In vivo activation of leukocyte GPR120/FFAR4 by PUFAs has minimal impact on atherosclerosis in LDL receptor knockout mice

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Abstract G protein-coupled receptor (GPR)120/FFA receptor (FFAR4) (GPR120/FFAR4) activation by n-3 PUFAs attenuates inflammation, but its impact on atherosclerosis is unknown. We determined whether in vivo activation of leukocyte GPR120/FFAR4 by n-3 versus n-6 PUFAs is atheroprotective. Leukocyte GPR120/FFAR4 WT or KO mice in the LDL receptor KO background were generated by bone marrow transplantation. Mice were fed one of the four atherogenic diets containing 0.2% cholesterol and 10% calories as palm oil (PO) + 10% calories as: 1) PO, 2) fish oil (FO; 20:5 n-3 and 22:6 n-3 enriched), 3) echium oil (EO; 18:4 n-3 enriched), or 4) borage oil (BO; 18:3 n-6 enriched) for 16 weeks. Compared with PO, mice fed BO, EO, and FO had significantly reduced plasma cholesterol, TG, VLDL cholesterol, hepatic neutral lipid, and atherosclerosis that were equivalent for WT and KO mice. In BO-, EO-, and FO-fed mice, but not PO-fed mice, lack of leukocyte GPR120/FFAR4 resulted in neutrophilia, pro-inflammatory Ly6Chigh mice, but not PO-fed mice, lack of leukocyte GPR120/FFAR4 WT or KO mice. In vivo, selective activation of GPR120/FFAR4 by n-3 PUFAs is anti-inflammatory and insulin sensitizing (7, 8). High-fat diet-fed GPR120/FFAR4 KO mice versus WT counterparts supplemented with n-3 PUFA or a selective GPR120/FFAR4 agonist (cpdA) (9) have: 1) increased adipose tissue F4/80+ macrophage infiltration and pro-inflammatory/M1 gene expression, 2) increased M1 gene expression in lipopolysaccharide-stimulated peritoneal macrophages, and 3) increased insulin resistance. Collectively, these findings highlight the anti-inflammatory potential of macrophage GPR120/FFAR4 activation by n-3 PUFA. However, the impact of GPR120/FFAR4 expression on atherosclerosis progression, particularly in the context of dietary FA composition, is unknown.

Supplementary key words fatty acid • G proteins • inflammation • omega-3 fatty acids • macrophages/monocytes • G protein-coupled receptor 120/free fatty acid receptor 4 • polyunsaturated fatty acids • low density lipoprotein

Deorphanization of FFA receptors (FFARs) has allowed further understanding of FFAs as signaling molecules. These class A G protein-coupled receptors (GPRs) include GPR40 (FFAR1), GPR43 (FFAR2), GPR41 (FFAR3), GPR84, and GPR120/FFAR4 that are activated by short-, medium-, or long-chain FAs (1–6). GPR120/FFAR4 is highly expressed in intestine, adrenals, lung, adipose tissue, and macrophages, and is described as the n-3 PUFA receptor (7). Upon activation by n-3 PUFA, GPR120/FFAR4 inhibits transforming growth factor β-activated kinase 1 activation, resulting in attenuation of IKKB/NF-κB and JNK/AP1 signaling (7). GPR120/FFAR4 expression regulates obesity in mice and humans; a nonsynonymous mutation (p.R270H) inhibited GPR120/FFAR4 signaling activity, resulting in increased risk of obesity in European populations (8). In vivo, selective activation of GPR120/FFAR4 by n-3 PUFAs is anti-inflammatory and insulin sensitizing (7, 8). High-fat diet-fed GPR120/FFAR4 KO mice versus WT counterparts supplemented with n-3 PUFA or a selective GPR120/FFAR4 agonist (cpdA) (9) have: 1) increased adipose tissue F4/80+ macrophage infiltration and pro-inflammatory/M1 gene expression, 2) increased M1 gene expression in lipopolysaccharide-stimulated peritoneal macrophages, and 3) increased insulin resistance. Collectively, these findings highlight the anti-inflammatory potential of macrophage GPR120/FFAR4 activation by n-3 PUFA. However, the impact of GPR120/FFAR4 expression on atherosclerosis progression, particularly in the context of dietary FA composition, is unknown.

Abbreviations: AA, arachidonic acid; AD, atherogenic diet; ALA, α-linolenic acid; AUC, area under the curve; BM, bone marrow; BMT, bone marrow transplantation; BO, borage oil; CE, cholesteryl ester; EO, echium oil; FADS2, fatty acid desaturase-2; FC, free cholesterol; FFAR, FFA receptor; FO, fish oil; FPLC, fast protein LC; GLA, γ-linolenic acid; GPR, G protein-coupled receptor; LA, linoleic acid; LDLrKO, LDL receptor KO; PO, palm oil; RBC, red blood cell; TC, total cholesterol.

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The n-3 PUFAs as well as the n-6 PUFAs are atheroprotective in mice, nonhuman primates, and humans (10–19). In humans, dietary n-3 PUFAs, EPA (20:5 n-3), and DHA (22:6 n-3) found in fish oil (FO) are anti-inflammatory and lower plasma TG, but not plasma LDL cholesterol, a primary risk factor for atherosclerosis in humans (20, 21). However, FO consumption is low in the USA (14). Dietary linoleic acid (LA; 18:2 n-6) is cardioprotective in humans, such that a 5% increase in energy intake from LA is associated with a 10% and 13% lower risk of coronary heart disease events and deaths, respectively (17). Nonhuman primates and mice fed diets enriched in LA are protected from atherosclerosis compared with their counterparts fed diets enriched in saturated/monounsaturated fat (22–24).

Concerns that increased n-6 PUFA consumption may result in elevated membrane arachidonic acid (AA; 20:4 n-6), increased cellular inflammation, and atherosclerosis exacerbation lack support in humans (16). Moreover, LA-enriched diets do not enrich AA in plasma and tissue lipid fractions (24–26), likely due to inefficient FA desaturase-2 (FADS2) conversion of LA to AA (27, 28). Although some of the atheroprotective effects by n-6 PUFAs is likely due to plasma lipid lowering, diets enriched in desaturation (via FADS2)-elongation products of LA, such as dihomo-γ-linolenic acid (20:3 n-6), have anti-inflammatory potential through generation of prostaglandin E1, a potent thromboxane A2 inhibitor that reduces leukocyte endothelial cell adherence (29, 30). Currently, in North American diets, plant-derived LA and ω-linolenic acid (ALA; 18:3 n-3) constitute the majority of PUFA intake (15, 17). Despite high consumption of LA and ALA, tissue enrichment of their longer chain bioactive products, AA and EPA, respectively, is limited due to inefficiency of the rate-limiting FADS2 enzyme (16, 17). To circumvent this, we have identified botanical oils enriched in n-3 or n-6 PUFAs beyond FADS2, 18:4 n-3 (stearidonic acid)-enriched echium oil (EO) and 18:3 n-6 (ω-linolenic acid)-enriched borage oil (BO). We previously demonstrated that EO and BO are as atheroprotective and hepatoprotective as FO, compared with a diet enriched in saturated/monounsaturated fat [palm oil (PO)] (31).

Based on available information, we hypothesized that leukocyte GPR120/FFAR4 activation contributes to the atheroprotective effects of n-3 PUFAs, but not n-6 PUFAs. Selective activation of leukocyte GPR120/FFAR4 by n-3 PUFAs should result in decreased leukocyte inflammation and atherosclerosis. We tested this hypothesis by feeding PO, FO, EO, or BO diets to leukocyte GPR120/FFAR4 WT and KO mice in the C57BL/6/LDL receptor KO (LDLrKO) background generated by bone marrow transplantation (BMT). To our knowledge, this is the first study to determine the in vivo role of leukocyte GPR120/FFAR4 in the context of n-3 versus n-6 PUFA-induced atheroprotection.

MATERIALS AND METHODS

Dietary oils

The seed oil of *Bongo officinalis* L., a member of the Boraginaceae family, was generously donated by Nordic Naturals (Watsonville, CA). The seed oil of *Echium plantagineum* L., a member of the Boraginaceae family was a generous gift from Croda Europe Ltd. (Leek, Staffordshire, UK). All oils were authenticated by the Wake Forest University Center for Botanical Lipids and Inflammatory Disease Prevention. A certificate of analysis is on file for reference along with retention samples deposited at the Wake Forest School of Medicine. The seed oil of palm, *Elaeis guineensis* Jacq., a member of the Areaceae family, was purchased from Shay and Co. (Portland, OR). A certificate of analysis is on file for reference along with retention samples deposited at the Wake Forest School of Medicine. The FO source was *Brevoortia tyrannis* Latrobe, a member of the Clupeidae family, which was manufactured and generously provided by Omega Protein (Houston, TX) with a report of analysis on file for reference.

Animals and atherogenic diets

Female LDLrKO (C57BL/6 background) mice (5–6 weeks of age) were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in a specific pathogen-free facility on a 12 h light/dark cycle. Mice were allowed to acclimate to in-house animal resource facilities for 1–2 weeks. At 8 weeks of age, mice received bone marrow (BM) from either WT or KO GPR120/FFAR4 male mice (see below). Bones from WT or GPR120/FFAR4 KO mice were generously provided by Drs. Jerrold Olefsky and Da Young Oh (University of San Diego, La Jolla, CA). After ~6 weeks of recovery from BMT and at 13–14 weeks of age, mice were randomly assigned to one of four atherogenic diet (AD) groups (n = 15 per diet group) containing 10% calories as PO and 0.2% cholesterol, supplemented with an additional 10% of calories as: 1) PO, 2) BO (18:3 n-6 enriched), 3) EO (18:4 n-3 enriched), or 4) FO (20:5 n-3 and 22:6 n-3 enriched) for an additional 16 weeks. All protocols and procedures were approved by the Institutional Animal Care and Use Committee. ADs were prepared by the diet kitchen in the Department of Pathology at Wake Forest School of Medicine as previously described (32). Detailed composition and quality control data for similar ADs have been published (31, 33).

BMT

BM cells were harvested from cleaned femurs and tibias of male GPR120/FFAR4 WT and GPR120 KO mice and resuspended in serum-free RPMI 1640 medium. Female LDLrKO recipient mice (Jackson Laboratories) were fasted overnight and received a sublethal dose of radiation (900 rad) 4 h prior to BM injection. BM cells (~7 × 10^6 per mouse) were injected into the retro-orbital venous plexus of anesthetized 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 weeks after the transplantation. Mice were then given autoclaved acidified water until the end of the study, as described previously (34).

Repopulation of blood leukocytes by transplanted GPR120/FFAR4 WT or KO hematopoietic stem cells in female LDLrKO recipients was evaluated after 6 weeks of recovery by determining percentage expression of the Y-chromosome-associated sex-determining region Y gene (Sry) in genomic DNA obtained from white blood cells. Additionally, white blood cell GPR120/FFAR4 WT and KO genotype was also confirmed using PCR. Briefly, genomic DNA from whole blood was extracted with a Wizard genomic DNA purification kit (Promega). Sry was amplified by PCR to a linear amplification phase under the following conditions: 95°C for 3 min followed by 30 cycles of 94°C for 30 s, 61°C for 1 min, and 72°C for 1 min. Sterol O-acetyltransferase 2 (SOAT2) was amplified by PCR as an internal control. The PCR products were separated on 0.8% agarose gels and visualized with ethidium bromide. A series of male and female genomic DNA mixtures...
(100, 50, 25, and 0% male DNA) were made and used to construct a standard curve of male:female DNA ratio versus Sry:SOAT2 ratio, determined by quantification of density of PCR bands. Male genomic DNA in the whole blood samples of transplanted mice was estimated using the standard curve.

**Body weight gain and organ weights**

Mice were weighed one day prior to and daily for three consecutive days after BMT and then on a weekly basis for 6 weeks until they were started on the ADs. During the 16 weeks of AD feeding, mice were weighed every 2–4 weeks. At necropsy, terminal body weights were recorded after 4 h of fasting. Mice were then anesthetized using ketamine-xylazine (intramuscularly) and perfused through the left ventricle using cold PBS at the rate of ~3 ml/min for 3–4 min prior to organ harvesting. After perfusion, liver, spleen, and adipose wet weight were measured and normalized to terminal body weight.

**FA analysis**

Lipid extraction of holphilized diets and red blood cells (RBCs) was performed using the Bligh-Dyer method (35). The lipid extracts were trans-methylated using boron trifluoride (BF3), and percentage FA composition was quantified by gas-liquid chromatography as described previously (32).

**Plasma lipid and lipoprotein analysis**

Blood was collected from 4 h-fasted mice by tail bleeding and plasma was isolated by low speed centrifugation. Plasma total cholesterol (TC) and free cholesterol (FC) (Wako) concentrations were determined using enzymatic assays, as described earlier (36), at baseline and after 2, 4, 8, and 16 weeks of AD feeding. Cholesterol ester (CE) content was calculated as (TC × FC) ÷ 1.67; the multiplication by 1.67 corrects for loss of FA during the cholesterol esterase step of the assay. Data were expressed as area under the curve (AUC), which integrates plasma lipid concentrations over the 16 weeks of AD feeding, representing a time average estimate of hypercholesterolemia throughout atherosclerosis progression. Plasma lipoprotein cholesterol mass distribution was determined using fast protein LC (FPLC) fractionation on a Superose 6 10/300 column (GE Healthcare). Three equal volume plasma samples from five mice per group were pooled and subjected to FPLC fractionation on a Superose 6 10/300 column (GE Healthcare). Three equal volume plasma samples from five mice per group were pooled and subjected to FPLC fractionation on a Superose 6 10/300 column (GE Healthcare).

**Liver lipids**

Livers were harvested at necropsy, flash-frozen in liquid N2, and stored at −80°C. Liver lipids were quantified using a detergent-based enzymatic assay as described earlier (37).

**Flow cytometry**

Peripheral blood was obtained for circulating leukocyte analysis. For splenic cells, tissue was digested with an enzyme cocktail as published (38). The cell suspension was subsequently passed through a 70 μm nylon cell strainer (BD Falcon). RBCs were removed from flow cytometry preparations by treatment with ACK lysis buffer (Gibco). The remaining white blood cells were incubated with the following mAbs: CD11b-APC-Cy7, clone: M1/70 (BioLegend); CD11c-APC, clone: AF589; Ly6C-PE, clone: A18; CD11c-PE-Cy7, clone: H1L3; and Ly6G-FITC or PE clone: AL-21 (BD Pharmingen). Data were acquired on a BD FACScanto II instrument (BD Biosciences) and analyzed using FACSDiva software (BD Biosciences).

**Monocyte labeling**

Monocytes were labeled following the Gr-1lo method as described by Tacke and colleagues (39, 40). One micron Fluoresbrite Yellow Green microspheres (2.5% solids (wt/vol); Polysciences, Inc., Warrington, PA) were diluted in PBS (1:4) and retro-orbitally injected into anesthetized mice. Blood was harvested from mice 24 h after labeling for evaluation of bead-containing monocytes. The method labels predominantly Ly6Cmo monocytes (39).

**Quantification of monocyte recruitment**

Monocyte recruitment was evaluated as reported previously (18). Briefly, aortic root sections were taken as described below and the number of beads per section in atherosclerotic lesions was counted manually at 20× magnification. A total of eight sections representing the length of the aortic root were analyzed per mouse. Because of slight variations in monocyte labeling (6–8%) among animals, data were normalized for individual mice based on the percentage of blood monocytes labeled. This adjustment gave a normalized value, which represented the actual number of monocytes entering the lesion (i.e., normalized frequency).

**Quantification of aortic root lesion area**

Aortic root atherosclerosis was assessed according to the method of Daugherty and Whitman (41). Aortic roots were embedded in optimal cutting temperature (TissueTek) media in a plastic mold, frozen, and cut at 8 μm intervals. Sections were collected from the aortic root moving toward the apex of the heart and sequentially

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**TABLE 1. AD percentage FA composition and percentage total energy equivalence of individual FAs**

| FA                  | PO  | Percent FA | Percent EE | PO  | Percent FA | Percent EE | PO  | Percent FA | Percent EE | PO  | Percent FA | Percent EE | PO  | Percent FA | Percent EE | PO  | Percent FA | Percent EE | PO  | Percent FA | Percent EE |
|---------------------|-----|------------|------------|-----|------------|------------|-----|------------|------------|-----|------------|------------|-----|------------|------------|-----|------------|------------|-----|------------|------------|
| Palmitic acid (C16:0) | 43.2| 8.89       | 30.6       | 6.89| 25.6       | 5.27       | 26.0| 5.20       |
| Palmitoleic acid (C16:1) | 0.37| 0.08       | 4.2        | 0.95| 0.4        | 0.02       | 0.4 | 0.08       |
| Stearic acid (C18:0)  | 4.5 | 0.95       | 4.5        | 1.01| 0.84       | 0.35       | 0.5 | 0.7        |
| Oleic acid (C18:1 n-9) | 37.3| 7.68       | 24.9       | 5.61| 26.1       | 5.37       | 25.1| 5.02       |
| LA (C18:2 n-6)       | 11.1| 2.29       | 8.2        | 1.85| 15.4       | 3.17       | 25.0| 5.0        |
| ALA (C18:3 n-3)      | 0.37| 0.08       | 1.0        | 0.23| 13.8       | 2.84       | 0.4 | 0.08       |
| γ-Linolenic acid (C18:3 n-6) | 5.0 | 1.03       | 11.40      | 2.28|            |            |     |            |
| SDA (C18:4 n-3)      | 1.5 | 0.34       | 6.0        | 1.23| 0.2        | 0.04       | 0.94| 0.18       |
| Erucic acid (C22:1 n-9) | 0.2 | 0.04       | 7.9        | 1.58| 0.3        | 0.06       | 0.3 | 0.06       |
| EPA (C20:5 n-3)      | 0.1 | 0.02       | 6.9        | 1.55| 0.3        | 0.06       | 0.3 | 0.06       |
| DHA (C22:6 n-3)      | 0.2 | 0.04       | 7          | 1.58| 0.3        | 0.06       | 0.3 | 0.06       |

ADs contained 0.2% cholesterol + 10% calories as PO + 10% calories as PO, BO, EO, or FO. Percent FA composition was measured using gas-liquid chromatography. Percent total energy equivalence (EE) for individual FA was calculated using total energy derived from FAs (i.e., 20%) divided by diet and percent FA composition of the respective diet. SDA, stearidonic acid.

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**Figure 1.** Aortic root atherosclerosis was assessed according to the method of Daugherty and Whitman (41). Aortic roots were embedded in optimal cutting temperature (TissueTek) media in a plastic mold, frozen, and cut at 8 μm intervals. Sections were collected from the aortic root moving toward the apex of the heart and sequentially...
placed on six slides, such that each slide had sections 48 μm apart. The sections were 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 DigitalSight DSFi1 camera and quantified using NIS Elements (Nikon) software. Intimal area measurements were obtained with ImagePro 6.2 software (Media Cybernetics Inc., Rockville, MD). We defined intimal area as the lesion area between the internal elastic lamina and the luminal surface of the aorta. Aortic root sections (n = 8–10) were quantified from six to eight mice per group. The mean coefficient of variation for these measurements from individual mice on ADs was 15.0%.

Sirius red staining for collagen

Fresh frozen aortic root sections were fixed (10% buffered formalin, 10 min), washed (DI water, 5 min), and dehydrated in 50, 75, and 80% ethanol, sequentially, for 1 min each. Sections were stained using hematoxylin, and then stained in 1% Sirius red dye (Sigma Aldrich #365548) in saturated solution of picric acid (1.3% picric acid in water; Sigma-Aldrich #P6744) for 60 min. After staining with Sirius red, sections were washed in acidified water (5% glacial acetic acid) for 5 min and dehydrated in increasing ethanol concentrations, cleaned in xylene, and cover slipped using xylene-based mounting media. Images were acquired under plane polarized light using a Nikon DigitalSight DSFi1 camera fitted with T-A2-DIC analyzer (Nikon #MEN51921) and T-P2 DIC polarizer (Nikon #MEN51951).

Immunohistochemistry

Aortic root cross-sections and liver sections were fixed and incubated with anti-CD68 antibody (AbDSerotec; clone FA11), anti-CD11c antibody (BD Pharmingen; clone HL3), or anti-cleaved caspase-3 antibody (Abcam; polyclonal #ab2302) (1:200, overnight) and quantified as described previously (18).

Analysis of aortic cholesterol content

Aortic TC, FC, and CE content was measured as described earlier (18).

Statistical analyses

Data are presented as mean ± SEM. All data were tested for significant differences using one-way or two-way ANOVA (diet vs. genotype) with post hoc Tukey’s multiple comparison test. All the analyses were performed with Statistica software.

RESULTS

Systemic response to ADs enriched in n-3 and n-6 PUFAs

Eight-week-old female LDLrKO mice were irradiated and then received BM from male GPR120/FFAR4 WT or KO mice. LDLrKO recipients recovered from radiation/BMT-induced body weight loss within 1 week and showed a similar and positive weight gain pattern during 6 weeks of recovery. Transplantation efficiency was ~95% based on expression of the male Syr gene in blood leukocytes (data not shown). Blood leukocyte GPR120/FFAR4 alleles (WT and KO) were verified using PCR analysis (data not shown). Eight weeks after BMT, WT and KO mice were switched from chow to one of the four ADs containing PO, BO, EO, or FO for an additional 16 weeks. Dietary FA compositions showed relative enrichment of 18 carbon FAs beyond FADS2 in the BO (11.4% 18:3 n-6) and EO (6% 18:4 n-3) ADs. AD FA compositions are given in Table 1 and are similar to those published previously (31), except that γ-linolenic acid content (2.3% energy) was approximately half that of the previous study (3.9% energy) because a different source of BO was used. AD feeding over 16 weeks resulted in uniform food consumption (~3–4 g/day/mouse), body weight gain, and terminal liver, spleen, and gonadal adipose/body weight ratios among all groups irrespective of leukocyte GPR120/FFAR4 expression (data not shown). Additionally, leukocyte GPR120/FFAR4 did not affect percentage RBC FA enrichment. Because WT and KO mice fed the same AD had similar percentage RBC FA enrichment, data from both genotypes were pooled and are plotted together (Fig. 1). We previously demonstrated that percentage FA enrichment in plasma and liver phospholipids was similar to that in RBCs (31).

Leukocyte GPR120/FFAR4 does not affect plasma lipids and cholesterol lipoprotein distribution

Consistent with previous reports, FO, EO, and BO reduced total plasma cholesterol (Fig. 2A, B), plasma TG...
compared with WT mice (Fig. 4B). FO KO mice versus WT mice also had reduced expression of macrophage marker genes, CD68 and F4/80, and anti-inflammatory genes, CD206 and arginase-1 (data not shown), whereas other diet groups did not.

**Leukocyte GPR120/FFAR4 does not affect hepatosteatosis**

In general, leukocyte GPR120/FFAR4 expression had no effect on hepatic neutral lipid content (Fig. 4A). Liver sections of WT and KO mice fed the same AD had similar morphological appearance by hematoxylin and eosin and CD68 immunohistochemistry staining (data not shown). Thus, reduced hepatosteatosis in FO-, EO-, and BO-fed mice versus PO-fed mice was independent of leukocyte GPR120/FFAR4 expression and likely occurs via reduced SREBP1 activation and lipogenic gene expression (31). Previous reports have shown a macrophage phenotype switch from M2 to M1 in GPR120/FFAR4 KO versus WT mice fed a high-fat diet containing n-3 PUFAs or a selective GPR120/FFAR4 agonist (7, 9). We determined the extent to which leukocyte GPR120/FFAR4 affects hepatic inflammatory gene expression. PO-fed mice had similar hepatic inflammatory gene expression regardless of leukocyte GPR120/FFAR4 expression (Fig. 4B). However, FO-, EO-, and BO-fed GPR120/FFAR4 KO mice had increased M1 (MCP-1, TNF-α, IL-6, and IL-1β) gene expression compared with WT mice (Fig. 4B). FO KO mice versus WT mice also had reduced expression of macrophage marker genes, CD68 and F4/80, and anti-inflammatory genes, CD206 and arginase-1 (data not shown), whereas other diet groups did not.

**PUFAs attenuate neutrophilia and monocytosis via leukocyte GPR120/FFAR4**

Hypercholesterolemia induces leukocytosis, primarily by elevating circulating monocytes (monocytosis) and neutrophils (neutrophilia), both of which are positively associated with atherosclerosis in mice and humans (42, 43). The percentage of circulating neutrophils (CD11b+, CD115−, Ly6G+) was decreased with FO, EO, and BO feeding relative to PO feeding, which was reversed in the absence of leukocyte GPR120/FFAR4 (Fig. 5A). The spleen is an extramedullary reservoir for circulating undifferentiated monocytes, which upon recruitment to a site of injury/inflammation may differentiate into macrophages, dendritic cells, or other tissue descendants (44, 45). Splenic neutrophilia (Fig. 5B) and monocytosis (percentage CD11b+, CD115−, Ly6G−) (Fig. 5C) were significantly attenuated by dietary FO, EO, and BO in mice expressing leukocyte GPR210, but not those lacking leukocyte GPR120/FFAR4, relative to PO-fed mice. Splenic Ly6Chigh monocytosis was also significantly attenuated in FO-, EO-, and BO-fed WT mice, relative to PO-fed mice, but not KO mice, suggesting that leukocyte GPR120/FFAR4 plays a significant role in maintaining a favorable Ly6Clow monocyte profile in FO-, EO-, and BO-fed mice (Fig. 5D). Despite leukocyte GPR120/FFAR4 expression and dietary

![Fig. 2.](image) Leukocyte GPR120/FFAR4 expression does not affect plasma lipids. Irradiated LDLrKO mice received BM from WT or GPR120/FFAR4 KO donors and were fed ADs (PO, FO, EO, or BO) for 16 weeks. Fasting (4 h) plasma TC (A) and TG (C) concentrations were measured by enzymatic assays. Plasma TC (B) and TG (D) concentrations were integrated over time (0–14 weeks) and expressed as AUC. Data are expressed as mean ± SEM; n = 13–15 per diet. Groups with different letters are significantly different (P < 0.05) by two-way ANOVA and Tukey’s post hoc analysis.
Leukocyte GPR120/FFAR4 expression and atherosclerosis

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Macrophage chemotaxis ex vivo and result in reduced aortic root CD68+ intimal area in LDLrKO mice (31). We hypothesized that activated leukocyte GPR120/FFAR4 may limit monocyte trafficking into aortic root intima and performed monocyte-labeling experiments to test our hypothesis. Blood monocytes were phagocytically labeled with 1 μm fluorescent beads using the procedure developed by Randolph and colleagues (39, 40) and previously used by our laboratory (18, 31). Using this procedure, ~1% of monocytes were phagocytosed and labeled.

PUFA effects on splenic monocytosis, neither affected the percentage of circulating monocytes or Ly6C<sup>hi</sup> monocytes (data not shown).

**Leukocyte GPR120/FFAR4 attenuates monocyte infiltration into atherosclerotic lesions**

GPR120/FFAR4 negatively regulates macrophage chemotaxis ex vivo (7). We previously showed that ADs containing FO, EO, and BO, compared with PO, reduce macrophage chemotaxis ex vivo and result in reduced aortic root CD68+ intimal area in LDLrKO mice (31). We hypothesized that activated leukocyte GPR120/FFAR4 may limit monocyte trafficking into aortic root intima and performed monocyte-labeling experiments to test our hypothesis. Blood monocytes were phagocytically labeled with 1 μm fluorescent beads using the procedure developed by Randolph and colleagues (39, 40) and previously used by our laboratory (18, 31). Using this procedure, ~1% of monocytes were phagocytosed and labeled.

**Fig. 3.** Leukocyte GPR120/FFAR4 does not affect plasma lipoprotein cholesterol distribution. Fasted (4 h) plasma was harvested from AD-fed LDLrKO mice transplanted with WT or GPR120/FFAR4 KO BM and fractionated by FPLC to determine cholesterol distribution among VLDL (A), LDL (C), and HDL (E) fractions. Plasma VLDL cholesterol (VLDL-c) (B), LDL cholesterol (LDL-c) (D), and HDL cholesterol (HDL-c) (F) concentrations were integrated over time (0–14 weeks) and expressed as AUC. Data are expressed as mean ± SEM; n = 3 equal volume pooled samples from four mice per group. Groups with different letters are significantly different (P < 0.05) by two-way ANOVA and Tukey's post hoc analysis.

**Fig. 4.** Effect of leukocyte GPR120/FFAR4 expression on hepatic lipid content and pro-inflammatory gene expression. Livers were harvested from 16 week AD-fed LDLrKO mice transplanted with WT or GPR120/FFAR4 KO BM to measure lipid content and mRNA abundance. A: Hepatic neutral lipid content. Neutral lipid = CE + TG. Liver was lipid extracted and TC, FC, and TG were quantified by enzymatic assays. CE mass was calculated as (TC − FC) × 1.67 and normalized to protein content. B: Macrophage pro-inflammatory gene expression. Data are expressed as mean ± SEM; n = 6. Groups with different letters are significantly different (P < 0.05) by two-way ANOVA and Tukey's post hoc analysis.
blood leukocytes were labeled with beads (bead+) and of these, 60–80% were monocytes, resulting in a range of 6–8% monocyte labeling among the groups (data not shown). Monocyte recruitment into aortic root intima was significantly reduced for mice consuming FO, EO, and BO, relative to PO, as anticipated; however, recruitment for all three PUFA groups was equivalent to PO in mice lacking leukocyte GPR120/FFAR4 (Fig. 6A). Additionally, splenic Ly6C<sup>hi</sup> monocytosis positively correlated with monocyte infiltration into aortic root intima (Fig. 6B).

**Leukocyte GPR120/FFAR4 expression does not affect atherosclerosis in LDLrKO mice**

Aortic root intimal area was significantly lower (500–700 μm<sup>2</sup> vs. 950–1,050 × 10<sup>3</sup> μm<sup>2</sup>) in FO-, EO-, and BO-fed mice versus PO-fed mice (Fig. 7A), as was aortic root intimal macrophage content (CD68+) (Fig. 7B). Leukocyte GPR20 expression did not affect aortic root intimal area or intimal macrophage content. Intimal collagen was similar among all groups of mice except for a increase in KO mice fed FO (data not shown). CD11c<sup>+</sup> intimal area was also similar among all groups of mice except for an increase in KO mice fed FO (data not shown). We also found that the number of cleaved caspase-3<sup>+</sup> apoptotic cells was similar among all groups of mice (data not shown). To examine atherosclerosis at another site, we measured whole aorta FC (Fig. 8A) and CE (Fig. 8B) content, which was significantly lower in FO-, EO-, and BO-fed mice (~2-fold reduction in FC; ~3 to 4-fold reduction in CE) versus PO-fed mice. In agreement with aortic root intimal area results, leukocyte GPR120/FFAR4 expression had no impact on this measurement of atherosclerosis, indicating that PUFA-induced atheroprotection is not significantly affected by leukocyte GPR120/FFAR4 expression.

**DISCUSSION**

Whether in vivo leukocyte GPR120/FFAR4 activation by PUFAs is atheroprotective is unknown. In this study, we determined the extent to which leukocyte GPR120/FFAR4 expression contributes to atheroprotective effects of n-3 versus n-6 PUFAs, because in vitro studies suggest that n-3 PUFAs preferentially activate GPR120/FFAR4 (7). Our study led to several novel observations. First, we show that leukocyte GPR120/FFAR4 deletion had minimal effects on plasma lipid concentrations, lipoprotein cholesterol distribution, hepatic neutral lipid content, aortic root intimal area, and aortic cholesterol content regardless of dietary fat type fed to the mice. Second, in vivo activation of leukocyte GPR120/FFAR4 by PUFA-enriched diets limits: 1) hepatic macrophage (i.e., Kupffer cell) pro-inflammatory gene expression, 2) splenic and blood neutrophilia, 3) splenic Ly6C<sup>hi</sup> monocytosis, and 4) monocyte recruitment into aortic root atherosclerotic lesions. Third, there was no distinction between
Leukocyte GPR120/FFAR4 expression and atherosclerosis

We investigated whether GPR120/FFAR4 activation by n-3 PUFAs would reduce atherogenesis based on its role in attenuating inflammation and insulin resistance in n-3 versus n-6 PUFAs with regard to in vivo activation of GPR120/FFAR4. These data suggest that dietary n-3 and n-6 PUFAs, though atheroprotective in LDLrKO mice relative to PO, have GPR120-independent (i.e., attenuation of hypercholesterolemia, hepatosteatosis, and aortic atherosclerosis) and GPR120-dependent (i.e., attenuation of pro-inflammatory gene expression, neutrophilia, splenic monocytosis, and monocyte trafficking) atheroprotective roles.

We investigated whether GPR120/FFAR4 activation by n-3 PUFAs would reduce atherogenesis based on its role in attenuating inflammation and insulin resistance in

Fig. 6. Effect of leukocyte GPR120/FFAR4 on monocyte recruitment into aortic root intima. Blood monocytes were labeled using 1 μm Fluoresbrite microparticles in LDLrKO mice transplanted with WT or GPR120/FFAR4 KO BM and fed AD for 15 weeks. The percentage of FITC bead-labeled blood monocytes was measured by flow cytometry 24 h after injection. Five days later, the number of FITC+ beads was quantified in the intimal area of 8 μm-thick aortic root sections and normalized by the percentage of FITC+ blood monocytes to obtain the normalized frequency of monocytes recruited into aortic intima (A). Data are expressed as mean ± SEM; n = 6–7 per group. Groups with different letters are significantly different (P < 0.05) by two-way ANOVA and Tukey’s post hoc analysis. The percentage of splenic Ly6C+ monocytes was plotted versus monocyte recruitment into aortic intima (B). Each data point represents an individual animal and symbols represent their respective groups, n = 6–7 per group. The line of best fit, determined by linear regression analysis, is also plotted.

Fig. 7. Histological quantification of aortic root atherosclerotic lesions. LDLrKO mice transplanted with WT or GPR120/FFAR4 KO BM were fed AD for 16 weeks. At necropsy, hearts were frozen in OCT, and aortic roots were serially sectioned. A: Quantification of aortic root Oil Red O-positive intimal area (lesion area). B: Quantification of percentage aortic root lesional area occupied by CD68+ cells (macrophages); n = 6–8 per group, each data point represents an individual mouse. Horizontal lines denote the mean for each diet group. Groups with different letters are significantly different (P < 0.05) by two-way ANOVA and Tukey’s post hoc analysis. Representative images for each group of mice are presented below each plot.
diet-induced obesity (7, 9). GPR120/FFAR4 is highly expressed in macrophages, which are important inflammatory cells in the pathogenesis of obesity and atherosclerosis (7). Mice fed a high-fat diet have increased monocyte recruitment to adipose tissue, adipose tissue macrophage inflammation, and insulin resistance (7). This phenotype was reversed in mice fed a high-fat diet in which FO was isocalorically (27% of calories) substituted for saturated fat; however, this was not the case for GPR120/FFAR4 KO mice or mice transplanted with GPR120/FFAR4 KO BM, suggesting that most of the beneficial effect of FO was due to macrophage GPR120/FFAR4 activation (7). Oh et al. (7) have shown that activation of GPR120/FFAR4 results in a β-arrestin 2-mediated internalization of GPR120/FFAR4 and binding to TAB1, preventing its activation of TAK1 and blunting a common node of inflammatory signaling for Toll-like receptors, TNFα receptor, and inflammasome activation. However, another study using a different GPR120/FFAR4 gene targeting construct showed that reversal of insulin resistance by feeding a high n-3 PUFAs-containing diet was independent of GPR120/FFAR4 expression, suggesting the in vivo metabolic impact of GPR120/FFAR4 expression is not fully elucidated (46).

Central to the pathogenesis of atherosclerosis are lipid-laden macrophages that initiate a chronic inflammatory state (47). Dietary PUFAs are atheroprotective in mice, nonhuman primates, and humans (10–19); however, the extent to which this atheroprotection is related to GPR120/FFAR4 expression, in general, and macrophage GPR120/FFAR4 expression, in particular, is unknown. Our results show that dietary PUFA-induced atheroprotection was independent of BM GPR120/FFAR4 expression by several measurements, including aortic root intimal area and whole aorta cholesterol content. There may be several explanations for this unexpected outcome. First, inflammation, while important in the initiation and progression of atherosclerosis, likely plays a less important role in the LDLrKO mouse model, in which apoB lipoproteins are elevated in plasma. Past studies from our laboratory and others have shown that atherosclerosis is highly associated with plasma VLDL cholesterol concentrations in LDLrKO mice (31, 48) and leukocyte GPR120/FFAR4 expression did not affect plasma VLDL concentrations in our study. Thus, the elevation of plasma VLDL likely overwhelmed any beneficial effect of BM GPR120/FFAR4 expression in our study. Second, cells outside the BM compartment (i.e., endothelial and smooth muscle cells) that express GPR120/FFAR4 may play important atheroprotective roles in the context of dietary PUFA feeding and the expression of GPR120/FFAR4 in these cells would not have been affected in our study. Finally, the intake of dietary PUFAs may not have been sufficient to activate GPR120/FFAR4 in vivo, as our study used much lower n-3 PUFA-enriched diets compared with previous studies (7, 46).

To determine whether diet enrichment of PUFAs was sufficient for in vivo activation of macrophage GPR120/FFAR4 in our study, we made several measurements of leukocyte function. Mice fed the PUFA-containing diets had decreased percentage of splenic Ly6Chigh monocytes and decreased trafficking of blood monocytes into aortic root atherosclerotic lesions relative to PO-fed mice and these trends were reversed in mice transplanted with GPR120/FFAR4 KO versus WT BM, even though aortic root macrophage content was uniformly reduced in PUFA-fed mice. Likely, decreased macrophage proliferation in advanced atherosclerotic lesions (49–51) or increased macrophage trafficking out of atherosclerotic lesions (52), as described in other studies, may have negated the increased monocyte trafficking into lesions in the absence of BM GPR120/FFAR4 expression in PUFA-fed mice. Alternatively, monocyte recruitment may play a small role in lesional macrophage content in an atherosclerosis mouse model (i.e., LDLrKO) driven by elevated plasma VLDL concentrations. Blood and splenic neutrophilia were also suppressed in mice transplanted with WT BM and fed PUFAs, but not PUFA-fed mice transplanted with GPR120/FFAR4 KO BM. In addition, PUFA-fed mice lacking BM GPR120/FFAR4 had higher hepatic pro-inflammatory gene expression than their WT counterparts. Because liver has low GPR120/FFAR4 expression (4, 8), whereas macrophages have relatively high expression (7), these results suggest that hepatic Kupffer cells, as well as circulating monocytes and neutrophils, display dietary PUFA-mediated GPR120/FFAR4 activation in vivo. Although hypercholesterolemia is associated with monocytosis (18), our outcomes were unrelated to plasma cholesterol concentrations, which were equivalent among all PUFA-fed mice regardless of BM GPR120/FFAR4 expression. Past studies showed in vivo GPR120/FFAR4 activation (i.e., decreased inflammation, increased insulin sensitivity) at much higher super physiological intakes of FO (7). As discussed in our previous publication (31), the dietary PUFA intake levels in our study were in a
physiological range that could be achievable for human consumption. Whether diminished monocytecyctosis and inflammation will occur in humans consuming diets with a similar PUFA enrichment is unknown. However, adipose tissue GPR120/FFAR4 expression is increased in obese humans and a GPR120/FFAR4 coding variant that inhibits its activation increases obesity risk in Europeans (8). Collectively, our results support the conclusion that sufficient PUFA enrichment occurred in vivo to activate GPR120/FFAR4, but the anticipated GPR120/FFAR4-mediated reduction in atherosclerosis was not observed because the plasma lipid lowering effect of the PUFA diets, which was GPR120/FFAR4 independent, was overwhelming. Although not investigated in our study, we speculate that much higher levels of PUFA intake may be necessary to observe GPR120/FFAR4-dependent improvements in metabolic health observed with diet-induced obesity (7).

Our study also demonstrated that the in vivo activation of GPR120/FFAR4 was not specific for n-3 PUFAs, but was equally effective with dietary n-6 PUFAs. The FA specificity of GPR120/FFAR4 activation has been investigated primarily using in vitro techniques and results are contradictory. Some report a preference of n-3 PUFAs for GPR120/FFAR4 activation, whereas other studies report broader fatty acyl specificity (4, 7, 53). In vivo studies also have yielded conflicting results. Oh et al. (7) showed that a high-fat diet enriched in FO reduced glucose intolerance in a GPR120/FFAR4-dependent manner, whereas Bjursell et al. (46) demonstrated that a FO-containing high-fat diet reversed glucose intolerance in both WT and GPR120/FFAR4 KO mice. The explanation for these disparate outcomes has been discussed at length by Bjursell et al. (46); possibilities include different gene targeting constructs, mouse backgrounds, and dietary n-3 PUFA content and length of diet feeding. It is possible that the higher dietary n-3 PUFA content and the longer length of diet feeding in the Bjursell study may have overwhelmed any beneficial effect of GPR120/FFAR4 expression. Although our primary outcome, which was atherosclerosis progression, was not affected by leukocyte GPR120/FFAR4 expression, similar to results published by Bjursell et al. (46) on glucose intolerance, our results clearly showed an impact of leukocyte GPR120/FFAR4 expression on pro-inflammatory gene expression, neutrophilia, splenic monocytosis, and monocyte trafficking with both n-3 and n-6 PUFA diets, suggesting the in vivo specificity for leukocyte GPR120/FFAR4 activation is equal for both classes of PUFAs.

Whole body deletion of GPR120/FFAR4 has led to increased hepatosteatosis in some (7, 8), but not all, studies (46). In our study, PUFA-containing diets uniformly reduced hepatic neutral lipid content relative to PO regardless leukocyte GPR120/FFAR4 expression. The lack of difference in hepatic lipid content between our study and that of Oh et al. (7) may relate to global versus BM KO of GPR120/FFAR4 or differences in dietary fat content and length of feeding of n-3 PUFAs. LDLrKO mice transplanted with GPR120/FFAR4 KO versus WT BM had increased liver inflammatory gene expression, which we attribute to Kupffer cells (see above), but only in the PUFA-fed groups. The reason for a PUFA-induced increase in inflammatory gene expression only in mice lacking leukocyte GPR120/FFAR4 and its relationship to hepatic lipid content is unknown. We speculate that hepatic Kupffer cell GPR120/FFAR4 activation by PUFA-enriched diets normally suppresses inflammation, perhaps initiated by increased PUFA oxidation. However, leukocyte GPR120/FFAR4 deletion in our study was not sufficient to significantly affect hepatic neutral lipid content.

In summary, we have shown that leukocyte GPR120/FFAR4 expression is neither necessary nor sufficient for athroprotection in LDLrKO mice fed n-6 or n-3 PUFA-enriched diets. We also show that in vivo GPR120/FFAR4 activation can be achieved by both n-3 and n-6 dietary PUFAs at intake levels that are reasonable for human consumption. Future studies will be necessary to determine the impact of dietary fat quality on GPR120/FFAR4 activation in humans.

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