

# Effects of n-3 fatty acid treatment on monocyte phenotypes in humans with hypertriglyceridemia



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## KEYWORDS:

Hypertriglyceridemia;  
Eicosapentaenoic acid;  
Docosapentaenoic acid;  
n-3 polyunsaturated fatty acids;  
Monocytes;  
Atherosclerosis

**BACKGROUND:** Hypertriglyceridemia increases risk for atherosclerotic cardiovascular disease and may contribute to atherosclerosis by changing circulating monocyte phenotypes. High-dose n-3 polyunsaturated fatty acids reduce blood triglyceride levels. Effects of triglyceride-lowering therapy on monocyte phenotypes are not well known.

**OBJECTIVE:** We examined effects of n-3 polyunsaturated fatty acid treatments (eicosapentaenoic acid [EPA] plus docosapentaenoic acid [MAT9001] vs EPA ethyl esters [EPA-EE]) on monocyte phenotypes in individuals with hypertriglyceridemia.

**METHODS:** Individuals with triglycerides 200 to 400 mg/dL were recruited. Subjects received 2 treatments in randomized order for 14 days each: MAT9001 and EPA-EE, at 4 g/d. At 2 days before the start of, and on the last day of, each treatment, Nile red staining for lipids and phenotypes of each monocyte subset were examined by flow cytometry after an overnight fast and postprandially after a high-fat meal.

**RESULTS:** Treatment with MAT9001 or EPA-EE reduced fasting triglyceride levels and decreased proportions of intermediate monocytes. Only MAT9001 decreased postprandial blood triglyceride levels, lowered fasting Nile red levels, indicating less lipid in classical and intermediate monocytes, and reduced postprandial CD11c levels on nonclassical monocytes. MAT9001 and EPA-EE each reduced fasting and postprandial CD11c and CD36 levels on classical and intermediate monocytes and postprandial CCR5 levels on intermediate and nonclassical monocytes, with no significant differences between the 2 treatments.

**CONCLUSIONS:** Treatment with MAT9001 in individuals with hypertriglyceridemia reduced fasting Nile red staining for lipids in classical and intermediate monocytes. MAT9001 and EPA-EE each improved fasting and postprandial monocyte phenotypes, which could potentially help to protect against atherosclerosis.

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## Introduction

Hypertriglyceridemia has been identified as an independent causal risk factor for atherosclerotic cardiovascular disease (ASCVD).<sup>1–4</sup> However, the mechanisms whereby hypertriglyceridemia contributes to ASCVD remain incompletely understood.

Atherosclerosis is an inflammatory disease characterized by accumulation of lipid-laden macrophages (foam cells) in arterial walls.<sup>5–8</sup> Monocyte infiltration from the circulation into arterial walls and differentiation into macrophages, which take up modified lipoproteins and become foam cells, are important steps in the development of atherosclerosis.<sup>5,8,9</sup> Monocytes are heterogeneous and include several subsets, which may exert differential roles in inflammation and atherosclerosis. Based on surface markers CD14 and CD16, human monocytes have been classified into CD14<sup>+</sup>/CD16<sup>−</sup> classical, CD14<sup>+</sup>/CD16<sup>+</sup> intermediate, and CD14<sup>dim</sup>/CD16<sup>+</sup> nonclassical monocytes.<sup>10–13</sup> Under healthy conditions, classical monocytes are the dominant monocyte population, accounting for ~80% to 90% of total monocytes, while intermediate and nonclassical monocytes each account for 5% to 10% of total monocytes.<sup>11,14</sup> Nevertheless, results from most studies show that compared with CD16<sup>−</sup> classical monocytes, CD16<sup>+</sup>, particularly intermediate, monocytes play more important roles in inflammation.<sup>10,14</sup>

While the traditional paradigm in atherosclerosis has focused on macrophage foam cell formation in arterial walls,<sup>9</sup> our studies and others have shown that hyperlipidemia also causes formation of foamy monocytes, monocytes with intracellular lipid droplets, in the circulation in both mice and humans.<sup>15–22</sup> Foamy monocytes infiltrate into arterial walls and contribute to atherosclerosis.<sup>19,23</sup> Hypertriglyceridemia in humans with obesity and metabolic syndrome is also associated with increased proportions of CD16<sup>+</sup> intermediate or nonclassical monocytes<sup>12,24</sup> and with phenotypic changes in monocytes and subsets, including upregulation of adhesion molecules, toll-like receptors, inflammatory molecules, and oxidative stress.<sup>15,20,25–28</sup> Moreover, postprandial elevations in triglyceride (TG) levels after a single high-fat meal promote monocyte (subset) phenotypic changes, particularly in subjects with elevated fasting TGs and metabolic syndrome.<sup>20</sup> Formation of foamy monocytes with phenotypic changes in hyperlipidemia may accelerate monocyte (subset) contributions to atherosclerosis and therefore serve as an important link between hypertriglyceridemia and the development of ASCVD.<sup>19,21,23</sup>

Because of the causal role of hypertriglyceridemia in ASCVD, reducing TG levels may be an important strategy for ASCVD prevention.<sup>4,29</sup> Treatment with large doses of n-3 polyunsaturated fatty acids (PUFAs; generally 2–4 g/d) reduces blood TG levels, including fasting and postprandial TG levels.<sup>30,31</sup> Eicosapentaenoic acid (EPA) and docosahexaenoic acid have been the most commonly used n-3 PUFAs for TG lowering.<sup>32</sup> More recently, supplementation

with n-3 docosapentaenoic acid (DPA) revealed incorporation of DPA in various plasma lipid fractions, further reducing plasma TG levels.<sup>33</sup> Compared with EPA alone, EPA plus DPA resulted in greater reductions in fasting blood TG levels in subjects with hypertriglyceridemia.<sup>34</sup> Moreover, n-3 PUFAs may exert direct anti-inflammatory effects through several pathways independent of changes in plasma TG levels.<sup>35</sup> However, potential effects of n-3 PUFA treatment and reduced TG levels on monocyte (subset) lipid accumulation and phenotypes have not been well studied, particularly in individuals with hypertriglyceridemia. In the present study, we examined effects of treatment with EPA plus DPA, compared with EPA alone, on fasting and postprandial monocyte subset lipid accumulation and phenotypes in subjects with hypertriglyceridemia.

## Subjects and methods

### Study population and design

This was a substudy of a clinical trial ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02310022) identifier: NCT02310022) examining lipid effects of EPA plus DPA (MAT9001; Matinas BioPharma, Inc, Bedminster, NJ) vs EPA ethyl esters (EPA-EE) alone (VASCEPA, icosapent ethyl; Amarin Pharma, Inc, Bedminster, NJ) in subjects with hypertriglyceridemia.<sup>34</sup> The study was performed in a clinical research unit (Pharma Medica Research, Inc) located in Mississauga, ON, Canada, and has been detailed previously.<sup>34</sup> Briefly, the study included men and women aged 18 to 70 years, each with body mass index of 19.0 to 40.0 kg/m<sup>2</sup> and fasting TG levels of 200 to 400 mg/dL. Exclusion criteria included total cholesterol levels >300 mg/dL; nonstable use of statin therapy within 8 weeks before study drug; use of the highest recommended dose of any statin; use of TG-targeted drugs, or nonsteroidal anti-inflammatory drugs within 30 days before study drug; known history or presence of diabetes, CVD, impaired cardiovascular function, clinically significant gastrointestinal disease, or malabsorption; regular consumption of more than 1 meal containing fish or shellfish per week for 6 months before drug administration; or following a special diet within 30 days before the study.

The study had an open-label crossover design with two 14-day treatment periods (4 g/d MAT9001 vs 4 g/d EPA-EE) in randomized order, separated by a ≥35-day washout period. At 2 days before the start of each treatment and on the last day of each treatment, a postprandial study with a high-fat high-calorie breakfast was performed after a ≥12-hour fasting time. The total calorie content of the breakfast was 1241 kcal, of which 45% was from fat ([Supplemental Table 1](#)). Blood samples were collected by venipuncture before the breakfast (after fasting ≥12 hours) and at 4 and 6 hours after the breakfast.

The study protocol was approved by an ethics review board (Optimum Clinical Research Inc, Oshawa, ON, Canada). All subjects signed informed consent forms to participate in the study.

## Blood collection and experimental procedures

Blood samples for monocyte phenotyping were collected while fasting and at 4 and 6 hours postprandially into Cyto-Chex BCT Vacutainer Tubes (Streck, Omaha, NE)<sup>36</sup> and mixed with anticoagulant by inverting gently 8 times.<sup>37</sup> After collection, the blood samples were shipped overnight in a temperature-stabilizing container with temperature of 20 to 25°C (around 22°C)<sup>37</sup> to the investigator's laboratory in Baylor College of Medicine. Samples usually arrived in the laboratory within 24 hours after collection. On receipt, samples were processed immediately for monocyte phenotype analysis as described in the following. In addition, separate tubes of blood were collected by Alpha Laboratories Inc (Toronto, ON, Canada) for analysis of a complete blood count and lipoprotein lipids, including TG levels, which were measured using a Cobas 6000 analyzer series module c501 (Roche Diagnostics, Indianapolis, IN).

## Experimental protocol for monocyte phenotyping

Two 100- $\mu$ L aliquots of whole blood were incubated at room temperature with combinations of multiple fluorescent antibodies for the following molecules or Nile red in 2 protocols: (1) CD14 + CD16 + CD36 + scavenger receptor type A (SRA) + CD11c + CCR2 + CX3CR1; (2) CD14 + CD16 + CCR5 + Nile red. All the antibodies were purchased commercially: anti-human CD14 (clone: RMO52, Krome Orange-conjugated; Beckman Coulter, Brea, CA); anti-human CD16 (clone: 3G8, PE-Cy7-conjugated; Beckman Coulter); anti-human CD36 (clone: FA6.152, FITC-conjugated; Beckman Coulter); anti-human SRA (clone: 351615, PE-conjugated; R & D Systems, Minneapolis, MN); anti-human CD11c (clone: B-ly6, V450-conjugated; BD Biosciences, San Jose, CA); anti-human CCR2 (clone: K036C2, PerCP/Cy5.5-conjugated; Biolegend, San Diego, CA); anti-human CX3CR1 (clone: 2A9-1, APC-conjugated; Biolegend); anti-human CCR5 (clone: J418F1, APC/Cy7-conjugated; Biolegend).

For protocol 1, whole blood was incubated with the previously mentioned combinations of antibodies for 30 minutes in the dark. Stained blood was processed in a Coulter TQ-prep Workstation (Beckman Coulter) using ImmunoPrep Reagent (Beckman Coulter), which lysed red blood cells and fixed the samples. The processed samples were then washed twice with phosphate buffered saline (PBS) and resuspended in 300  $\mu$ L PBS for analysis of monocyte phenotypes (see in the following). For protocol 2, blood was first incubated with the previously mentioned antibodies for 30 minutes. Stained blood was processed in the TQ-prep Workstation and washed twice with PBS. The sample was then stained with Nile red for lipids using Lipid Droplets Fluorescence Assay Kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer's instructions. After

washing twice with PBS, the sample was resuspended in 300  $\mu$ L PBS for analysis of monocyte lipid staining and phenotypes. A Coulter Gallios flow cytometer (Beckman Coulter) was used to collect data.

Kaluza software (Beckman Coulter) was used to analyze data. Total leukocytes were first gated based on forward and side scatter patterns. Then, monocytes were defined within the gated leukocytes by CD14 expression in combination with the side scatter pattern. Based on surface expression levels of CD14 and CD16, monocytes were categorized into 3 subsets: CD14<sup>+</sup>/CD16<sup>-</sup> classical, CD14<sup>+</sup>/CD16<sup>+</sup> intermediate, and CD14<sup>dim</sup>/CD16<sup>+</sup> nonclassical monocytes. Each subset was examined for expression levels indicated by mean fluorescence intensity of the markers as described in the previously mentioned protocols ([Supplemental Fig. 1](#)).

The proportion of each monocyte subset was calculated based on the flow cytometric data and presented as percentage in total monocytes. The count of each monocyte subset was also calculated based on the percentage of each monocyte subset and total monocyte count obtained by a complete blood count.

## Statistical analysis

SAS, version 9.3 (SAS Institute, Cary, NC), was used for statistical analyses. Data analyses of monocyte phenotypes were blinded and exploratory. Descriptive statistics were generated for TG levels, proportions of each monocyte subset, and mean fluorescence intensity levels of various markers on each monocyte subset while fasting and at 4 and 6 hours postprandially before and after each treatment. Total area under the curve (AUC) for TG levels from 0 to 6 hours was calculated using the trapezoidal rule before and after each treatment.<sup>38</sup> Data are presented as mean  $\pm$  standard error of the mean unless stated otherwise. Differences between treatments were analyzed by repeated measures analysis of covariance with treatment as a factor and baseline (pretreatment) as a covariate in each model. Differences were considered significant at  $P \leq .05$ . The distribution of residuals for each statistical model was tested for normality with the Shapiro–Wilk test.<sup>39</sup> Parametric analyses were used for data that were normally distributed, and rank-transformed analyses were used for data that were not normally distributed (Shapiro–Wilk  $P < .01$ ). Pairwise comparisons were completed using linear contrasts with Bonferroni adjustments of the alpha levels. Within-treatment comparisons (pre- to post-treatment) for TG levels and monocyte phenotypes were analyzed before the meal and at 4 or 6 hours after the meal using paired t-test or Wilcoxon signed-rank test.

Possible carryover was assessed by evaluating the responses in each sequence group and by assessing treatment-by-sequence interactions in statistical models. No marked differences in responses were present between the 2 treatment sequence groups. No treatment-by-sequence interaction terms had  $P$  values  $< .02$ , and the number with  $P$  values

<.05 was well within the range expected given the number of comparisons, so values for both treatment sequences were pooled.

## Results

### Effects of treatments on postprandial TG levels

Twenty-seven subjects were included in this substudy. The baseline demographic data and pretreatment fasting lipid levels are shown in [Supplemental Tables 2 and 3](#).

As reported in the parent study, treatment with either MAT9001 or EPA-EE lowered fasting blood TG levels in subjects with hypertriglyceridemia, with greater reductions by MAT9001 than by EPA-EE alone.<sup>34</sup>

As expected, a high-fat meal increased TG levels before and after each treatment ([Fig. 1A](#)). Only MAT9001 significantly reduced postprandial TG levels and TG AUC from pre- to post-treatment ([Fig. 1A and B](#)). The posttreatment vs pretreatment changes (reductions) in postprandial TG levels and TG AUC were significantly greater for MAT9001 than EPA-EE ( $P < .01$  for TG levels at 6 hours after the high-fat meal, and  $P < .05$  for percent changes in TG AUC for MAT9001 vs EPA-EE).

### Effects of treatments on counts and proportions of monocyte subsets

Analyses of monocyte subset counts showed that compared with the fasting state, a high-fat meal increased or tended to increase the count of each monocyte subset ([Fig. 2A–C](#)). However, the percentage of most monocyte subsets was not significantly altered by a high-fat meal either before or after each treatment ([Fig. 2A–C](#)). Nevertheless, compared with pretreatment, treatment with MAT9001 or EPA-EE decreased the percentage and count of intermediate monocytes and increased the percentage and count of

classical monocytes in the fasting and postprandial states ([Fig. 2A and B](#)). EPA-EE treatment also slightly, but significantly, lowered the percentage of nonclassical monocytes in the postprandial state ([Fig. 2C](#)).

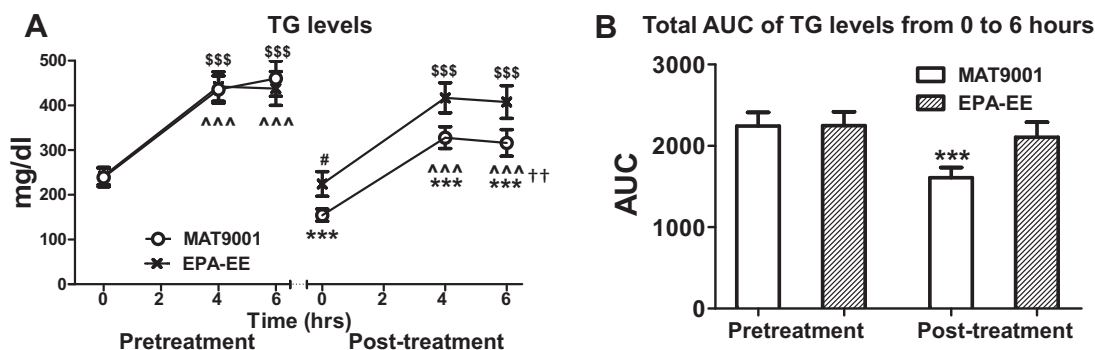
### Effects of treatments on Nile red staining of lipids in monocytes (subsets)

Fasting and postprandial hypertriglyceridemia is associated with increased lipid content in monocytes and with formation of foamy monocytes in the circulation,<sup>15–17,20</sup> which may contribute to ASCVD.<sup>19</sup> Reductions in TG levels in subjects with hypertriglyceridemia by MAT9001 were associated with significantly reduced Nile red levels, indicating less lipid, in both classical and intermediate monocytes in the fasting state compared with pretreatment ([Fig. 3A and B](#)). Compared with the fasting state, a high-fat meal increased Nile staining of lipids in all monocyte subsets after, but not before, MAT9001 treatment, and Nile red staining of lipids in monocytes (subsets) in the postprandial state was similar before and after MAT9001 treatment ([Fig. 3A–C](#)). These findings were consistent with our previous observations that compared with obese subjects with hypertriglyceridemia and metabolic syndrome, control subjects with normal fasting TG levels had lower proportions of foamy monocytes in the fasting state but showed greater increases in postprandial Nile red staining of lipids in monocytes.<sup>20</sup> In contrast to MAT9001 treatment, treatment with EPA-EE did not significantly alter Nile red levels in any monocyte subsets in either the fasting or postprandial state ([Fig. 3A–C](#)).

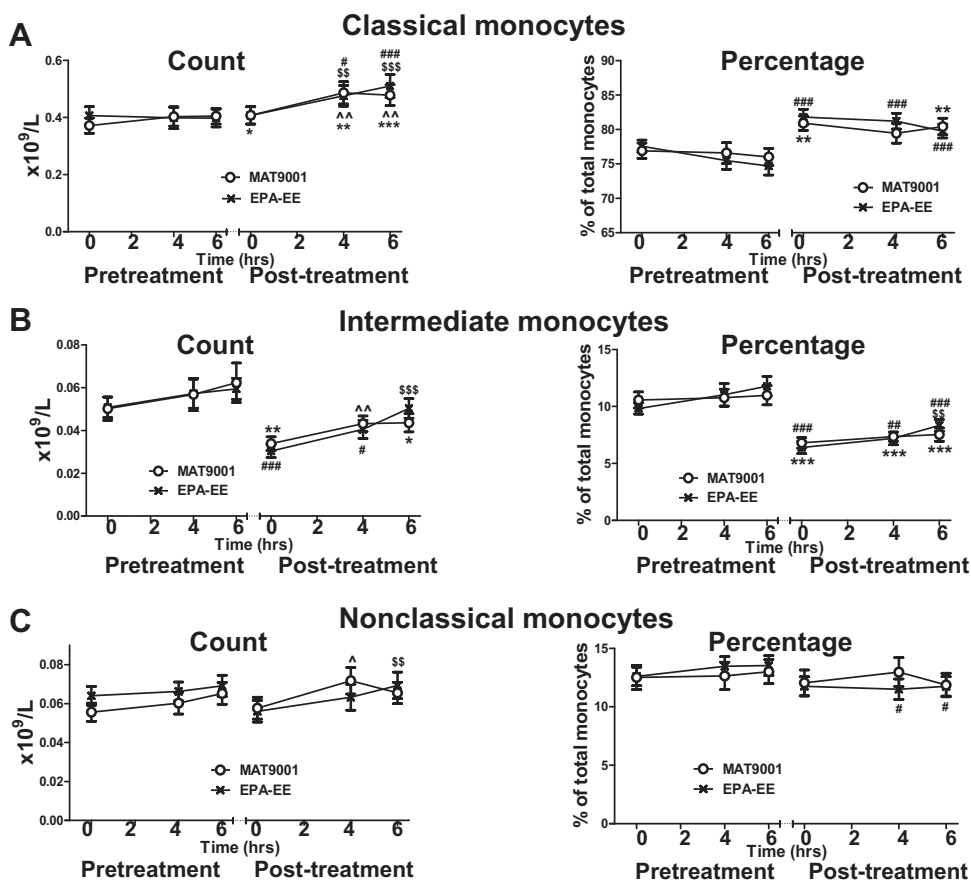
### Effects of treatments on monocyte (subset) phenotypes

#### Effects on CD11c expression

Consistent with previous studies,<sup>20</sup> levels of CD11c, a  $\beta 2$  integrin that mediates monocyte adhesion to endothelial cells



**Figure 1** Effects of MAT9001 or EPA-EE treatment on postprandial TG levels. (A) Blood triglyceride (TG) levels were measured fasting (0 hour) and postprandially (4 and 6 hours after the high-fat meal) just before (pretreatment) and at the end of (post-treatment) each treatment. (B) For each intervention, total area under the curve (AUC) of TG levels from 0 to 6 hours was compared between pre- and post-treatment values. \*\*\* $P < .001$  for posttreatment vs pretreatment with MAT9001; # $P < .05$  for posttreatment vs pretreatment with EPA-EE; \$\$\$ $P < .001$  for 4 or 6 hours vs 0 hour of MAT9001 treatment; \$\$\$ $P < .001$  for 4 or 6 hours vs 0 hour of EPA-EE treatment. †† $P < .01$  for MAT9001 vs EPA-EE for posttreatment vs pretreatment.



**Figure 2** Effects of MAT9001 or EPA-EE treatment on monocyte subset counts and proportions. Counts and percentages of each monocyte subset (classical [A], intermediate [B], and nonclassical [C]) were examined by flow cytometry in combination with a complete blood count in the fasting (0 hour) and postprandial (4 and 6 hours after the high-fat meal) states just before and at the end of each treatment. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$  for posttreatment vs pretreatment of MAT9001; # $P < .05$ , ## $P < .01$ , ### $P < .001$  for posttreatment vs pretreatment of EPA-EE;  $P < .05$ , ~ $P < .01$  for 4 or 6 hours vs 0 hour of MAT9001 treatment; \$\$\$ $P < .01$ , \$\$\$ $P < .001$  for 4 or 6 hours vs 0 hour of EPA-EE treatment.

and contributes to the development of atherosclerosis,<sup>15,20,26,27,40</sup> were higher on nonclassical and intermediate monocytes than on classical monocytes within individuals, and a high-fat meal increased CD11c levels on nonclassical, but not classical or intermediate, monocytes in subjects with hypertriglyceridemia (Fig. 4A–C). Compared with pretreatment, treatment with MAT9001 or EPA-EE reduced fasting and postprandial CD11c levels on classical and intermediate monocytes (Fig. 4A and B), with no significant differences between MAT9001 and EPA-EE treatments. However, while EPA-EE treatment did not significantly change fasting or postprandial CD11c levels on nonclassical monocytes, MAT9001 treatment significantly reduced nonclassical CD11c levels at 6 hours after a high-fat meal (Figure 4C). The change (reduction) in postprandial CD11c levels on nonclassical monocytes was greater with MAT9001 treatment than with EPA-EE ( $P < .05$ ).

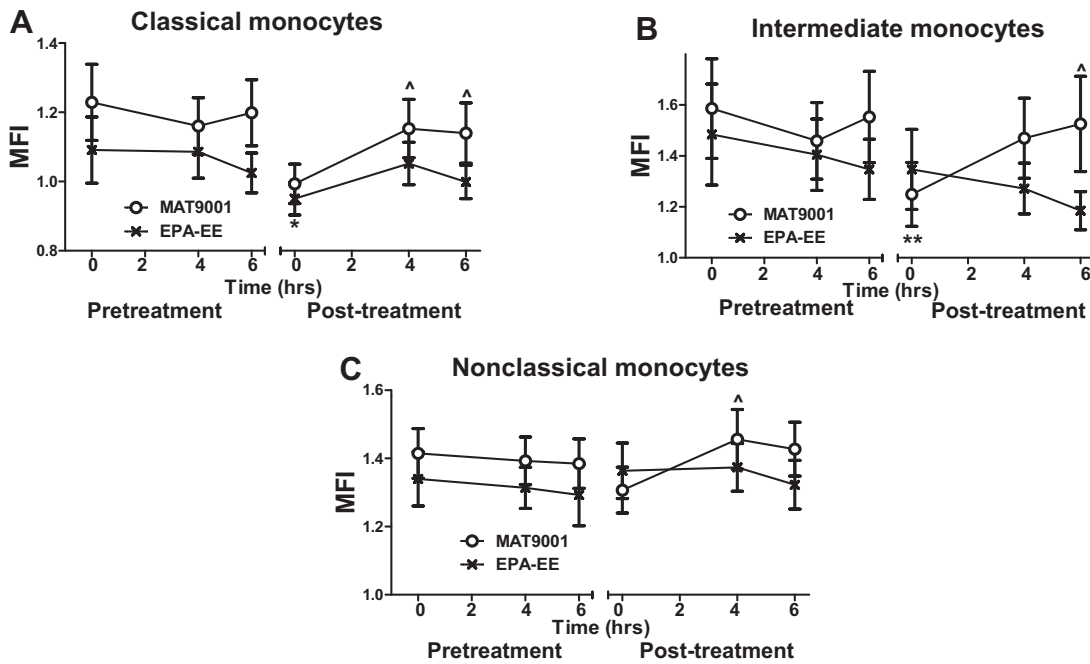
#### Effects on scavenger receptors

Levels of CD36, a scavenger receptor implicated in macrophage or monocyte uptake of modified lipoproteins and foamy cell formation,<sup>19,41</sup> were higher on classical and

intermediate monocytes than on nonclassical monocytes within individuals (Fig. 5A–C). Compared with fasting, a high-fat meal either lowered or did not change CD36 levels on monocytes (subsets; Fig. 5A–C). Treatment with MAT9001 or EPA-EE reduced or tended to reduce fasting and postprandial CD36 levels on classical and intermediate, but not nonclassical, monocytes (Fig. 5A–C). Treatment with MAT9001 or EPA-EE either tended to increase or did not change monocyte (subset) levels of SRA (Supplemental Fig. 2), another scavenger receptor implicated in macrophage foamy cell formation.<sup>41</sup> However, our data indicated that SRA levels were generally low on all 3 monocyte subsets as examined by flow cytometry.

#### Effects on chemokine receptors

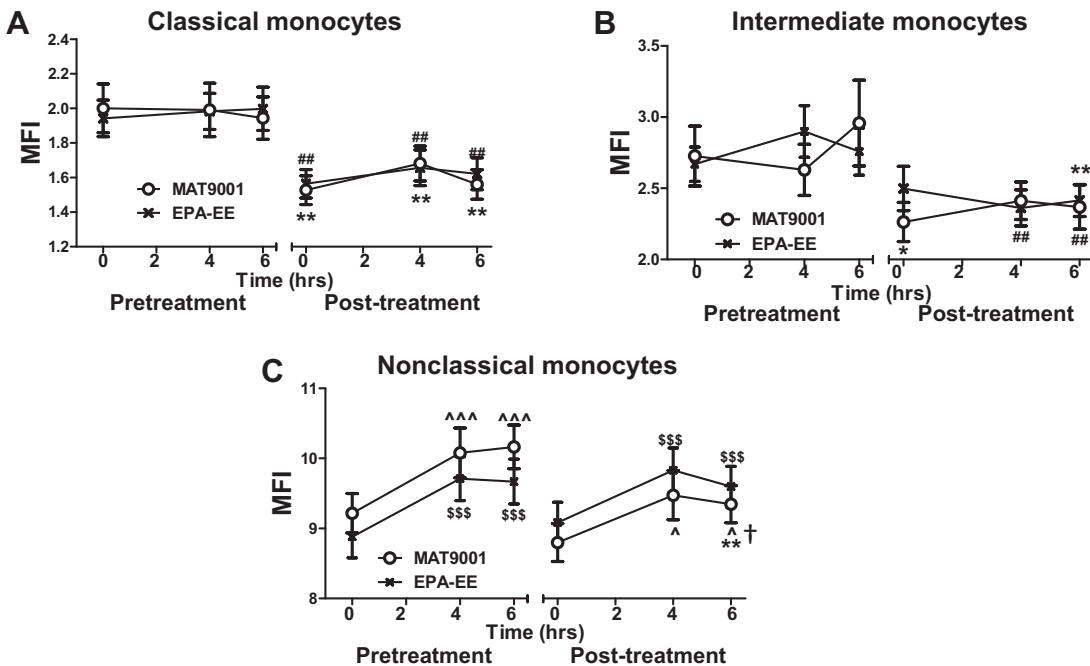
CCR5, a receptor for CCL3, CCL4, and CCL5, was expressed at higher levels on intermediate and nonclassical monocytes than on classical monocytes within individuals (Fig. 6A–C). Treatment with MAT9001 or EPA-EE lowered or tended to lower postprandial levels of CCR5 on monocytes, particularly on intermediate and nonclassical monocyte subsets (Fig. 6A–C), with no significant differences between



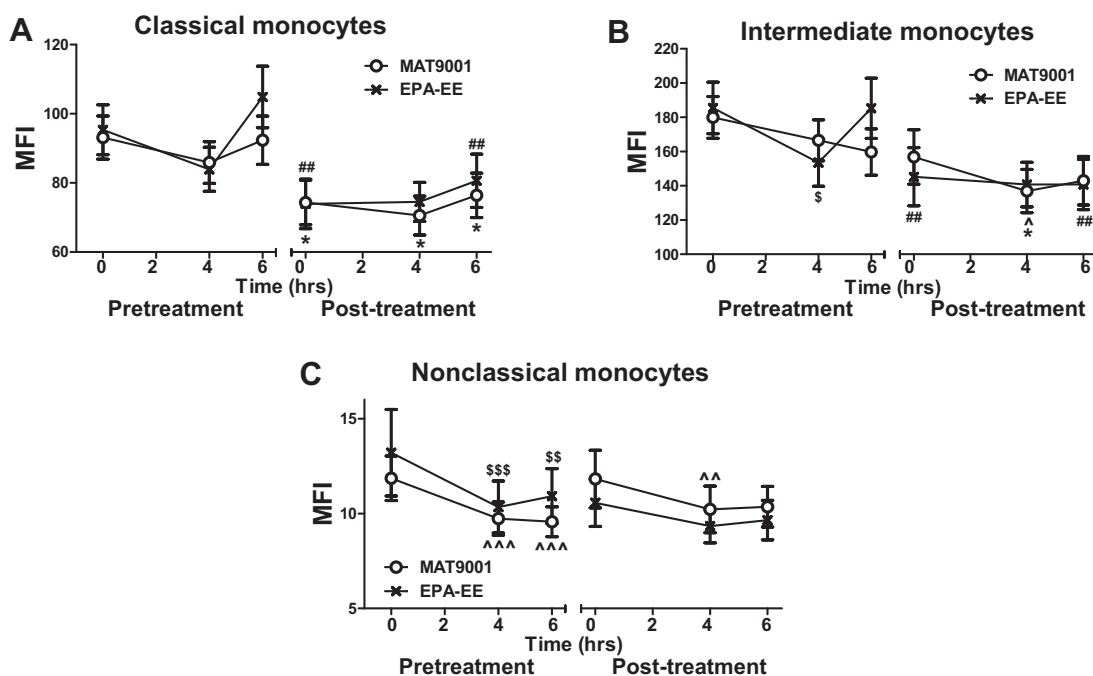
**Figure 3** Effects of MAT9001 or EPA-EE treatment on monocyte Nile red staining of lipids. Nile red staining of lipids was performed in monocytes (subsets) by flow cytometry in the fasting (0 hour) and postprandial (4 and 6 hours after the high-fat meal) states and presented as mean fluorescence intensity (MFI) of Nile red staining on each monocyte subset: classical (A), intermediate (B), and nonclassical (C). \* $P < .05$ , \*\* $P < .01$  for posttreatment vs pretreatment of MAT9001;  $P < .05$  for 4 or 6 hours vs 0 hour of MAT9001 treatment.

MAT9001 and EPA-EE. CCR2, a receptor for CCL2, was expressed at higher levels on classical and intermediate monocytes than on nonclassical monocytes within individuals. Treatment with MAT9001 or EPA-EE increased or tended to

increase fasting and postprandial CCR2 levels on classical monocytes (Supplemental Fig. 3A). While EPA-EE decreased fasting CCR2 levels on nonclassical monocytes, MAT9001 or EPA-EE did not reduce CCR2 levels on intermediate



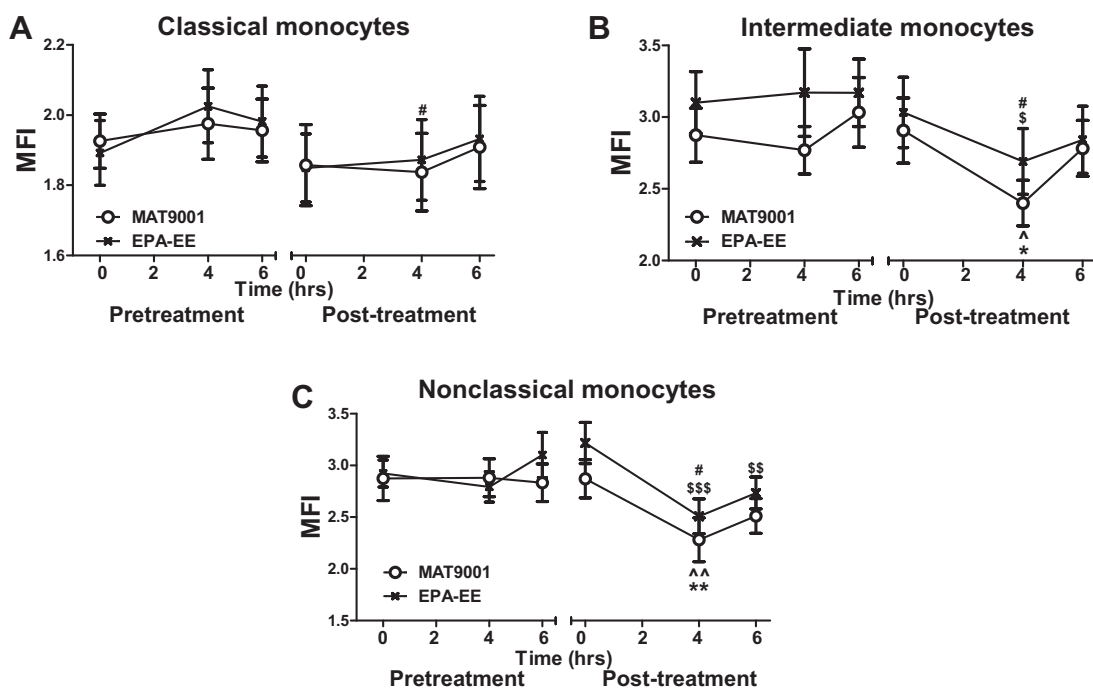
**Figure 4** Effects of MAT9001 or EPA-EE treatment on monocyte CD11c. CD11c levels were examined on monocytes (subsets) by flow cytometry in the fasting (0 hour) and postprandial (4 and 6 hours after the high-fat meal) states and presented as MFI levels on each monocyte subset: classical (A), intermediate (B), and nonclassical (C). \* $P < .05$ , \*\* $P < .01$  for posttreatment vs pretreatment of MAT9001; ### $P < .01$  for posttreatment vs pretreatment of EPA-EE;  $P < .05$ , ^^ $P < .001$  for 4 or 6 hours vs 0 hour of MAT9001 treatment; \$\$\$ $P < .001$  for 4 or 6 hours vs 0 hour of EPA-EE treatment; † $P < .05$  for MAT9001 vs EPA-EE for posttreatment vs pretreatment.



**Figure 5** Effects of MAT9001 or EPA-EE treatment on monocyte CD36. CD36 MFI levels were examined on monocytes (subsets: classical [A], intermediate [B], and nonclassical [C]) by flow cytometry in the fasting (0 hour) and postprandial (4 and 6 hours after the high-fat meal) states. \* $P < .05$  for posttreatment vs pretreatment of MAT9001; ### $P < .01$  for posttreatment vs pretreatment of EPA-EE;  $P < .05$ ,  $^{**}P < .01$ ,  $^{***}P < .001$  for 4 or 6 hours vs 0 hour of MAT9001 treatment;  $^{*}P < .05$ ,  $^{**}P < .01$ ,  $^{***}P < .001$  for 4 or 6 hours vs 0 hour of EPA-EE treatment.

monocytes (Supplemental Fig. 3B and C). CX3CR1, a receptor for CX3CL1, was expressed at higher levels on nonclassical monocytes than on classical and intermediate

monocytes in the fasting state within individuals and increased or tended to increase after a high-fat meal, particularly on nonclassical monocytes (Supplemental Fig. 4). However,



**Figure 6** Effects of MAT9001 or EPA-EE treatment on monocyte CCR5. CCR5 MFI levels were examined on monocytes (subsets: classical [A], intermediate [B], and nonclassical [C]) by flow cytometry in the fasting (0 hour) and postprandial (4 and 6 hours after the high-fat meal) states. \* $P < .05$ , \*\* $P < .01$  for posttreatment vs pretreatment of MAT9001; # $P < .05$  for posttreatment vs pretreatment of EPA-EE;  $P < .05$ ,  $^{*}P < .01$  for 4 or 6 hours vs 0 hour of MAT9001 treatment;  $^{*}P < .05$ ,  $^{**}P < .01$ ,  $^{***}P < .001$  for 4 or 6 hours vs 0 hour of EPA-EE treatment.

treatment with MAT9001 or EPA-EE did not significantly alter fasting or postprandial levels of CX3CR1 on any monocyte subsets (Supplemental Fig. 4).

## Discussion

Although MAT9001 and EPA-EE both reduced fasting TG levels in subjects with hypertriglyceridemia,<sup>34</sup> this sub-study showed that MAT9001, but not EPA-EE, significantly lowered postprandial TG levels and TG AUC. MAT9001 also reduced fasting Nile red levels for lipids in classical and intermediate monocytes. MAT9001 and EPA-EE each decreased the counts and the relative ratio of intermediate monocytes in total monocytes, fasting and postprandial levels of CD11c and CD36 on classical and intermediate monocytes, and postprandial levels of CCR5 on intermediate and nonclassical monocytes, whereas only MAT9001 significantly reduced postprandial levels of CD11c on nonclassical monocytes.

Increased proportions and counts of CD16<sup>+</sup> monocytes, particularly intermediate monocytes, and elevated monocyte (subset) levels of lipids and other markers such as CD11c, CD36, and CCR5 have been associated with hyperlipidemia and may be a link to the development of atherosclerosis.<sup>10,12,14–20,24</sup> Therefore, reductions in the proportions and counts of intermediate monocytes and in the monocyte (subset) markers as described in subjects with hypertriglyceridemia by treatment with n-3 PUFAs may help reduce risk for ASCVD.

In mouse models, initial studies indicated a preferential increase in Ly-6C<sup>high</sup> classical monocytes in apoE<sup>-/-</sup> mice on Western diet.<sup>42,43</sup> More recent studies including ours also showed a significant increase of Ly-6C<sup>low</sup> monocytes or a similar ratio of Ly-6C<sup>low</sup> and Ly-6C<sup>high</sup> monocytes in mice with hypercholesterolemia and/or obesity.<sup>18,19,26,44,45</sup> Importantly, Ly-6C<sup>low</sup>, but few Ly-6C<sup>high</sup>, monocytes express CD11c and become foamy (and positive for Nile red and oil red O staining) in the circulation of apoE<sup>-/-</sup> or LDLR<sup>-/-</sup> mice with hypercholesterolemia.<sup>18,19</sup> CD11c<sup>+</sup> foamy monocytes infiltrate into arterial walls and contribute to atherosclerosis.<sup>19</sup> A study showed that dietary supplementation with n-3 PUFAs (fish oil or echium oil) reduced atherosclerosis in LDLR<sup>-/-</sup>, but not apoE<sup>-/-</sup>, mice, although fish oil decreased the proportion of Ly-6C<sup>high</sup> monocytes in the circulation of apoE<sup>-/-</sup>, but not LDLR<sup>-/-</sup>, mice. Interestingly, the reduced atherosclerosis in LDLR<sup>-/-</sup> mice with n-3 PUFAs was associated with decreased Ly-6C<sup>high</sup> monocytes in spleens and reduced Ly-6C<sup>low</sup> monocyte infiltration into atherosclerotic lesions.<sup>46</sup>

Our previous study showed that in the fasting state, Nile red staining of monocytes for lipids was elevated in human subjects with hypertriglyceridemia and metabolic syndrome compared with control subjects with normal TG levels and was correlated with TG levels. However, compared with subjects with hypertriglyceridemia, control subjects with normal TG levels showed greater postprandial increases in Nile red staining for lipids in monocytes,<sup>20</sup>

supporting a hypothesis that high lipid content in monocytes in the fasting state in subjects with hypertriglyceridemia may prevent further increases in postprandial lipid accumulation within circulating monocytes. This may also explain our current observations that reductions in TGs by MAT9001 in subjects with hypertriglyceridemia were associated with reduced Nile red staining of lipids in monocytes in the fasting state and that a high-fat meal increased Nile red staining of lipids in monocytes (subsets) after (with lower TG levels), but not before (with elevated TG levels), MAT9001 treatment. The nonsignificant effects of EPA-EE on fasting monocyte Nile red levels may be because of smaller reductions in blood levels of lipids, particularly TG, by EPA-EE alone as shown in the parent study.<sup>34</sup> Our current observation also indicated that monocyte accumulation of lipids may be reversible with lipid-lowering therapy (by MAT9001). The same is true for CD11c levels on monocyte subsets. Obesity with metabolic syndrome and hypertriglyceridemia increased CD11c on monocyte subsets,<sup>20</sup> and, in the present study, n-3 PUFA treatment in subjects with hypertriglyceridemia decreased CD11c levels on monocyte subsets.

We previously observed that hypertriglyceridemia in obesity significantly increased fasting CD11c levels on classical and intermediate, but not nonclassical, monocytes and that a high-fat diet increased CD11c levels on nonclassical monocytes in subjects with hypertriglyceridemia, but not in control subjects.<sup>20</sup> These observations pointed to the potential that long-term “chronic” hypertriglyceridemia increases CD11c levels on classical and intermediate monocytes in the fasting state and may “prime” nonclassical monocytes for greater CD11c response to an acute TG increase induced by a high-fat challenge. Consistent with this hypothesis, reductions in fasting TG levels by n-3 PUFAs were associated with decreased CD11c levels on classical and intermediate monocytes in the fasting (and postprandial) state, while the greater reduction in fasting TG by MAT9001 was associated with a smaller response in nonclassical monocyte CD11c to a high-fat meal challenge.

CD36 has been associated with hyperlipidemia and may contribute to atherogenesis by mediating monocyte/macrophage uptake of modified low-density lipoprotein and foam cell formation.<sup>19,41</sup> Reductions in monocyte CD36 levels with n-3 PUFA treatment are expected to decrease monocyte uptake of atherogenic lipoproteins, thereby reducing foam cell formation and protecting against atherogenesis. CCR5 levels increase on monocytes (subsets) with hyperlipidemia and may contribute to atherosclerosis by mediating monocyte recruitment.<sup>20,42</sup> Therefore, reductions of CCR5 levels on monocytes by n-3 PUFA treatment would also be expected to protect against atherogenesis by reducing monocyte recruitment.

In contrast to our current report, Schirmer et al reported that n-3 PUFA treatment did not change monocyte subset proportions and total monocyte levels of CD11b, CD14, and CCR2 in subjects with coronary artery disease.<sup>47</sup> The reasons for the discrepancy are not known, but may include



that the subjects in Schirmer's study had relatively normal baseline TG levels and that the investigators examined markers on total monocytes instead of monocyte subsets.<sup>47</sup> Indeed, as discussed previously, baseline TG levels significantly impact monocyte phenotypes and monocyte response to a high-fat diet challenge. Furthermore, monocyte subsets differ in numerous markers.<sup>10,20</sup> It would be ideal to compare markers on each monocyte subset rather than on total monocytes.

We have focused on the lipid-lowering effect of n-3 PUFAs as the main mediator for improvements in monocyte inflammatory phenotypes in subjects with hypertriglyceridemia. Our finding that EPA-EE reduced several monocyte markers in the postprandial state without a significant impact on postprandial TG levels may be because EPA-EE had already reduced fasting TG levels. Indeed, fasting TG levels influence monocyte response to a high-fat meal.<sup>20,27</sup> However, n-3 PUFAs may also exert anti-inflammatory effects through several additional pathways: (1) n-3 PUFAs reduce the generation of intracellular secondary inflammatory messengers such as diacylglycerol and ceramide, thereby decreasing the downstream cell inflammatory response<sup>48</sup>; (2) incorporation of n-3 PUFAs into cell membrane phospholipids alters lipid rafts, suppressing protein kinase C recruitment into lipid rafts and downregulating protein kinase C-mediated signaling inflammatory cascades, including suppression of NF- $\kappa$ B activation and inhibition of inflammatory gene transcription<sup>48</sup>; (3) n-3 PUFAs bind to and activate peroxisome proliferator-activated receptors, with subsequent anti-inflammatory effects.<sup>35,49</sup> These mechanisms may all contribute to "TG-independent" effects of EPA alone on postprandial monocyte phenotypes in these subjects with hypertriglyceridemia. In addition, besides the greater reductions in TG levels, the DPA content of MAT9001 may also help improve monocyte phenotypes given the potential direct anti-inflammatory effects of DPA.<sup>50</sup> While our present study focused on effects of n-3 PUFAs on circulating monocyte phenotypes, previous studies showed that n-3 PUFAs may promote macrophage polarization from M1-like proinflammatory to M2-like phenotypes,<sup>51-53</sup> which may also provide benefit on the prevention and treatment of ASCVD.

Limitations of this study include descriptive phenotyping of monocytes as part of an exploratory substudy of a clinical trial. However, our work provides more careful characterization of phenotypic changes in monocyte subsets in the fasting and postprandial states than was previously available. The current data, in conjunction with our prior work,<sup>20</sup> highlight the complexity of phenotypic changes in each monocyte subset in patients with hypertriglyceridemia and the effects of interventions with different n-3 PUFA formulations on both fasting and postprandial TG levels as well as on monocyte subset phenotypic changes. These data are hypothesis generating but may be useful in designing future studies to understand better how hypertriglyceridemia alters monocyte subset phenotypes and how therapies that alter TG metabolism improve monocyte subset phenotypes and function, thereby benefiting prevention and treatment of ASCVD.

## Conclusions

Treatment with both MAT9001 and EPA-EE in individuals with hypertriglyceridemia was associated with reductions in the relative ratio of intermediate monocytes, in fasting and postprandial levels of CD11c and CD36 on classical and intermediate monocytes and in postprandial levels of CCR5 on intermediate and nonclassical monocytes. MAT9001, but not EPA-EE, significantly reduced fasting Nile red staining for lipids in classical and intermediate monocytes and postprandial CD11c on nonclassical monocytes. These changes in monocyte phenotypes with n-3 PUFA treatment may benefit subjects with hypertriglyceridemia by potentially reducing development and/or progression of ASCVD.

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Authors' contributions: H.W., K.C.M., and G.B. designed the study. X.-Y.D.P. and H.W. acquired data. X.-Y.D.P., Z.L., G.B., M.R.D., K.C.M., and H.W. analyzed and interpreted the data and contributed to drafting, critical revision, and approval of the article.

## Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jacl.2017.08.011>.

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