SUPPLEMENTARY DATA

The primers in real-time quantitative PCR using human monocytes

The primers used were as follows: TNFα (forward primer: 5'-AAG AGT TCC CCA GGG ACC TCT-3', reverse primer: 5'-CCT GGG AGT AGA TGA GGT ACA-3'), IL-6 (forward primer: 5'-GGA CGG CTT TTA CTT AAA CGC CAA GG-3', reverse primer: 5'-ATC TTC CCT AGT TAC CCA GGT TCA GC-3'), IL-10 (forward primer: 5'-AAC AAG AGC AAG GCC GTG G-3', reverse primer: 5'-GAA GAT GTC AAA CTC ACT CAT GGC-3') (1, 2).

Cell culture

The preparation and characterization of eicosapentaenoic acid (EPA) used *in vitro* were as reported elsewhere (3, 4). Fetal bovine serum (FBS) and penicillin-streptomycin (P/S) were obtained from GIBCO (Great Island, NY, USA). RPMI1640 and phosphate- buffered saline were purchased from Nacalai Tesque (Kyoto, Japan) and Cosmo Bio (Tokyo, Japan), respectively. PPARα antagonist, MK886, and PPARγ antagonists, GW9662 and T0070907, were purchased from Cayman Chemical (Ann Arbor, MI, USA). The human THP-1 cells used were purchased from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium supplemented with 10% FBS and 1% P/S at 37°C in a 5% CO_2 humidified atmosphere.

Human monocytic THP-1 cells with a density of 1×10^6 cells/well in 6-well plates were incubated with 10 or 50 μM EPA for 24 h, stimulated with 20 ng/ml lipopolysaccharide (LPS) for 6 h, and then harvested. In experiments using PPAR antagonists, THP-1 monocytes were incubated in RPMI1640 containing 0.5% FBS, 1% P/S, and 5 mg/ml BSA for 24 h and then preincubated with 10 μM PPAR antagonists (MK886, GW9662, or T0070907) 1 h before EPA treatment. Thereafter, 50 μM EPA was added to cells and incubated for 24 h, and then cells were stimulated with LPS for 6 h and harvested. Total RNA was extracted from the cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The expression of IL-10 was analyzed with a real-time quantitative PCR method using the above-mentioned primers (2). The IL-10 levels in the culture supernatant were determined employing the above-mentioned ELISA kit (R&D Systems, Minneapolis, MN, USA) (5).

PPARα and PPARγ silencing by small interfering RNA

Human PPARα– and PPARγ–specific small interfering RNA (siRNA) and scrambled control siRNA oligonucleotides (FlexiTube siRNA Premix Ctrl_Control_1, Hs_PPARA_5, Hs_PPARA_6, Hs_PPARG_1, Hs_PPARG_2) were purchased from QIAGEN (Valencia, CA, USA). In this analysis, THP-1 cells were differentiated into adherent, well-spread macrophages by the addition of 100 nM phorbol myristate acetate. THP-1 cells were re-plated in 12-well plates at a density of 2×10^5 cells/well in RPMI1640 containing 10% FBS and 1% P/S. The medium was changed to OPTI-MEM I (GIBCO, Great Island, NY, USA), and each siRNA premix was added at 25 nM. The cells were incubated for 48 h, the medium was changed again to RPMI1640 containing 0.5% FBS, 1% P/S, and 5 mg/ml BSA, and then 50 μM EPA was added and incubated for 24 h. The cells were stimulated with LPS for 6 h, and then harvested.

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Transient transfection and luciferase assay

The luciferase reporter assay was performed as previously described (6), using the luciferase reporter constructs for the human IL-10 promoter (7). Fragments of the human IL-10 promoter were cloned from THP-1 genomic DNA by PCR using the following primers: -384F 5'-GGG GTA CCG AAT GAG AAC CCA CAG CTG-3', -421F 5'- GGG GTA CCG GCA ATT TGT CCA CGT CAC-3' and 120R 5'-CCG CTC GAG GGC AGG TTG CCT GGG AAG-3', yielding two fragments spanning from -384 to +120 and -421 to +120 of the IL-10 promoter sequence. Fragments were subcloned into the pGL3-Basic (Promega) vector to produce pGL3-P384 and pGL3-P421, and the sequences were checked. pGL3-P421, not pGL3-P384, contained the (-406/-390) IL-10-peroxisome proliferator response element (PPRE).

THP-1 monocytes were transiently transfected using FuGENE6 (Promega, Madison, WI, USA) with either pGL3-P384 or pGL3-P421 and a control plasmid pRL-TK (Promega). After 24 h, cells were treated with 50 μM EPA for 24 h, and then stimulated by LPS for 6 h. Luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega) with a luminometer, and the results were normalized against the *Renilla* luciferase control. Student's *t*-test was performed to determine significant values for treatments compared with the vehicle controls.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as described by Suganami T et al (8). using an anti-PPARγ antibody (sc-7196, Santa Cruz Biotechnologies). THP-1 cells were treated with 50 μM EPA for 24 h, and stimulated by LPS (20 ng/ml) for 6 h. Protein-DNA complexes were cross-linked in 1% formaldehyde for 10 min at room temperature. Cross-linking was stopped by the addition of 1 M glycine for 10 min at 4°C. Cells were lysed and sonicated to shear DNA into 0.1-1.0-kb fragments and cellular debris was removed by centrifugation. Chromatin aliquots were pre-cleared with 80 μl of 50% protein A sepharose suspension. The immune-complexes were precipitated with either an antibody against PPARγ or normal rabbit IgG (sc-2027, Santa Cruz Biotechnologies) overnight at 4°C with rotation. Before immunoprecipitation, "input" samples were removed from the lysates. The immunocomplexes were mixed with protein A sepharose suspension followed by incubation for 1 h at 4°C with rotation. Beads were collected by brief centrifugation and the immunocomplexes were eluted by elution buffer (1% SDS, 0.1 M $NaHCO_3$, 10 mM DTT). Precipitated complexes were reverse cross-linked with 0.2 mM NaCl overnight at 65°C. DNA fragments were purified with the QIAquick Spin kit (Qiagen, Hilden, Germany), and the purified DNA was used for quantitative real-time PCR employing the following primers, converting the -462 to -323 region of the human IL-10 promoter containing the (-406/-390) IL-10-PPRE: sense primer 5'-GGG GGA CCC AAT TAT TTC TC-3' and anti-sense primer 5'-TGA TTT CCT GGG GAG AAC AG-3'; or part of the GAPDH gene: sense primer 5'- TGC ACC ACC AAC TGC TTA GC-3' and anti-sense primer 5'- GGA TGC AGG GAT GAT GTT CTG-3' as a negative control. ChIP experiments were run in triplicate.

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Supplementary Table 1. Baseline characteristics of obese patients with or without dyslipidemia

	Obese patients without dyslipidemia			Obese patients with dyslipidemia			P-value*
n (Male/Female)	11/15			53/37			
Age (years)	50.1	±	10.3	51.2	±	12.8	0.688
BMI (kg/m^2)	31.2	±	5.6	30.9	±	4.8	0.781
Systolic blood pressure (mmHg)	147	±	19	141	±	19	0.149
Diastolic blood pressure (mmHg)	87.6	±	12.8	85.5	±	11.1	0.402
EPA (μg/ml) [a]	66 [38 - 113]			53 [35 - 97]			0.629
EPA/Arachidonic acid [a]	0.5 [0.3 - 0.8]			0.3 [0.2 - 0.6]			0.188
Fasting plasma glucose (mmol/l) [a]	6.9 [5.4 - 8.3]			5.9 [5.1 - 8.0]			0.121
HbA1c (%)[a]	7.0 [5.7 - 8.0]			6.2 [5.6 - 7.4]			0.318
IRI (pmol/l) [a]	62 [39 - 106]			73 [49 - 127]			0.117
Triglycerides (mmol/l) [a]	1.2 [0.9 - 1.3]			2.2 [1.8 - 2.9]			<0.001
HDL-C (mmol/l)	1.6	±	0.2	1.4	±	0.3	<0.001
LDL-C (mmol/l)	3.1	±	0.7	3.4	±	0.7	0.060
Adiponectin (μg/ml) [a]	8.2 [5.6 - 12.1]			6.0 [4.2 - 7.7]			0.010
CRP (μg/ml) [a]	0.96 [0.42 - 1.85]			0.73 [0.46 - 2.06]			0.870
IL-10 (pg/ml)	5.62	±	1.59	4.82	±	1.27	0.047
PWV (cm/sec)	1375	±	224	1416	±	311	0.555
Expression in monocytes							
TNFα(arbitrary units) [a]	3.1 [2.5 - 3.7]			2.7 [1.6 - 4.1]			0.349
IL-6 (arbitrary units) [a]	7.3 [5.2 - 11.0]			8.0 [5.8 - 10.1]			0.634
IL-10 (arbitrary units) [a]	6.6 [3.8 - 10.2]			4.4 [2.9 - 7.5]			0.030
CD163/CD14 (%)	59.0	±	12.1	55.3	±	13.1	0.169
Proportion of							
Diabetes (%)	57.7			44.4			0.233
Hypertension (%)	61.5			51.7			0.658

BMI, body mass index; EPA, eicosapentaenoic acid; IRI, immunoreactive insulin; HDL-C, high-density-lipoprotein cholesterol; LDL-C, low-density-lipoprotein cholesterol; CRP, high-sensitive C-reactive protein; PWV, pulse wave velocity. Data are shown as the mean ± S.D. or median and interquartile range, as appropriate. *Student's *t*-test, the non-parametric Wilcoxon test and Chi-square test were used to compare mean, median and proportion, respectively, in obese patients without dyslipidemia vs. obese patients with dyslipidemia groups. [a] Data were non-normally distributed and analyzed by non-parametric Wilcoxon test.

SUPPLEMENTARY DATA

Supplementary Table 2. Baseline correlations related to the expression of TNFα,IL-6, IL-10, and CD163/CD14 in peripheral blood monocytes and PWV

	Expression in monocytes				PWV
	TNFα[a]	IL-6 [a]	IL-10 [a]	CD163/CD14	
	r	*r*	*r*	*r*	*r*
Age	0.30**	-0.07	-0.09	0.05	0.57**
BMI	0.01	0.08	-0.05	-0.22*	-0.19*
Systolic blood pressure	-0.01	-0.12	-0.02	-0.17	0.42**
EPA [a]	0.23*	-0.11	0.03	0.04	0.09
HbA1c [a]	0.02	0.03	-0.21*	-0.24**	-0.02
Triglycerides [a]	0.04	0.02	-0.16	-0.18	0.04
HDL-C	0.12	-0.06	-0.01	0.02	0.13
Adiponectin [a]	0.28**	-0.08	0.02	0.13	-0.10
IL-10	-0.04	0.09	0.07	0.03	0.17
PWV	0.08	-0.02	-0.19*	-0.26**	-
Expression in monocytes					
TNFα [a]	-	0.17	-0.10	-0.18*	0.08
IL-6 [a]	0.17	-	-0.04	-0.09	-0.02
IL-10 [a]	-0.10	-0.04	-	0.11	-0.19*
CD163/CD14	-0.18*	-0.09	0.11	-	-0.26**

In 116 obese patients, correlations related to changes in monocyte IL-10 and PWV at the baseline were examined. The abbreviations used in this table are the same as those in Table 1. *r*, correlation coefficient. * $P < 0.05$, ** $P < 0.01$. Data are Pearson's correlation coefficient of the expression of cytokines in monocytes and PWV with all variables at the baseline. [a] Logarithmic transformation was performed since the variable had a skewed distribution. $n = 116$ obese patients.

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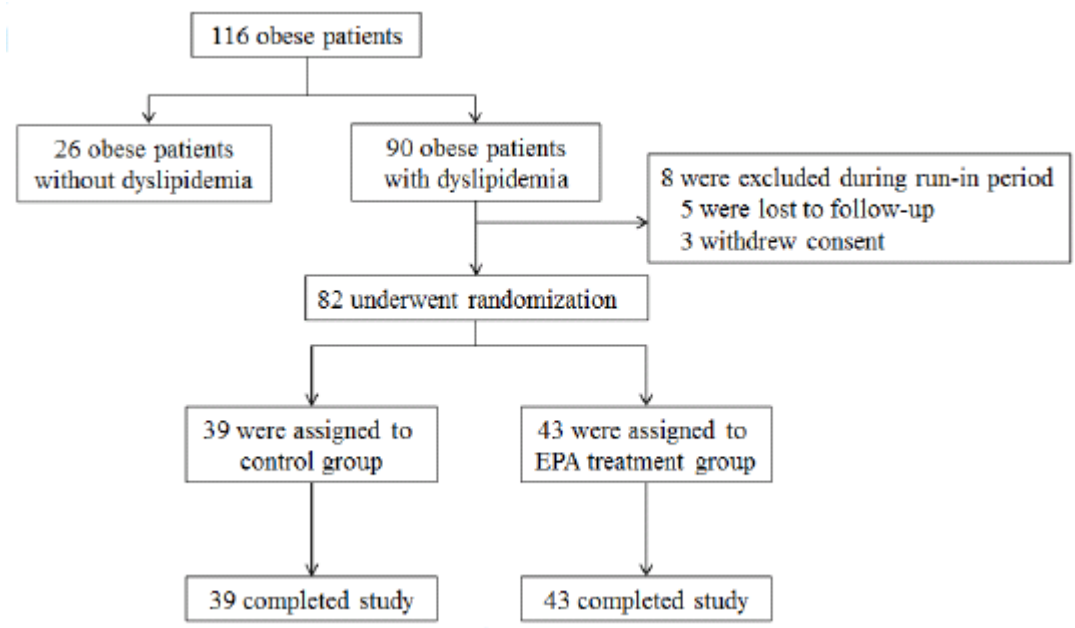
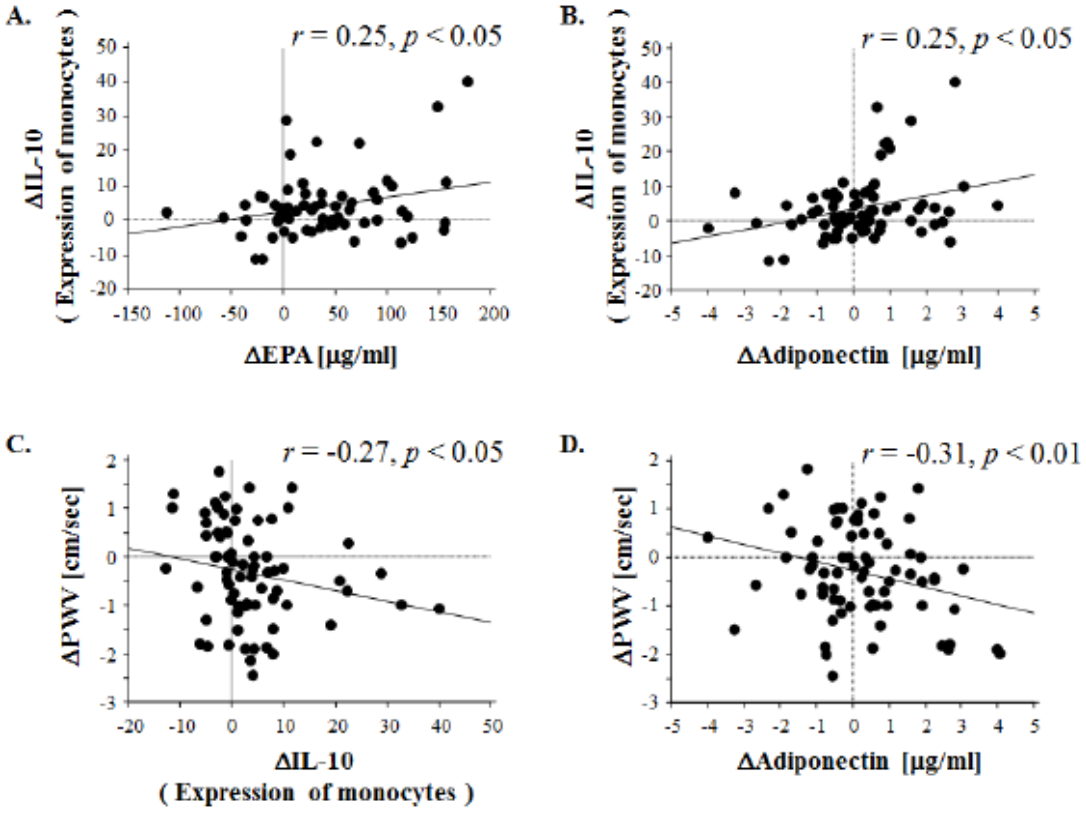
Supplementary Figure 1. Study Design and Enrollment Flowchart shows the numbers of patients who were enrolled in the study, underwent randomization, and completed the study.



Supplementary Figure 2. Correlations related to changes in IL-10 in peripheral blood and PWVduring treatment with EPA Correlations between ΔIL-10 and ΔEPA (A), ΔIL-10 and Δadiponectin (B), ΔPWV and Δ IL-10 (C), and ΔPWV and Δadiponectin (D).



SUPPLEMENTARY DATA

Results

Baseline correlations among metabolic syndrome-related variables, M1/M2 markers in peripheral blood monocytes, and PWV

In all subjects, Pearson's correlation coefficient showed that ln TNFα mRNA levels in peripheral blood monocytes were positively correlated with age and ln adiponectin expression ($P < 0.01$), and ln EPA and ln EPA/AA ($P < 0.05$), and negatively correlated with CD163 in monocytes ($P<0.05$). In this study, the ln IL-6 mRNA level in monocytes was not correlated with any variable. However, the ln IL-10 mRNA level in monocytes was negatively correlated with ln FPG, ln HbA1c, and PWV ($P < 0.05$). CD163 expression in monocytes was negatively correlated with BMI, ($P < 0.05$), ln HbA1c, ln IRI, PWV ($P < 0.01$), and the ln TNFα mRNA level in monocytes ($P < 0.05$). PWV showed positive correlations with age and SBP ($P < 0.01$) and negative correlations with BMI and ln IL-10 expression in monocytes ($P < 0.05$), and CD163 expression in monocytes ($P < 0.01$) (Supplemental Table II).

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