MATERIALS AND METHODS

Melanocortin 1 Receptor Deficiency Promotes Atherosclerosis in Apolipoprotein E $^{-/-}$ Mice

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Mice

Recessive yellow (Mc1r$^{e/e}$) mice (Jackson Laboratory, strain# 000060, Bar Harbor, ME, USA) were intercrossed with apolipoprotein E deficient (Apoe$^{-/-}$) mice (Jackson Laboratory, strain# 002052) to generate double mutant Apoe$^{-/-}$ Mc1r$^{e/e}$ mice. Mc1r$^{e/e}$ and Apoe$^{-/-}$ mice were originally on the same C57Bl/6J background. Wild-type Apoe$^{-/-}$ mice were used as controls and they were generated from the same crossings as Apoe$^{-/-}$ Mc1r$^{e/e}$ mice. All experiments were performed on adult (4-6 months) male Apoe$^{-/-}$ and Apoe$^{-/-}$ Mc1r$^{e/e}$ mice. Animals were housed on a 12 h light/dark cycle and fed ad libitum a regular chow diet or a cholesterol-rich diet (RD Western Diet, D12079B, Research Diets Inc, NJ, USA) for the indicated times to promote atherosclerosis.

All animal experiments were approved by the local ethics committee (Animal Experiment Board in Finland, License Number: ESAVI/6280/04.10.07/2016) and conducted in accordance with the institutional and national guidelines for the care and use of laboratory animals.

Histology and Immunohistochemistry

Spleen and aortic root samples were fixed overnight in formalin, embedded in paraffin and then cut in 4 µm-thick serial sections. Sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome to measure atherosclerotic plaque area, the size of necrotic core and plaque collagen content. Aortic sections were also immunostained with anti-mouse Mac-2 (1:400, Cedarlane Labs, Burlington, ON, Canada), α-SMA (1:200, Sigma-Aldrich, St. Louis, MO, USA), iNOS (1:100, Abcam, Cambridge, United Kingdom) and CD206 (1:500, Abcam) antibodies followed by detection with a horseradish peroxidase-conjugated secondary antibody and diaminobenzidine (ABC kit, Vector Labs, Burlingame, USA) to determine macrophage- and smooth muscle cell-positive areas, respectively. For immunofluorescence, sections were stained with antibodies against iNOS (1:100, Abcam), VCAM-1 (1:200, Abcam), Mac-2 (1:400, Cedarlane) and/or CD11b (1:50, Biolegend) followed by detection with fluorochrome-conjugated secondary antibodies (anti-rabbit Alexa Fluor 647 and anti-rat Alexa Fluor 488, Jackson ImmunoResearch, West Grove, USA). Sections were counterstained with hemotoxylin (CarlRoth) or DAPI (Fluoroshield mounting medium, Abcam), coverslipped and then scanned with a digital slide scanner (Pannoramic 250 or Pannoramic Midi, 3DHISTECH Kft., Budapest, Hungary). Negative controls were obtained by incubating tissue sections with the corresponding secondary antibodies only (**Supplemental Figure X**). Images were analyzed using automated image analysis software (ImageJ, Fiji, NIH, Bethesda, MD, USA) and a color deconvolution method.[1-3]

En face Staining of the Aorta
Aortas from the iliac bifurcation to the aortic root were excised and the adventitia was removed. Samples were fixed in 10 % neutral-buffered formalin for 24 hours and stored in PBS at 4 °C until further use. The fixed samples were opened longitudinally, pinned flat, and stained with Sudan IV (Sigma-Aldrich) to quantify atherosclerotic lesion are. Samples were first immersed in 70% ethanol for 5 min, stained with filtered 0.5 % (wt/vol) Sudan IV solution for 6 min, destained with 80 % ethanol for 3 min and finally, washed with PBS. For quantitative analysis, images were captured using Zeiss Stemi 2000-C stereomicroscope and the atherosclerotic plaque area per total intimal area was determined using image analysis software (ImageJ, National Institutes of Health, Bethesda, Maryland, USA).

Liver Histology and Lipid Analysis
Whole liver was excised and weighed. A transverse piece of the left lobe was embedded in O.C.T compound (Tissue-Tek®, Sakura Finetek USA Inc, Torrance, CA, USA) for cryosectioning. Liver sections were therafter stained with Oil-Red-O and H&E. A separate piece of the left lobe (100 mg) was homogenized in 500 µL of PBS with 0.1 % NP-40 using TissueLyser and then centrifuged to remove insoluble material. Cholesterol was quantified in the liver homogenate using CHOD-PAP reagent (Roche, Basel, Switzerland) according to manufacturer's instructions.[3]

***In vivo* Reverse Cholesterol Transport (RCT) Assay**
Bone marrow cells from the femurs and tibiae of $Apoe^{-/-}$ were isolated and allowed to differentiate into bone marrow-derived macrophages (BMDM) for 7 days using IMDM-high glucose medium supplemented with 10 % FBS, penicillin (100 U/mL), streptomycin (100 µg/mL) and recombinant M-CSF (Biolegend). BMDMs were then labeled with ^{3}H-cholesterol (5 µCi/mL, PerkinElmer) and acetylated LDL (30 µg/mL, BioRad) in complete medium for 48 h. After labeling, cells were washed twice with PBS, equilibrated in IMDM medium containing 0.2 % BSA for 2 h, washed again twice with PBS and then suspended in PBS at a concentration of 10 million cells/mL. Labeled BMDMs (typically $4x10^6$ cells containing 3.5 $x10^6$ counts per minute (cpm)) were intraperitoneally injected into individually housed $Apoe^{-/-}$ and $Apoe^{-/-}$ $Mc1r^{e/e}$ mice. At 48 hours after the injection, mice were euthanized using an overdose of CO_2 and the blood was withdrawn by cardiac puncture. Remaining blood was flushed by transcardial perfusion with PBS and the whole liver was thereafter excised and weighed. Feces were collected, dried and weighed.

For the liquid scintillation counting (LSC), blood was centrifuged (10 min, 400 g) and 50 µl aliquots of the resulting plasma was directly mixed with 9 mL of LSC cocktail (Optiphase Hisafe, PerkinElmer) and measured in duplicate using a Wallac model 1410 liquid scintillation counter (Wallac, Turku, Finland). A 50 mg piece of the liver was homogenized with 1 mL of SOLVABLE (PerkinElmer) for 60 min at 50-60 °C. Sample was cooled to RT and 0.2 mL of 30 % H_2O_2 was added and then heated for 30 min at 50-60 °C. Twelve mL of LSC cocktail was added to the sample and then counted for radioactivity. Fecal samples (20 mg of dry weight) were first rehydrated and then mixed with 1 mL of SOLVABLE (PerkinElmer) and incubated at 50 °C for 2 hours. After cooling to RT, 30 % H_2O_2 (0.2 mL) was added dropwise and allowed to stand for 30 min at RT. Samples were heated again at 50 °C for 1 hour and after cooling to RT, 16mL of the LSC cocktail was added and the samples were finally

measured for radioactivity. RCT to plasma, liver and feces was calculated as a percentage of total injected radioactivity.

Analysis of Bile Acids and Neutral Sterols by Gas–Liquid Chromatography
Feces were collected over 48 h from individually housed mice. Bile was also collected from the same mice after euthanasia *via* CO_2 asphyxiation. Fecal samples were dried for 24 h at 50 °C, weighed and pulverized. Total bile acids were extracted and analyzed by a gas-liquid chromatography system equipped with a nonpolar Ultra 1 capillary column for bile acids and Ultra 2 column for neutral sterols (Agilent 6890N Network GC System, Agilent Technologies, Santa Clara, CA, USA). Standards (Sigma-Aldrich) were run to identify the following individual bile acids: cholic acid, chenodeoxycholic acid, β-muricholic acid, deoxycholic acid, epideoxycholic acid, litocholic acid, isolitocholic acid and ursodeoxycholic acid; the following neutral sterols: cholesterol and coprostanol; and following plant sterols: campesterol, campestanol, stigmasterol, sitosterol, sitostanol and avenasterol.[4,5] Quantification of total bile acids also included unidentified peaks.

Quantitative RT-PCR
Total RNA from aorta, bone marrow and liver was extracted (Qiagen, Venlo, Netherlands) and reverse-transcribed (Takara Clontech). Quantitative RT-PCR was performed using SYBR Green protocols (Kapa Biosystems, MA, USA) on an Applied Biosystems 7300 Real-Time PCR system.[3,6] Samples were run in duplicate. Primer sequences are given in Supplemental Table I. Target gene mRNA expression levels were normalized to the geometric mean of ribosomal protein S29 and β-actin using the comparative ΔCt method and are presented as relative transcript levels (2-ΔΔCt).

Plasma Analysis
Plasma was obtained from EDTA-anticoagulated whole blood after centrifugation. Plasma total cholesterol concentration was determined using a colorimetric assay (CHOD-PAP, Roche, Basel, Switzerland). Furthermore, plasma HDL and LDL/VLDL fractions were separated using LDL/VLDL precipitation buffer (Abcam) and cholesterol concentration in the resulting fractions was measured with a fluorometric assay kit (catalogue number: 10007640, Cayman Chemical). Pro-inflammatory cytokines and chemokines in the plasma were quantified with ProcartaPlex™ Multiplex Immunoassay (Chemokine 20-Plex Mouse Panel 1, catalogue number: EPX200-26090-901, eBioscience).

Flow Cytometry
Total leukocytes and their subsets in the aorta, spleen, bone marrow and whole blood samples from $Apoe^{-/-}$ and $Apoe^{-/-}$ $Mc1r^{e/e}$ were measured by flow cytometry, as previously descibed.[3] Briefly, aortas were flushed with PBS, excised from aortic root to iliac bifurcation and digested with an enzymatic cocktail (Collagenase I, 450 U/mL; Collagenase XI, 250 U/mL; Hyaluronidase, 120 U/mL; DNase I, 120 U/mL; Sigma Aldrich) for 60 min at 37°C and thereafter, single cell suspensions were prepared by filtering the aortic tissue lysate through a 50-µm cell strainer (BD Biosciences). For the analysis of aortic endothelial cells, aortas were digested with a cocktail of collagenase type 4 (Worthington Biochemical Corporation) and DNase I for 40 min at 37°C, and thereafter prepared for staining as described above. Spleen, bone marrow and peripheral blood samples were prepared for staining as previously described.

Subsequently, cells were stained for 30 min at 4°C with fluorochrome-conjugated antibodies against CD11b (clone M1/70, BioLegend), CD11c (clone N418, Biolegend), CD18 (clone M18/2, eBioscience), CD31 (clone MEC13.3, BD Biosciences), CD45.2 (clone 30-F11, BD Biosciences), CD49d (clone R1-2, eBioscience), CD54/ICAM-1 (clone 3E2, BD Biosciences), CD62L (clone MEL-14, BD Biosciences), CD62P (clone RMP-1, Biolegend), CD106/VCAM-1 (clone MVCAM.A, BD Biosciences), CD115 (clone AFS98, BioLegend), CD162/PSGL-1 (clone2PH1, BD Biosciences), F4/80 (clone BM8, BioLegend), Ly6C (clone AL-21, BD Biosciences), Ly6G (clone 1A8, BD Biosciences). Discrimination between live and dead cells was done by staining with a fixable viability dye for 15 min at RT (Zombie Aqua, Biolegend) before proceeding to the leukocyte staining. Data were acquired on LSRFortessa (BD Biosciences) and the results were analyzed using Flowjo v.10 (Flowjo, LLC, Ashland, USA).

Sorting of Splenic Monocytes

Monocyte subsets from the spleen of were isolated by fluorescence-activated cell sorting (FACS) using FACSAria IIu cell sorter (BD Biosciences). $Ly6C^{low}$ and $Ly6C^{high}$ were identified as $CD45^+$ $CD11b^+$ $Ly6G^-$ $CD115^+$ $Ly6C^{low}$ and $CD45^+$ $CD11b^+$ $Ly6G^-$ $CD115^+$ $Ly6C^{high}$ cells. From each mouse, ~ 50 000 $Ly6C^{low}$ and $Ly6C^{high}$ monocytes were collected directly into RNA stabilization buffer (RNAlater®, ThermoFisher) of the PicoPure RNA isolation kit (Arcturus). Total RNA was subsequently extracted using PicoPure® RNA isolation kit according to the manufacturer's instructions (Arcturus, ThermoFisher).

Integrin Binding Assay

Peripheral blood was obtained by cardiac puncture after euthanasia with an overdose of CO_2. Erythrocytes were first lysed in the whole blood samples and thereafter the remaining leukocytes were suspended in adhesion buffer (Hanks Balanced Salt Solution containing calcium chloride and magnesium chloride, and supplemented with 1 % bovine serum albumin). Leukocytes were left untreated or stimulated with CCL2 (Peprotech, 5 µg/mL) for 5 min at 37 °C in 100 µL of adhesion buffer containing soluble VCAM-1-Fc (R&D Systems, 5 µg/mL) and PE-conjugated anti-human IgG1 (Southern Biotechnology, Fc-specific, 1 µg/mL). After washing, cells were stained with fluorochrome-conjugated antibodies against CD45.2 (clone 30-F11, BD Biosciences), CD11b (clone M1/70, BioLegend), CD115 (clone AFS98, BioLegend), and Ly6C (clone AL-21, BD Biosciences) to identify $Ly6C^{high}$ monocytes. Binding of $Ly6C^{high}$ monocytes to VCAM-1 was measured by flow cytometry (LSRFortessa, BD Biosciences) and data were analyzed with FlowJo Software (10.1 Flowjo LLC).

Statistical Analyses

Statistical analyses were performed with GraphPad Prism 6 software (La Jolla, CA, USA). Comparisons between the mouse genotypes were made by unpaired Student's t test. Data that did not pass D'Agostino and Pearson normality test or had fewer than 8 samples per group were analyzed using the Mann-Whitney U test. Unpaired t test with Welch's correction was used for data that revealed unequal variances with the F test. For two independent factors, two-way analysis of variance (ANOVA) was used followed by Bonferroni *post hoc* tests. Data that had two independent factors and did not follow normal distribution were analyzed using the Kurskal-Wallis test followed by comparisons of the mean ranks of preselected pairs

of sample groups. All data are presented as mean ± standard error of the mean. A two-tailed *P* value of less than 0.05 was considered statistically significant.

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