are expressed as mean $\pm$ SEM. n = 7–8 per group. *, $p < 0.05$; **, $p < 0.01$; ns: not significant.