Palmitoylethanolamide Promotes a Proresolving Macrophage Phenotype and Attenuates Atherosclerotic Plaque Formation

Petteri Rinne,* Raquel Guillamat-Prats,* Martina Rami, Laura Bindila, Larisa Ring, Leo-Pekka Lyytikäinen, Emma Raitoharju, Niku Oksala, Terho Lehtimäki, Christian Weber, Emiel P.C. van der Vorst, Sabine Steffens

Objective—Palmitoylethanolamide is an endogenous fatty acid mediator that is synthetized from membrane phospholipids by *N*-acyl phosphatidylethanolamine phospholipase D. Its biological actions are primarily mediated by PPAR-α (peroxisome proliferator-activated receptors α) and the orphan receptor GPR55. Palmitoylethanolamide exerts potent anti-inflammatory actions but its physiological role and promise as a therapeutic agent in chronic arterial inflammation, such as atherosclerosis remain unexplored.

Approach and Results—First, the polarization of mouse primary macrophages towards a proinflammatory phenotype was found to reduce *N*-acyl phosphatidylethanolamine phospholipase D expression and palmitoylethanolamide bioavailability. *N*-acyl phosphatidylethanolamine phospholipase D expression was progressively downregulated in the aorta of apolipoprotein E deficient ($ApoE^{-/-}$) mice during atherogenesis. *N*-acyl phosphatidylethanolamine phospholipase D mRNA levels were also downregulated in unstable human plaques and they positively associated with smooth muscle cell markers and negatively with macrophage markers. Second, $ApoE^{-/-}$ mice were fed a high-fat diet for 4 or 16 weeks and treated with either vehicle or palmitoylethanolamide (3 mg/kg per day, 4 weeks) to study the effects of palmitoylethanolamide on early established and pre-established atherosclerosis. Palmitoylethanolamide treatment reduced plaque size in early atherosclerosis, whereas in pre-established atherosclerosis, palmitoylethanolamide promoted signs of plaque stability as evidenced by reduced macrophage accumulation and necrotic core size, increased collagen deposition and downregulation of M1-type macrophage markers. Mechanistically, we found that palmitoylethanolamide, by activating GPR55, increases the expression of the phagocytosis receptor MerTK (proto-oncogene tyrosine-protein kinase MER) and enhances macrophage efferocytosis, indicative of proresolving properties.

Conclusions—The present study demonstrates that palmitoylethanolamide protects against atherosclerosis by promoting an anti-inflammatory and proresolving phenotype of lesional macrophages, representing a new therapeutic approach to resolve arterial inflammation.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2018;38:2562-2575. DOI: 10.1161/ATVBAHA.118.311185.)**

Key Words: atherosclerosis ■ cholesterol ■ fatty acids ■ inflammation ■ macrophages

Biologically active lipids play pivotal roles in acute inflammatory responses, resolution of inflammation, as well as chronic inflammation.[1] Chronic inflammation in atherosclerosis is driven by persistent lipid overload, immune activation, and failed resolution of inflammation. Hypercholesterolemia leads to accumulation of LDL (low-density lipoprotein) and its modified forms in the artery wall, which elicits vascular inflammation that promotes subendothelial monocyte-derived macrophage accumulation. Macrophages that engulf lipids and transform into foam cells are the main innate effector cell within atherosclerotic plaques. An imbalance between proinflammatory signals and endogenous signals that resolve inflammation will lead to plaque destabilization, linked to enhanced smooth muscle cell (SMC) apoptosis, secondary necrosis because of impaired apoptotic cell clearance and necrotic core expansion.[2] Recent advances in the field of chronic inflammatory disorders have identified diverse functions of lipid mediators, including proinflammatory and anti-inflammatory, as well as proresolving properties. Promising findings in animal models of atherosclerosis suggest a possible

Received on: April 13, 2018; final version accepted on: September 11, 2018.

From the Institute for Cardiovascular Prevention (IPEK), Ludwig Maximilians University (LMU) of Munich, Germany (P.R., R.G.-P., M.R., L.R., C.W., E.P.C.v.d.V., S.S); Institute of Biomedicine and Turku Center for Disease Modeling, University of Turku, Finland (P.R.); Institute for Physiological Chemistry, University Medical Center, Johannes Gutenberg University Mainz, Germany (L.B.); Department of Clinical Chemistry, Fimlab Laboratories and Finnish Cardiovascular Research Center Tampere, Faculty of Medicine and Life Sciences, University of Tampere, Finland (L.-P.L., E.R., N.O., T.L.); Department of Surgery, Tampere University Hospital, Finland (N.O.); Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, The Netherlands (C.W.); and German Centre for Cardiovascular Research (DZHK), Partner Site Munich Heart Alliance, Germany (C.W., S.S.).

*These authors contributed equally to this article.

The online-only Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/ATVBAHA.118.311185.

Correspondence to Sabine Steffens, PhD, Institute for Cardiovascular Prevention (IPEK), Ludwig Maximilians University (LMU) of Munich, Munich, Germany Pettenkoferstraße 8a, München 80336, Germany. Email sabine.steffens@med.uni-muenchen.de

Nonstandard Abbreviations and Acronyms	
BMDM	bone marrow-derived mouse macrophages
CCL2	C-C motif chemokine ligand 2
HDL	high-density lipoprotein
HFD	high-fat diet
ICAM-1	intercellular adhesion molecule 1
IFN-γ	interferon-γ
IL4	interleukin 4
iNOS	inducible nitric oxide synthase
LDL	low-density lipoprotein
MerTK	proto-oncogene tyrosine-protein kinase MER
NAAA	*N*-acylethanolamine acid amidase
NAE	N-acylethanolamines
NAPE-PLD	*N*-acyl phosphatidylethanolamine phospholipase D
PPAR-α	peroxisome proliferator-activated receptor α
PRRT2	proline-rich transmembrane protein 2
SMC	smooth muscle cell
SR-BI	scavenger receptor class B member 1
TNF-α	transforming growth factor α
TVS	Tampere Vascular Study
VCAM-1	vascular cell adhesion molecule 1
VLDL	very-LDL

therapeutic exploitation of bioactive lipids for plaque stabilization and limiting plaque progression.[3,4]

Palmitoylethanolamide is an endogenous lipid mediator of the *N*-acylethanolamines (NAE) family, which shares the same biosynthetic pathways with the endocannabinoid anandamide.[5] In mice and humans, it is synthesized via hydrolysis of a membrane phospholipid precursor through the catalytic action of *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD). The subsequent hydrolysis of palmitoylethanolamide to palmitic acid and ethanolamine is catalyzed by fatty acid amide hydrolase and more specifically by *N*-acylethanolamine acid amidase (NAAA).[5] Palmitoylethanolamide is not only produced in mammals, but it also occurs as a natural compound of plant- and animal-derived food sources, such as soybeans and chicken egg yolk.[6] In contrast to anandamide, palmitoylethanolamide exhibits only weak binding efficacy at the classical cannabinoid receptors CB1 and CB2. Instead, the direct molecular targets that mediate the biological effects of palmitoylethanolamide are PPAR-α (peroxisome proliferator-activated receptor α) and the orphan receptor GPR55.[7,8] Although the precise molecular mechanisms of action of palmitoylethanolamide are not fully elucidated, there is an emerging interest into its use as potential analgesic, neuroprotective, and anti-inflammatory drug.[5] So far, clinical data about the therapeutic benefit of palmitoylethanolamide for pain treatment are still limited because of the small number of clinical studies and patients enrolled, but at least they suggest that it is well tolerated.[9]

Numerous in vitro and in vivo studies have documented anti-inflammatory effects of palmitoylethanolamide in different disease models,[5,10] including contact allergic dermatitis,[11] colitis,[12] kidney ischemia-reperfusion injury,[13] and diabetic retinopathy.[14] The first evidence to support the anti-inflammatory action of palmitoylethanolamide came from a study showing that palmitoylethanolamide suppresses mast cell activation and degranulation.[15] However, palmitoylethanolamide has multiple effects and the ability to act on various cell types, including astrocytes, microglia, and macrophages, making it an attractive therapeutic tool to treat pathologies characterized by neurodegeneration and inflammation. The neuroprotective and anti-inflammatory effects of palmitoylethanolamide are primarily based on the same molecular mechanism, that is PPAR-α activation, which reduces the transcription of proinflammatory genes.[7,16]

Despite the well-established anti-inflammatory actions of palmitoylethanolamide, possible therapeutic benefits in preventing atherosclerotic plaque development and arterial inflammation have not been studied. Furthermore, the regulation of palmitoylethanolamide metabolism in atherosclerosis remains unexplored. This prompted us to investigate how atherosclerosis-associated inflammation modulates the biosynthesis of palmitoylethanolamide and further to determine the effects of chronic systemic administration of palmitoylethanolamide on early plaque formation, as well as on plaque progression at more advanced stage in the apolipoprotein E deficient (ApoE$^{-/-}$) mouse model of atherosclerosis.

Materials and Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request. Please see the Major Resources Table in the online-only Data Supplement for additional experimental details.

Mice

Adult C57Bl/6J mice and GPR55 deficient (GPR55$^{-/-}$) mice on a C57Bl/6J background were used to obtain bone marrow-derived macrophages (BMDMs). For the in vivo pharmacological experiment, female ApoE$^{-/-}$ mice (The Jackson Laboratory) were fed a high-fat diet (HFD, TD88137 mod./Western diet, Ssniff Spezialdiäten GmbH, Soest, Germany) for 4 or 16 weeks and randomly assigned to receive either vehicle or palmitoylethanolamide (3 mg/kg per day, IP, 4 weeks, Tocris Bioscience, Bristol, United Kingdom). Vehicle solution contained 5% ethanol and 5% Tween-80 in PBS. The dose selection was based on previous studies and on our pilot experiment showing that 3 mg/kg of palmitoylethanolamide is needed to induce anti-inflammatory effects and to promote macrophage polarization towards M2-type phenotype in leukocyte-rich lymphoid organs (data not shown).[17,18] Mice were group housed during the pharmacological experiment to avoid stress caused by single housing. Female mice were used because male mice tend to show aggressive behavior against their littermates during repeated handling. All animal experiments were approved by the local Ethics committee (District Government of Upper Bavaria, Germany; License number: 55.2-1-54- 2532-111-13) and conducted in accordance with the institutional and national guidelines for the care and use of laboratory animals.

Human Endarterectomy Samples

TVS (Tampere Vascular Study) endarterectomy samples were obtained from carotid arteries, abdominal aortas, and femoral arteries as previously described.[19,20] The left internal thoracic artery samples, obtained during coronary artery bypass surgery, served as controls. Gene expression was analyzed from carotid (n=29), abdominal aortic (n=15), and femoral (n=24) plaques (cases), and atherosclerosis-free left internal thoracic arteries (n=28). The expression levels were analyzed with Illumina HumanHT-12 v3 Expression BeadChip (Illumina).[20,21] The study was approved by the Ethics Committee of Tampere Hospital District and conducted according to the declaration of Helsinki, and the study subjects gave written informed consent.

Bone Marrow-Derived Macrophages

BMDMs were cultured in RPMI (Roswell Park Memorial Institute)-1640 medium supplemented with 10% FCS, penicillin

(100 U/mL), streptomycin (100 µg/mL; all Gibco Life Technologies), and 10% filtered L-929 cell-conditioned medium as previously described.[22] For polarization experiments, BMDMs at a confluence of 80% were washed with PBS and, thereafter, left untreated or stimulated with IFN-γ (interferon-γ; 10 ng/mL, R&D), IL4 (interleukin 4; 20 ng/mL, Cedarlane Labs), or dexamethasone (20 nmol/L, Sigma-Aldrich) in 250 µL RPMI medium without any other supplement. After 6 hours, cells and their supernatants were harvested and stored at −80°C until further analysis.

Measurement of NAE by Liquid Chromatography-Tandem Mass Spectrometry

Concentrations of anandamide and palmitoylethanolamide were measured in plasma and lipid extracts of aortas[23] of ApoE$^{-/-}$ mice and in supernatants from BMDMs that were polarized in serum-free medium as described above. Samples were allowed to thaw on ice water, and 50 µL aliquots were transferred to 1.5 mL centrifugation tubes. After adding 300 µL of ice-cold ethyl acetate/hexane (9:1, v/v) containing the deuterated NAEs as internal standards, tubes were vortexed for 30 seconds and immediately centrifuged for 15 minutes at 20 000 g at 4°C. The upper organic phase was removed, evaporated to dryness under a gentle stream of nitrogen at 37°C, and reconstituted in 50 µL acetonitrile/water (1:1, v/v). Concentrations of anandamide and palmitoylethanolamide were determined by liquid chromatography-multiple reaction monitoring as previously described.[24] Values of anandamide and palmitoylethanolamide were normalized to the total volume of supernatant.

Vascular SMC and Endothelial Cell Culture

Rat aortic vascular SMCs (A7r5, ATCC CRL-1444) were cultured in DMEM supplemented with 10% FCS, penicillin (100 U/mL), and streptomycin (100 µg/mL). A7r5 cells were serum-starved (0.5 % FCS) overnight and then treated with TNF-α (tumor necrosis factor α; 0.1–50 ng/mL) for 0.5 to 8 hours. Immortalized murine endothelial cells (SVEC, ATCC CRL-2181) were cultured as previously described.[22] SVECs were left untreated or treated with palmitoylethanolamide (1 µmol/L) for 30 minutes before stimulation with TNF-α (10 ng/mL) for 4 hours.

Histology and Immunohistochemistry

For the quantification of lesion size and necrotic core size, aortic roots were cut in 5 µm-thick serial cryosections and stained with Oil-Red O or hematoxylin and eosin, respectively. In accordance with the guidelines for experimental atherosclerosis studies by the American Heart Association,[25] lesion size was analyzed in a blinded manner and quantified as an average of 4 to 5 sections that were separated by 100 µm to cover the entire aortic root. For conventional immunohistochemistry, mouse aortic root cryosections and human paraffin-embedded atherosclerosis samples were incubated with primary antibodies (NAPE-PLD, Mac-2, CD68, and α-smooth muscle actin) followed by colorimetric detection with a horseradish peroxidase (brown color) or alkaline phosphatase (red color) based systems or by fluorometric detection with fluorochrome-conjugated secondary antibodies. To quantify apoptosis in atherosclerotic lesions, aortic root sections were stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (DeadEnd, Catalog number: G3250, Promega) according to the manufacturer's protocol.

Lipoprotein Profile and Blood Count Analysis

Plasma samples were subjected to fast-performance liquid chromatography (gel filtration on a Superose 6 column; GE Healthcare, Chicago, IL). Different lipoprotein fractions (VLDL [very-LDL], LDL, and HDL [high-density lipoprotein]) were separated and evaluated based on flow-through time. Cholesterol levels were quantified using an enzymatic assay (Roche) according to the manufacturer's protocol. Freshly obtained EDTA (ethylenediaminetetraacetic acid) blood harvested by cardiac puncture was used to analyze leukocyte counts using an animal blood counter (scil Vet ABC Hematology Analyzer).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA from BMDMs, A7r5 cells, SVECs, or from lysed tissue samples was extracted (peqGold Trifast and Total RNA kit, Peqlab, and Norgen biotek kit, Catalog number: 48200) and reverse-transcribed (PrimeScript RT reagent kit, Clontech).[22] Real-time polymerase chain reaction was performed with the 7900HT Sequence Detection System (Applied Biosystems) using the KAPA PROBE FAST Universal quantitative polymerase chain reaction kit (Peqlab) and predesigned primer and probe mix (TaqMan Gene Expression Assays, Life Technologies). Target mRNA expression was normalized to hypoxanthine guanine phosphoribosyl transferase or glyceraldehyde-3-phosphate dehydrogenase, and the fold induction was calculated using the comparative ΔCt method and are presented as relative transcript levels ($2^{-\Delta\Delta Ct}$).

Western Blotting

Total protein from aortic lysates was extracted using a Norgen Biotek kit according to the manufacturer's protocol (Catalog number: 48200, Norgen Biotek Corp). Aliquots of total protein were then size-fractioned by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking for 1 hour in Tris-buffered saline containing 0.1% Tween 20 and 5% skim milk (Carl Roth), membranes were incubated with primary antibodies against VCAM-1 (vascular cell adhesion molecule 1), MerTK (proto-oncogene tyrosine-protein kinase), or SR-B1 (scavenger receptor class B member 1) followed by detection with horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence. Target protein expression was normalized to glyceraldehyde-3-phosphate dehydrogenase to correct for loading.

Adoptive Transfer of Monocytes

Monocytes were isolated from femurs, tibias, and spines of female ApoE$^{-/-}$ mice and enriched with a monocyte isolation kit (Miltenyi Biotec MACS, Catalog number: 130-100-629). The purity of isolated monocytes was 90.5±2.1 %. Monocytes were then fluorescently labeled (eBioscience efluor 670, Catalog number: 65-0840-85) and transferred into female ApoE$^{-/-}$ mice (1×10^6 cells, IP) that were fed an HFD and treated with vehicle or palmitoylethanolamide (3 mg/kg per day) for 4 weeks. After 24 hours, mice were euthanized and the transferred monocytes (identified as CD45$^+$, CD11b$^+$, CD115$^+$, and efluor 670$^+$) were quantified in the blood, spleen, and aorta by flow cytometry.

In Vitro Phagocytosis Assays and Flow Cytometry

BMDMs were treated with palmitoylethanolamide (1 µmol/L) for 24 hours. After the treatment, cells were washed with PBS and incubated with fluorescent beads (fluoresbrite carboxylate microspheres, Catalog number: 15702, Polysciences, Inc) for 15 minutes at 37°C. Cells were thereafter stained with fluorochrome-conjugated antibodies against CD11b and F4/80. Phagocytosis was also measured using THP-1 monocytes (ATCC TIB-202) that were stained with calcein (1 mg/mL, 30 minutes) and treated with a mixture of actinomycin D, camptothecin, cycloheximide, dexamethasone, and etoposide to induce apoptosis (Apoptosis Inducer Kit, Abcam, Catalog number: ab102480) for 2 hours. BMDMs were incubated with the apoptotic THP-1 cells for 30 minutes and then stained as described above. In other experiments, cells were additionally stained with an unconjugated antibody against SR-B1 or MerTK and subsequently with an anti-rabbit or anti-goat antibody conjugated with Alexa Fluor488 (Ref: 711-545-152; Jackson ImmunoResearch Laboratories). Data were acquired on a fluorescence-activated cell sorter Canto II flow cytometer (BD Biosciences) and analyzed with FlowJo v10.2 software (FlowJo, LLC). Percentage and mean fluorescence intensity of F4/80 and CD11b positive cells were analyzed for efferocytosis efficiency.

Statistical Analyses

For human atherosclerosis samples, statistical significance of differences in gene expression was assessed using the nonparametric Mann-Whitney *U* test and log-transformed data. Pearson or nonparametric Spearman correlation coefficients were calculated for gene associations based on D'Agostino-Pearson omnibus normality test results. In vitro and in vivo mouse data were analyzed by unpaired Student *t* test, 1-way ANOVA, or by 2-way ANOVA followed by Bonferroni post hoc tests. Data that did not pass D'Agostino and Pearson omnibus K2 normality test ($P<0.05$) or had <8 samples per group were analyzed using the Mann-Whitney *U* test or Kruskal-Wallis test. Unpaired *t* test with Welch's correction was used for data that revealed unequal variances with the *F* test ($P<0.05$). All data are presented as mean±SEM. A 2-tailed $P<0.05$ was considered statistically significant.

Results

Inflammation Reduces Palmitoylethanolamide Levels and NAPE-PLD Expression in Mouse Primary Macrophages

The production of palmitoylethanolamide has been previously shown to be compromised in RAW264.7 macrophages after a short 6-hour treatment with the bacterial endotoxin lipopolysaccharide.[26] This was attributable to reduced transcription and bioactivity of NAPE-PLD, the enzyme synthesizing NAEs including palmitoylethanolamide. To investigate whether the levels of NAEs are also affected in primary mouse macrophages and whether they are dependent on macrophage polarity, we treated BMDMs with IFN-γ, IL4, or dexamethasone to polarize the cells towards M1-, M2a-, or M2c-type phenotype, respectively. Macrophage polarization did not affect anandamide levels, whereas palmitoylethanolamide levels were reduced in M1 and M2a macrophages and increased in reparative M2c macrophages (Figure 1A). In line with the reduced palmitoylethanolamide level, NAPE-PLD expression was significantly downregulated in M1-type macrophages (Figure 1B). This was accompanied by a drastic induction of the palmitoylethanolamide-degrading enzyme NAAA (Figure 1C). NAAA expression was also slightly increased in M2a-type macrophages (Figure 1C). Providing in vivo support for these findings, a previous study has shown that HFD-induced inflammation selectively reduces tissue palmitoylethanolamide levels in the aorta and visceral adipose tissue of ApoE$^{-/-}$ mice, whereas the tissue levels of anandamide and oleoylethanolamine are unchanged or increased, if anything.[27] We were, therefore, interested to investigate whether the decline in tissue palmitoylethanolamide levels is paralleled by reduced expression of the palmitoylethanolamide-synthetizing NAPE-PLD and by increased expression of the palmitoylethanolamide-degrading enzyme NAAA. Interestingly, shifting from normal chow diet to atherogenic HFD progressively reduced NAPE-PLD expression in ApoE$^{-/-}$ mice with the most robust reduction occurring in the aorta (Figure 1D). In contrast, no clear pattern of expressional changes was evident for NAAA (Figure 1E). Possibly accounting for the robust reduction of NAPE-PLD expression in the aorta, we found that inflammatory stimulus with TNF-α downregulates NAPE-PLD expression also in cultured vascular SMCs (Figure I in the online-only Data Supplement). These data suggest that the decline in tissue palmitoylethanolamide level is mainly caused by reduced NAPE-PLD expression and that NAPE-PLD could be used as a biomarker for palmitoylethanolamide availability. To investigate the localization of NAPE-PLD in atherosclerotic lesions, we performed immunohistochemical staining for aortic root sections obtained from ApoE$^{-/-}$ mice. Conventional immunohistochemistry confirmed the presence of NAPE-PLD in the SMC layer, as well as in the atherosclerotic plaque, particularly in close proximity to the arterial wall (Figure 1F). Double immunofluorescence further revealed that NAPE-PLD colocalizes with the macrophage marker Mac-2 in the lesion (Figure 1G). Collectively, these findings demonstrate that proinflammatory stimuli downregulate the expression of NAPE-PLD, which in turn, is likely to selectively suppress the synthesis and bioavailability of palmitoylethanolamide.

NAPE-PLD Expression Is Reduced in Unstable Human Atherosclerotic Plaques

We then analyzed NAPE-PLD expression as a surrogate marker of palmitoylethanolamide bioavailability in human atherosclerosis. Consistent with the mouse data, NAPE-PLD was markedly downregulated in human atherosclerotic plaques compared with histologically normal control arteries (Figure 2A). Interestingly, further analysis of type V and VI lesions revealed that NAPE-PLD transcript levels were reduced in unstable advanced plaques compared with stable plaques (Figure 2B). Immunohistochemical stainings confirmed the expression of NAPE-PLD in human plaques (Figure 2C) and also showed that NAPE-PLD distinctly colocalizes in CD68-positive monocytes/macrophages (Figure 2D) and to a lesser extent in α-smooth muscle actin expressing SMCs (Figure 2E). Using previously published gene signatures,[22,28] we found that NAPE-PLD correlated negatively with macrophage markers and positively with SMC markers in carotid plaques (Figure 2F and 2G). In contrast, these gene association patterns were less prominent in abdominal and femoral plaques (Figures IIA and IC in the online-only Data Supplement), which are more fibrotic lesions compared with macrophage-rich carotid lesions. No clear-cut correlations between NAPE-PLD and macrophage and SMC markers were observed in control arteries (Figures IIB and ID in the online-only Data Supplement). The strongest individual correlations were observed for the M2-type macrophage markers (SR-BI encoded by the *SCARB1* gene) and MerTK and for the SMC marker PRRT2 (proline-rich transmembrane protein 2; Figure III in the online-only Data Supplement). Taken together, the NAPE-PLD expression is associated with SMC markers and inversely with macrophages markers in the human atherosclerotic plaque, supporting the finding of reduced NAPE-PLD transcript levels in unstable advanced lesions.

Chronic Treatment With Palmitoylethanolamide Reduces Plaque Formation in Early Atherosclerosis

To assess the therapeutic benefits of palmitoylethanolamide on atherosclerotic plaque burden and inflammation during the early phases of the disease, ApoE$^{-/-}$ mice were fed an HFD for 4 weeks and randomly allocated to receive daily IP injections of vehicle or palmitoylethanolamide (3 mg/kg). The dose selection was based on previous studies and on our pilot experiment showing that 3 mg/kg of palmitoylethanolamide

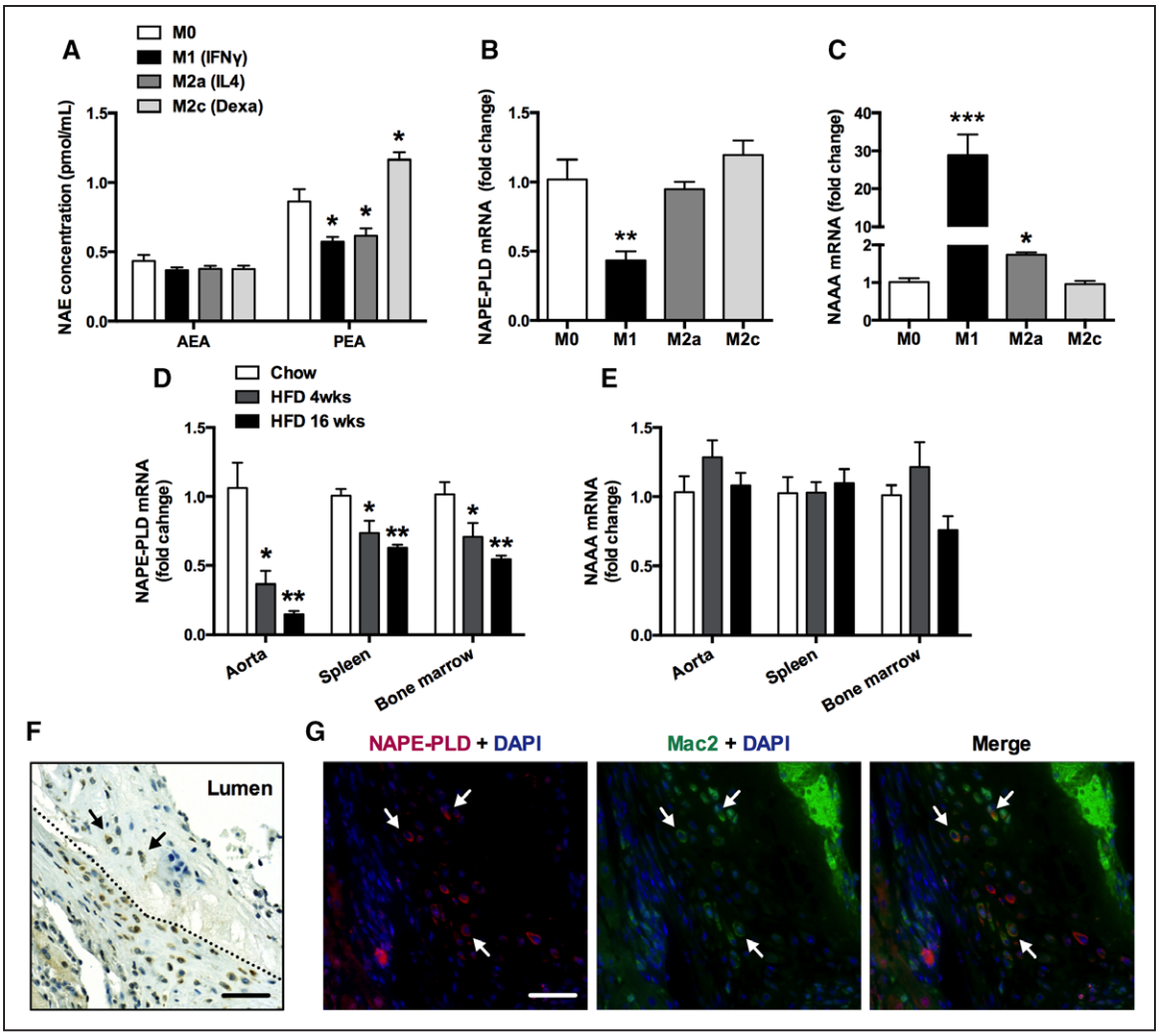

Figure 1. Proinflammatory stimuli disturb palmitoylethanolamide (PEA) metabolism. **A**, Quantification of anandamide (AEA) and PEA levels in cell culture supernatants of bone marrow-derived macrophages (BMDMs). BMDMs were left untreated (M0) or polarized for 6 h with IFN-γ (interferon-γ), IL4 (interleukin 4), or dexamethasone (Dexa) and thereafter the supernatants were analyzed for AEA and PEA concentration. **B** and **C**, Quantitative polymerase chain reaction (PCR) analysis of *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) and N-acylethanolamine acid amidase expression (NAAA) in polarized BMDMs. **D** and **E**, Quantitative PCR analysis of NAPE-PLD and NAAA mRNA levels in the aorta, spleen, and bone marrow of ApoE$^{-/-}$ mice fed either a normal chow diet or high-fat diet (HFD). **F**, NAPE-PLD immunostaining (brown color) of aortic root section of chow-fed ApoE$^{-/-}$ mouse. Black arrows indicate NAPE-PLD-positive cells within atherosclerotic plaque. The dotted line indicates the boundary between the plaque and arterial wall. Scale bar, 50 μm. **G**, Immunofluorescence staining for NAPE-PLD and Mac-2 in aortic root section of chow-fed ApoE$^{-/-}$ mouse. White arrows indicate NAPE-PLD/Mac-2 double-positive cells. Nuclei are stained with DAPI (4',6-diamidine-2'-phenylindole dihydrochloride). Scale bar, 50 μm. **P*<0.05, ***P*<0.01, and ****P*<0.001 vs M0 macrophages or chow-fed mice. Data are mean±SEM, n=4–5 per group from female donors. NAE indicates *N*-acylethanolamines.

is needed to induce anti-inflammatory effects and to promote macrophage polarization towards M2-type phenotype in leukocyte-rich lymphoid organs (data not shown).[17,18] Pharmacokinetic analysis of palmitoylethanolamide tissue levels after a single IP injection (3 mg/kg) demonstrated that the administration results in a rapid and subtle increase in plasma palmitoylethanolamide concentration, which was not detectable in aortic tissue (Figure IV in the online-only Data Supplement). With the selected dose and dosage regimen, chronic palmitoylethanolamide treatment had no effect on body weight or plasma total cholesterol levels (Figure 3A and 3B). However, palmitoylethanolamide treatment significantly reduced atherosclerotic lesion size at the level of the aortic root (Figure 3C and 3D). Because early plaque formation predominantly relies on monocyte recruitment and accumulation in inflamed arteries, we assessed plaque macrophage content by immunohistochemistry and found that palmitoylethanolamide-treated mice tended (*P*=0.05) to have less Mac-2-positive cells in the aortic root sections (Figure 3E), but the relative plaque macrophage content was unaffected (Figure 3F). Furthermore, palmitoylethanolamide treatment did not affect total or differential leukocyte counts in the blood (Table I in the online-only Data Supplement).

Administration of Palmitoylethanolamide Reduces Vascular Inflammation and Promotes Plaque Stability in Advanced Atherosclerosis

The atheroprotective effect of palmitoylethanolamide as a preventive therapy in early atherosclerosis led us to investigate whether palmitoylethanolamide treatment could reduce disease burden also in established atherosclerosis. To this end, ApoE$^{-/-}$ mice were first fed an HFD for 12 weeks

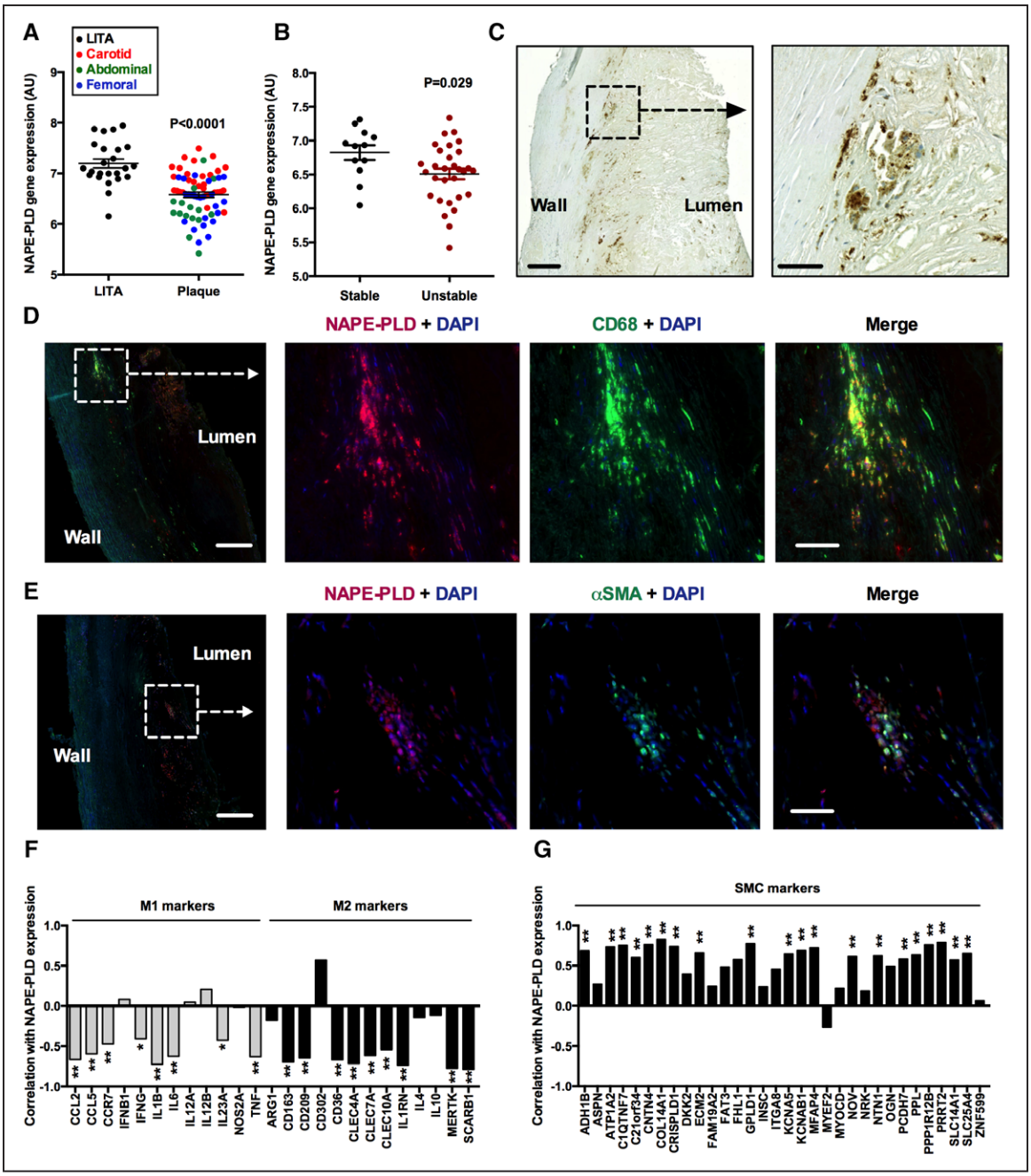

Figure 2. The expression of the palmitoylethanolamide (PEA)-producing enzyme N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) is down-regulated in unstable human atherosclerotic plaques. **A**, NAPE-PLD expression (brown color) in atherosclerosis-free control arteries (left internal thoracic artery; LITA) and in endarterectomy samples from the carotid artery, abdominal aorta, and femoral artery. **B**, NAPE-PLD expression in stable and unstable plaque phenotypes in a subgroup of advanced plaques (stage V and VI). Exact *P* values are given in the graphs. **C**, NAPE-PLD immunostaining of carotid endarterectomy sample. **D** and **E**, Immunofluorescence staining for NAPE-PLD and CD68 or α-smooth muscle actin (α-SMA). Nuclei are stained with DAPI (4',6-diamidine-2'-phenylindole dihydrochloride). Scale bars, 500 and 50 μm. **F** and **G**, Correlation between NAPE-PLD mRNA levels and established M1/M2 macrophage and smooth muscle cell (SMC) markers in carotid endarterectomy samples (n=29). Spearman correlation coefficient (*r*) values are shown. **P*<0.05 and ***P*<0.01 for correlation significances. CCL indicates C-C motif chemokine ligand; IL, interleukin; IFN, interferon; and TNF, tumor necrosis factor.

and then treated with palmitoylethanolamide for 4 weeks in parallel with HFD. Similarly to the early atherosclerosis model, palmitoylethanolamide administration did not affect body weight, plasma total cholesterol, or blood leukocyte counts (Figure 4A and 4B; Table I in the online-only Data Supplement). Given that PPAR-α activation with established therapies, such as fibrates are known to increase plasma HDL level while modestly reducing LDL cholesterol,[29] we further analyzed the plasma lipoprotein profile in palmitoylethanolamide-treated mice. However, no changes were noted in plasma VLDL, LDL, or HDL cholesterol fractions (Figure 4C and 4D). In contrast to the effects in early atherosclerosis, palmitoylethanolamide treatment did not limit atherosclerotic plaque size in the aortic root (Figure 4E and 4F), but it significantly reduced macrophage accumulation in the lesions (Figure 4G). Plaque SMC content was

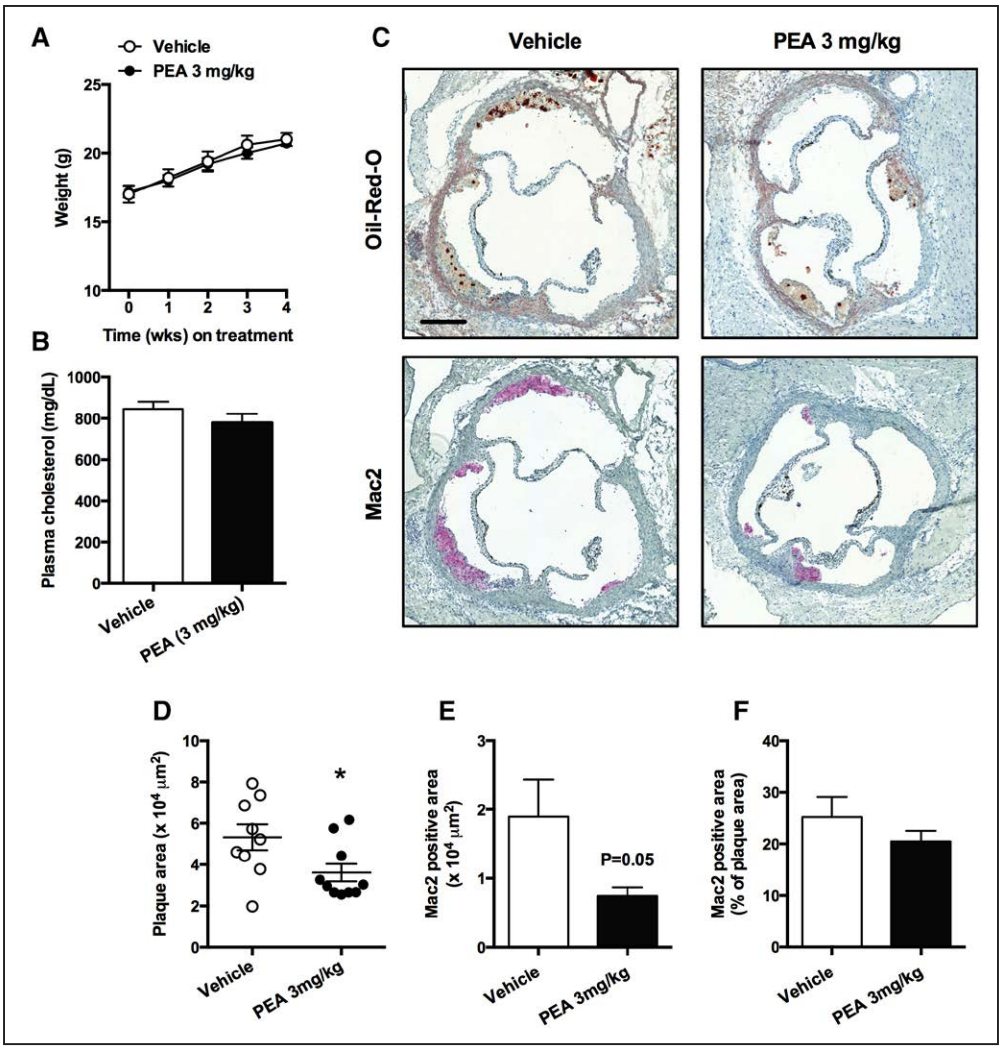

Figure 3. Chronic treatment with palmitoylethanolamide (PEA) limits early plaque formation in atherosclerotic ApoE$^{-/-}$ mice. Body weight development (**A**) and plasma cholesterol levels (**B**) in vehicle- and PEA-treated mice. **C**, Representative Oil-Red-O and Mac-2-stained aortic root sections. Scale bar, 200 μm. The quantification of total plaque area (**D**), as well as absolute (**E**) and relative (**F**) Mac-2 positive area in aortic root sections. *P<0.05 vs vehicle treated. Data are mean±SEM, n=9–10 mice per group in each graph.

unaffected by palmitoylethanolamide treatment (Figure V in the online-only Data Supplement). Further analysis of plaque composition revealed that palmitoylethanolamide treatment increased plaque collagen content and reduced necrotic core size (Figure 4H and 4I), indicative of more stable plaques. Taken together, these data indicate that palmitoylethanolamide restrains plaque progression in advanced atherosclerosis without affecting plasma cholesterol levels.

In agreement with the established anti-inflammatory actions of palmitoylethanolamide, gene expression analysis of aortic lysates showed reduced mRNA levels of proinflammatory cytokines and adhesion molecules in palmitoylethanolamide-treated mice (Figure 5A). Specifically, ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 mRNA levels were reduced in the aorta of palmitoylethanolamide-treated mice (Figure 5A). The reduction of VCAM-1 expression was also evident at the protein level (Figure 5B and 5C). Furthermore, iNOS (inducible nitric oxide synthase) and CCL2 (C-C motif chemokine ligand 2), phenotypic markers of M1-type macrophages, were downregulated by palmitoylethanolamide treatment (Figure 5A). We also conducted in vitro experiments with endothelial cells and found that palmitoylethanolamide treatment–reduced VCAM-1 and CCL2 mRNA expression in TNF-α-stimulated cells, suggesting that the therapeutic effects of palmitoylethanolamide are not only mediated by macrophages but also by endothelial cells (Figure VI in the online-only Data Supplement). In line with reduced vascular inflammation, adoptive transfer of monocytes into vehicle or palmitoylethanolamide-pretreated ApoE$^{-/-}$ mice revealed a lower number of the transferred monocytes remaining in the blood of palmitoylethanolamide-treated mice 24 hours after cell transfer. This was paralleled with a more increased accumulation of injected monocytes in the spleen, whereas we unexpectedly did not find differences in aortas (Figure VII in the online-only Data Supplement). We may speculate that adoptively transferred monocytes preferentially home to the spleen and that this effect is more pronounced in palmitoylethanolamide-treated mice, which have a less inflamed arterial endothelium. To identify whether the downregulation of iNOS is localized to macrophages, we did

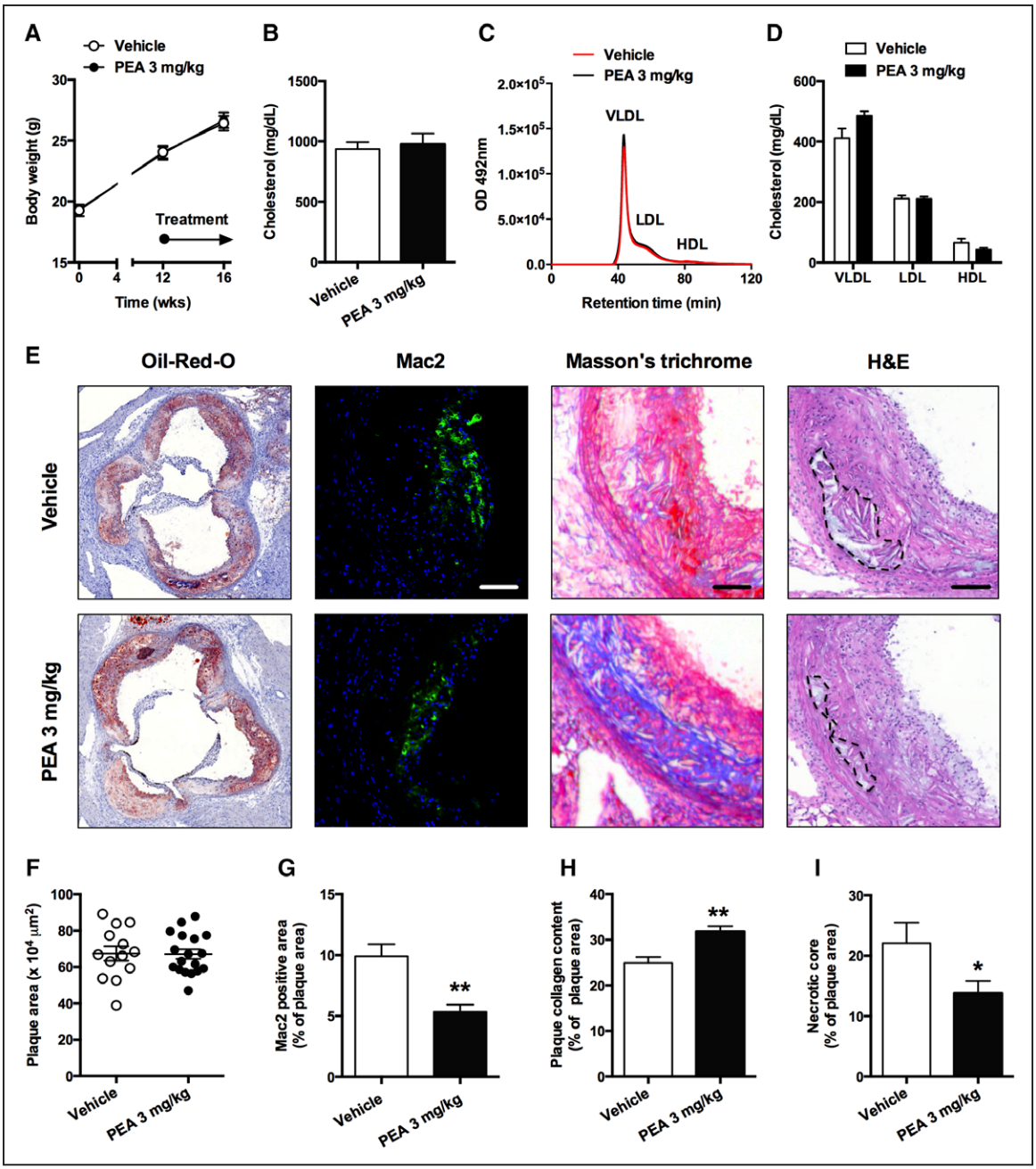

Figure 4. Palmitoylethanolamide (PEA) promotes plaque stability in advanced atherosclerosis. Body weight development (**A**) and plasma total cholesterol levels (**B**) in vehicle- and PEA-treated ApoE$^{-/-}$ mice after 16 wk of high-fat diet (HFD). n=13–20 mice per group. **C**, Representative histograms high-performance liquid chromatography–fractionated plasma lipoprotein profiles from vehicle- and PEA-treated mice. **D**, Quantification of plasma VLDL (very-low-density lipoprotein), LDL, and HDL (high-density lipoprotein) cholesterol levels in vehicle- and PEA-treated mice. n=4–5 per group after pooling of 8–10 samples **E**, Representative Oil-Red-O, Mac-2, Masson's trichrome, and hematoxylin and eosin (H&E)-stained aortic root sections. Scale bars, 500 μm (Oil-Red-O) and 50 μm (Mac-2, Masson's trichrome, and H&E). Quantification of total plaque area (**F**) and relative Mac-2 positive-area (**G**) in aortic root sections. Quantification of relative collagen content (**H**) and necrotic core size (**I**) in atherosclerotic plaques. **F**, n=13–18 mice per group; **G** through **I**, n=7–10 mice per group. *P<0.05 and **P<0.01 vs vehicle treated. Data are mean±SEM.

double immunofluorescence staining with iNOS and Mac-2 for aortic root sections. Indeed, palmitoylethanolamide-treated mice had reduced number of iNOS-positive lesional macrophages (Figure 5D and 5F). To gain further insights into the possible atheroprotective mechanisms, we turned our attention to MerTK and SR-BI, which showed the strongest association with NAPE-PLD expression in human atherosclerotic plaques. Western blot analysis of aortic lysates revealed that MerTK and SR-BI protein expression were increased in palmitoylethanolamide-treated mice (Figure 5B and 5C). MerTK is expressed predominantly by M2-type reparative macrophages and it mediates phagocytosis of apoptotic cells, that is efferocytosis, in atherosclerotic lesion, thus limiting the expansion of necrotic core.[30–32] We, therefore, quantified the number of apoptotic TUNEL^{+} cells in aortic root lesions. In line with the reduced necrotic core and increased MerTK

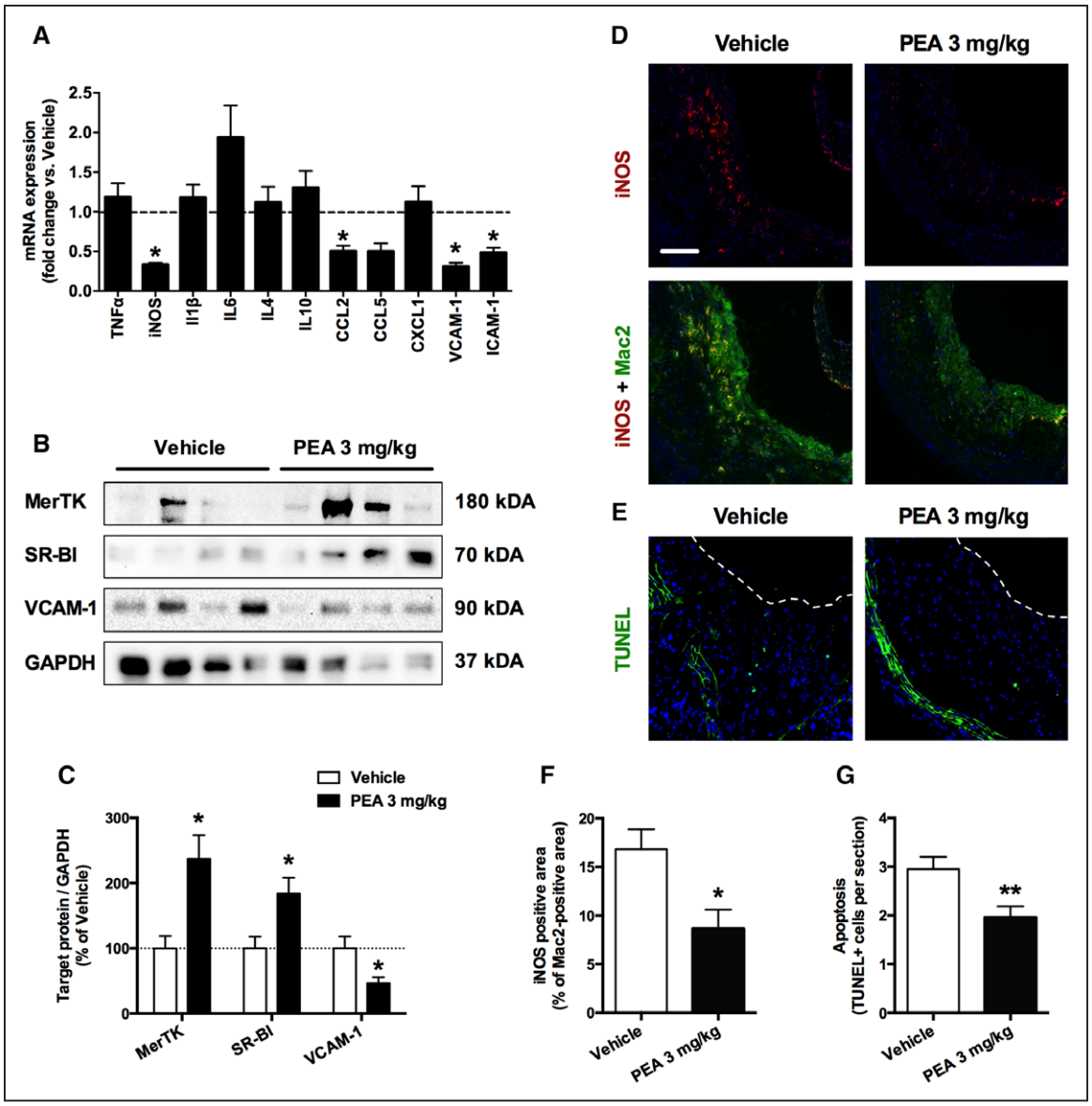

Figure 5. Palmitoylethanolamide (PEA) enhances an anti-inflammatory and proresolving plaque phenotype in advanced atherosclerosis. **A**, Aortic mRNA expression levels of cytokines and adhesion molecules normalized to hypoxanthine phosphoribosyltransferase and expressed as fold change compared with vehicle-treated mice. **B**, Representative Western blots for MerTK (proto-oncogene tyrosine-protein kinase MER), SR-BI (scavenger receptor class B member 1), VCAM-1 (vascular cell adhesion molecule 1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; loading control) in the aortic lysates of vehicle- and PEA-treated mice. **C**, Quantification of aortic MerTK, SR-BI, and VCAM-1 protein expression (normalized to GAPDH). **D**, Representative double immunofluorescent staining of iNOS (inducible nitric oxide synthase) and Mac-2 in the aortic roots of vehicle- and PEA-treated ApoE$^{-/-}$ mice. Scale bar, 100 μm. **E**, Representative images of aortic root sections in which apoptotic cells were labeled by TUNEL (green) and nuclei by DAPI (4',6-diamidine-2'-phenylindole dihydrochloride; blue). Scale bar, 100 μm. **F**, Quantification of iNOS-positive macrophage area (% of Mac-2-positive area) in the aortic sinuses. **G**, Quantification of TUNEL^{+} cells in aortic root plaques of vehicle- and PEA-treated mice. *P<0.05 and **P<0.01 vs vehicle treated. Data are mean±SEM, n=5–13 mice per group in each graph. CCL indicates C-C motif chemokine ligand; ICAM, intercellular adhesion molecule 1; IL, interleukin; and TNF, tumor necrosis factor.

expression, plaques of palmitoylethanolamide-treated mice contained less TUNEL^{+} cells (Figure 5E and 5G).

Palmitoylethanolamide Increases MerTK Expression and Enhances Efferocytosis Through the Activation of GPR55

Having noted the therapeutic effect of palmitoylethanolamide on plaque necrosis and MerTK expression, we next investigated whether palmitoylethanolamide directly enhances the efferocytotic capacity of macrophages. To address this, we first used fluorescently labeled beads and measured the efficiency of BMDMs to clear these beads after 24-hour treatment with palmitoylethanolamide. In particular, we studied the specific effect of palmitoylethanolamide on macrophages that was first polarized to M1, M2a, or M2c-type phenotype. Palmitoylethanolamide substantially enhanced phagocytosis of fluorescent beads in all BMDM phenotypes with the strongest effect appearing in M2c-type macrophages (Figure 6A). This effect was evidenced by the increased percentage of phagocytotic BMDMs, as well as by the increased amount of engulfed beads (ie, mean fluorescence intensity of fluorescein isothiocyanate in CD11b^{+}, F4/80^{high} cells;

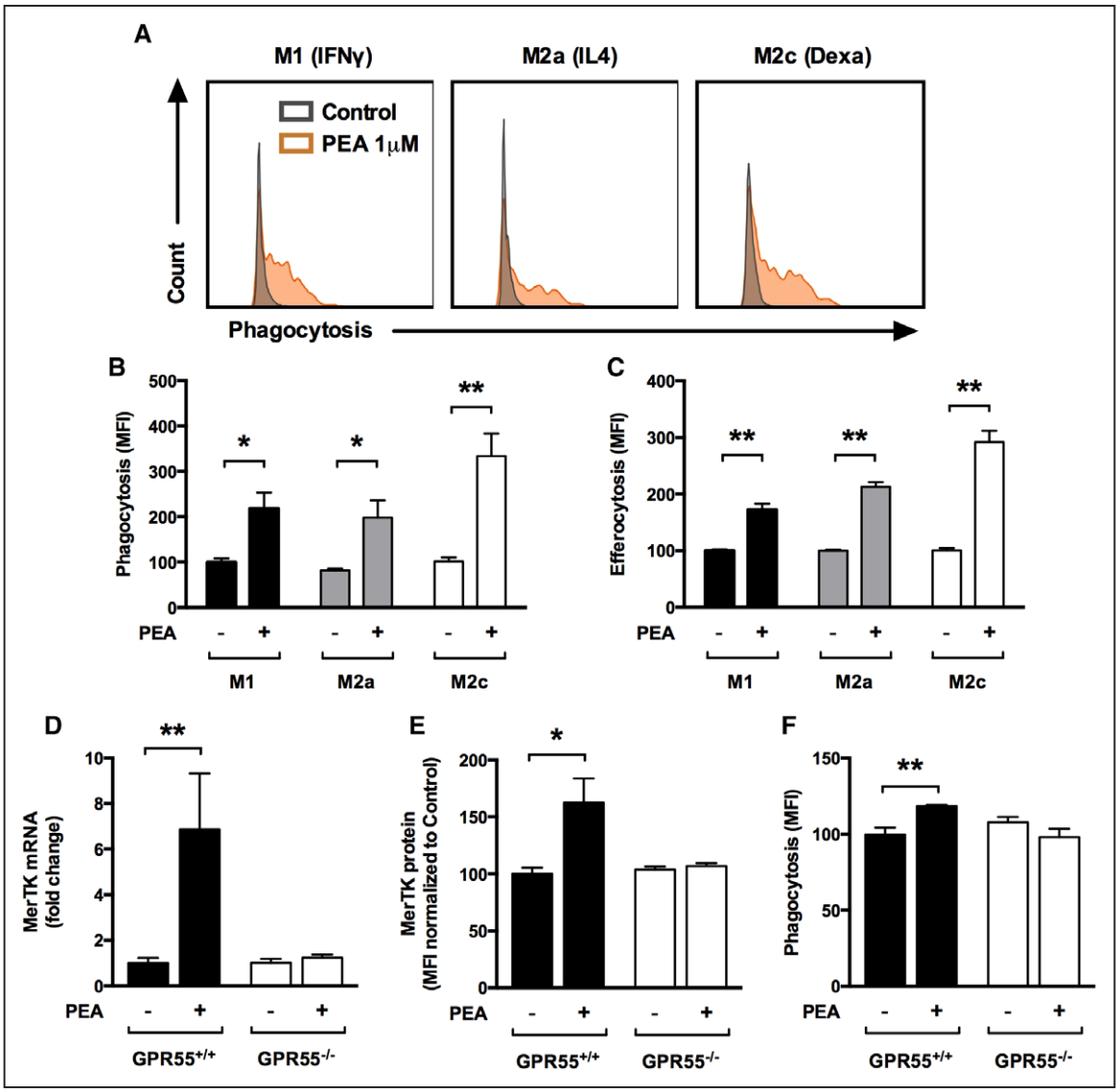

Figure 6. Palmitoylethanolamide (PEA) enhances MerTK (proto-oncogene tyrosine-protein kinase MER) expression and phagocytosis in cultured macrophages. **A**, Representative histograms for phagocytosis of fluorescent beads in control and PEA-treated bone marrow-derived macrophages (BMDMs). Cells were first polarized with IFN-γ (interferon-γ), IL4 (interleukin 4), or dexamethasone (Dexa) towards M1-, M2a-, or M2c-type phenotype, respectively, and thereafter treated with PEA (1 μmol/L, 24 h) or left untreated. **B**, Quantification of phagocytosis capacity, expressed as mean fluorescence intensity (MFI) for fluorescein isothiocyanate-labeled beads, in M1-, M2a-, and M2c-polarized BMDMs ($CD11b^+$, $F4/80^{high}$ cells). **C**, Efferocytosis of apoptotic, calcein-stained THP-1, expressed as MFI for calcein, in control and PEA-treated macrophages ($CD11b^+$, $F4/80^{high}$ cells). **D**, Quantitative real-time polymerase chain reaction analysis of MerTK mRNA expression in BMDMs derived from $GPR55^{+/+}$ and $GPR55^{-/-}$ mice. **E**, Quantification of MerTK surface expression by flow cytometry in $GPR55^{+/+}$ and $GPR55^{-/-}$ BMDMs. **F**, Quantification of phagocytosis capacity in PEA-treated (1 μmol/L, 24 h) unpolarized $GPR55^{+/+}$ and $GPR55^{-/-}$ BMDMs. *P<0.05 and **P<0.01 vs untreated control. Data are mean±SEM, n=3–6 per group in each graph. Female donors were used except in **D** through **F** (see Major Resources Table in the online-only Data Supplement for details).

Figure 6B; Figure VIIIC in the online-only Data Supplement). Second, BMDMs were incubated with apoptotic THP-1 monocytes to study the effect of palmitoylethanolamide on efferocytosis. Similarly to the phagocytosis of fluorescent beads, palmitoylethanolamide-treated macrophages cleared more efficiently apoptotic cells, and the effect was most prominent in M2c-type macrophages (Figure 6C). We hypothesized that the underlying mechanism for enhanced efferocytosis capacity in response to palmitoylethanolamide is a direct induction of MerTK expression in macrophages possibly via GPR55 receptor signaling. To test this hypothesis, we used BMDMs derived from wild-type and $GPR55^{-/-}$ mice, treated them with palmitoylethanolamide and analyzed the expression of MerTK. Quantitative polymerase chain reaction and flow cytometric analyses showed that in wild-type cells, palmitoylethanolamide treatment upregulated MerTK expression at the mRNA and protein level, whereas in $GPR55^{-/-}$ macrophages, these effects were completely absent (Figure 6C and 6D). Likewise, $GPR55^{-/-}$ macrophages did not show any response to palmitoylethanolamide treatment in terms of phagocytosis of fluorescent beads or apoptotic THP-1 cells (Figure 6E; Figure IX in the online-only Data Supplement). Palmitoylethanolamide also upregulated SR-BI mRNA and protein expression in BMDMs, but the effect was independent of GPR55 activation (Figure X in the online-only Data Supplement).

Discussion

The present study demonstrates that palmitoylethanolamide limits atherosclerosis and improves plaque stability. The underlying mechanism is likely to be multifactorial involving PPAR-α-mediated anti-inflammation and promotion of proresolving properties of macrophages through the activation of GPR55. The clinical relevance of these findings is supported by the reduced expression of the palmitoylethanolamide-synthesizing enzyme NAPE-PLD in unstable human plaques. Thus, counteracting the inflammation-driven decline in endogenous palmitoylethanolamide level by pharmacological means could serve as an intriguing therapeutic strategy for atherosclerosis.

Several lines of evidence indicate that inflammation causes an imbalance in palmitoylethanolamide metabolism characterized by reduced biosynthesis and increased degradation.[10,26,33,34] The ensuing reduction in the bioavailability then dampens the counterbalancing effect of palmitoylethanolamide on inflammatory processes. Our findings corroborate this concept and suggest that changes in palmitoylethanolamide metabolism may occur also in diet-induced inflammation and atherosclerosis. We used quantification of NAPE-PLD expression as an indirect marker of palmitoylethanolamide bioavailability, representing a more feasible approach compared with mass spectrometric analyses of palmitoylethanolamide level. Admittedly, a considerably limitation of this approach is that it does not allow concluding that reduced NAPE-PLD expression is associated with reduced palmitoylethanolamide level. Furthermore, the effect on other NAEs, including oleoylethanolamine and anandamide, is unclear. However, studies on mice with global and tissue-specific deficiency of NAPE-PLD have shown that the gene deletion leads to a marked reduction in palmitoylethanolamide and oleoylethanolamine levels without affecting anandamide level, highlighting the existence of alternative synthesis pathways for anandamide.[34,35] This notion is further supported by the present findings of unaltered anandamide levels and significantly reduced palmitoylethanolamide levels in proinflammatory M1-type macrophages. Thus, considering that palmitoylethanolamide and oleoylethanolamine both activate PPAR-α and suppress inflammation, reduction in their bioavailability is likely to have a parallel and synergistic effect on inflammatory signaling. The reduced NAPE-PLD expression in unstable advanced plaques and the positive association between NAPE-PLD and SMC markers prompted us to study whether palmitoylethanolamide or oleoylethanolamine holds anti-atherosclerotic properties. However, considering that the therapeutic potential of palmitoylethanolamide has gained wider attention and also fueled the development of new pharmaceutical formulations and their clinical testing, we chose to administer palmitoylethanolamide as therapeutic strategy for atherosclerotic ApoE$^{-/-}$ mice.

Our pharmacokinetic data, revealing a rapid and subtle increase in plasma palmitoylethanolamide concentration, reflect the rapid metabolism of palmitoylethanolamide and are in line with a previous report on the pharmacokinetic properties of palmitoylethanolamide.[36] In a mouse model of epilepsy, a transient increase of palmitoylethanolamide in the plasma and hippocampus was detectable after intraperitoneal injection of a much higher palmitoylethanolamide dose (40 mg/kg). Unfortunately, it was impossible to detect significant increases of palmitoylethanolamide in aortic tissue, likely because of the much lower dose used in this study. We may speculate that palmitoylethanolamide is rapidly taken up by circulating leukocytes and endothelial cells; however, it was technically not possible to measure palmitoylethanolamide in cellular subfractions of aortas. It needs to be also acknowledged that the highest plasma concentration after palmitoylethanolamide administration was much lower (<30 nmol/L) than the concentration used in in vitro experiments (1 μmol/L).

We found that chronic palmitoylethanolamide treatment reduced early atherosclerosis independent of body weight and plasma cholesterol level. In the advanced stage of the disease, palmitoylethanolamide-reduced plaque inflammation and improved signs of plaque stability. The current finding of reduced iNOS, CCL2, ICAM-1, and VCAM-1 expression in aortas of palmitoylethanolamide-treated mice is directly supported by earlier studies showing the same effects in various in vitro and in vivo experiments.[10] For example, a recent study that has the closest relevance to our work demonstrated that palmitoylethanolamide, as a combination treatment with an antioxidant (polydatin), attenuated the upregulation of iNOS, VCAM-1, and ICAM-1 associated with vascular injury.[37] As a major contributing factor in atherogenesis, we found that aortic iNOS expression, as a signature marker of M1-type macrophages, as well as the number of iNOS^{+} lesional macrophages was reduced by palmitoylethanolamide treatment. Proinflammatory M1 macrophages, which outnumber the anti-inflammatory M2 macrophages in advanced lesions, feed plaque inflammation by secreting cytokines, such as IL6, CCL2, and CCL5.[38] Second, palmitoylethanolamide treatment increased the aortic expression of the phenotypic M2 markers MerTK and SR-BI, supporting the shift of plaque macrophages towards an anti-inflammatory and proresolving phenotype. Although our in vitro experiments with endothelial cells suggest that palmitoylethanolamide may inhibit monocyte recruitment, we could not detect a reduced number of adoptively transferred monocyte counts in aortas. This might be because of the fact that only a minor number of the adoptively transferred monocytes is recruited to aortas and consequently small changes might not be detectable by flow cytometric analysis of aortic lysates. Taken together, the present study further validates the anti-inflammatory effects of palmitoylethanolamide and opens a new therapeutic horizon for its applicability in arterial inflammation associated with atherosclerosis.

Cholesterol is an important risk factor and driving force of atherosclerosis. It also triggers arterial inflammation that exacerbates atherosclerosis. Because palmitoylethanolamide did not change plasma total cholesterol level or lipoprotein profile, the explanation for reduced plaque size and improved plaque stability lies in the ability of palmitoylethanolamide to locally promote an anti-inflammatory and proresolving milieu in the advanced plaque. Palmitoylethanolamide most likely and primarily acts on plaque macrophages, which are the dominant effector cells in atherosclerosis,[38,39] and favor their switch to M2-type phenotype. Aside from this notion, VCAM-1 and CCL2, which recruit and guide monocytes into atherosclerotic plaques, were found to be downregulated in

palmitoylethanolamide-treated mice. Supporting these findings, we observed reduced macrophage accumulation in the advanced plaques of palmitoylethanolamide-treated mice. These data highlight that palmitoylethanolamide is acting locally on macrophages to induce M2 polarization and possibly also on endothelial cells to reduce monocyte entry and consequent macrophage accumulation in the lesions.

Of particular importance, we identified the induction of MerTK expression and efferocytosis as a new atheroprotective mechanism for palmitoylethanolamide. Mounting in vivo evidence demonstrates that efferocytosis is impaired in advanced atherosclerosis, which in turn, aggravates key features of clinically dangerous plaques, including expansion of necrotic core and thinning of a fibrous cap that overlies the core.[40–42] Conversely, induction of MerTK and enhanced efferocytosis has been linked to resolution of plaque inflammation and to stabilization of advanced plaques.[30] The in vitro finding that palmitoylethanolamide enhances macrophage MerTK expression and efferocytosis provides a mechanistic explanation for the reduced necrotic core size and increased collagen deposition in palmitoylethanolamide-treated ApoE$^{-/-}$. The phenotype of palmitoylethanolamide-treated ApoE$^{-/-}$ mice closely resembles a mouse model displaying higher macrophage MerTK and improved efferocytosis because of a cleavage-resistant variant of MerTK.[30] Mice with increased macrophage MerTK exhibit smaller necrotic cores and increased collagen content in atherosclerotic plaques and no change in plaque size or in lesional macrophage or SMC numbers. From a drug development perspective, measures to enhance MerTK function and efferocytosis serve as an attractive strategy to prevent the progression of plaque necrosis, thus further extending the therapeutic repertoire of palmitoylethanolamide.

Using GPR55 deficient macrophages, we were able to pinpoint the receptor-level mechanism by which palmitoylethanolamide enhances MerTK expression and efferocytosis. Although the nuclear receptor PPAR-α is thought to primarily mediate the anti-inflammatory effects of palmitoylethanolamide, it seems that palmitoylethanolamide induces MerTK expression by activating the orphan receptor GPR55. Despite showing a low homology with CB1 and CB2 receptors, GPR55 has been introduced as a novel cannabinoid receptor that can be directly activated by the endocannabinoids 2-AG and anandamide, as well as by low concentrations of palmitoylethanolamide.[8,43,44] An earlier in vitro study demonstrated that selective CB2 receptor activation also improves efferocytosis partly through a mechanism involving MerTK induction.[45] However, considering that palmitoylethanolamide has only weak affinity for this receptor,[46] it is unlikely that palmitoylethanolamide shares the same mode of action. This notion is supported by the complete lack of MerTK upregulation in palmitoylethanolamide-treated GPR55$^{-/-}$ macrophages. In contrast, palmitoylethanolamide appeared to upregulate macrophage SR-BI independent of GPR55. This effect might be mediated by PPAR-α, which has been previously shown to regulate SR-BI in cultured human macrophages and in lesional macrophages of ApoE$^{-/-}$ mice.[47] SR-BI is a multifunctional membrane protein that regulates selective uptake of cholesterol esters from mature HDL particles in the liver, thus being viewed as the HDL receptor.[48,49] It also mediates bidirectional lipid transport in macrophages.[50] Although the physiological role of SR-BI in macrophage cholesterol transport is unclear,[50] it is associated with the development of atherosclerosis and the risk of coronary heart disease.[51] Overexpression of SR-BI protects against atherosclerosis,[52,53] whereas BM-specific deficiency accelerates atherosclerosis.[54,55] Given these characteristics, the induction of SR-BI by palmitoylethanolamide might provide additional therapeutic benefits in the treatment of atherosclerosis.

In conclusion, the present results demonstrate that the anti-inflammatory potential of palmitoylethanolamide could be therapeutically expanded to atherosclerosis, which is characterized by chronic arterial inflammation. In addition to reducing plaque size, we observed that palmitoylethanolamide exhibits a plaque-stabilizing effect by increasing collagen content and by limiting plaque necrosis. These effects were linked to GPR55-mediated enhancement of MerTK expression and macrophage efferocytosis, which can further amplify the anti-inflammatory properties of palmitoylethanolamide and hence, synergistically promote resolution of plaque inflammation. With the advent of the first clinical outcome data on anti-inflammatory therapy in high-risk atherosclerosis patients,[56] palmitoylethanolamide and its improved formulations represent a new anti-inflammatory treatment option to resolve inflammation in atherosclerosis.

Acknowledgments

We thank Diana Wagner, Hanna Haukkala, and Aya Bouazza for excellent technical assistance.

Sources of Funding

This work was supported by grants from the Academy of Finland (grant 274852 to P. Rinne), the Finnish Foundation for Cardiovascular Research (to P. Rinne), the Paavo Nurmi Foundation (to P. Rinne), the Deutsche Forschungsgemeinschaft (STE-1053/3-1 to S. Steffens and as part of the SFB1123 TP A1 to C. Weber), the Else Kroener Fresenius Foundation (2013_A114 to S. Steffens), the German Centre for Cardiovascular Research (DZHK MHA VD1.2 to C. Weber and DZHK B18-015-SE to S. Steffens and L. Bindila), the European Research Council ERC (AdG 692511 to C. Weber), and the Alexander von Humboldt Foundation (to E.P.C. van der Vorst). Tampere Vascular Study was supported with grants from the Competitive Research Funding of the Tampere University Hospital (Grant 9M048 and 9N035 for T. Lehtimäki), the Emil Aaltonen Foundation (to T. Lehtimäki and N. Oksala), the Pirkanmaa Regional Fund of the Finnish Cultural Foundation, the Research Foundation of Orion Corporation, the Jenny and Antti Wihuri Foundation, and the Academy of Finland (Grant no. 286284 for T. Lehtimäki), the Finnish Foundation for Cardiovascular Research, the Yrjö Jahnsson Foundation, European Union 7th Framework Program (grant 201668 for AtheroRemo), and EU Horizon 2020 (grant 755320 for TAXINOMISIS).

Disclosures

None.

References

1. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. *Nature*. 2014;510:92–101. doi: 10.1038/nature13479
2. Silvestre-Roig C, de Winther MP, Weber C, Daemen MJ, Lutgens E, Soehnlein O. Atherosclerotic plaque destabilization: mechanisms, models, and therapeutic strategies. *Circ Res*. 2014;114:214–226. doi: 10.1161/CIRCRESAHA.114.302355

3. Viola JR, Lemnitzer P, Jansen Y, Csaba G, Winter C, Neideck C, Silvestre-Roig C, Dittmar G, Döring Y, Drechsler M, Weber C, Zimmer R, Cenac N, Soehnlein O. Resolving lipid mediators maresin 1 and resolvin D2 prevent atheroprogression in mice. *Circ Res*. 2016;119:1030–1038. doi: 10.1161/CIRCRESAHA.116.309492
4. Petri MH, Laguna-Fernandez A, Arnardottir H, Wheelock CE, Perretti M, Hansson GK, Bäck M. Aspirin-triggered lipoxin A4 inhibits atherosclerosis progression in apolipoprotein E-/- mice. *Br J Pharmacol*. 2017;174:4043–4054. doi: 10.1111/bph.13707
5. Petrosino S, Di Marzo V. The pharmacology of palmitoylethanolamide and first data on the therapeutic efficacy of some of its new formulations. *Br J Pharmacol*. 2017;174:1349–1365. doi: 10.1111/bph.13580
6. Gabrielsson L, Mattsson S, Fowler CJ. Palmitoylethanolamide for the treatment of pain: pharmacokinetics, safety and efficacy. *Br J Clin Pharmacol*. 2016;82:932–942. doi: 10.1111/bcp.13020
7. Lo Verme J, Fu J, Astarita G, La Rana G, Russo R, Calignano A, Piomelli D. The nuclear receptor peroxisome proliferator-activated receptor-alpha mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol Pharmacol*. 2005;67:15–19. doi: 10.1124/mol.104.006353
8. Ryberg E, Larsson N, Sjögren S, Hjorth S, Hermansson NO, Leonova J, Elebring T, Nilsson K, Drmota T, Greasley PJ. The orphan receptor GPR55 is a novel cannabinoid receptor. *Br J Pharmacol*. 2007;152:1092–1101. doi: 10.1038/sj.bjp.0707460
9. Artukoglu BB, Beyer C, Zuloff-Shani A, Brener E, Bloch MH. Efficacy of palmitoylethanolamide for pain: a meta-analysis. *Pain Physician*. 2017;20:353–362.
10. Alhouayek M, Muccioli GG. Harnessing the anti-inflammatory potential of palmitoylethanolamide. *Drug Discov Today*. 2014;19:1632–1639. doi: 10.1016/j.drudis.2014.06.007
11. Petrosino S, Cristino L, Karsak M, Gaffal E, Ueda N, Tüting T, Bisogno T, De Filippis D, D'Amico A, Saturnino C, Orlando P, Zimmer A, Iuvone T, Di Marzo V. Protective role of palmitoylethanolamide in contact allergic dermatitis. *Allergy*. 2010;65:698–711. doi: 10.1111/j.1398-9995.2009.02254.x
12. Esposito G, Capoccia E, Turco F, Palumbo I, Lu J, Steardo A, Cuomo R, Sarnelli G, Steardo L. Palmitoylethanolamide improves colon inflammation through an enteric glia/toll like receptor 4-dependent PPAR-α activation. *Gut*. 2014;63:1300–1312. doi: 10.1136/gutjnl-2013-305005
13. Di Paola R, Impellizzeri D, Mondello P, Velardi E, Aloisi C, Cappellani A, Esposito E, Cuzzocrea S. Palmitoylethanolamide reduces early renal dysfunction and injury caused by experimental ischemia and reperfusion in mice. *Shock*. 2012;38:356–366. doi: 10.1097/SHK.0b013e318267bbb9
14. Paterniti I, Di Paola R, Campolo M, Siracusa R, Cordaro M, Bruschetta G, Tremolada G, Maestroni A, Bandello F, Esposito E, Zerbini G, Cuzzocrea S. Palmitoylethanolamide treatment reduces retinal inflammation in streptozotocin-induced diabetic rats. *Eur J Pharmacol*. 2015;769:313–323. doi: 10.1016/j.ejphar.2015.11.035
15. Mazzari S, Canella R, Petrelli L, Marcolongo G, Leon A. N-(2-hydroxyethyl)hexadecanamide is orally active in reducing edema formation and inflammatory hyperalgesia by down-modulating mast cell activation. *Eur J Pharmacol*. 1996;300:227–236.
16. Mattace Raso G, Russo R, Calignano A, Meli R. Palmitoylethanolamide in CNS health and disease. *Pharmacol Res*. 2014;86:32–41. doi: 10.1016/j.phrs.2014.05.006
17. Borrelli F, Romano B, Petrosino S, Pagano E, Capasso R, Coppola D, Battista G, Orlando P, Di Marzo V, Izzo AA. Palmitoylethanolamide, a naturally occurring lipid, is an orally effective intestinal anti-inflammatory agent. *Br J Pharmacol*. 2015;172:142–158. doi: 10.1111/bph.12907
18. Vaia M, Petrosino S, De Filippis D, Negro L, Guarino A, Carnuccio R, Di Marzo V, Iuvone T. Palmitoylethanolamide reduces inflammation and itch in a mouse model of contact allergic dermatitis. *Eur J Pharmacol*. 2016;791:669–674. doi: 10.1016/j.ejphar.2016.10.005
19. Levula M, Airla N, Oksala N, et al. ADAM8 and its single nucleotide polymorphism 2662 T/G are associated with advanced atherosclerosis and fatal myocardial infarction: Tampere Vascular Study. *Ann Med*. 2009;41:497–507. doi: 10.1080/07853890903025945
20. Oksala N, Pärssinen J, Seppälä I, Raitoharju E, Kholova I, Ivana K, Hernesniemi J, Lyytikäinen LP, Levula M, Mäkelä KM, Sioris T, Kähönen M, Laaksonen R, Hytönen V, Lehtimäki T. Association of neuroimmune guidance cue netrin-1 and its chemorepulsive receptor UNC5B with atherosclerotic plaque expression signatures and stability in human(s): Tampere Vascular Study (TVS). *Circ Cardiovasc Genet*. 2013;6:579–587. doi: 10.1161/CIRCGENETICS.113.000141
21. Raitoharju E, Seppälä I, Lyytikäinen LP, Levula M, Oksala N, Klopp N, Illig T, Laaksonen R, Kähönen M, Lehtimäki T. A comparison of the accuracy of Illumina HumanHT-12 v3 expression BeadChip and TaqMan qRT-PCR gene expression results in patient samples from the Tampere Vascular Study. *Atherosclerosis*. 2013;226:149–152. doi: 10.1016/j.atherosclerosis.2012.10.078
22. Rinne P, Rami M, Nuutinen S, Santovito D, van der Vorst EPC, Guillamat-Prats R, Lyytikäinen LP, Raitoharju E, Oksala N, Ring L, Cai M, Hruby VJ, Lehtimäki T, Weber C, Steffens S. Melanocortin 1 receptor signaling regulates cholesterol transport in macrophages. *Circulation*. 2017;136:83–97. doi: 10.1161/CIRCULATIONAHA.116.025889
23. Jehle J, Hoyer FF, Schöne B, Pfeifer P, Schild K, Jenniches I, Bindila L, Lutz B, Lütjohann D, Zimmer A, Nickenig G. Myeloid-specific deletion of diacylglycerol lipase α inhibits atherogenesis in ApoE-deficient mice. *PLoS One*. 2016;11:e0146267. doi: 10.1371/journal.pone.0146267
24. Bindila L, Lutz B. Extraction and simultaneous quantification of endocannabinoids and endocannabinoid-like lipids in biological tissues. *Methods Mol Biol*. 2016;1412:9–18. doi: 10.1007/978-1-4939-3539-0_2
25. Daugherty A, Tall AR, Daemen MJAP, Falk E, Fisher EA, García-Cardeña G, Lusis AJ, Owens AP III, Rosenfeld ME, Virmani R; American Heart Association Council on Arteriosclerosis, Thrombosis and Vascular Biology; and Council on Basic Cardiovascular Sciences. Recommendation on design, execution, and reporting of animal atherosclerosis studies: a scientific statement from the American Heart Association. *Arterioscler Thromb Vasc Biol*. 2017;37:e131–e157. doi: 10.1161/ATV.0000000000000062
26. Zhu C, Solorzano C, Sahar S, Realini N, Fung E, Sassone-Corsi P, Piomelli D. Proinflammatory stimuli control N-acylphosphatidylethanolamine-specific phospholipase D expression in macrophages. *Mol Pharmacol*. 2011;79:786–792. doi: 10.1124/mol.110.070201
27. Montecucco F, Matias I, Lenglet S, Petrosino S, Burger F, Pelli G, Braunersreuther V, Mach F, Steffens S, Di Marzo V. Regulation and possible role of endocannabinoids and related mediators in hypercholesterolemic mice with atherosclerosis. *Atherosclerosis*. 2009;205:433–441. doi: 10.1016/j.atherosclerosis.2008.12.040
28. Puig O, Yuan J, Stepaniants S, et al. A gene expression signature that classifies human atherosclerotic plaque by relative inflammation status. *Circ Cardiovasc Genet*. 2011;4:595–604. doi: 10.1161/CIRCGENETICS.111.960773
29. Fruchart JC. Peroxisome proliferator-activated receptor-alpha activation and high-density lipoprotein metabolism. *Am J Cardiol*. 2001;88:24N–29N.
30. Cai B, Thorp EB, Doran AC, Sansbury BE, Daemen MJ, Dorweiler B, Spite M, Fredman G, Tabas I. MerTK receptor cleavage promotes plaque necrosis and defective resolution in atherosclerosis. *J Clin Invest*. 2017;127:564–568. doi: 10.1172/JCI90520
31. Ait-Oufella H, Pouresmail V, Simon T, Blanc-Brude O, Kinugawa K, Merval R, Offenstadt G, Lesèche G, Cohen PL, Tedgui A, Mallat Z. Defective mer receptor tyrosine kinase signaling in bone marrow cells promotes apoptotic cell accumulation and accelerates atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2008;28:1429–1431. doi: 10.1161/ATVBAHA.108.169078
32. Thorp E, Cui D, Schrijvers DM, Kuriakose G, Tabas I. MerTK receptor mutation reduces efferocytosis efficiency and promotes apoptotic cell accumulation and plaque necrosis in atherosclerotic lesions of apoE-/- mice. *Arterioscler Thromb Vasc Biol*. 2008;28:1421–1428. doi: 10.1161/ATVBAHA.108.167197
33. Solorzano C, Zhu C, Battista N, Astarita G, Lodola A, Rivara S, Mor M, Russo R, Maccarrone M, Antonietti F, Duranti A, Tontini A, Cuzzocrea S, Tarzia G, Piomelli D. Selective N-acylethanolamine-hydrolyzing acid amidase inhibition reveals a key role for endogenous palmitoylethanolamide in inflammation. *Proc Natl Acad Sci USA*. 2009;106:20966–20971. doi: 10.1073/pnas.0907417106
34. Geurts L, Everard A, Van Hul M, et al. Adipose tissue NAPE-PLD controls fat mass development by altering the browning process and gut microbiota. *Nat Commun*. 2015;6:6495. doi: 10.1038/ncomms7495
35. Leung D, Saghatelian A, Simon GM, Cravatt BF. Inactivation of N-acyl phosphatidylethanolamine phospholipase D reveals multiple mechanisms for the biosynthesis of endocannabinoids. *Biochemistry*. 2006;45:4720–4726. doi: 10.1021/bi060163l
36. Post JM, Loch S, Lerner R, Remmers F, Lomazzo E, Lutz B, Bindila L. Antiepileptogenic effect of subchronic palmitoylethanolamide treatment in a mouse model of acute epilepsy. *Front Mol Neurosci*. 2018;11:67. doi: 10.3389/fnmol.2018.00067
37. Gugliandolo E, Fusco R, Biundo F, D'Amico R, Benedetto F, Di Paola R, Cuzzocrea S. Palmitoylethanolamide and polydatin combination reduces inflammation and oxidative stress in vascular injury. *Pharmacol Res*. 2017;123:83–92. doi: 10.1016/j.phrs.2017.06.014
38. Moore KJ, Sheedy FJ, Fisher EA. Macrophages in atherosclerosis: a dynamic balance. *Nat Rev Immunol*. 2013;13:709–721. doi: 10.1038/nri3520

39. Ley K, Miller YI, Hedrick CC. Monocyte and macrophage dynamics during atherogenesis. *Arterioscler Thromb Vasc Biol*. 2011;31:1506–1516. doi: 10.1161/ATVBAHA.110.221127
40. Tabas I. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. *Arterioscler Thromb Vasc Biol*. 2005;25:2255–2264. doi: 10.1161/01.ATV.0000184783.04864.9f
41. Schrijvers DM, De Meyer GR, Kockx MM, Herman AG, Martinet W. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2005;25:1256–1261. doi: 10.1161/01.ATV.0000166517.18801.a7
42. Tabas I. Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol*. 2010;10:36–46. doi: 10.1038/nri2675
43. Sharir H, Console-Bram L, Mundy C, Popoff SN, Kapur A, Abood ME. The endocannabinoids anandamide and virodhamine modulate the activity of the candidate cannabinoid receptor GPR55. *J Neuroimmune Pharmacol*. 2012;7:856–865. doi: 10.1007/s11481-012-9351-6
44. Pertwee RG. GPR55: a new member of the cannabinoid receptor clan? *Br J Pharmacol*. 2007;152:984–986. doi: 10.1038/sj.bjp.0707464
45. Jiang L, Chen Y, Huang X, Yuan A, Shao Q, Pu J, He B. Selective activation of CB2 receptor improves efferocytosis in cultured macrophages. *Life Sci*. 2016;161:10–18. doi: 10.1016/j.lfs.2016.07.013
46. Sugiura T, Kondo S, Kishimoto S, Miyashita T, Nakane S, Kodaka T, Suhara Y, Takayama H, Waku K. Evidence that 2-arachidonoylglycerol but not N-palmitoylethanolamine or anandamide is the physiological ligand for the cannabinoid CB2 receptor. Comparison of the agonistic activities of various cannabinoid receptor ligands in HL-60 cells. *J Biol Chem*. 2000;275:605–612.
47. Chinetti G, Gbaguidi FG, Griglio S, Mallat Z, Antonucci M, Poulain P, Chapman J, Fruchart JC, Tedgui A, Najib-Fruchart J, Staels B. CLA-1/SR-BI is expressed in atherosclerotic lesion macrophages and regulated by activators of peroxisome proliferator-activated receptors. *Circulation*. 2000;101:2411–2417.
48. Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science*. 1996;271:518–520.
49. Jian B, de la Llera-Moya M, Ji Y, Wang N, Phillips MC, Swaney JB, Tall AR, Rothblat GH. Scavenger receptor class B type I as a mediator of cellular cholesterol efflux to lipoproteins and phospholipid acceptors. *J Biol Chem*. 1998;273:5599–5606.
50. Rosenson RS, Brewer HB Jr, Davidson WS, Fayad ZA, Fuster V, Goldstein J, Hellerstein M, Jiang XC, Phillips MC, Rader DJ, Remaley AT, Rothblat GH, Tall AR, Yvan-Charvet L. Cholesterol efflux and atheroprotection: advancing the concept of reverse cholesterol transport. *Circulation*. 2012;125:1905–1919. doi: 10.1161/CIRCULATIONAHA.111.066589
51. Zanoni P, Khetarpal SA, Larach DB, et al; CHD Exome+ Consortium; CARDIoGRAM Exome Consortium; Global Lipids Genetics Consortium. Rare variant in scavenger receptor BI raises HDL cholesterol and increases risk of coronary heart disease. *Science*. 2016;351:1166–1171. doi: 10.1126/science.aad3517
52. Covey SD, Krieger M, Wang W, Penman M, Trigatti BL. Scavenger receptor class B type I-mediated protection against atherosclerosis in LDL receptor-negative mice involves its expression in bone marrow-derived cells. *Arterioscler Thromb Vasc Biol*. 2003;23:1589–1594. doi: 10.1161/01.ATV.0000083343.19940.A0
53. Van Eck M, Bos IS, Hildebrand RB, Van Rij BT, Van Berkel TJ. Dual role for scavenger receptor class B, type I on bone marrow-derived cells in atherosclerotic lesion development. *Am J Pathol*. 2004;165:785–794. doi: 10.1016/S0002-9440(10)63341-X.
54. Kozarsky KF, Donahee MH, Glick JM, Krieger M, Rader DJ. Gene transfer and hepatic overexpression of the HDL receptor SR-BI reduces atherosclerosis in the cholesterol-fed LDL receptor-deficient mouse. *Arterioscler Thromb Vasc Biol*. 2000;20:721–727.
55. Arai T, Wang N, Bezouevski M, Welch C, Tall AR. Decreased atherosclerosis in heterozygous low density lipoprotein receptor-deficient mice expressing the scavenger receptor BI transgene. *J Biol Chem*. 1999;274:2366–2371.
56. Ridker PM, Everett BM, Thuren T, et al; CANTOS Trial Group. Antiinflammatory therapy with canakinumab for atherosclerotic disease. *N Engl J Med*. 2017;377:1119–1131. doi: 10.1056/NEJMoa1707914

Highlights

- Palmitoylethanolamide-synthesizing enzyme *N*-acyl phosphatidylethanolamine phospholipase D is downregulated in proinflammatory M1-type mouse macrophages and in the aorta of ApoE$^{-/-}$ mice during atherogenesis.
- *N*-acyl phosphatidylethanolamine phospholipase D expression is also reduced in human atherosclerotic plaques and particularly in advanced unstable lesions.
- Chronic palmitoylethanolamide treatment promotes atherosclerotic plaque stability in ApoE$^{-/-}$ mice as evidenced by reduced macrophage accumulation and necrotic core size, increased collagen deposition and downregulation of M1-type macrophage markers.
- Palmitoylethanolamide induces MerTK (proto-oncogene tyrosine-protein kinase MER) expression and promotes macrophage efferocytosis by activating GPR55.