Supplemental Material

Interleukin-17A deficiency accelerates unstable atherosclerotic plaque formation in apolipoprotein E-deficient mice

Keiko Danzaki, Yutaka Matsui, Masahiro Ikesue, Daichi Ohta, Koyu Ito, Masashi Kanayama, Daisuke Kurotaki, Junko Morimoto, Yoichiro Iwakura, Hideo Yagita, Hiroyuki Tsutsui, and Toshimitsu Uede

Manuscript ID#: ATVB/2011/229997

Expanded Materials and Methods

Mice

IL-17A-deficient mice used in this study were created as described previously¹. C57BL/6 ApoE-deficient male mice (ApoE⁺⁻) (backcrossed 10 times; The Jackson Laboratory, Bar Harbor, ME) were bred with IL-17A-deficient (IL-17A⁻⁻) female mice on a C57BL/6 background (backcrossed 10 times). Heterozygous F1 progeny were
interbred to yield F2 genotypes. IL-17A wild-type (WT) and IL-17A-deficient mice among ApoE-deficient mice were designated as ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$, respectively. These mice were intercrossed to yield ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$, which served as subjects in this experiment on a C57BL/6 background. ApoE deficiency in these mice was defined by a phenotype of elevated serum cholesterol in blood as described previously$^2$. IL-17A genotyping was performed by polymerase chain reaction analysis of tail DNA as described previously$^1$. All animal protocols were approved by the committee on animal experimentation of Hokkaido University.

**Diet and experimental design in the high-fat diet model**

Male ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice whose body weights were between 16.0 and 22.0 g were weaned at 6-8 weeks of age and fed an atherogenic high-fat diet (HFD) (0.15% cholesterol and 21% milk fat, 57BD; TestDiet, Richmond, USA) or normal chow diet ad libitum. Eight or 16 weeks after HFD or normal chow diet feeding, the mice were killed, and atherosclerosis was determined using an en face method$^2$. The heart and aorta from the aortic root to the iliac branch were removed; aortae were fixed in 10% phosphate-buffered formalin for histopathology. Aortic roots were embedded in OCT compound (Sakura Finetek) and stored at -80°C. The blocks were sectioned at 10
or 6 μm thickness and fixed for 10 min in 10% phosphate-buffered formalin at R.T. or acetone at -20°C.

**Analysis of atherosclerotic lesions**

The degree of atherosclerosis was determined by quantifying oil red O staining in en face lesions in pinned-out aortae

Briefly, the mice were perfused with PBS followed by 10% phosphate-buffered formalin. The aorta was opened longitudinally from the aortic root to the iliac branch and from the iliac bifurcation to a point equidistant from the aortic valve. The brachiocephalic artery was removed, pinned out flat on a black wax surface, and stained with oil red O. The aortas were then photographed, and the total surface and entire lesion areas were measured by planimetry.

**Histopathology**

Serial 6-μm sections were taken from the aortic valve or abdominal aorta area. Sections were fixed for 10 min in acetone at -20°C and used for immunohistochemistry. In another experiment, sections (10 μm thick) were fixed for 10 min in 10% phosphate-buffered formalin and oil red O stain or hematoxylin and eosin (H&E) to determine the plaque area.
Immunohistochemistry

A monoclonal antibody against α-smooth muscle actin (α-SMA), clone 1A4, was purchased from Sigma-aldrich. A monoclonal antibody against macrophages, MOMA-2, was purchased from Serotec. A rabbit polyclonal antibody against type I collagen was purchased from Abcam (ab21286). The sections were stained with MOMA-2 or 1A4 followed by biotin-conjugated goat anti-rat IgG (for MOMA-2) or rabbit anti-mouse IgG (for α-SMA, 1A4) followed by streptavidin-biotin peroxidase complex (Histofine kit; Nichirei). The sections were stained with an antibody against type I collagen, followed by horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG. The sections were subsequently counterstained with hematoxylin.

Lipid measurements and serum immunoglobulin titration

Serum levels of total cholesterol (Determiner TC555; KYOWA MEDEX), triglycerides (Determiner TG; KYOWA MEDEX), and high density lipoprotein (HDL) cholesterol (Determiner HDL; KYOWA MEDEX) were measured. Serum immunoglobulin (Ig) isotypes (IgG, IgG1, IgG2a, and IgM) and malondialdehyde (MDA)-low density lipoprotein (LDL)-specific antibodies were measured as described previously. To quantify MDA-LDL-specific antibodies, plates were coated with 100 μg/mL
MDA-LDL, washed, and blocked; sera were subsequently added at an optimized 1:100, 1:1,000 or 1:10,000 dilutions, and specific detection antibodies for IgG₁, IgG₂a (BETHYL), IgG, and IgM (Jackson ImmunoResearch Laboratories) were added. MDA-LDL was prepared as described previously.

**Cytokine analysis**

Eight weeks or 16 weeks after HFD feeding, spleen cells were removed and single-cell suspensions were obtained by passing the cells through a 100-μm cell strainer. CD4⁺ T cells were isolated from these cells using MACS separation columns (25 MS) with CD4 microbeads (L3T4; Miltenyi Biotec). These cells were stimulated in microwell cultures (6 x 10⁵/well) with 20 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 250 ng/mL ionomycin (Sigma-Aldrich) in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Wako). Supernatants were collected at 72 h and analyzed by ELISA for IFN-γ, IL-5, IL-4, IL-6, IL-10 (BD Biosciences), IL-17A (R & D Systems) and IL-17C (Uscn Life Science Inc).

**Intracellular cytokine staining**
Murine splenic CD4$^+$ T cells were cultured with 20 ng/mL PMA, 250 ng/mL ionomycin, and 1 μL/mL GolgiPlug (BD Biosciences) for 5.5 h. The cells were washed with FACS buffer (0.5% bovine serum albumin and 0.05% NaN$_3$ in PBS) and blocked with rat anti-mouse Fc receptor antibody, CD16/CD32 (2.4G2; BD Biosciences). The cells were then labeled with FITC-anti-CD3ε (145-2C11; BioLegend) and PE/Cy5-anti-CD4 (RM4-5; BioLegend). Next, the cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences). Intracellular cytokines were stained with PE-anti-IL-17A (BD Biosciences) and Alexa Fluor 647-anti IFN-γ (XMG1.2; BioLegend). All analyses were performed on a FACS Calibur flow cytometer (BD Biosciences) with FlowJo software (Tree Star).

**Experimental design for exogenous IL-17A administration**

ApoE$^{-/-}$ mice at ages of 6-8 weeks, fed the HFD for 12 weeks were used in this study. Additionally, ApoE$^{-/-}$IL-17A$^{-/-}$ mice at ages of 5 weeks, fed the HFD for 10 weeks were used. Both groups of mice were treated with recombinant mouse IL-17A (eBioscience) (2 μg/mouse) diluted in PBS 0.05% mouse albumin (Sigma) (200 μL/mouse) or PBS 0.05% albumin twice a week during HFD feeding.
Statistical analysis

Results are expressed as mean (SEM). Statistical significance between groups was estimated using Student’s $t$-test; $p < 0.05$ was considered statistically significant.
Supplemental Figure Legends

Supplemental Figure I. IL-17A deficiency did not affect atherosclerotic plaque formation in ApoE<sup>−/−</sup> mice under normal chow diet feeding.

Atherosclerotic plaque formation was quantitatively analyzed by staining of aortae with oil red O. ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> mice: day 0 (n = 5; each group), 8 weeks after normal chow diet feeding (normal diet 8w; n = 4 and 3, respectively), 16 weeks after normal chow diet feeding (normal diet 16w; n = 6; each group). *p < 0.05. **p < 0.005. N.S. denotes difference between two groups is not significantly different.

Supplemental Figure II. Atherosclerotic plaque area of abdominal aorta area was decreased in ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> mice compared to ApoE<sup>−/−</sup> mice.

A. Representative microphotographs of abdominal aortic sections stained with H&E in ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> mice after 8 weeks of HFD feeding. Scale bars indicate 30 μm. B. Quantitative analysis of plaque areas in both ApoE<sup>−/−</sup> (n= 8) and ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> (n= 8) mice. *p < 0.05.
Supplemental Figure III. Type I collagen-positive area was decreased in ApoE\textsuperscript{-/-}IL-17A\textsuperscript{-/-} mice compared to ApoE\textsuperscript{-/-} mice

A, Representative microphotographs of aortic root sections stained with type I collagen in ApoE\textsuperscript{-/-} and ApoE\textsuperscript{-/-}IL-17A\textsuperscript{-/-} mice after 8 weeks of HFD feeding. Scale bars in upper panels indicate 300 \( \mu \)m and in under panels indicate 50 \( \mu \)m. B, Quantitative analysis of the percentage of type I collagen-positive areas in both ApoE\textsuperscript{-/-} (n= 4) and ApoE\textsuperscript{-/-}IL-17A\textsuperscript{-/-} (n= 11) mice. *p < 0.05.

Supplemental Figure IV. IL-17A deficiency did not significantly affect IL-4, IL-6, IL-10, and IL-17C production in ApoE\textsuperscript{-/-} mice.

Quantitative analysis of IL-4 (A), IL-6 (B), IL-10(C), and IL-17C (D) production in the supernatants of splenic CD4-positive T cells from ApoE\textsuperscript{-/-} and ApoE\textsuperscript{-/-}IL-17A\textsuperscript{-/-} mice before (n= 4 and 6, respectively) and after 8 (n= 5 and 8, respectively) or 16 (n= 4 and 7, respectively) weeks of HFD feeding. Splenic CD4-positive T cells were cultured \textit{in vitro} with PMA and ionomycin; culture supernatants were examined by ELISA. Data were obtained from at least three independent experiments. *p < 0.05. N.D. denotes not detectable.
Supplemental Figure V. IL-17A deficiency did not significantly affect production of MDA-LDL-specific IgG2a, IgG, and IgM antibodies in ApoE<sup>−/−</sup> mice after HFD feeding.

Quantitative analysis of titers of MDA-LDL-specific antibodies, IgG2a(A), IgG(B), and IgM(C) in ApoE<sup>−/−</sup> (n= 21) and ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> (n= 35) mice before and after 8 or 16 weeks of HFD feeding. Values are indicated by the relative protein levels against MDA-LDL-specific antibody titers of ApoE<sup>−/−</sup> mice at day 0 and value at day 0 was set as 1. *p < 0.05. ***p < 0.0005. N.S., not significantly different. Note that only IgM class of anti-MDA-LDL antibody was reduced in ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> compared to ApoE<sup>−/−</sup> mice at 16 weeks after HFD feeding.
References


Supplemental Figure I

plaque area (%)

N.S.  N.S.

*  **  \( p=0.1 \)

day 0  normal diet  normal diet

8w  8w  16w
Supplemental Figure II
Supplemental Figure III

A

B

Supplemental Figure III

ApoE-/- type I collagen-positive area (%)
ApoE-/-/IL-17A-/-

B

Supplemental Figure III

ApoE-/- type I collagen-positive area (%)
ApoE-/-/IL-17A-/-

*
Supplemental Figure IV