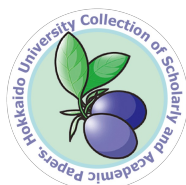




HOKKAIDO UNIVERSITY

Title	Interleukin-17A Deficiency Accelerates Unstable Atherosclerotic Plaque Formation in Apolipoprotein E-Deficient Mice
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Supplemental Material

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

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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 previously². IL-17A genotyping was performed by polymerase chain reaction analysis of tail DNA as described previously¹. 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 method². 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^{2,3}. 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, IgG_{2a}, and IgM) and malondialdehyde (MDA)-low density lipoprotein (LDL)-specific antibodies were measured as described previously.⁴ To quantify MDA-LDL-specific antibodies, plates were coated with 100 μ g/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, IgG_{2a} (BETHYL), IgG, and IgM (Jackson ImmunoResearch Laboratories) were added. MDA-LDL was prepared as described previously⁵.

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 μ L/mL GolgiPlug (BD Biosciences) for 5.5 h. The cells were washed with FACS buffer (0.5% bovine serum albumin and 0.05% NaN₃ 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-CD3 ϵ (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 μ g/mouse) diluted in PBS 0.05% mouse albumin (Sigma) (200 μ L/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^{-/-} mice under normal chow diet feeding.

Atherosclerotic plaque formation was quantitatively analyzed by staining of aortae with oil red O. ApoE^{-/-} and ApoE^{-/-}IL-17A^{-/-} 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^{-/-}IL-17A^{-/-} mice compared to ApoE^{-/-} mice.

A, Representative microphotographs of abdominal aortic sections stained with H&E in ApoE^{-/-} and ApoE^{-/-}IL-17A^{-/-} mice after 8 weeks of HFD feeding. Scale bars indicate 30 μm. B, Quantitative analysis of plaque areas in both ApoE^{-/-} (n = 8) and ApoE^{-/-}IL-17A^{-/-} (n = 8) mice. **p* < 0.05.

Supplemental Figure III. Type I collagen-positive area was decreased in

ApoE^{-/-}IL-17A^{-/-} mice compared to ApoE^{-/-} mice

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

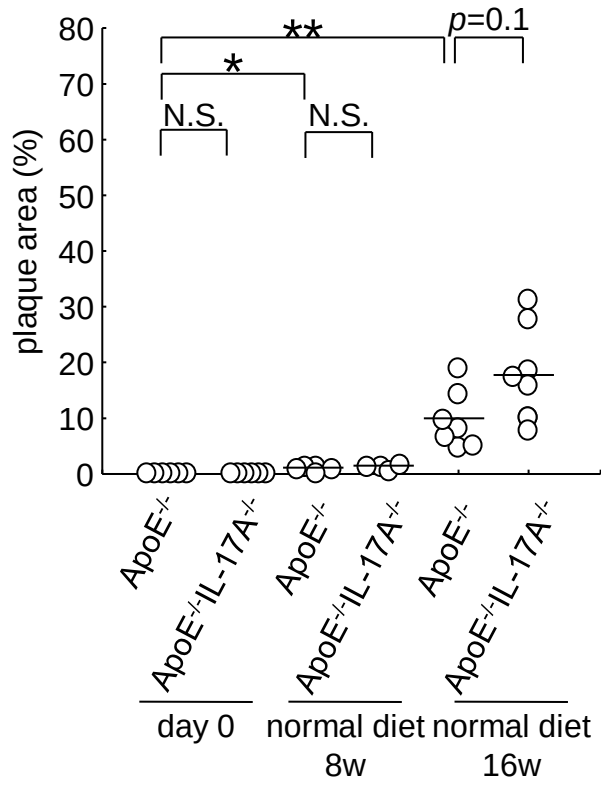
Supplemental Figure V. IL-17A deficiency did not significantly affect production of MDA-LDL-specific IgG_{2a}, IgG, and IgM antibodies in ApoE^{-/-} mice after HFD feeding.

Quantitative analysis of titers of MDA-LDL-specific antibodies, IgG_{2a}(A), IgG(B), and IgM(C) in ApoE^{-/-} (n= 21) and ApoE^{-/-}IL-17A^{-/-} (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^{-/-} 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^{-/-}IL-17A^{-/-} compared to ApoE^{-/-} mice at 16 weeks after HFD feeding.

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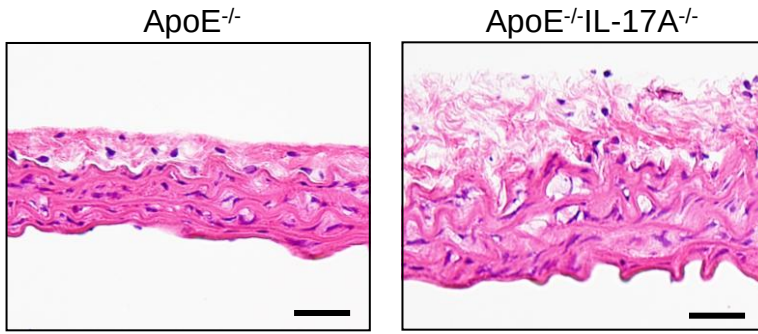
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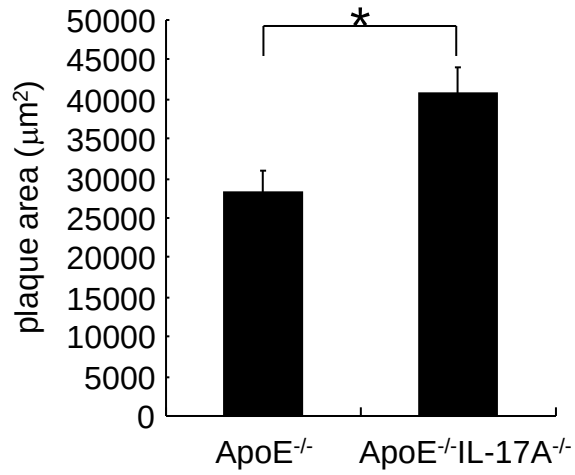


Supplemental Figure I

A

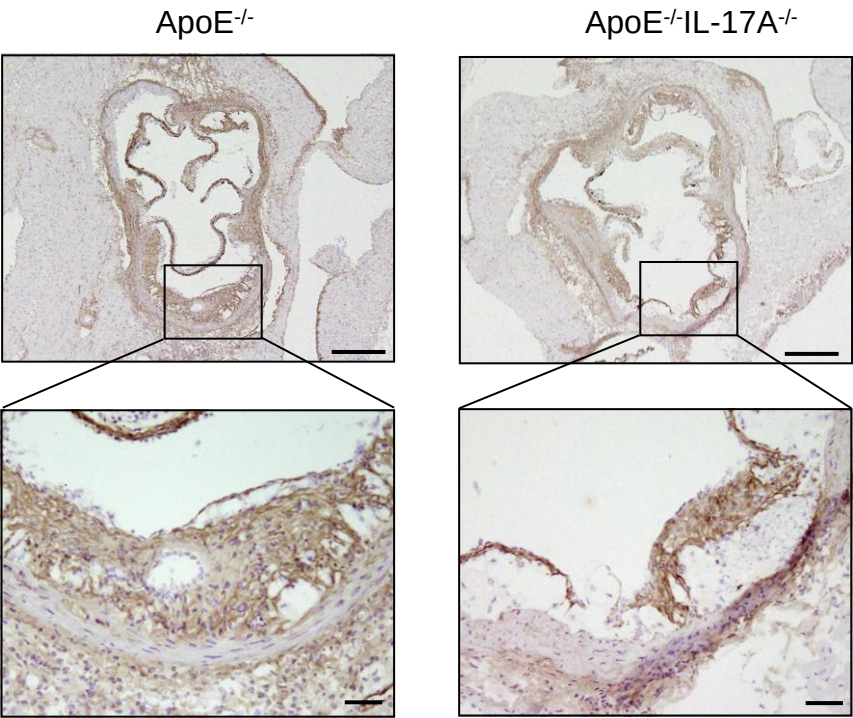


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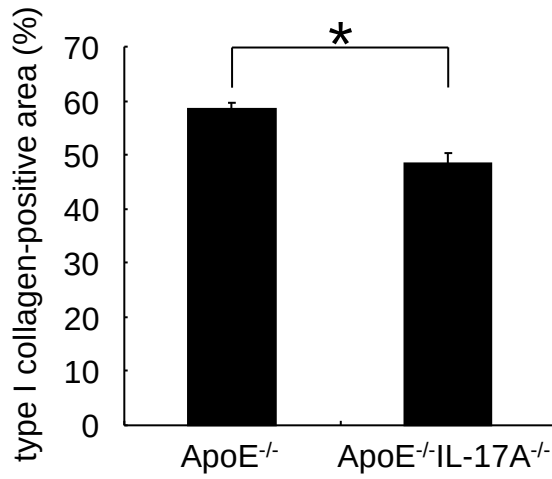


Supplemental Figure II

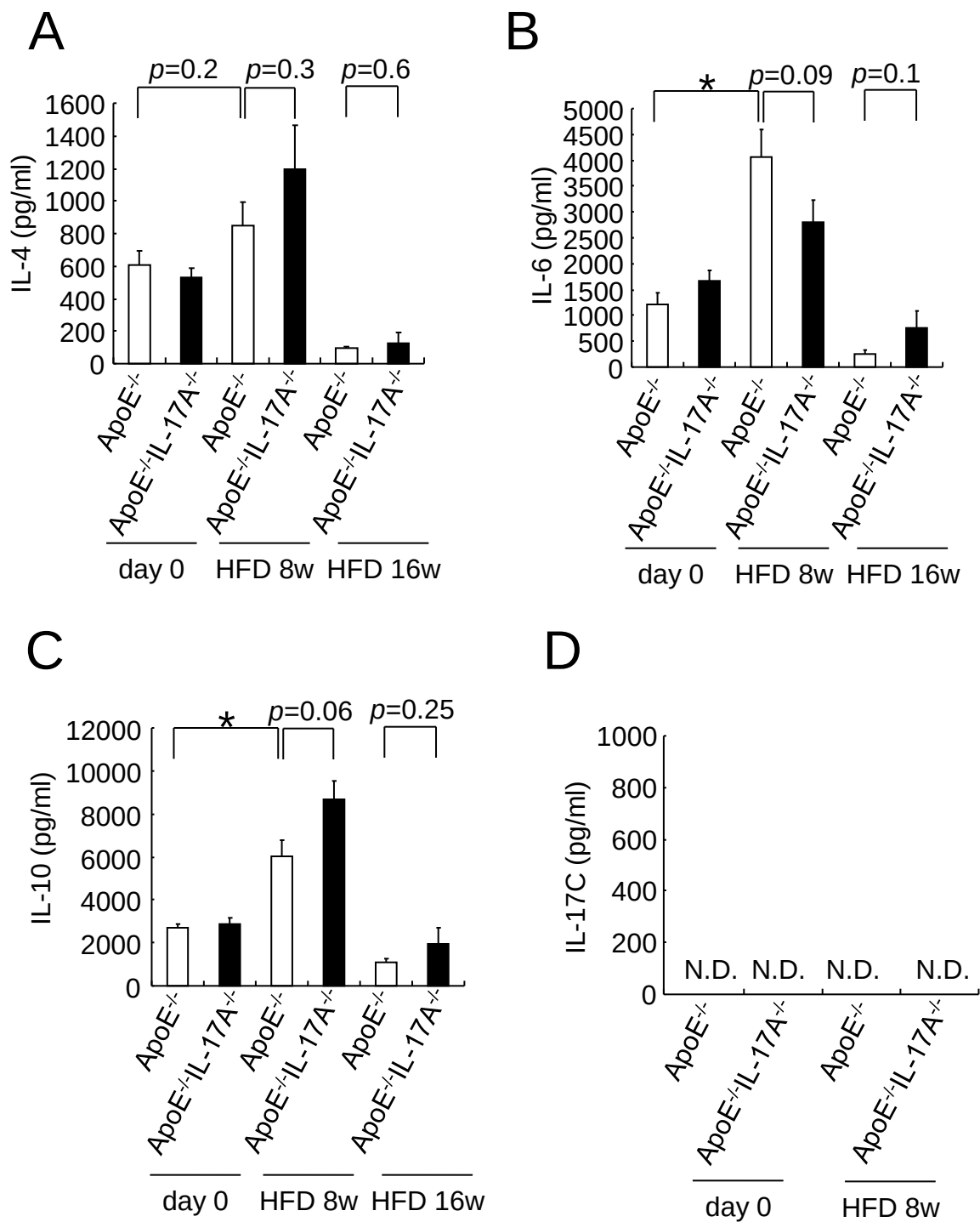
A



B

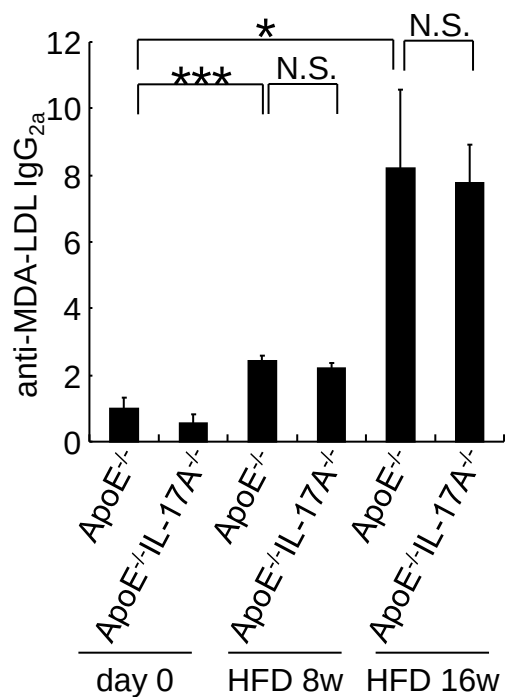


Supplemental Figure III

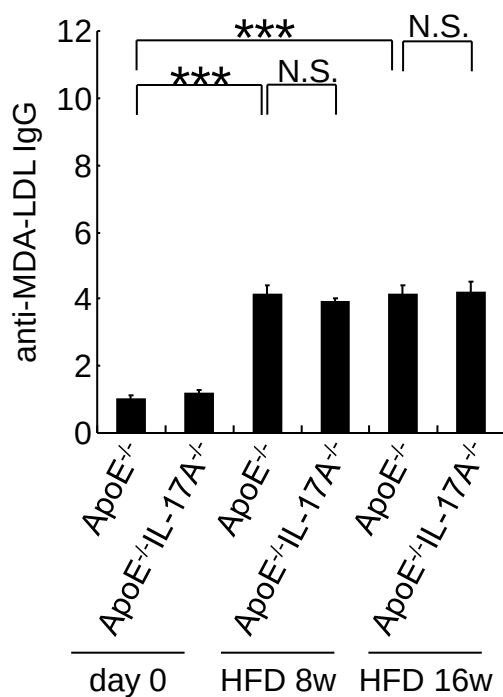


Supplemental Figure IV

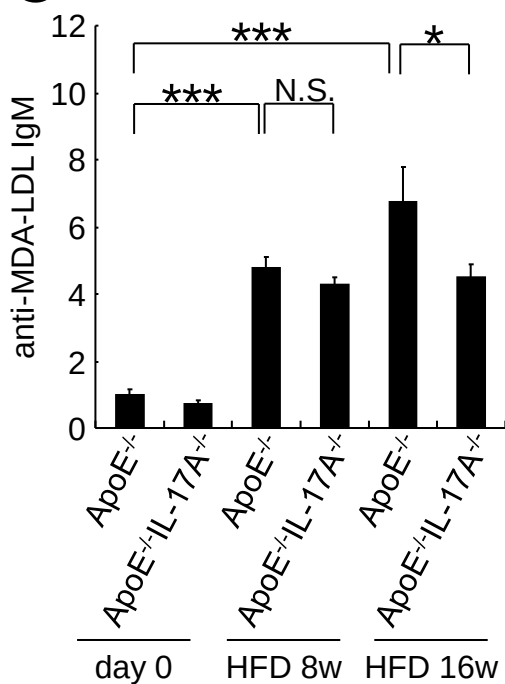
A



B



C



Supplemental Figure V