Omega-3 Fatty Acids Ameliorate Atherosclerosis by Favorably Altering Monocyte Subsets and Limiting Monocyte Recruitment to Aortic Lesions

Amanda L. Brown, Xuewei Zhu, Shunxing Rong, Swapnil Shewale, Jeongmin Seo, Elena Boudyguina, Abraham K. Gebre, Martha A. Alexander-Miller, John S. Parks

Objective—Fish oil, containing omega-3 fatty acids, attenuates atherosclerosis. We hypothesized that omega-3 fatty acid–enriched oils are atheroprotective through alteration of monocyte subsets and their trafficking into atherosclerotic lesions.

Methods and Results—Low–density lipoprotein receptor knockout and apolipoprotein E−/− mice were fed diets containing 10% (calories) palm oil and 0.2% cholesterol, supplemented with an additional 10% palm oil, echium oil (containing 18:4 n-3), or fish oil. Compared with palm oil–fed low–density lipoprotein receptor knockout mice, echium oil and fish oil significantly reduced plasma cholesterol, splenic Ly6C+ monocytois by ≈50%, atherosclerosis by 40% to 70%, monocyte trafficking into the aortic root by ≈50%, and atherosclerotic lesion macrophage content by 30% to 44%. In contrast, atherosclerosis and monocyte trafficking into the artery wall was not altered by omega-3 fatty acids in apolipoprotein E−/− mice; however, Ly6C+ splenic monocytes positively correlated with aortic root intimal area across all diet groups. In apolipoprotein E−/− mice, fish oil reduced the percentage of blood Ly6C+ monocytes, despite an average 2-fold higher plasma cholesterol relative to palm oil.

Conclusion—The presence of splenic Ly6C+ monocytes parallels the appearance of atherosclerotic disease in both low–density lipoprotein receptor knockout and apolipoprotein E−/− mice. Furthermore, omega-3 fatty acids favorably alter monocyte subsets independently from effects on plasma cholesterol and reduce monocyte recruitment into atherosclerotic lesions. (Arterioscler Thromb Vasc Biol. 2012;32:2122-2130.)

Key Words: echium oil ◼ fish oil ◼ inflammation ◼ Ly6C ◼ monocyosis ◼ omega-3 fatty acids

Omega-3 fatty acids (n-3 FAs) are atheroprotective molecules1-3 that exert potent triglyceride-lowering effects in animals and humans.4-5 But in humans, n-3 FAs rarely affect plasma cholesterol levels, the primary factor leading to atherosclerosis.4-5 However, atherosclerosis has a marked inflammatory component, making it necessary to understand how the anti-inflammatory properties of n-3 FAs5-9 impact this disease. Thus, we sought to elucidate other potential mechanisms by which n-3 FAs reduce atherosclerosis, directing attention to their anti-inflammatory properties.

n-3 FAs reportedly reduce macrophage staining in atherosclerotic plaques in animal models of atherosclerosis and humans,10-12 suggesting a potential mechanism by which n-3 FAs exhibit atheroprotection because of the critical role macrophages, and their precursor monocytes, play in this disease.13-15 The accumulation of blood monocytes in the artery wall correlates with lesion development, and there is constant influx of these cells into developing plaques throughout the progression of atherosclerosis.16 Two major subclasses of monocytes have been identified in mammals,17,18 designated as classical and nonclassical monocytes. They are differentiated by a number of cell-surface receptors in mice, including CCR2, CX3CR1, CD62L, CD11c, and Ly6C. Ly6C classical monocytes coexpress CCR2 and CD62L and are actively recruited to the sites of inflammation. In contrast, Ly6C classical monocytes express higher levels of CX3CR1 and CD11c compared with Ly6C classical cells. This population patrols the circulation, eventually entering tissues to replenish resident dendritic cells and macrophages.17 Recent studies suggest that Ly6C classical monocytes increase in hyperlipidemic apolipoprotein E knockout (apoE−/−) mice and migrate into atherosclerotic plaques to become lesional macrophages.19-21 However, Ly6C classical monocytes, which preferentially express CD11c, may also contribute to atherogenesis by accumulating lipids in the circulation and transporting them to the developing plaque.22 Thus, both monocyte subsets have the potential to impact atherosclerosis.
We hypothesized that n-3 FAs would reduce atherosclerosis and concomitantly alter monocyte subsets and monocyte recruitment to the developing lesion. We tested this hypothesis using botanical and marine sources of n-3 FAs in 2 well-characterized mouse models of atherosclerosis, low-density lipoprotein receptor (LDLr<sup>−/−</sup>) knockout and apoE<sup>−/−</sup> mice. Furthermore, we also sought to determine whether hypercholesterolemia-associated monocytosis of Ly6C<sup>+</sup> cells occurs in the LDLr<sup>−/−</sup> background, as has been described in apoE<sup>−/−</sup> mice, and whether n-3 FAs could inhibit this process.

**Materials and Methods**

**Mice and Diets**

LDLr<sup>−/−</sup> and apoE<sup>−/−</sup> mice (C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a specific pathogen-free facility with a 12-hour light/dark cycle. All protocols and procedures were approved by the Institutional Animal Care and Use Committee of Wake Forest School of Medicine. Female mice aged 6 to 8 weeks were fed atherogenic diets containing 10% calories palm oil (PO) and 0.2% cholesterol, supplemented with an additional 10% of calories as PO, echium oil (EO; containing 18: 4 n-3), or fish oil (FO) for 16 (LDLr<sup>−/−</sup>) or 12 (apoE<sup>−/−</sup>) weeks. See the Methods section in the online-only Data Supplement for a detailed description of the oils. The synthetic diets were prepared by the diet kitchen in the Department of Pathology at Wake Forest School of Medicine as previously described. The FA composition of the PO, EO, and FO diets has been previously published. A standard chow diet, ProlabsIsoPro RMH 3000 (LabDiet, Purina, St. Louis, MO) was used as an additional control.

**Quantification of Aortic Root Lesion Area**

The aortic root was sequentially sectioned after an adaptation of the method of Daugherty et al. Once the aortic valves were visible, 8-μm aortic root sections were retained for the length of 640 μm. The sections were placed on a series of 10 slides. This resulted in 8 sections per slide, with a distance of 80 μm apart. Oil red O and hematoxylin staining was performed to assist in tissue visualization. Images were acquired with a Nikon DigitalSight DSI1 camera using NIS Elements (Nikon, Tokyo, Japan) software. Intimal area measurements were obtained with Image Pro 6.2 software (Media Cybernetics, Bethesda, MD). We defined intimal area as the lesion area within the internal elastic lamina and the luminal surface of the aorta. Three 8-μm aortic root sections were blindly quantified from individual mice on atherogenic diets was 10.7%.

**Plasma Lipid and Lipoprotein Analyses**

Blood was collected by submandibular vein puncture in nonfasted mice in the presence of heparin. Plasma from these blood samples was isolated using a table top centrifuge (10 000rpm for 15 minutes at 4°C). Plasma cholesterol or triglyceride concentrations were measured enzymatically using commercially available kits (Wako, Richmond, VA). Plasma lipoproteins were fractionated by fast protein liquid chromatography, and cholesterol distribution among lipoproteins was measured by enzymatic assay.

**Flow Cytometry**

Peripheral blood was obtained by submandibular vein puncture for circulating leukocyte analysis. For splenic cells, tissue was digested with an enzyme cocktail as published. The cell suspension was subsequently passed through a 70-μm nylon cell strainer (BD Falcon, Becton, Dickinson and Company, Franklin Lakes, NJ). Red blood cells were removed from flow cytometry preparations by treatment with ammonium, chloride, potassium lysing buffer ( Gibco, Invitrogen Corporation, Carlsbad, CA). The remaining white blood cells were incubated with the following monoclonal antibodies CD11b-APC-Cy7 clone: M1/70 (BioLegend, San Diego, CA), CD115-APC clone: AFS98, CD62L-PerCP-Cy5.5 clone: MEL-14 (eBioscience, San Diego, CA), Ly6G-PE clone: 1A8, CD11c-PE-Cy7 clone: HL3, Ly6C-FITC, or PE clone: AL-21 (BD Pharmingen, Becton, Dickinson and Company, Franklin Lakes, NJ). 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).

**Monocyte Labeling and Quantification of Monocyte Recruitment**

Monocytes were labeled using the Gr-1<sup>−</sup> method of Tacke et al. Briefly, 1-μm Fluoresbrite Yellow Green microspheres (2.5% solids [wt/vol], Polysciences, Inc., Warrington, PA) were diluted 1:4 in PBS and injected retroorbitally into anesthetized mice. Blood was drawn from recipient mice the day after labeling to evaluate the total number of bead-containing monocytes per mouse. This approach predominately labels Ly6C<sup>+</sup> monocytes, although some transient labeling of Ly6C<sup>−</sup> monocytes has been observed.

Aortic root tissue sections were taken as described above and the number of beads confined to atherosclerotic lesions was quantified per section. A total of 8 sections from a single slide representative of the length of the aortic root was analyzed per mouse. Beads were counted manually, at 20x magnification. To adjust for slight variations in labeling, data were normalized for individual mice based on the percentage of blood monocytes labeled. For example, if an individual mouse had 11% of blood monocytes labeled and 15 bead-containing cells per section were counted, the calculation would be as follows: 15/0.11=136. This adjustment gave a normalized value, which represented the actual number of monocytes expected to have entered the lesion.

**Immunohistochemistry**

Aortic roots were mounted in optimal cutting temperature compound (Tissue-Tek, Ted-Pella, Inc., Redding, CA) and sectioned as described under the Quantification of Aortic Root Lesion Area section of the Materials and Methods. Aortic root cross-sections were fixed with acetone and incubated with an anti-C668 antibody (1:75; overnight) from AbDSerotec, clone: FA11, (Raleigh, NC), followed by staining with an alkaline phosphatase–conjugated secondary antibody (goat anti-rat; 1:50; 30 minutes) from AbDSerotec; control slides contained no primary antibody. Four 8-μm aortic root sections from each mouse were blindly analyzed for the percent CD68-positive area.

**Statistical Analyses**

All data were subjected to parametric analysis and passed the D’Agostino and Pearson omnibus normality test. Data are presented as mean±SEM and were tested for significant differences (P<0.05) using 1-way ANOVA with Tukey post hoc analysis. Pearson correlation coefficient was calculated after the line of best fit was determined using linear regression analysis. All of the analyses were performed with GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, CA).

**Results**

**n-3 FAs Reduced Atherosclerosis in LDLr<sup>−/−</sup>, but Not ApoE<sup>−/−</sup> Mice**

As previously described, n-3 FAs reduced atherosclerosis development in LDLr<sup>−/−</sup> mice (Figure 1A and 1B). We observed a stepwise decrease in atherosclerosis that correlated with the degree of enrichment of long chain n-3 FAs after either EO or FO supplementation for 16 weeks. Standard rodent chow has negligible amounts of cholesterol, and as expected, this group...
had the lowest intimal lesion area. Interestingly, parallel studies in apoE−/− mice resulted in no difference in atherosclerosis among diet groups after 12 weeks of diet consumption (Figure 1A and 1C). As expected, apoE−/−chow-fed mice had reduced atherosclerosis compared with mice fed atherogenic diets. We also measured aortic cholesterol content in the entire aorta (arch, thoracic, and abdominal) in LDLr−/− and apoE−/− mice and observed similar trends as with aortic root intimal area (Figure IA and IB in the online-only Data Supplement). Furthermore, aortic root intimal area positively correlated with aortic cholesterol ester content in mice of both backgrounds (Figure IC and ID in the online-only Data Supplement). Thus, dietary n-3 FAs reduced atherosclerosis in LDLr−/− but not apoE−/− mice.

**Differential Plasma Cholesterol Responses to Diet in LDLr−/− and ApoE−/− Mice**

In agreement with previous reports, n-3 FAs blunted hypercholesterolemia in the LDLr−/− mice (Figure 2A and 2C), with equivalent reductions in EO- and FO-fed mice compared with PO-fed mice. Plasma cholesterol measurements in apoE−/− mice uncovered an unexpected finding (Figure 2B). The FO-fed mice had the highest exposure to cholesterol over time as illustrated using area under the curve analysis for total plasma cholesterol, with all other groups exhibiting similar levels (Figure 2D). Fast protein liquid chromatography size fractionation of plasma revealed that the increase in total plasma cholesterol concentrations in the FO diet group was primarily because of an increase in very low-density lipoprotein size particles (Figure IIIB in the online-only Data Supplement). Therefore, n-3 FAs reduced atherosclerosis in LDLr−/− but not apoE−/− mice.

**Plasma Cholesterol Does Not Correlate With Disease Burden in ApoE−/− Mice**

Atherosclerosis was similar among PO-, EO-, and FO-fed apoE−/− mice (Figure 1C, Figure IB in the online-only Data Supplement), despite 2-fold higher plasma cholesterol area under the curve in the FO group (Figure 2D). Furthermore, linear regression analysis demonstrated a significant association between intimal area and plasma cholesterol in the LDLr−/− mice (Figure 2E) but not in apoE−/− mice (Figure 2F). These results suggest a unique property of FO that counterbalanced the hypercholesterolemia induced by FO feeding in apoE−/− mice to promote atherosclerosis progression.

**n-3 FAs Favorably Alter Ly6Chi Monocytes in Atherosclerotic Mice**

Despite a substantial lowering of triglycerides, n-3 FAs typically do not alter plasma cholesterol in humans and may, in fact, increase low-density lipoprotein (LDL) cholesterol and lower high-density lipoprotein cholesterol in some individuals. We investigated the possibility that n-3 FAs could reduce the Ly6C60 monocyte subset, which infiltrate into atherosclerotic lesions because reduced macrophage staining has been reported with n-3 FA supplementation.

We identified monocytes as CD11b+Ly6G−CD115+ (Figure IIIA in the online-only Data Supplement). In agreement with other findings, Ly6C60 cells preferentially expressed CD62L, whereas CD11c expression was found primarily on Ly6C60 monocytes (Figure IIIB in the online-only Data Supplement). We observed no significant difference in the percentage of blood monocytes in LDLr−/− mice during the 16-week atherosclerosis progression experiment based on the

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Figure 1. Dietary omega-3 fatty acids (FAs) reduce atherosclerosis in low-density lipoprotein receptor (LDLr−/−), but not apolipoprotein E knockout (apoE−/−) mice. Female mice aged 6 to 8 weeks were fed chow or diets containing 10% (calories) palm oil (PO) and 0.2% cholesterol, supplemented with an additional 10% of calories as PO, echium oil (EO), or fish oil (FO) for 16 (LDLr−/−) or 12 (apoE−/−) weeks. A, Representative photomicrographs of Oil red O and hematoxylin-stained cross-sections of the aortic root (magnification ×4). Quantitative analysis of aortic root intimal area in LDLr−/− (B) or apoE−/− mice (C). Data points shown in B and C represent individual mice; the horizontal line denotes the group mean. Values not sharing a common superscript differ significantly (P<0.05).
type of fat in the atherogenic diet. However, chow-fed mice had significantly less monocytosis compared with PO- and EO-fed mice (Figure IVA and IV C in the online-only Data Supplement). In agreement with another 12-week study in apoE−/− mice,19 the saturated fat–containing atherogenic diet (ie, PO) did not significantly alter the percentage of monocytes in the circulation compared with chow-fed controls (Figure IVB and IVD in the online-only Data Supplement). However, using area under the curve analysis, we did observe an increase in percentage of monocytes in FO-fed mice compared with PO- and chow-fed mice (Figure IVD in the online-only Data Supplement). With regard to subset distribution, the proportion of monocytes in the Ly6Chi subset was not altered over 16 weeks in LDLr−/− mice (Figure 3A and 3D). In the apoE−/− background, only the mice fed chow were different than the PO group (Figure 3E). In fact, the changes in the Ly6Chi subset were strikingly similar to changes in intimal lesion area (compare Figures 3E and 1C). Furthermore, we found a significant positive association between percentage of Ly6Chi splenic monocytes and intimal area in both LDLr−/− and apoE−/− mice (Figure 3F and 3G).

n-3 FAs Protect Against Neutrophilia in Hypercholesterolemic Mice

Recent advances in the field have also highlighted the importance of hypercholesterolemia-induced neutrophilia in atherosclerosis.36 We measured blood neutrophils in LDLr−/− and apoE−/− mice after 16 and 12 weeks of atherogenic diets, respectively (Figure VA and VB in the online-only Data Supplement). Just as n-3 FAs dissociated hypercholesterolemia from Ly6C hi monocytosis, we also observed dissociation of hypercholesterolemia from neutrophilia after 16 weeks in the LDLr−/− mice, where n-3 FAs reduced blood neutrophils to levels of chow-fed mice. In apoE−/− mice, we detected a decrease in neutrophils with both EO- and FO-fed mice after 6 and 9 weeks on the diet. Similar findings were observed...
in the spleen (Figure VC and VD in the online-only Data Supplement), a compartment that may reflect cumulative effects of dietary FAs on systemic neutrophil levels.

n-3 FAs Reduce Monocyte Infiltration into Atherosclerotic Lesions

To determine whether n-3 FAs would decrease monocyte trafficking into atherosclerotic lesions, we performed monocyte-labeling experiments after maintaining LDLr−/− or apoE−/− mice on the diet for 16 or 12 weeks, respectively. Using the procedure developed by Randolph et al., we phagocytically labeled monocytes with 1-μm fluorescent beads. Within 24 hours of injection, 0.7% to 1.2% of blood leukocytes were labeled in the LDLr−/− diet groups, and 1.0% to 1.2% of blood leukocytes were labeled in the apoE−/− groups. Of these bead+ cells, 63% to 81% (mean, 79.4%) were monocytes in LDLr−/− mice, and 78% to 90% (mean, 85.3%) were monocytes in the apoE−/− mice. This translated to 6.5% to 13.3% (mean, 9%) of monocytes carrying a fluorescent label in LDLr−/− mice. In apoE−/− mice, 6.0% to 11.6% (mean, 8%) of monocytes were bead+. The remainder of bead-labeled nonmonocytic cells in blood were B cells (CD19+; 66%) and neutrophils (Ly6-G+; 34%), similar to previous findings using this technique.

The number of recruited monocytes (visualized as fluorescent beads, see Figure VI in the online-only Data Supplement) within the lesion area of the aortic root in mice fed PO, EO, or FO diets was compared with chow-fed controls in LDLr−/− mice (Figure 4A) and apoE−/− mice (Figure 4B). Because of slight variations in labeling (6.5%–13.3% in LDLr−/− mice; 6.0%–11.6% in apoE−/−), 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 (ie, normalized frequency). Normalization revealed a decrease in bead+ cells...
per intimal area per section in all groups compared with PO in LDLr−/− mice (Figure 4C). In contrast, bead+ monocytes did not differ among diet groups in the apoE−/− background (Figure 4D), although there was a trend toward a decrease in chow- versus PO-fed mice. Of interest, the number of monocytes trafficking to lesions was higher in the apoE−/− compared with the LDLr−/− (compare scales in Figure 4A and 4C versus 4B and 4D), perhaps reflecting the heightened inflammatory response in the apoE−/− model.37,38 Together, these data indicate that n-3 FAs reduce infiltration of monocytes into atherosclerotic lesions to an extent related to the amount of atherosclerosis.

**n-3 FAs Decrease Macrophage Staining in Atherosclerotic Lesions**

In concordance with our monocyte infiltration data, analysis of CD68 immunohistochemistry in LDLr−/− mice (Figure 5A and 5B) revealed that n-3 FA-fed mice exhibit reduced (30%–44%) macrophage staining compared with PO-fed mice, equivalent to chow-fed mice.

**Discussion**

n-3 FAs have been appreciated as cardioprotective molecules for decades.39 Much of the focus regarding their mode of action has been centered on plasma lipid–lowering effects.4,5,40 However, in humans, n-3 FAs rarely have an effect on plasma cholesterol levels, a bona fide promoter of atherosclerosis.4,6 Thus, we sought to elucidate other potential mechanisms by which n-3 FAs reduce atherosclerosis, directing our attention to their anti-inflammatory properties.41 We used LDLr−/− mice to examine development of hypercholesterolemia-associated monocytosis and neutrophilia, in studies not previously performed in this model. Using the apoE−/− model enabled us to compare the relative contributions of nonplasma cholesterol parameters, such as monocyte phenotype, on atherosclerosis outcome because in apoE−/− mice n-3 FAs did not reduce plasma cholesterol and even increased it in the case of FO.

As anticipated, dietary n-3 FAs reduced atherosclerosis in LDLr−/− mice. Reductions in plasma cholesterol are a potential mechanism for the decrease in atherosclerosis. Indeed, both EO and FO reduced total plasma cholesterol to a similar level over the 16-week study (Figure 2A and 2C); however, FO-fed mice had significantly lower aortic root intimal area compared with those fed EO (Figure 1B). We attribute this to the higher levels of atheroprotective n-3 FAs in FO-fed mice compared with EO-fed mice, particularly docosahexaenoic acid.24 Therefore, even in LDLr−/− mice, in which n-3 FAs are associated with consistently lower plasma lipids, we have evidence that n-3 FAs reduce atherosclerosis independently of plasma lipid reduction. Nonetheless, based on linear regression analysis in the LDLr−/− mice, ≈70% of the variability in atherosclerosis can be explained by plasma cholesterol levels (Figure 2E). Thus, in this model, it is difficult to state with certainty that reductions in atherosclerosis and changes in monocyte subset distribution are because of the anti-inflammatory potential of n-3 FAs versus their hypolipidemic properties.

Our findings in apoE−/− mice allow for different interpretations because dietary fat did not lower plasma cholesterol. Interestingly, only chow-fed mice had reduced atherosclerosis compared with PO-fed mice. However, this could not be ascribed to significantly decreased exposure to cholesterol over time in chow-fed versus PO-fed mice (Figure 2B and 2D). In fact, no significant correlation between atherosclerosis and plasma cholesterol was observed in apoE−/− mice (Figure 2F). By contrast, the FO group had twice the plasma cholesterol exposure as their PO-fed counterparts (Figure 2D), yet similar atherosclerosis compared with PO-fed mice (Figure 1C). This illustrates that n-3 FAs can moderate atherosclerosis apart from effects on plasma cholesterol, as has been consistently reported in humans.5,6

Changes in plasma lipids, namely triglycerides, were not expected with n-3 FAs in apoE−/− mice because apoE is thought to play an important role in the increased clearance of triglyceride-rich lipoproteins that occurs with n-3 FAs.

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**Figure 4.** Omega-3 fatty acids (FAs) reduce infiltration of monocytes into atherosclerotic lesions. Labeled monocyte trafficking to the aortic root lesion area in low–density lipoprotein receptor (LDLR−/−) (A) or apolipoprotein E knockout (apoE−/−) (B) mice after 16 or 12 weeks on diet, respectively, was quantified as beads per section. Each bead represents a labeled monocyte. C and D, Normalized data adjusted for individual animal differences in the percentage of labeled monocytes in the circulation of LDLR−/− or apoE−/− mice, respectively. Values not sharing a common superscript differ significantly, and panels lacking superscripts indicate statistical differences were not observed at P=0.05.
Interestingly, in the apoE−/− mice, the correlate with plasma cholesterol measurements but instead changes in the Ly6C hi subset paralleled lesion area more closely than did plasma cholesterol. This finding suggests that Ly6Chi monocytosis can be dissociated from hypercholesterolemia, particularly in the context of reduced inflammation, as would be expected with n-3 FA supplementation.

Likely, the elevated cholesterol levels in FO-fed animals can be attributed to decreased activity of the LDL receptor clearance pathway. Our group and others have noted a decrease in LDL receptor activity and binding affinity of lipoproteins after FO feeding. Thus, because of the role of the LDL receptor in clearing remnant particles, any alterations could have a profound effect in the apoE−/+ model, in which plasma remnant particles are elevated. Thus, in apoE−/+ mice, where the LDL receptor pathway is intact, FO may reduce the clearance of remnant particles, resulting in an increase in cholesterol compared with PO-fed controls.

Our data indicated that factors other than plasma lipids contribute to the antatherogenic properties of n-3 FAs. Therefore, we tested the hypothesis that n-3 FAs could alter Ly6C monocyte subsets, which have important implications in atherosclerosis. In the LDLr−/− mice, analysis of the expression of Ly6C on blood monocytes showed similar subset distribution among diet groups. In contrast, apoE−/+ mice exhibited a decrease in the Ly6C hi subset to levels of chow-fed mice, despite the high plasma cholesterol observed with FO feeding. This finding suggests that Ly6C hi monocytes can be dissociated from hypercholesterolemia, particularly in the context of reduced inflammation, as would be expected with n-3 FA supplementation.

Because the spleen can serve as a reservoir for monocytes in the periphery and may reflect cumulative changes during dietary treatment, we assessed this compartment for the effects of n-3 FAs. Proportions of total monocytes in the spleen did not change as a function of dietary fat in either LDLr−/− or apoE−/+ mice. However, contrary to the blood monocyte data, n-3 FAs reduced the Ly6C hi subset compared with PO to the level of chow-fed mice in the LDLr−/− model. This is likely because of the fact that blood monocytes represent a transient pool of cells that turn over rather quickly, whereas the spleen reflects what may be occurring in the body over time. Furthermore, in both LDLr−/− and apoE−/+ mice, the Ly6C hi profile of splenic monocytes did not appear to correlate with plasma cholesterol measurements but instead with atherosclerosis. Interestingly, in the apoE−/+ mice, the changes in the Ly6C hi subset paralleled lesion area more closely than did plasma cholesterol. Our data with blood and splenic neutrophils resulted in a similar conclusion, as we also observed reductions in this cell type in EO- and FO-fed mice compared with PO-fed mice that were not reflective of changes in hypercholesterolemia per se. These observations support the notion that monocyte distribution skewing toward the Ly6C hi subset, as well as neutrophilia, correlate better with atherosclerosis disease burden than cholesterol levels.

We also sought to determine whether decreased macrophage abundance in atherosclerotic lesions after n-3 FA treatment is because of decreased monocyte trafficking. We observed decreased recruitment of monocytes to plaques coupled with decreased macrophage staining in EO-, FO-, and chow-fed mice compared with PO-fed LDLr−/− mice, suggesting that monocyte trafficking could be the reason for fewer macrophages in lesions after n-3 FA treatment. The finding that n-3 FA supplementation reduces monocyte recruitment to lesions is important because recent evidence suggests that decreased monocyte recruitment leads to regression of atherosclerotic disease.

Overall, our data support the notion that n-3 FAs reduce atherosclerosis, in part, by favorably altering monocyte subsets and reducing trafficking of monocytes to atherosclerotic plaques. In addition, several points from this study and others converge to make an argument for the Ly6C hi monocyte profile as a relevant marker for disease progression as opposed to plasma cholesterol. First, cholesterol feeding the wild-type mice increases plasma cholesterol, yet these animals do not develop monocytosis or exhibit changes in the distribution of Ly6C monocytes. Second, although results observed after statin treatment have been used to support an association between hypercholesterolemia and Ly6C hi monocytosis, statins have known immunomodulatory and anti-inflammatory properties that could impact blood monocyte numbers and subset distribution. Furthermore, any treatment (such as statin therapy) that alters plasma cholesterol will impact atherosclerotic disease progression, making it impossible to conclude that alterations in plasma cholesterol alone are affecting Ly6C hi monocytosis. Third, in our studies, hypercholesterolemia, monocytosis, and the distribution of the Ly6C subset do not correlate across time. Using LDLr−/− mice as an example, plasma cholesterol peaks around 4 weeks of dietary intervention, yet the blood monocytes...
gradually increase over 16 weeks. In further support of this, plasma cholesterol was not related to the percentage of Ly6C<sup>hi</sup> monocytes in the circulation or the spleen in either LDLr<sup>−/−</sup> or apoE<sup>−/−</sup> mice. Interestingly, plasma cholesterol in EO- and FO-fed mice was intermediate between PO- and chow-fed controls in the LDLr<sup>−/−</sup> background, yet splenic Ly6C<sup>hi</sup> monocytes and monocyte trafficking were reduced to the extent of chow-fed mice, further supporting the idea that beneficial effects on Ly6C<sup>hi</sup> monocytosis are independent of alterations in plasma cholesterol.

It is noteworthy that the beneficial effects of anti-inflammatory n-3 FAs were partially blunted in apoE<sup>−/−</sup> mice, which generally exhibit a higher inflammatory state than LDLr<sup>−/−</sup> mice.<sup>37,38</sup> Part of this blunted response may be related to the absence of plasma lipid lowering by n-3 FAs in apoE<sup>−/−</sup> mice.<sup>39</sup> In addition, apoE is a critical component of the macrophage sterol-responsive network in macrophage foam cells and is upregulated in the artery wall by atheroprotective substances, such as thiazolidinediones and statins.<sup>52</sup> In the absence of apoE, the beneficial effects of n-3 FA on reducing macrophage inflammation and foam cell formation may be partially lost.

Future studies may examine whether any of the mechanisms described in this current study could occur through FA sensors, such as the recently discovered GPR120 receptor.<sup>9</sup> This receptor regulates the ability of n-3 FAs to decrease M1 (classical) proinflammatory gene expression and increase M2 (alternative) gene expression. It is plausible that GPR120 plays an additional role in the alterations of Ly6C monocyte subsets observed with n-3 FA treatment. Another potential mechanism might involve n-3 FA regulation of low-density lipoprotein receptor–related protein 1, which has recently been shown to decrease Ly6C<sup>hi</sup> monocytosis in an apoE-independent fashion.<sup>53</sup>

In conclusion, n-3 FAs reduce atherosclerosis, alter monocyte subsets, and reduce monocyte trafficking to atherosclerotic lesions of LDLr<sup>−/−</sup> mice. The combination of fewer circulating Ly6C<sup>hi</sup> monocytes and a reduced percentage of circulating neutrophils maintained atherosclerotic disease burden in apoE<sup>−/−</sup> mice fed n-3 FAs to levels comparable to those fed saturated fat, despite the 2-fold higher plasma cholesterol exposure in the FO group. Reductions in atherosclerosis in LDLr<sup>−/−</sup> mice likely occurred through similar mechanisms, with the additional benefit that n-3 FAs decreased monocyte recruitment to the artery wall. Finally, our data provide evidence that the inflammatory profile of splenic monocytes parallels atherosclerosis, as opposed to plasma cholesterol levels. This finding has important implications regarding the interactions between hyperlipidemia and innate immunity in the context of atherosclerosis and should be considered when evaluating the impact of hyperlipidemia on immune cells in models of atherosclerosis.

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**Disclosures**

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SUPPLEMENTAL MATERIAL

Omega-3 Fatty Acids Ameliorate Atherosclerosis by Favorably Altering Monocyte Subsets and Limiting Monocyte Recruitment to Aortic Lesions

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Supplemental Methods

**Dietary oils** - The seed oil of *Echium plantagineum* L., a member of the Boraginaceae family was a generous gift from Croda Europe Ltd. (Leek, Staffordshire, UK) and 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 Arecaceae family, was purchased from Shay and Company (Portland, OR, USA). A certificate of analysis is on file for reference. The fish oil source was *Brevoortia tyrannis* Latrobe, a member of the Clupeidae family, was manufactured and generously provided by Omega Protein (Reidsville, VA, USA) with a report of analysis on file for reference.
Supplemental Figure Legends

**Supplemental Figure I.** Aortic cholesterol ester (CE) content. Aortic cholesterol ester (CE) content per mg protein for LDLr<sup>−/−</sup> (A) or apoE<sup>−/−</sup> mice (B) was measured as previously described<sup>1</sup>. Linear regression analysis of intimal area vs. aortic CE content for LDLr<sup>−/−</sup> (C) or apoE<sup>−/−</sup> (D) mice. In panels C and D, each point represents an individual animal of the denoted diet group and the correlation coefficient is shown for entire data set.

**Supplemental Figure II.** Plasma lipoprotein cholesterol distribution. Cholesterol distribution in a separate cohort of LDLr<sup>−/−</sup> mice (A) fed PO, EO, FO or chow for 20 weeks. Data represent the mean ± SEM of 3-5 mice per group. B. Cholesterol distribution in apoE<sup>−/−</sup> mice after 9 weeks on diet. Plasma lipoproteins were separated by fast protein liquid chromatography and lipoprotein (VLDL, LDL, or HDL size range) cholesterol concentrations were measured enzymatically as described in the Methods section. Data were obtained from an equal volume pool of plasma from 3 mice per diet group.

**Supplemental Figure III.** Representative dot plots and histograms of apoE<sup>−/−</sup> mice after 12 weeks of consumption of the indicated diets. Live cells were gated and further identified as monocytes by expression of CD11b and CD115. A. CD11b<sup>+</sup>Ly6G<sup>−</sup>CD115<sup>+</sup> monocytes (dot plots) were analyzed for expression of Ly6C (histograms) after 12
weeks on diet for apoE<sup>−/−</sup> mice. Percentages of Ly6C<sup>hi</sup> monocytes are shown as mean ± SD. 

**B.** Representative dot plots illustrating expression of CD11c and CD62L versus Ly6C expression on blood monocytes of apoE<sup>−/−</sup> mice after 12 weeks on the indicated diets. Percentages of monocytes in each quadrant are shown as mean ± SD.

**Supplemental Figure IV.** N-3 FAs favorably alter the Ly6C<sup>hi</sup> monocyte subset in the circulation of apoE<sup>−/−</sup> mice. The percentage of monocytes in the circulation over time was measured in LDLr<sup>−/−</sup> (A) and apoE<sup>−/−</sup> (B) mice. Area under the curve (AUC; i.e., percentage of monocytes vs. time) for individual mice was calculated for LDLr<sup>−/−</sup> (C) or apoE<sup>−/−</sup> mice (D). The percentage of circulating monocytes in the Ly6C<sup>hi</sup> subset was measured over time in LDLr<sup>−/−</sup> (C) and apoE<sup>−/−</sup> (D) mice. Data shown in panels A-F represent the mean ± SEM of 6-10 mice per group; values not sharing a common superscript differ significantly. Panels lacking superscripts indicate statistical differences were not observed at p=0.05.

**Supplemental Figure V.** EO and FO protect against neutrophilia in hypercholesterolemic mice. The percentage of CD11b<sup>+</sup>Ly6G<sup>+</sup>CD115<sup>−</sup> neutrophils in the circulation was measured in LDLr<sup>−/−</sup> mice after 16 weeks of diet feeding (A), and after 12 weeks of diet feeding in apoE<sup>−/−</sup> mice (B). (C) The percentage of neutrophils in the spleen was calculated after 16 weeks on diet in LDLr<sup>−/−</sup> mice and after 12 weeks in apoE<sup>−/−</sup> mice (D). Data represent mean ± SEM from 6-10 mice per group; values not sharing a common superscript differ significantly.
**Supplemental Figure VI.** Beads within the aortic lesion colocalize with CD68+ cells. A. Representative photomicrograph (20X) of an aortic root cross-section from an LDLr<sup>−/−</sup> mouse after 16 weeks on diet illustrating the presence of bead<sup>+</sup> monocyte-derived cells. Red: CD68+ cells; blue: DAPI-stained nuclei; green: beads (fluorescent microspheres). Bead<sup>+</sup> cells within the lesion area are indicted by white arrows. lu: aortic lumen. Sections were stained with an anti-CD68 antibody as described in the Methods section using a Cy3-conjugated secondary antibody (goat anti-rat) from Jackson ImmunoResearch.

**Supplemental References**

1. Furbee JW, Jr., Parks JS. Transgenic overexpression of human lecithin: Cholesterol acyltransferase (lcat) in mice does not increase aortic cholesterol deposition. Atherosclerosis. 2002;165:89-100
Supplemental Figure II

(A) LDLr<sup>-/-</sup>

(B) ApoE<sup>-/-</sup>