Orally Administered Eicosapentaenoic Acid Induces Rapid Regression of Atherosclerosis Via Modulating the Phenotype of Dendritic Cells in LDL Receptor-Deficient Mice

Kenji Nakajima, Tomoya Yamashita, Tomoyuki Kita, Masafumi Takeda, Naoto Sasaki, Kazuyuki Kasahara, Masakazu Shinoohara, Yoshiyuki Rikitake, Tatsuro Ishida, Mitsuhiro Yokoyama, Ken-ichi Hirata

Objective—Eicosapentaenoic acid (EPA) has been shown to have beneficial effects on cardiovascular diseases, although the precise mechanism is unknown. We investigated the effect of EPA on the regression of atherosclerosis.

Methods and Results—LDL-receptor–deficient mice were fed a high-cholesterol diet for 8 weeks to build up aortic sinus atherosclerotic lesions and then were fed a normal diet with or without 5% EPA for 4 weeks. Atherosclerotic lesions were histologically assessed, and immunologic assays were performed. EPA treatment significantly regressed atherosclerosis (−22.7%, P<0.05) and decreased the content of macrophages, CD4+ T cells, and dendritic cells (DCs) in atherosclerotic lesions, though only changing the chow never induced the regression. Flow cytometric analysis revealed that EPA increased immature DCs (CD11c+CD80−CD86−), increased the indoleamine 2,3-dioxygenase (IDO) in DCs, and decreased the number of CD4+ T cells. In the presence of the IDO inhibitor, the beneficial effects of EPA on regression were inhibited, suggesting that the effect of EPA was mainly mediated through IDO.

Conclusion—In addition to lowering plasma cholesterol, EPA regressed atherosclerosis probably due to modulation of DC phenotype and reduction in T cell numbers. The present findings might partly explain the beneficial effects of EPA in clinics and support clinical evidence. (Arterioscler Thromb Vasc Biol. 2011;31:1963-1972.)

Key Words: Atherosclerosis ■ Immune system ■ dendritic cells ■ lymphocytes ■ regression

Evidence for atherosclerosis regression in humans has been reported.1,2 Although lowering of plasma lipid levels is a major driving force, the mechanisms that promote lesion regression are not clear. Recent clinical evidence indicated that lowering elevated high-sensitive C-reactive protein levels in plasma with 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) significantly reduced the incidence of major cardiovascular events in normolipidemic healthy persons.3 These data indicate that statins, in addition to their aggressive lipid lowering effect, may also be a hopeful therapeutic strategy for inhibiting cardiovascular events via stabilization or regression of atherosclerotic plaques through their antiinflammatory actions.

See accompanying articles on page 1939 and 1943

Eicosapentaenoic acid (EPA), a long-chain n-3 fatty acid, was reported to have beneficial effects on cardiovascular diseases.4-5 We have also demonstrated that the use of highly purified EPA, in addition to statins, prevented cardiovascular events in Japanese hypercholesterolemic patients.6 On the basis of these findings, we hypothesized that EPA could regress atherosclerosis. Recent studies demonstrated that a metabolite of EPA inhibited the maturation of dendritic cells (DCs), downregulated costimulatory molecules, and induced indoleamine 2,3-dioxygenase (IDO).7,8 IDO, an enzyme involved in tryptophan catabolism, breaks down tryptophan required for T cell proliferation, and its induction is associated with T cell immune suppression.8,9 Here, we investigated the effects of highly purified EPA in an atherosclerosis regression mouse model. The present results indicate that the intervention to inflammatory responses, such as the induction of tolerogenic DCs, could be therapeutic methods for atherosclerotic plaque regression. A better understanding of the underlying mechanisms involved in atherosclerosis regression will allow us to develop novel therapeutic
interventions to enhance plaque regression and reduce cardiovascular events.

Methods

Please see the Online Appendix for a detailed description of Methods, available at http://atvb.ahajournals.org.

Animals and Experimental Design

Low-density lipoprotein receptor knockout (LDLР−/−) mice were kept in a specific pathogen-free animal facility at Kobe University Institute. All animal experiments were conducted according to the Guidelines for Animal Experiments at Kobe University School of Medicine. Six-week-old male LDLР−/− mice received a high-fat and cholesterol diet (21% fat, 1.25% cholesterol, and 0.5% cholate; Oriental Yeast, Tokyo, Japan) for 8 weeks. At 14 weeks of age, their diet was changed to fish powder-free diet with or without 5% ultrapure EPA ethylester (>99% purity; Mochida Pharmaceutical Co, Ltd, Tokyo, Japan) and continued until euthanized. In several experiments, mice were treated with the IDO inhibitor (1-methyl-DL-tryptophan, 1-MT; Sigma, St. Louis, MO) or its solvent. Mice were euthanized at 14, 16, 18, and 22 weeks of age, and atherosclerotic lesions were assessed as previously described.11

Cell Isolation and Flow Cytometry Analyses

Purified CD11с+ DCs and CD4+ T cells were isolated from mesenteric lymph nodes (LN)s and spleens using an AutoMACS separator (Miltenyi Biotec, Inc, Auburn, CA) according to the manufacturer’s instructions and were used for flow cytometry analyses, cell culture experiments, and total RNA extraction.

Assessment of mRNA Expression

Total RNA was extracted from CD11с+ DCs and mouse aortic roots after perfusion with RNAlater (Ambion, Austin, TX) using TRIzol reagent (Invitrogen, Carlsbad, CA). Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was performed as described previously.11

Statistical Analysis

Data were expressed as means±SEM. Mann-Whitney U test was used to detect significant differences between two groups. Kruskal-Wallis test was used to detect significant differences when 3 groups were compared. Statistical values of P<0.05 were considered statistically significant.

Results

EPA Induces Regression of Established Atherosclerotic Lesions

Our original regression mouse model and the design of experiments presented here are outlined in Figure 1A. In brief, 6-week-old LDLР−/− mice were fed a high-cholesterol and high-fat diet for 8 weeks to build up atherosclerotic lesions at the aortic sinus. When mice were 14 weeks old, the diet was changed to normal food with (EPA) or without 5% EPA (control) and continued for 4 weeks. Changing the diet markedly reduced plasma cholesterol levels as shown in Table. Orally administered EPA further decreased plasma cholesterol levels as previously observed.12 For baseline levels, atherosclerosis was assessed when mice were 14 weeks (baseline; 5.50±0.46×105 μm²) and 18 weeks old for each group (Figure 1B and 1C). There was no observable decrease in plaque area in mice who were switched to the normal diet at 18 weeks of age (control; 5.38±0.33×10⁵ μm²) or at 22 weeks of age (Figure 1C). Addition of EPA to the normal diet normalized plasma cholesterol and signifi- canently regressed established atherosclerosis at 18 weeks of age (EPA; 4.25±0.33×10⁵ μm²; −22.7% versus baseline and −20.9% versus control, P<0.05; Figure 1B and 1C), although further EPA treatment for 4 weeks showed no additional effects. As EPA significantly reduced plasma cholesterol level, we examined EPA-treated group with adjusted plasma cholesterol level to control group to rule out the effect of lowering cholesterol on the regression. As shown in Supplemental Figure 1, EPA could induce the regression compared with control group even under the same plasma cholesterol condition.

Qualitative Analyses of Atherosclerotic Plaques Before and After Regression

Immunohistochemical analysis of the aortic root (baseline, control, and EPA) was performed to determine changes in atherosclerotic plaques before and after regression. Macrophage accumulation and CD4+ T cell content was markedly decreased with EPA treatment compared with those in the baseline and control groups (Figure 1D and 1E). In addition, smooth muscle cell content, as determined by α-smooth muscle actin staining and collagen content assessed by Masson Trichrome staining, was increased by EPA compared with those in the baseline and control groups (Figure 1D and 1E).

Next, we performed the assays focusing on DCs in atherosclerotic lesions. CD86 is recognized as an important costimulatory molecule related to maturation of DCs. We examined the number of CD11с+ and CD11с+ CD86+ mature DCs using immunohistochemical studies, and mRNA expression of CD11c and costimulatory molecules with quantitative RT-PCR in atherosclerotic lesions. Lowering cholesterol significantly reduced the number of DCs, and EPA further markedly decreased both the number of DCs and the expression of costimulatory molecules (Figure 2A). The same observation applied to the mRNA expression of CD11c and costimulatory molecules (Figure 2B). Taken together, these results indicate that EPA decreased the number of mature DCs in the plaque at 18 weeks.

To assess the mechanism of regression of the atherosclerotic plaque, we examined mRNA expression of cytokines, chemokines, matrix metalloproteinases (MMPs), and adhesion molecules in atherosclerotic plaques by quantitative RT-PCR (Figure 2C). We found that proinflammatory cytokines and chemokines, such as interferon (IFN)-γ, interleukin (IL)-12p40, monocyte chemoattractant protein (MCP)-1, and tumor necrosis factor (TNF)-α, were significantly reduced in the EPA group compared with those in the baseline or control groups. MMPs, which degrade the extracellular matrix, were also reduced in the EPA group. On the other hand, the levels of the antiinflammatory cytokine transforming growth factor (TGF)-β were increased, but IL-10 levels were decreased in the EPA group. Levels of adhesion molecules such as intracellular adhesion molecule (ICAM)-1 were reduced in the EPA group compared with those in the baseline or control groups, but vascular cell adhesion molecule (VCAM)-1 levels were not affected by EPA treatment.
Figure 1. Eicosapentaenoic acid (EPA) induces rapid regression of established atherosclerosis in male low-density lipoprotein receptor knockout (LDLR−/−) mice. A, Experimental design. B, Representative photomicrographs of aortic sinus atherosclerotic lesions stained by Oil Red O. A black bar represents 200 μm. C, Time course of atherosclerotic lesion size in the aortic sinus of LDLR−/− mice at 14 weeks old (baseline; closed square) and after changing to normal chow with (EPA; open squares) or without EPA (control; gray squares). D, Representative sections of aortic sinus stained with antibodies specific for MOMA-2 for macrophages, CD4+ T cells, and α-smooth muscle actin for smooth muscle cells in male LDLR−/− mice at 14 weeks (baseline) and 18 weeks (control and EPA) of age. The fibrous area was stained by Masson’s trichrome. A white bar represents 200 μm. E, Quantitative analyses of the immunohistochemical and Masson’s trichrome stainings. Data represent means±SEM of n=10 at 14 weeks old, n=5 at 16 weeks old, n=9 at 18 weeks old, and n=8 at 22 weeks old in both groups. *P<0.05 vs control group, and #P<0.05 vs baseline.
Effect of EPA on DCs in Systemic Lymphatic Organs

We performed flow cytometry analyses using CD11c⁺ DCs from spleens and LNs after positive selection with anti-CD11c microbeads in each group and found that CD11c⁺ DCs in the EPA group expressed lower levels of CD80, CD86, CD40, and major histocompatibility complex class II compared with levels in the control groups (Figure 3A, 3B, and 3C). We also confirmed another maturation marker CD83 was reduced in EPA group (data not shown). These results indicate that EPA systemically induces immature DCs characterized by low expression of costimulatory molecules.

We assessed the expression of IDO in CD11c⁺ DCs by flow cytometry analysis. IDO is an enzyme that catabolizes the amino acid tryptophan and plays an important role in T cell proliferation and apoptosis.9 We observed significant increase in IDO expression in CD11c⁺ DCs from EPA-treated mice compared with those from the baseline and control groups (P<0.05; Figure 3D). We next examined whether the DC-expressed IDO from EPA-treated mice was functional. IDO activity assessed by the levels of kynurenine, a metabolite of tryptophan, in the culture supernatant of DCs from spleens indicated higher values in EPA-treated group than that in the baseline or control group (P<0.01; Figure 3E). We also

Table. Effect of EPA on Body Weight and Plasma Lipid Profile

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<th>Body Weight (g)</th>
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<th>HDL Cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
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<td>17.0±4.2</td>
<td>72.0±16.4</td>
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<td>61.3±2.0</td>
<td>64.3±6.1</td>
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<tr>
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<td>160.4±8.7*</td>
<td>36.9±2.0*</td>
<td>73.9±7.1</td>
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<tr>
<td>Control+1-MT</td>
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<td>65.0±6.2</td>
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<tr>
<td>EPA+1-MT</td>
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<td>Control+Solvent</td>
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<td>160.9±11.0#</td>
<td>38.0±2.8#</td>
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Results are expressed as means±SEM. *P<0.05 vs control, $P<0.05$ vs control+1-MT, ##P<0.05 vs control+solvent.

Figure 2. Phenotype of dendritic cells (DCs) in atherosclerotic lesions in the aortic sinus. A, Representative photomicrographs of CD11c⁺ (green), CD86 (red), and CD11c⁺CD86⁺ mature DCs (yellow) in the plaques of the aortic sinus from 14-week-old (baseline) and 18-week-old (control and EPA) mice. A white bar represents 20 μm. B, Quantitative analyses of mRNA expression of CD11c and costimulatory molecules (CD80, CD86, and CD40) related to maturation of DCs were shown. n=6 in baseline, n=9 in control, and n=6 in eicosapentaenoic acid (EPA) group. C, Cytokines and chemokines (interferon [IFN]-γ, interleukin [IL]-10, transforming growth factor (TGF)-β, IL-12p40, monocyte chemoattractant protein [MCP]-1), activated macrophage and DC-releasing materials (tumor necrosis factor [TNF]-α, matrix metalloproteinases [MMP]2, MMP9), and adhesion molecules (intracellular adhesion molecule [ICAM]-1, vascular cell adhesion molecule [VCAM]-1) in atherosclerotic aortas were quantified by quantitative real-time reverse transcription polymerase chain reaction and normalized to glyceraldehyde 3-phosphate dehydrogenase. Total RNA was extracted from aortas of 14-week-old (baseline) and 18-week-old (control and EPA) mice. Fold changes relative to each baseline group are shown. At least n=4 in baseline, n=7 in control, and n=5 in EPA group. *P<0.05 and **P<0.01 vs control, ##P<0.05 and ###P<0.01 vs baseline.
Figure 3. Effect of eicosapentaenoic acid (EPA) on dendritic cells (DCs) in vivo. A, Representative results of CD80, CD86, CD40, and major histocompatibility complex (MHC) class II expression in CD11c⁺ DCs isolated from spleens and lymph nodes (LNs) of 14-week old (baseline) and 18-week old (control and EPA) mice assessed by flow cytometry. In the representative histograms, isotype control stains are given as under black line area. Surface marker expression on CD11c⁺ DCs of baseline (black shaded area), control (gray area), and EPA (under blue line area) is shown. B and C, Quantitative analysis of surface expression for each marker was determined as mean fluorescence intensity (MFI). n=5 in baseline, n=7 in control, and n=7 in EPA group. D, Cells from spleens and LNs of 14-week-old (baseline) and 18-week-old (control and EPA) mice were prepared, stained with fluorescein isothiocyanate-conjugated anti-CD11c and anti-indoleamine 2,3-dioxygenase (IDO) antibody followed by allophycocyanin-labeled secondary antibody, and assessed by flow cytometry. The histograms were gated on CD11c⁺ DCs. Quantitative analysis of expression for IDO was determined as MFI. n=4 in baseline, n=7 in control, and n=7 in EPA group. E, The IDO activity was assessed by kynurenine production in the culture supernatant of CD11c⁺ DCs isolated from spleens of each group, which were incubated in Hanks balanced salt solutions for 8 hours. n=4 per group. F, Total RNA was extracted from CD11c⁺ DCs isolated from spleens of each group. Expressions of interleukin (IL)-6, IL-12p40, IL-10, transforming growth factor (TGF)-β were quantified by quantitative real-time reverse transcription polymerase chain reaction and normalized to glyceraldehyde 3-phosphate dehydrogenase. Fold changes relative to each baseline group are shown. n=6 per group. *P<0.05 and **P<0.01 vs control. #P<0.05 and ##P<0.01 vs baseline.
assessed mRNA levels of DC-associated cytokines in CD11c\(^+\) DCs from spleens and found a significant decrease in mRNA levels of proinflammatory cytokines IL-6 and IL-12p40 compared with control, whereas mRNA levels of antiinflammatory cytokines IL-10 and TGF-\(\beta\) were upregulated in the EPA group (Figure 3F). Therefore, we could say that oral administration of EPA makes the phenotype of DCs immature and tolerogenic in vivo.

**Effect of EPA on T Lymphocytes in Systemic Lymphatic Organs**

Next, we investigated the effect of EPA on T cells. Orally administered EPA decreased the number of CD4\(^+\) T cells in spleens and LNs (Figure 4A). DCs with immunosuppressive properties have less capacity to induce T cell proliferation. We performed in vitro proliferation assays of CD4\(^+\) T cells from Balb/c mice cocultured with and stimulated by CD11c\(^+\) dendritic cells from spleens of 14-week-old (baseline) or 18-week-old (control and EPA) mice. CD4\(^+\) T cell proliferation was suppressed when cocultured with DCs from the EPA group compared with DCs from the baseline or control groups \((P<0.05)\), suggesting that DCs from EPA-treated mice had less proliferation activity of T lymphocytes. To determine whether EPA directly contributes to the suppressive potential of lymphoid cells, we performed an in vitro proliferation assay of CD4\(^+\) T cells from spleens after positive selection with anti-CD4 antibody in each group in response to anti-CD3/anti-CD28 antibodies. As shown in Figure 4C,
we observed no reduction in CD4+ T cell proliferation in the EPA group compared with that in the control group. It is unlikely that EPA directly suppresses T cell proliferation. Tolerogenic DCs have been previously shown to inhibit T cell proliferation through the induction of Tregs.13 Most Tregs are characterized by CD25 and Foxp3 expression and, for some subsets, by increased IL-10 or TGF-β production.14 We observed no increase in CD25 or Foxp3 expression in the CD4+ T cell population in spleens and LNs of EPA-treated mice at 18 weeks (Figure 4D). To assess whether EPA induces the apoptosis in T cells or not, we investigated the annexin V assays. There was no difference in the expression of annexin V in splenocytes and LN cells between the two groups (Figure 4E). Previous studies have shown that DCs generated in the presence of EPA metabolites are poor stimulators of activated T cells but do not induce CD4+ Foxp3+ T cells.7 As such, our data imply that tolerogenic DCs induced by EPA treatment may systemically suppress activated immune reactions without inducing Tregs. Further, to determine the effects of EPA on the activation of T lymphocytes in vivo, we examined the surface expression of the activation marker CD44hi and CD62Llow by flow cytometry analysis. These activation markers on T cells were not changed or slightly increased in EPA-treated mice compared with controls (Figure 4F). Next, to determine whether EPA-treatment affects the cytokine production from T cells, we analyzed IFN-γ, IL-4, IL-10, and IL-17 production or expression by intracellular cytokine staining. We could not detect any differences in the ex vivo cytokine production from T cells of spleens among the three groups (Figure 4G). Taken together, EPA might not directly affect the function of T cells in vivo but inhibited T cell proliferation via DC-dependent manner and resulted in decreasing the number of T cells.

Role of IDO in the Beneficial Action of EPA

To determine whether IDO expressed in DCs is directly involved in regression of established atherosclerotic plaques after EPA treatment, we treated mice with an IDO inhibitor, 1-MT, which blocks the function of IDO.10 Administration of 1-MT significantly increased atherosclerotic lesions (Figure 5A and 5B), macrophage contents, and CD4+ T cell contents (Supplemental Figure IIA and IIB) in the EPA-treated group compared with the solvent-treated EPA group, although 1-MT never affected plasma cholesterol levels (Table). In the presence of 1-MT, there were no significant differences in atherosclerotic lesions between the EPA and control groups. 1-MT treatment did not significantly affect the IDO expression in DCs (Figure 5C). The IDO activity was assessed by the kynurenine production in the culture supernatant of DCs from spleens of each group. 1-MT treatment significantly decreased the kynurenine level in DCs only in EPA group (Figure 5D). We found that the number of CD4+ T cells was significantly increased in spleens and LNs in the presence of 1-MT (Figure 5E). There were no differences in DC-mediated T cell proliferation between 1-MT–treated control and 1-MT–treated EPA groups (Figure 5F), suggesting 1-MT treatment could partly cancel the effect of EPA on DC-mediated T cell proliferation. These results suggest that the beneficial effects of EPA on the regression of atherosclerosis in this model were mediated at least in part by IDO. Moreover, inhibition of T cell proliferation was mainly dependent on the activity of IDO likely expressed in DCs.

Discussion

The major findings of the present study are as follows: (1) EPA administration to mice with normalized plasma cholesterol levels induced regression of atherosclerosis in LDLR−/− mice over a 4-week period, and (2) we demonstrated a possible mechanism associated with the regression of atherosclerosis. Increasing IDO expression in DCs is highly associated with regression, and decreasing the number of CD4+ T lymphocytes is another therapeutic candidate to induce atherosclerosis regression.

Epidemiological and clinical evidence suggests an increased intake of EPA protects against death from coronary artery disease.4,5 We demonstrated that additive EPA treatment with statins reduced the frequency of major coronary events without affecting plasma lipid values.6 To provide a molecular mechanism for the epidemiological evidence, several animal studies have demonstrated the antiatherogenic effects of EPA. Matsumoto et al demonstrated that EPA reduced progression of atherosclerotic lesions in apolipoprotein E-deficient mice through its antiinflammatory effects, including attenuation of some adhesion molecules and chemokines.11 Another article indicated that a high content of dietary long-chain n-3 fatty acids minimizes atherosclerosis and inflammatory responses in LDLR−/− mice.15 On the basis of these findings, we investigated the effect of EPA treatment with normalized plasma cholesterol levels on atherosclerotic plaques in LDLR−/− mice. We demonstrated for the first time that EPA treatment results in a rapid regression of atherosclerosis, supporting the clinical beneficial effects of EPA on secondary prevention of cardiovascular diseases in patients. Transfer of high-fat and cholesterol-fed LDLR−/− mice to a normal diet stopped further progression of atherosclerosis and led to a less inflammatory plaque phenotype, but it was not sufficient to induce the regression of aortic sinus lesions. In contrast, a marked reduction in aortic plaque area was found in EPA-treated mice, suggesting that EPA facilitated the net removal of inflammatory cells and lipids probably due to antiinflammatory actions and increase in reverse cholesterol transfer. In the present study, we demonstrated that the number of infiltrated cells and mRNA expression of several chemokines, cytokines, and adhesion molecules were significantly reduced in atherosclerotic lesions in EPA-treated mice.

An in vitro study by Wang et al demonstrated that EPA inhibited LPS-induced DC maturation, decreased IL-12 and TNF-α levels, and reduced T cell stimulatory capacity.16 IL-12 induces IFN-γ production and is involved in the differentiation of T cells into potent type 1 helper T cells.17 In this study, we found that EPA systemically reduced the maturation of DCs (expression of CD80,
CD86, CD40, and major histocompatibility complex class II) and induced tolerogenic DCs, which was characterized by low expression of costimulatory molecules, less T cell stimulatory capacity, and minimal secretion of IL-12 in vivo. We also demonstrated that EPA markedly decreased the number of mature DCs in atherosclerotic plaques and DC-releasing material of plaques quantified by real-time RT-PCR as compared with the control. Although several investigators showed the role of DCs in the cholesterol metabolism and plaque formation to the extent of control with IDO inhibitor, 1-methyl-DL-tryptophan (1-MT) or its solvent, from 14 weeks of age when the diet was changed to normal chow with or without 5% EPA and continued for 4 weeks. Representative photomicrographs of Oil Red O staining were shown. A black bar represents 200 μm. B, Quantitative analyses of atherosclerotic lesion size in the aortic sinus of LDLR−/− mice at 18 weeks of age. n = 7 per group. C, Cells from spleens and lymph nodes (LNs) of 18-week-old (control and EPA with 1-MT or its solvent) mice were prepared, stained with FITC-conjugated anti-CD11c and anti-IDO antibody followed by APC-labeled secondary antibody, and assessed by flow cytometry. Quantitative analysis of expression for IDO was determined as MFI. n = 6 in control with solvent, n = 5 in control with 1-MT, n = 6 in EPA with solvent, n = 5 in EPA with 1-MT. D, The IDO activity was assessed by kynurenine production in the culture supernatant of CD11c+ dendritic cells (DCs) isolated from spleens of each group, which were incubated in HBSS for 8 hours, n = 4 per group. E, CD4+ lymphocytes from spleens and LNs of 18-week-old (control and EPA with 1-MT or its solvent) mice were prepared, stained with FITC-conjugated anti-CD4, and counted by flow cytometry. F, Proliferation of CD4+ T cells from spleens of Balb/c mice cocultured and stimulated by CD11c+ DCs from spleens of 18-week-old (control and EPA with 1-MT or its solvent) mice assessed by [3H]-thymidine incorporation; n = 3 per group. *P < 0.05 and **P < 0.01 vs corresponding controls (control and EPA with solvent), #P < 0.05 and ##P < 0.01 vs control with solvent.

Vassiliou et al demonstrated that a metabolite of EPA inhibited T cell proliferation through increasing IDO expression in DCs.7 IDO is an enzyme involved in tryptophan catabolism. IDO breaks down tryptophan, which is necessary for T cell growth, resulting in the inhibition of T cell proliferation by inducing cell cycle arrest.8,9 Intriguingly, we detected an increase in IDO expression in DCs from the spleen and LNs of EPA-treated mice in vivo and demonstrated that the IDO enzyme activity, assessed by the production of kynurenine (a metabolite of tryptophan), was also increasing. In particular, IDO expression in DCs was indispensable for atherosclerotic plaque regression because administration of an IDO inhibitor canceled the beneficial effects of EPA and increased inflammatory cell infiltration and plaque formation to the extent of control with IDO inhibitor. We showed that EPA reduces the maturation of

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**Figure 5.** Indoleamine 2,3-dioxygenase (IDO) inhibitor canceled the beneficial effects of eicosapentaenoic acid (EPA) on the regression of atherosclerosis. A, Male low-density lipoprotein receptor knockout (LDLR−/−) mice were treated with an IDO inhibitor, 1-methyl-γ-tryptophan (1-MT) or its solvent, from 14 weeks of age when the diet was changed to normal chow with or without 5% EPA and continued for 4 weeks. Representative photomicrographs of Oil Red O staining were shown. A black bar represents 200 μm. B, Quantitative analyses of atherosclerotic lesion size in the aortic sinus of LDLR−/− mice at 18 weeks of age. n = 7 per group. C, Cells from spleens and lymph nodes (LNs) of 18-week-old (control and EPA with 1-MT or its solvent) mice were prepared, stained with FITC-conjugated anti-CD11c and anti-IDO antibody followed by APC-labeled secondary antibody, and assessed by flow cytometry. Quantitative analysis of expression for IDO was determined as MFI. n = 6 in control with solvent, n = 5 in control with 1-MT, n = 6 in EPA with solvent, n = 5 in EPA with 1-MT. D, The IDO activity was assessed by kynurenine production in the culture supernatant of CD11c+ dendritic cells (DCs) isolated from spleens of each group, which were incubated in HBSS for 8 hours, n = 4 per group. E, CD4+ lymphocytes from spleens and LNs of 18-week-old (control and EPA with 1-MT or its solvent) mice were prepared, stained with FITC-conjugated anti-CD4, and counted by flow cytometry. F, Proliferation of CD4+ T cells from spleens of Balb/c mice cocultured and stimulated by CD11c+ DCs from spleens of 18-week-old (control and EPA with 1-MT or its solvent) mice assessed by [3H]-thymidine incorporation; n = 3 per group. *P < 0.05 and **P < 0.01 vs corresponding controls (control and EPA with solvent), #P < 0.05 and ##P < 0.01 vs control with solvent.
DCs and increases IDO expression in DCs resulting in decreased numbers of CD4+ T lymphocytes in vivo.

Recent work has demonstrated that natural Tregs, which show high expression factor Foxp3, play a protective role in atherogenesis in mice. Several articles demonstrated the relationship between IDO expression in DCs and the generation of Tregs. In this study, we found that CD4+ CD25+ Foxp3+ Tregs were not increased in spleens and LNs of EPA-treated mice, although IDO expression in DCs was increased. EPA was shown to inhibit T cell activation through a reduction in IL-12 secretion, which is critical for T cell proliferation and modification of the membrane lipid profile. It is possible that Tregs are not involved in rapid atherosclerotic plaque regression, at least in this model. Moreover, it is unlikely that the apoptosis is a cause of the reduced number of CD4+ T cells in EPA group. We demonstrated that T cell activation assessed by anti-CD3/anti-CD28 antibodies induced proliferation, surface markers, and cytokine production was not inhibited in EPA group. On the other hand, we also verified that DC-mediated T cell proliferation was significantly inhibited in EPA-treated group compared with that of control group, suggesting the inhibitory effects on the T cell proliferation was dependent on changes in phenotypes of DCs. Taken together, based on our experiments associated with T lymphocytes, EPA might not directly change the function of T cells but reduced the number of T cells possibly through the functional changes in DCs.

Systemic reduction of CD4+ T cells via induction of tolerogenic DCs is proposed to be involved in rapid plaque regression. An IDO-inhibitor administration canceled the tolerogenic DCs is proposed to be involved in rapid plaque generation of Tregs. In this study, we found that CD4+

atherosclerotic lesion with increasing the number of CD4+ T lymphocytes in vivo. Recent work has demonstrated that natural Tregs, which show high expression factor Foxp3, play a protective role in atherogenesis in mice. Several articles demonstrated the relationship between IDO expression in DCs and the generation of Tregs. In this study, we found that CD4+ CD25+ Foxp3+ Tregs were not increased in spleens and LNs of EPA-treated mice, although IDO expression in DCs was increased. EPA was shown to inhibit T cell activation through a reduction in IL-12 secretion, which is critical for T cell proliferation and modification of the membrane lipid profile. It is possible that Tregs are not involved in rapid atherosclerotic plaque regression, at least in this model. Moreover, it is unlikely that the apoptosis is a cause of the reduced number of CD4+ T cells in EPA group. We demonstrated that T cell activation assessed by anti-CD3/anti-CD28 antibodies induced proliferation, surface markers, and cytokine production was not inhibited in EPA group. On the other hand, we also verified that DC-mediated T cell proliferation was significantly inhibited in EPA-treated group compared with that of control group, suggesting the inhibitory effects on the T cell proliferation was dependent on changes in phenotypes of DCs. Taken together, based on our experiments associated with T lymphocytes, EPA might not directly change the function of T cells but reduced the number of T cells possibly through the functional changes in DCs.

Systemic reduction of CD4+ T cells via induction of tolerogenic DCs is proposed to be involved in rapid plaque regression. An IDO-inhibitor administration canceled the tolerogenic DCs is proposed to be involved in rapid plaque generation of Tregs. In this study, we found that CD4+

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Yokoyama and Ken-ichi Hirata

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Supplemental Methods

Administration of 1-MT

To prepare 1-MT for administration in drinking water, 1 g of 1-MT, 97% (Sigma) was added to a 15 ml conical tube with 7.8 ml Methocel/Tween [0.5% Tween 80/0.5% Methylcellulose (v/v in water; both from Sigma)]. The mixture was bead milled overnight by adding 1-2 ml by volume of 3 mm glass beads and mixing by inversion. The next day, the 1-MT concentration was adjusted to 85 mg/ml by adding an additional 4 ml Methocel/Tween and mixing again briefly. For administration in drinking water, 1-MT was prepared at 2 mg/ml in water as described above, supplemented with a small amount of aspartame (1 g/L) to improve acceptance by the mice, and filter sterilized. The solution was delivered in standard autoclaved drinking-water bottles.¹ Male LDLR⁻/⁻ mice of control and EPA groups were fed 1-MT or its solvent from 14 weeks of age when the diet was changed to fish powder free diet with or without 5% EPA and continued for 4 weeks.

Blood Analysis

After overnight fasting, blood was collected by the cardiac puncture under anesthesia using pentobarbital sodium (80 mg/kg intraperitoneal injection). Plasma was obtained through centrifugation and stored at -80°C. Concentrations of plasma total cholesterol, high density lipoprotein, low density lipoprotein and triglyceride were determined enzymatically using an automated chemistry analyzer.
Atherosclerotic Lesions Assessments

Mice were anesthetized and the aorta was perfused with saline. For aortic root lesion analysis, the samples were cut in the ascending aorta, and the proximal samples containing the aortic sinus were embedded in OCT compounds (Tissue-Tek; Sakura Finetek, Tokyo, Japan). Five consecutive sections (10 μm thickness), spanning 550 μm of the aortic sinus, were collected from each mouse and stained with Oil Red O (Sigma, St Louis, MO). For quantitative analysis of atherosclerosis, the total lesion area of 5 separate sections from each mouse was obtained with the use of the Image J (National Institutes of Health) as previously described.² ³ We assessed the aortic sinus mean plaque area in comparison among the groups. Lesion analysis was conducted by a single observer blinded to the group of the mice.

Immunohistochemical Analyses of Atherosclerotic Lesions

Immunohistochemistry was performed on acetone-fixed cryosections (10 μm) of mouse aortic roots using antibodies to identify macrophages (MOMA-2, 1:400, BMA Biomedicals, Augst, Switzerland), and T cells (CD4, 1:100, BD Biosciences, San Jose, CA), followed by detection with biotinylated secondary antibodies and streptavidin-horseradish peroxidase. For natural Tregs, acetone-fixed cryosections of mouse aortic roots were incubated with rat anti-Foxp3 antibody (clone FJK-16s, 1:100, eBioscience, San Diego, CA), followed by Alexa Fluor 588 anti-rat secondary antibody (1:500, Molecular Probes, Eugene, OR) as described.³ Smooth muscle cells were identified by immunostaining with fluorescein isothiocyanate (FITC)-conjugated primary antibody against α-smooth muscle actin (clone 1A4, 1:500; Sigma). For mature DCs, slides were stained by using TSA™- kit (PerkinElmer LAS, Inc, Boston, MA)
according to the manufacturer’s instructions. In brief, endogenous peroxidase activity was quenched 0.5% H₂O₂ for 30 minutes. Sections were blocked with TNB buffer (TSA™- kit) and hamster anti-CD11c antibody (clone N418, 1:200, BioLegend, San Diego, CA) and rat anti-CD86 (clone PO3.1, 1:200, eBioscience, San Diego, CA) of primary antibodies were applied for 1 hour at room temperature. Slides were washed and incubated with HRP-labeled goat-anti-rat IgG (American Qualex, San Clemente, CA) for 30 minutes. CD86 were detected with Cy3-Tyramide. Next, primary HRP was deactivated by treatment with 0.5% H₂O₂ for 30 minutes, and the sections were then incubated with biotinylated anti-hamster IgG (Vector Laboratories, Inc, Burlingame, CA). Slides were washed and incubated with streptavidine-HRP (TSA™- kit). Staining by hamster anti-CD11c was visualized by amplification of the signal with FITC-Tyramide. Nuclei were counterstained with DAPI (Molecular Probes). The appropriate fixation reagent depending on the primary antibodies was used. Negative controls were prepared with substitution with an isotype control antibody. Staining with Masson’s trichrome was used to delineate the fibrous area as previously described.² Sections were observed under an All-in-one Type Fluorescence Microscope (BZ-8000; Keyence, Osaka, Japan) using BZ Analyzer Software (Keyence). Stained sections were digitally captured, and the percentage of the stained area (the stained area per total atherosclerotic lesion area) was calculated. Quantification of CD4⁺ T cells in atherosclerotic lesions was done by counting positively stained cells, which was divided by total plaque area.

Cell Isolation of CD11c⁺ DCs and CD4⁺ T cells Subsets

Purified CD11c⁺ DCs and CD4⁺ T cells were isolated from mesenteric lymph
nodes or spleens. These cells were positively selected with anti-CD11c-microbeads (Miltenyi Biotec, Inc. Auburn, CA) or anti-CD4 (L3T4) microbeads (Miltenyi Biotec) using an AutoMACS separator (Miltenyi Biotec) according to the manufacturer’s instructions. Purified CD11c+ DCs were used for real-time reverse transcriptional polymerase chain reaction (RT-PCR), flow cytometry analyses and cell proliferation assays. Purified CD4+ T cells were used for cell proliferation assays.

**Flow Cytometry Analyses**

For fluorescent-activated cell sorter (FACS) analyses of lymphoid organs, lymph nodes (LNs) cells and splenocytes were isolated at 14 weeks, 16 weeks, 18 weeks of age. Cells were stained in PBS containing 2% FCS. FACS analysis was performed by FACSCalibur using CellQuest Pro software (BD Bioscience). The antibodies used were as follows; anti-CD16/CD32 (clone 2.4G2; BD Bioscience), FITC-conjugated anti-CD4 (clone H129.19; BD Bioscience), phycoerythrin (PE)-conjugated anti-CD25 (clone PC61; BD Bioscience), PE-anti-CD44 (clone IM7; BD Bioscience), PE-anti-CD62L (clone MEL-14; BD Bioscience), allophycocyanin (APC)-conjugated Foxp3 (clone FJK-16s; eBioscience), APC-conjugated CD40 (clone 1C10; eBioscience), APC-conjugated CD80 (clone 16-10A1; eBioscience), PE-conjugated CD86 (clone PO3.1; eBioscience), FITC-conjugated MHC class II (clone M5/114.15.2; Miltenyi Biotec), and isotype matched control antibodies. For CD40, CD80, CD86 and MHC class II staining, CD11c+ DCs were positively selected with anti-CD11c microbeads described above and were stained. Intracellular staining of Foxp3 was performed using the Foxp3 staining buffer set (eBioscience) and APC-conjugated Foxp3 antibody described above according to the manufacturer’s instructions. All staining procedures
were performed after blocking Fc receptor with anti-CD16/CD32 antibody. Surface stainings were performed according to standard procedures at a density of 1x10^6 cells per 100 μl, and volumes were scaled up accordingly. For analysis of IDO in CD11c^+ DCs, cells were stained with PE-conjugated CD11c (clone N418; Miltenyi Biotec), permeabilized, and were stained further with rat anti-IDO antibody (clone mIDO-48; BioLegend), followed by FITC-conjugated donkey anti-rat immunoglobulin.

**Intracellular Cytokine Staining**

Splenocytes were stimulated with 20 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 1 mmol/L ionomycin (Sigma) for 5 hours in the presence of a Golgi transport inhibitor (BD Bioscience). After staining with FITC-anti-CD4, intracellular staining of cytokines was performed using intracellular cytokine staining kit (BD Bioscience) and PE-anti-IL-4, PE-anti-IL-10, Alexa Fluor 647-anti-IL-17, and PE-anti-IFN-γ (all from BD Bioscience) according to the manufacturer’s instructions.

**Cell Proliferation and Apoptosis Assays**

In all cell culture experiments, we used RPMI 1640 medium (Sigma) supplemented with 10% FCS, 10 mmol/L Hepes, 50 μmol/L 2β-mercaptoethanol and antibiotics. For analysis of in vitro suppressive function of CD11c^+ DCs, CD11c^+ cells from spleens of baseline, EPA-treated and control mice were cultured with 1x10^5 of CD4^+ T cells from spleens of Balb/c mice at various ratio (total volume, 200 μl/well). These cells were cultured at various ratios in flat-bottomed 96-well plates at 37 °C with 5 % CO₂ for 72 hours. In these experiments, CD11c^+ DC were irradiation with 18.5 Gy before co-culture. CD4^+ T cells after positive selection with anti-CD4 (L3T4)
microbeads described above in each group were cultured at 5x10^5 cells/well (total volume, 200 μL/well) in flat-bottomed 96-well plates and stimulated with anti-CD3/anti-CD28-coated beads. (0.5 per cell; Dynabeads Mouse T-Activator CD3/CD28; Invitrogen Dynal AS, Oslo, Norway) at 37 °C with 5 % CO_2 for 72 hours. The cells were pulsed with 1 μCi of [³H]-thymidine (GE Healthcare, Buckinghamshire, UK) for the last 16 hours, and thymidine incorporation was assessed with a LS 6500 liquid scintillation counter (Beckman Coulter, Inc, Brea, CA). To assess whether EPA induces the apoptosis in T cells, cells from spleens and LNs of 14-week old (Baseline) or 18-week old (Control and EPA) mice were immediately prepared, stained with Annexin V and propidium iodide using Annexin V-Fluorescein Staining kit (Wako, Osaka, Japan). Stained cells were analyzed by FACS.

**Measurement of IDO enzymatic activity**

The IDO enzyme assay was performed as previously reported. In brief, CD11c^+ cells from spleens were washed, resuspended in sterile HBSS (Sigma) containing 500 μmol/L tryptophan (Sigma), and incubated for 8 hours. The supernatants were then harvested and assayed for kynurenine. For the assay, 30 μl of 30% trichloroacetic acid was added to 60 μl of culture supernatant and the mixture was vortexed and centrifuged at 10,000 × g (12,000 rpm) for 5 minutes. Then, the supernatant was added into an equal volume of Ehrlich reagent (5 μl of glacial acetic acid and p-dimethylaminobenzaldehyde). The OD was measured at 492 nm using a microplate reader. Purified L-kynurenine (0–500 μmol/L; Sigma) was used as a standard.

**RT-PCR Analysis**
At 18 weeks old, mice were anesthetized and the aortic roots were excised as described above. Total RNA was extracted from CD11c+ DCs, or from the aortas after perfusion with RNA later (Ambion, Austin, TX) using TRizol reagent (Invitrogen, Carlsbad, CA). Quantitative PCR was performed using One Step SYBR PrimeScript RT-PCR Kit (Takara, Shiga, Japan) and an ABI PRISM 7500 Sequence Detection system (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol as described previously. 2 The following primers were used to amplify CD11c, CD80, CD86, CD40, MMP2, MMP9, TNF-α, IL-6, IL-12p40, IL-10, TGF-β, and GAPDH:

**CD11c**, 5'-AGA CGT GCC AGT CAG CAT CAA C-3' and 5'-CTA TTC CGA TAG CAT TGG GTG AGT G-3'; **CD80**, 5'-AGT TTC CAT GTC CAA GGC TCA TTC-3' and 5'-TTG TAA CGG CA A GGC AGC AAT A-3'; **CD86**, 5'-TGG CAT ATG ACC GTT GTG TGT G-3' and 5'-ACG TTT GAG CAG ATG GAA ACT CTT G-3'; **CD40**, 5'-AAA GGT GGT CAA GAA ACC AAA GT-3' and 5'-GCA CTG GAG CAG CGG TGT TA-3'; **MMP2**, 5'-GAT AAC CTG GAT GCC GTC GTG-3' and 5'-TTG TAA CGG CA A GGC AGC AAT A-3'; **MMP9**, 5'-GCC CTG GAA CTC ACA CGA CA-3' and 5'-TTG TAA CGG CA A GGC AGC AAT A-3'; **TNF-α**, 5'-GAT AAC CTG GAT GCC GTC GTG-3' and 5'-TTG TAA CGG CA A GGC AGC AAT A-3'; **IL-6**, 5'-CCA CTT CAC AAG TCG GAG GCT TA-3' and 5'-GCA CTG GAG CAG CGA CA-3' and 5'-TTG TAA CGG CA A GGC AGC AAT A-3'; **IL12p40**, 5'-GCT CGC AGC AAA GCA AGG TAA-3' and 5'-CCA TGA GTG GAG ACA CCA GAA TTA-3'; **IL-10**, 5'-GAC CAG CTG GAC AAC ATA CTG CTA A-3' and 5'-GAT AAG GCT TGG CAA CCC AAG TAA-3'; **TGF-β**, 5'-CCA TGA GTG GAG ACA CCA GAA TTA-3'; **IFN-γ**, 5'-CGG CAC AGT CAT TGA AAG CCT A-3' and 5'-GAT AAG GCT TGG CAA CCC AAG TAA-3'; **GAPDH**, 5'-TGT GTC CGT GGA TCT GAA-3' and
5’-TTG CTG TTG AAG TCG CAG GAG-3’. Amplification reactions were performed in duplicate and fluorescence curves were analyzed with included software. GAPDH was used as an endogenous control reference.

References


Supplemental Figure II. Male LDLR⁻/⁻ mice were treated with an IDO inhibitor, 1-MT, from 14 weeks of age when the diet was changed to normal chow with or without 5% EPA and continued for 4 weeks. Quantitative analyses of (A) CD4⁺ T cells (B) MOMA-2 area (macrophage content) in the aortic sinus of LDLR⁻/⁻ mice at 18 weeks of age. n=7 per group. (C) Quantitative analyses of mRNA expression of CD11c in atherosclerotic aortas. n=5 per group. **P<0.01 versus EPA without IDO inhibitor, #P<0.05 versus control with solvent.
Supplemental Figure I. A, Experimental design for assessing the effect of EPA on the regression of atherosclerosis under a plasma cholesterol adjusted condition. B, Atherosclerotic lesion size in the aortic sinus of LDLR−/− mice at 4 weeks after changing to normal chow without EPA, or with 0.75% cholesterol, EPA, and EPA + 0.75% cholesterol. Horizontal bars represent means, n=6 per group at 18 weeks old and 14 weeks old (baseline). C, Body weight and plasma lipid profile. *P<0.05 and **P<0.01 versus control, #P<0.05 and ##P<0.01 versus baseline.