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

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Abstract

Objective: Interleukin-17A (IL-17A), an inflammatory cytokine, has been implicated in atherosclerosis, in which inflammatory cells within atherosclerotic plaques express IL-17A. However, its role in the development of atheroscelrosis remains to be controversial. **Methods and Results:** To directly examine the role of IL-17A in atherosclerosis, we generated apolipoprotein E (ApoE)/IL-17A double-deficient ($ApoE^{-/-}IL\text{-}17A^{-/-}$) mice. Mice were fed with high-fat diet (HFD) for either 8 or 16 weeks, both starting at ages of 6-8 weeks. We found that splenic $CD4^+$ T cells produced high amounts of IL-17A in $ApoE^{-/-}$ mice after HFD feeding for 8 weeks. Atherosclerosis was significantly accelerated in HFD-fed $ApoE^{-/-}IL\text{-}17A^{-/-}$ mice compared with $ApoE^{-/-}$ mice. Splenic $CD4^+$ T cells of $ApoE^{-/-}IL\text{-}17A^{-/-}$ mice after HFD feeding for 8 weeks, but not for 16 weeks exhibited increased IFN-γ and decreased IL-5 production. Importantly, formation of vulnerable plaque as evidenced by reduced numbers of vascular smooth muscle cells and reduced type I collagen deposition in the plaque was detected in $ApoE^{-/-}IL\text{-}17A^{-/-}$ mice after HFD feeding for 8 weeks. **Conclusions:** These results suggest that IL-17A regulates the early phase of atherosclerosis development after HFD feeding and plaque stability, at least partly if not all by modulating IFN-γ and IL-5 production from $CD4^+$ T cells.

Key Words: IL-17A, atherosclerosis, CD4 positive T cells, high fat diet, IFN-γ

Introduction

Atherosclerosis is characterized by chronic inflammation of vessel walls and is initiated by infiltration of monocytes and activated T cells into activated endothelium, followed by their migration into the intima and subsequent lipid accumulation within macrophages. Soluble mediators, such as inflammatory cytokines, produced by activated T cells also affect the development of atherosclerosis[1]. $CD4^+$ T cells are the predominant T-cell subset in atherosclerotic lesions in apolipoprotein E-deficient ($ApoE^{-/-}$) and low density lipoprotein (LDL) receptor-deficient ($LDLR^{-/-}$) mice[2]. Upon activation, $CD4^+$ T cells differentiate into different T helper (Th) cell subsets with different cytokine profiles and distinct effector functions[3]. Historically, $CD4^+$ T cells have been divided into Th1 and Th2 cells on the basis of the cytokines they produce. A third subset of interleukin-17A (IL-17A)-producing $CD4^+$ T, called Th17 cells, has been discovered[4]. Murine IL-17A is a 21-kDa glycoprotein[5] that is produced not only by $CD4^+$ T cells, but also by $CD8^+$ T cells, γδ T cells, neutrophils, and monocytes[6]. IL-17A induces the production of cytokines (e.g., IL-6, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor), chemokines (e.g., CXCL1, CXCL5, IL-8, CCL2, and CCL7), and matrix metalloproteinases (e.g., MMP-1, MMP-3, and MMP-13) from fibroblasts,

endothelial cells, and epithelial cells. This suggests that IL-17A plays an important role in inflammatory processes[6]. In addition, Th17 cells play a central role in the development of autoimmune diseases, such as experimental autoimmune encephalomyelitis and collagen-induced arthritis, which have been previously believed to be Th1 cell-mediated diseases[7, 8].

With regard to atherosclerosis, several previous papers reported the critical role of interferon gamma (IFN-γ). IFN-γ expression has been detected within human atherosclerotic lesions[9] and IFN-γ-deficient mice exhibit attenuated atherosclerosis, whereas injections of recombinant IFN-γ increase lesion size[10, 11]. Furthermore, Eid *et al*. reported the presence of both IL-17A and IFN-γ in clinical specimens of coronary atherosclerosis and the presence of IL-17A/IFN-γ dual-producing T cells within coronary plaques[12]. However, recent studies using atherosclerotic mouse models have indicated the crucial, but controversial role of IL-17A in the progression of atherosclerosis. Some researchers suggest that IL-17A promotes atherosclerotic plaque formation[13-17], or inhibition of IL-17A signaling does not alter lesion development in Th1-biased C57BL/6 ApoE$^{-/-}$ and LDLR$^{-/-}$ mice with already low levels of IL-17A production[18], whereas others suggest that IL-17A suppresses the development of atherosclerosis[19, 20]. Since it is still not clear why these studies found contradictory experimental results, we addressed whether IL-17A plays a role in

atherosclerosis by using mice which completely lack IL-17A (IL-17A-deficient ($IL\text{-}17A^{-/-}$) mice) by crossing them with ApoE-deficient mice, which is the most common mice model for human atherosclerotic disease[21, 22]. In this study, we determined whether and how IL-17A deficiency affects atherosclerotic plaque formation and discussed how our data can be incorporated into the previous controversial roles of IL-17A on atherosclerosis formation. It has been shown that premenopausal women have a significantly lower risk of developing atherosclerosis than age-matched men[23] and estrogen has anti-atherogenic role in animal models[24]. Therefore, we focused on male mice.

Materials and Methods

Expanded materials and methods are available in the supplemental files (available online at http://atvb.ahajournals.org).

Mice and induction of atherosclerosis

All animal protocols were approved by the committee on animal experimentation of Hokkaido University. IL-17A-deficient mice used in this study were created as described previously[25]. C57BL/6 ApoE-deficient male mice ($ApoE^{-/-}$) (backcrossed 10 times; The Jackson Laboratory, Bar Harbor, ME) were bred with IL-17A-deficient ($IL\text{-}17A^{-/-}$) female mice on a C57BL/6 background (backcrossed 10 times). IL-17A wild-type (WT) and IL-17A-deficient mice among ApoE-deficient mice were designated as $ApoE^{-/-}$ and $ApoE^{-/-}IL\text{-}17A^{-/-}$, respectively. Male $ApoE^{-/-}$ and $ApoE^{-/-}IL\text{-}17A^{-/-}$ mice 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) ad libitum for 8 or 16 weeks. Other $ApoE^{-/-}$ mice at ages of 6-8 weeks were fed with HFD for 12 weeks in the absence or presence of recombinant mouse IL-17 (eBioscience) (2 μg/mouse, twice per week).

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.

Results

IL-17A deficiency accelerated atherosclerotic plaque formation in ApoE$^{-/-}$ mice

Starting at ages of 6 or 8 weeks, male ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice were fed with HFD for 8 or 16 weeks and analyzed at ages of 14-16 or 22-24 weeks. Mean body weight (BW) was not significantly different between ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice before and after HFD feeding (BW, before diet, ApoE$^{-/-}$ = 20.2 ±0.2g, ApoE$^{-/-}$IL-17A$^{-/-}$ = 20.1±0.2g, 8 weeks after diet; ApoE$^{-/-}$ = 27.7±0.7g, ApoE$^{-/-}$IL-17A$^{-/-}$ = 29.6±0.9g, 16 weeks after diet; ApoE$^{-/-}$ = 31.4±0.7g, ApoE$^{-/-}$IL-17A$^{-/-}$ = 33.2±0.6g). The development of atherosclerosis in the entire aorta was carefully analyzed. Representative macroscopic findings in ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice are shown in Fig. 1; the acceleration of lesion formation (red-stained areas) is evident in ApoE$^{-/-}$IL-17A$^{-/-}$ mice compared with ApoE$^{-/-}$ mice at 8 or 16 weeks after HFD feeding (Fig. 1A and 1B, respectively). Individual data points are plotted by genotype in Fig. 1C.

Before HFD feeding, there was no obvious plaque formation in both ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice at ages of 6 or 8 weeks (Fig. 1C, day 0). Importantly, however, ApoE$^{-/-}$IL-17A$^{-/-}$ mice had significantly larger atherosclerotic lesions than ApoE$^{-/-}$ mice at both 8 and 16 weeks after HFD feeding, demonstrating that complete absence

of IL-17A in atherogenic prone mice, ApoE$^{-/-}$ further promotes the development of HFD-induced atherosclerosis. It should be noted that there was a tendency of slight increase of plaque area in ApoE$^{-/-}$IL-17A$^{-/-}$ mice compared with ApoE$^{-/-}$ mice at 16 weeks after normal chow diet feeding, however, there was no statistically significant difference between two groups (Supplemental Fig. I).

IL-17A deficiency did not significantly affect lipid metabolism in ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice

Lipid metabolism critically influences the complex processes of atherosclerosis [26, 27]. Total-, HDL-, LDL-cholesterol and triglycerides levels were determined in ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice before as well as at both 8 and 16 weeks after HFD feeding, to examine whether any of these factors were altered by the IL-17A genotype. Although the HFD significantly increased all cholesterol and triglycerides levels in ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice, the IL-17A genotype had no significant effect on any cholesterol and triglycerides levels in ApoE$^{-/-}$ mice before and even after HFD feeding (Fig. 2). These data indicate that the exacerbation in atherosclerotic lesions observed in ApoE$^{-/-}$IL-17A$^{-/-}$ mice is not attributable to the alterations in serum lipid metabolism.

Effect of IL-17A deficiency on the nature of atherosclerotic plaque in the aortic sections of ApoE$^{-/-}$ mice

To determine how IL-17A affects the nature of plaques, we investigated atherosclerotic plaques in the aortic root sections of mice fed with HFD for 8 weeks. Consistent with the data from en face method (Fig. 1), we found that IL-17A deficiency enhanced atherosclerotic plaque formation as defined by oil red O staining, in the aortic root sections of ApoE$^{-/-}$ mice (Fig. 3A). We also found that in not only aortic roots, but also abdominal aorta, atherosclerosis was prominent in ApoE$^{-/-}$IL-17A$^{-/-}$ mice at 8 weeks after HFD feeding (Supplemental Fig. II). In addition, MOMA-2-positive macrophage infiltration was greater in ApoE$^{-/-}$IL-17A$^{-/-}$ mice than ApoE$^{-/-}$ mice (Fig. 3B). More importantly, the α-SMA^{+} vascular smooth muscle cell (VSMC) number was significantly reduced at fibrous cap in ApoE$^{-/-}$IL-17A$^{-/-}$ mice compared to ApoE$^{-/-}$ mice (Fig. 3C). Furthermore, type I collagen-positive area was also decreased in ApoE$^{-/-}$IL-17A$^{-/-}$ mice compared to ApoE$^{-/-}$ mice (Supplemental Fig. III). These data demonstrate that IL-17A deficiency leads to the formation of atherosclerotic lesion that are composed of abundant macrophage, fewer α-SMA^{+}VSMC at fibrous cap, and lesser type I collagen deposition, suggesting that plaques formed in the absence of IL-17A are vulnerable atherosclerotic plaque.

Increased IFN-γ production and decreased IL-5 production in ApoE$^{-/-}$IL-17A$^{-/-}$ mice

Next, to assess the immunological profile of ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice, splenic CD4^{+} T cells were harvested from ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice before, 8 and 16 weeks after HFD feeding and cultured with PMA and ionomycin *in vitro*. Culture supernatants were collected and cytokine production was examined by ELISA. High amounts of IL-17A were detected in ApoE$^{-/-}$ mice 8 and 16 weeks after, but not before HFD feeding (Fig. 4A). Nevertheless, IL-17A was never detected in supernatants derived from ApoE$^{-/-}$IL-17A$^{-/-}$ mice before and after HFD feeding (data not shown). IFN-γ levels were higher in supernatants of the splenic CD4^{+} T cells from ApoE$^{-/-}$IL-17A$^{-/-}$ mice than those from ApoE$^{-/-}$ mice at 8, but not 16 weeks after HFD feeding (Fig. 4B). Supernatants from splenic CD4^{+} T cells of ApoE$^{-/-}$ mice after HFD feeding for 8 and 16 weeks showed elevated concentrations of IL-5 compared with those before HFD feeding, however, IL-5 production was significantly reduced in ApoE$^{-/-}$IL-17A$^{-/-}$ mice after HFD feeding for 8 and 16 weeks compared with ApoE$^{-/-}$ mice (Fig. 4C). Consistently, flow cytometry analysis showed that the number of IFN-γ positive splenic CD4^{+} T cells was greater in HFD-fed ApoE$^{-/-}$IL-17A$^{-/-}$ mice than that in HFD-fed ApoE$^{-/-}$ mice 8 weeks after HFD feeding (Fig. 4D and E). It should be noted that IFN-γ is important in the development of atherosclerosis,

whereas IL-5 has an atheroprotective role[28, 29]. On the other hand, IL-17A deficiency did not significantly affect IL-4, IL-6, IL-10, and IL-17C production in ApoE$^{-/-}$ mice (Supplemental Fig. IV). Therefore, these results suggest that IL-17A deficiency might modulate cytokines balance in the lymphoid tissue and induces a pro-atherogenic immunological response, thereby leading to the augmented atherosclerosis in HFD-fed ApoE$^{-/-}$ IL-17A$^{-/-}$ mice.

Reduced production of MDA-LDL-specific IgG$_1$ antibodies in sera of ApoE$^{-/-}$IL-17A$^{-/-}$ mice

We first assessed the levels of Ig subclasses in serum of mice before and after HFD feeding for 8 weeks. The rationale for doing this experiment is follows; a signature Th1 cytokine, IFN-γ promotes the development of arteriosclerosis and Th1 cells favor IgG$_{2a}$ production, while Th2 cells induce IgG$_1$ synthesis [30]. There was no significant difference in the production of total IgG, IgG$_1$, IgG$_{2a}$, or IgM between HFD-fed ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice (data not shown). We next assessed levels of MDA-LDL-specific antibodies, as they are likely to be some of the most prevalent presumptive autoantigens present in atherogenic mice model[31]. There is no significant difference in any subclasses of anti-MDA-LDL antibodies between ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice before HFD feeding (Fig. 5 and Supplementary Fig. V).

$ApoE^{-/-}IL\text{-}17A^{-/-}$ mice produced significantly lesser amounts of IgG_1 class of anti-MDA-LDL antibody compared to $ApoE^{-/-}$mice after HFD feeding for 8 weeks, but not for 16 weeks (Fig. 5). However, IgG_{2a} class of anti-MDA-LDL antibody did not differ between two groups (Supplementary Fig. V), indicating that the absence of IL-17A does not simply affect the balance between Th1 and Th2.

IL-17A treatment led to attenuated atherosclerosis plaque formation in $ApoE^{-/-}$ and $ApoE^{-/-}IL\text{-}17A^{-/-}$ mice

The results above suggest that IL-17A plays a protective role against the development of atherosclerosis. Therefore, we studied whether exogenous IL-17A prevents atherosclerotic plaque formation. Administration of IL-17A twice a week during HFD feeding led to a significant elevation of circulating IL-17A levels (data not shown). We analyzed $ApoE^{-/-}$ mice fed with the HFD for 12 weeks starting at ages of 8 weeks, treated with either IL-17A or mouse albumin/PBS during HFD feeding twice a week. Indeed, treatment with IL-17A resulted in attenuated atherosclerosis plaque formation in HFD-fed $ApoE^{-/-}$ mice (Fig. 6A). We also tested whether exogenous IL-17A, starting at ages of 5 weeks reduces the development of atherosclerosis in $ApoE^{-/-}IL\text{-}17A^{-/-}$ mice fed with the HFD for 10 weeks and found that IL-17A significantly reduced atherosclerotic plaque area in $ApoE^{-/-}IL\text{-}17A^{-/-}$ mice (Fig. 6B).

These results suggest that IL-17A plays a protective role against the development of atherosclerosis in this setting and that manipulation of IL-17A may be a therapeutic approach for the progression of atherosclerosis.

Discussion

Atherosclerosis is characterized as a chronic inflammatory process of vessel walls and $CD4^+$ T cells are predominant in both human and murine atherosclerotic lesions[9, 32]. Depletion of $CD4^+$ T or CD4 deficiency reduces fatty streak development in C57BL/6 mice fed with an atherogenic diet[33]. It has been shown that Th1 cell-derived IFN-γ or IL-12 is proatherogenic [10, 11, 34]. However, IL-17A-producing Th17 cells are also involved in the development of atherosclerosis; IL-17A is increased in the plasma of unstable angina and acute myocardial infarction patients[35] and IL-17A/IFN-γ dual-producing T cells are present within coronary plaques[12]. In addition, IL-17A induces MMP-9 production from macrophages, which is related to vulnerable plaque[36, 37] . However, recently conflicting roles of IL-17A on arteriosclerosis have been reported.

In contrary to our results, numbers of papers independently supported the notion that IL-17A is proatherogenic. Firstly, transfer of IL-17 signaling deficient bone marrow (BM) cells into atherosclerosis prone mice led to the significant reduction of atherosclerosis at aortic root, indicating that IL-17-signaling in BM-derived cells promotes the process of atherosclerosis[13]. Interestingly, plaque stability was unchanged. In their system, IL-17-signaling is lacking only in BM cells, while in our

system IL-17 signaling is lacking in both BM cells and vascular smooth muscle cells (VSMC). It is possible that VSMC in their system was able to respond to IL-17 and thus, these cells might produce MMPs, which might explain the unchanged plaque stability between mice that receive IL-17R$^{-/-}$BM and IL-17R^{+}BM cells. In our model, plaque became unstable in ApoE$^{-/-}$IL-17A$^{-/-}$ mice, indicating that the stimulation of VSMC by IL-17A may play a critical role in plaque stability. Secondly, ApoE$^{-/-}$ mice, starting at age of 8 weeks were fed with a normal chow diet for 12 weeks with anti-IL-17A antibody once a week and were analyzed at the age of 20 weeks[14]. Inhibition of IL-17A reduced atherosclerotic lesion area and induced stability of plaque. The basis for the discrepancy between their and our data is currently not known, however, blockade of IL-17A function is not complete in previous study[14], whereas in our study IL-17A is completely absent due to deletion of IL-17A gene. This may be reflected by the unchanged proportion of CD4^{+}IFN-γ^{+} Th1 cells against CD3^{+}T cells between anti-IL-17A antibody treated and non-treated groups in previous report. In our case, we found the significant increase of CD4^{+}IFN-γ^{+} Th1 cells and IFN-γ production in ApoE$^{-/-}$IL-17A$^{-/-}$ mice 8 weeks after HFD feeding (Fig. 4B, D, and E), consistent with the idea that IFN-γ is important in atherogenesis [38]. Thirdly, ApoE$^{-/-}$mice fed with HFD for 15 weeks exhibited increased Th17 cells when examined at age of 21 weeks[15]. The adenovirus-mediated blocking of IL-17 receptor

A (IL-17RA)-signaling reduced atherosclerosis and accumulation of macrophages at plaque. Importantly, IFN-γ levels did not differ between control and IL-17RA treated $ApoE^{-/-}$ mice[15] and it is possible that the adenovirus-mediated blocking of IL-17RA-signaling may not be complete and this may explain the discrepancy between our and their results. In another report, $ApoE^{-/-}$ mice were fed with HFD for 10 weeks, starting at age of 8 weeks and these mice were further treated with anti-IL-17A antibody for 4 weeks or recombinant IL-17A for 5 weeks, which resulted in the attenuation or exacerbation of atherosclerosis, respectively[16]. Exogenous IL-17A also promoted atherosclerotic lesions with instability of plaque[16]. Effects of IL-17A on atherosclerosis development was entirely opposite to what we found in this study. One possible explanation for this discrepancy may be the age of mice used and the timing of treatment intervention. It has been known that atherosclerosis process starts in childhood[39]. Thus under our experimental model, effect of IL-17A was tested during early stage of atherosclerosis development. While they examined the effect of IL-17A after atherosclerosis was already established or later phase of atherosclerosis development.

On the other hand, similar to what we found, there are reports that IL-17A is a protective against atherosclerosis. Firstly, Taleb *et al*. demonstrated that when SOCS3-deficient T cells with increased IL-17A production and reduced IFN-γ

production were given to HFD-fed LDLR$^{-/-}$ mice, the development of atherosclerotic plaque areas was significantly limited[19]. This cytokine profile reminded our finding that level of IL-17A and IFN-γ was inversely related in CD4^{+} T cells of HFD-fed ApoE$^{-/-}$ mice. Importantly, normal mouse aorta, VSMC of LDLR$^{-/-}$ that had received SOCS3-deficient T cells and VSMC in human arteriosclerosis expressed IL-17A. Along with progression of atherosclerosis, the IL-17A staining seems to be rapidly lost in VSMC, suggesting that vascular expression of IL-17A is associated with plaque stability. Similarly, HFD-fed ApoE$^{-/-}$IL-17A$^{-/-}$ mice, which completely lacks IL-17A in VSMC led to the formation of unstable plaque in our model (Fig.3). Therefore, IL-17A produced by not only T cells, but also vascular walls should be taken into consideration for the physiological and pathological role of IL-17A during development of arteriosclerosis. Secondly, CD20 antibody-mediated B-cell depletion in ApoE$^{-/-}$ and LDLR$^{-/-}$ mice led to skewing of T cell differentiation, more specifically toward Th17 cell differentiation, thus resulting in higher IL-17A and lesser IFN-γ production, and attenuated atherosclerosis[20]. Atherosclerotic lesions in IFN-γ- or IFN-γR-deficient mice were reduced, whereas IFN-γ-treated ApoE$^{-/-}$ mice showed enhanced atherosclerotic plaque areas[11, 40, 41], suggesting that IFN-γ has pro-atherogenic functions. In addition, IFN-γ produced vulnerable atherosclerotic plaque by inhibiting VSMC proliferation or collagen production[41, 42]. Therefore, it is

reasonable to speculate that IL-17A deficiency led to the increased IFN-γ production in splenic $CD4^+$ T cells, thereby facilitating the atherosclerotic plaque formation in HFD-fed $ApoE^{-/-}$ mice. In fact, we observed that MOMA-2^+ macrophages were increased and α-SMA^+VSMC were decreased in $ApoE^{-/-}IL\text{-}17A^{-/-}$ mice compared to $ApoE^{-/-}$ mice, supporting the idea that the increased production of IFN-γ is responsible for exacerbation of atherosclerosis and instability of atherosclerotic plaque in $ApoE^{-/-}IL\text{-}17A^{-/-}$ mice.

In addition, we also found that IL-5 and anti-MDA-LDL IgG_1 production was decreased in HFD-fed $ApoE^{-/-}IL\text{-}17A^{-/-}$ mice. Of note, anti-MDA-LDL antibody was reported to be anti-atherogenic in some studies[43]. In another report, IL-33 treatment reduced atherosclerotic plaque formation in $ApoE^{-/-}$ mice[44]. In that study, IL-33 promoted the production of IL-5 and anti-ox-LDL antibody and diminishing IFN-γ production[44] and played an anti-atherogenic role via production of IL-5. Therefore, it is possible that the impaired production of IL-5 led to the decreased anti-MDA-LDL IgG_1 production, thus accelerating atherosclerosis in HFD-fed $ApoE^{-/-}IL\text{-}17A^{-/-}$ mice[45, 46]. However, production of IL-4 by splenic $CD4^+$ T cells did not differ significantly between $ApoE^{-/-}$ and $ApoE^{-/-}IL\text{-}17A^{-/-}$ mice after HFD feeding in our model. Thus, our data indicate that the presence or absence of IL-17A does not simply favor the generation of Th2 cytokines in general.

During preparation of this manuscript, an important report using ApoE$^{-/-}$IL-17A$^{-/-}$ mice, which we also used in our study, was published[47]. They found that IL-17A did not influence atherosclerotic plaque development, however did influence some aspects of vascular inflammation and thus plaque stability[47]. They found no change in the extracellular matrix components in vascular walls by conventional Russell-Movat-pentachrome staining between ApoE$^{-/-}$IL-17A$^{-/-}$ and ApoE$^{-/-}$ after HFD feeding[47]. However, we found that type I collagen deposition (Supplemental Fig. III) and α-SMA^{+}VSMC (Fig. 3C) were decreased in plaque in the absence of IL-17A by using specific antibodies. They also found that ApoE$^{-/-}$IL-17A$^{-/-}$ mice were resistant to HFD-induced weight gain[47]. We found that both ApoE$^{-/-}$IL-17A$^{-/-}$ and ApoE$^{-/-}$ mice gained weight similarly after HFD feeding. Consistent with our data, Gao *et al*. reported that ApoE$^{-/-}$ mice gained significant weight after HFD feeding regardless of neutralization of IL-17A or addition of recombinant IL-17A[16]. The reason for this discrepancy between our data and their data[47] using the same ApoE$^{-/-}$IL-17A$^{-/-}$ mice is currently not known, however, we started feeding mice with HFD at earlier time compared to their treatment protocol[47]. Our data in which clear up-regulation of IFN-γ, reduction of IL-5, reduction of anti-MDA-LDL IgG_1, increased accumulation of macrophage and reduced appearance of α-SMA^{+}VSMC at fibrous cap of plaque are evident at HFD feeding for 8 weeks, indicating the possibility that IL-17A and thus

IFN-γ and IL-5 may play a role in early stage of atherosclerosis formation and once atherosclerosis was established, its progression or deterioration is not critically affected by IFN-γ.

In summary, IL-17A deficiency led to the formation of the unstable atherosclerotic plaque in HFD-fed $ApoE^{-/-}$ mice. These outcomes might be attributable to increased IFN-γ and decreased IL-5 productions in splenocytes and decreased IgG_1 antibody against MDA-LDL in sera. Moreover, treatment with IL-17A attenuated atherosclerosis progression in HFD-fed $ApoE^{-/-}$ mice. However, it should be reminded that the role of IL-17A in the development and nature (instability) of arteriosclerosis may be considerably influenced by multiple factors, such as cytokine profile (IFN-γ and IL-5), level of IL-17A (partial inhibition versus complete absence), IL-17A-signaling in VSMC and stage of atherosclerosis development. Therefore, the role of IL-17A on atherosclerosis development needs further investigation.

Figure Legends

Figure 1. IL-17A deficiency accelerates atherosclerotic plaque formation in ApoE$^{-/-}$ mice

Representative macroscopic image of aortae stained with oil red O in ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice 8 weeks (A) and 16 weeks (B) after HFD feeding. Scale bars indicate 10 mm. C, Quantitative analysis of oil red O-stained aortae. Horizontal bars indicate mean values. ApoE$^{-/-}$: day 0 (n = 5), 8 weeks (8w; n = 23), 16 weeks (16w; n = 34), ApoE$^{-/-}$IL-17A$^{-/-}$: day 0 (n = 5), 8 weeks (n = 22), 16 weeks (n = 24). $^{**}p < 0.005$. $^{***}p < 0.0005$. N.S., not significantly different.

Figure 2. IL-17A deficiency did not significantly affect lipid metabolism in ApoE$^{-/-}$ mice

To determine the effect of IL-17A deficiency on the level of serum cholesterol, we determined the concentrations of total cholesterol (A), triglycerides (B), HDL cholesterol (C), and LDL cholesterol in ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice before (day 0) and after HFD feeding (8 or 16 weeks). Day 0 and 8-week ApoE$^{-/-}$ (n = 13), day 0 and 8-week ApoE$^{-/-}$IL-17A$^{-/-}$ (n = 16), 16-week ApoE$^{-/-}$ (n = 16-19), 16-week

$ApoE^{-/-}IL\text{-}17A^{-/-}$ (n = 18-22). Open bars indicate $ApoE^{-/-}$ mice. Closed bars indicate $ApoE^{-/-}IL\text{-}17A^{-/-}$ mice. *$p < 0.05$. ***$p < 0.0005$. N.S., not significantly different.

Figure 3. Effect of IL-17A deficiency on the nature of atherosclerotic plaque in aortic root sections in $ApoE^{-/-}$ mice

A, Representative microphotographs of aortic root sections stained with oil red O in $ApoE^{-/-}$ (n= 11) and $ApoE^{-/-}IL\text{-}17A^{-/-}$ (n= 16) mice after 8 weeks of HFD feeding. Red-stained areas indicate lipid-rich plaque. Scale bars indicate 300 μm. Quantitative analysis of data was shown in right panel. B, Representative microphotographs of the aortic root sections stained with MOMA-2 in $ApoE^{-/-}$ (n= 11) and $ApoE^{-/-}IL\text{-}17A^{-/-}$ (n= 15) mice after 8 weeks of HFD feeding. Scale bars in upper panels indicate 300 μm and in lower panels indicate 50 μm. C, Representative microphotographs of aortic root sections stained with α-SMA in $ApoE^{-/-}$ (n= 11) and $ApoE^{-/-}IL\text{-}17A^{-/-}$ (n= 16) mice after 8 weeks of HFD feeding. Arrows indicate α-SMA positive areas. Scale bars in upper panels indicate 300 μm and in under panels indicate 50 μm. A, B and C, sections were counterstained with hematoxylin. Statistical evaluation of data shown in A, B and C were shown in right panels. *$p < 0.05$.

Figure 4. IFN-γ production was increased and IL-5 production was decreased in ApoE$^{-/-}$IL-17A$^{-/-}$ mice

Quantitative analysis of IL-17A (A), IFN-γ (B) and IL-5 (C) production in the supernatants of splenic CD4-positive T cells from ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice before (n= 4-6 per each group) and after 8 (n= 17-22 per each group) and 16 (n= 17-27 per each group) weeks of HFD feeding. Splenic CD4^{+} T cells were cultured *in vitro* with PMA and ionomycin; culture supernatants were examined by ELISA. D, Representative examples of intracellular IFN-γ and IL-17A staining of isolated splenocytes from ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice fed the HFD for 8 weeks. Plots are gated on CD3^{+} and CD4^{+} cells. E, Ratio of the number of IFN-γ-positive cells to the total number of splenic CD3^{+} and CD4^{+} T cells. Horizontal bars indicate mean values. ApoE$^{-/-}$ (n= 14), ApoE$^{-/-}$IL-17A$^{-/-}$ (n= 22). *$p < 0.05$. **$p < 0.005$. ***$p <$ 0.0005. N.S., not significantly different.

Figure 5. Production of MDA-LDL-specific IgG$_1$ antibodies was suppressed in the sera of ApoE$^{-/-}$IL-17A$^{-/-}$ mice

We examined the humoral response to IL-17A deficiency by quantifying MDA-LDL-specific antibody titers in sera. Quantitative analysis of the levels of MDA-LDL-specific antibodies, IgG$_1$ in ApoE$^{-/-}$ (n= 21) and ApoE$^{-/-}$IL-17A$^{-/-}$ (n= 35)

mice before and after 8 or 16 weeks of HFD feeding. *$p < 0.05$. **$p < 0.005$. ***$p < 0.0005$.

Figure 6. IL-17A treatment attenuated atherosclerosis plaque formation in ApoE$^{-/-}$ and ApoE$^{-/-}$IL-17A$^{-/-}$ mice

A, We determined whether exogenous IL-17A prevents atherosclerotic plaque formation. We treated ApoE$^{-/-}$ mice with recombinant mouse IL-17A (2 μg/mouse) diluted in PBS containing 0.05% mouse albumin or PBS containing 0.05% mouse albumin (albumin/PBS) twice a week during 12 weeks of HFD feeding. Representative macroscopic images of oil red O-stained aortae in ApoE$^{-/-}$ mice treated with (n= 8) or without (n= 10) IL-17A and quantitative analysis of oil red O-stained aortae. B, Additionally, we treated ApoE$^{-/-}$IL-17A$^{-/-}$ mice with recombinant mouse IL-17A (2 μg/mouse) or albumin/PBS twice a week during 10 weeks of HFD feeding. Representative macroscopic images of oil red O-stained aortae in ApoE$^{-/-}$ mice treated with (n= 9) or without (n= 7) IL-17A and quantitative analysis of oil red O-stained aortae. Scale bars indicate 10 mm. Horizontal bars indicate mean values. *$p < 0.05$.

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Disclosures

None.

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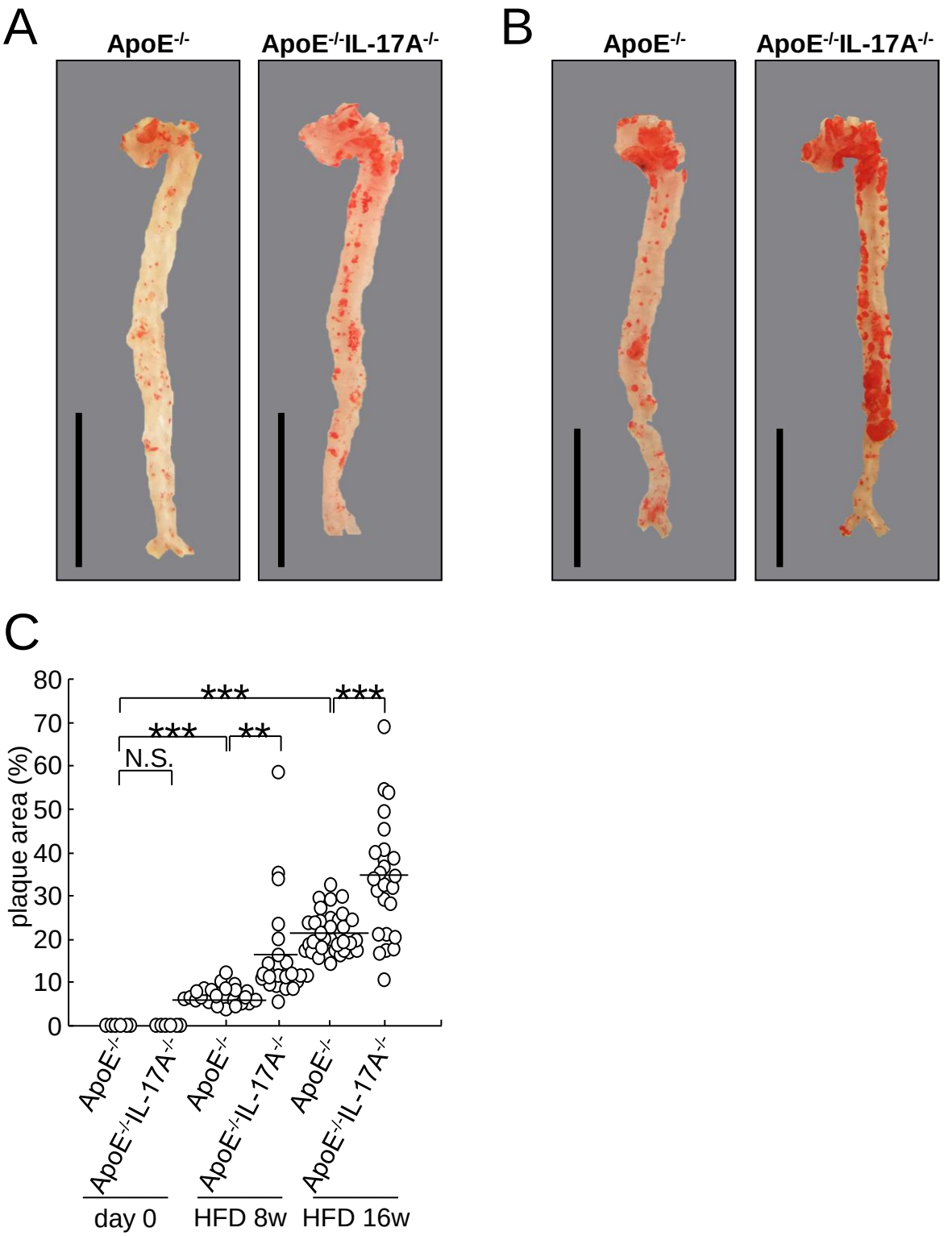

A
ApoE-/-
ApoE-/-IL-17A-/-
B
ApoE-/-
ApoE-/-IL-17A-/-
C
plaque area (%)
80
70
60
50
40
30
20
10
0
***
***
***
**
N.S.
ApoE-/-
ApoE-/-IL-17A-/-
ApoE-/-
ApoE-/-IL-17A-/-
ApoE-/-
ApoE-/-IL-17A-/-
day 0
HFD 8w
HFD 16w

Figure 1

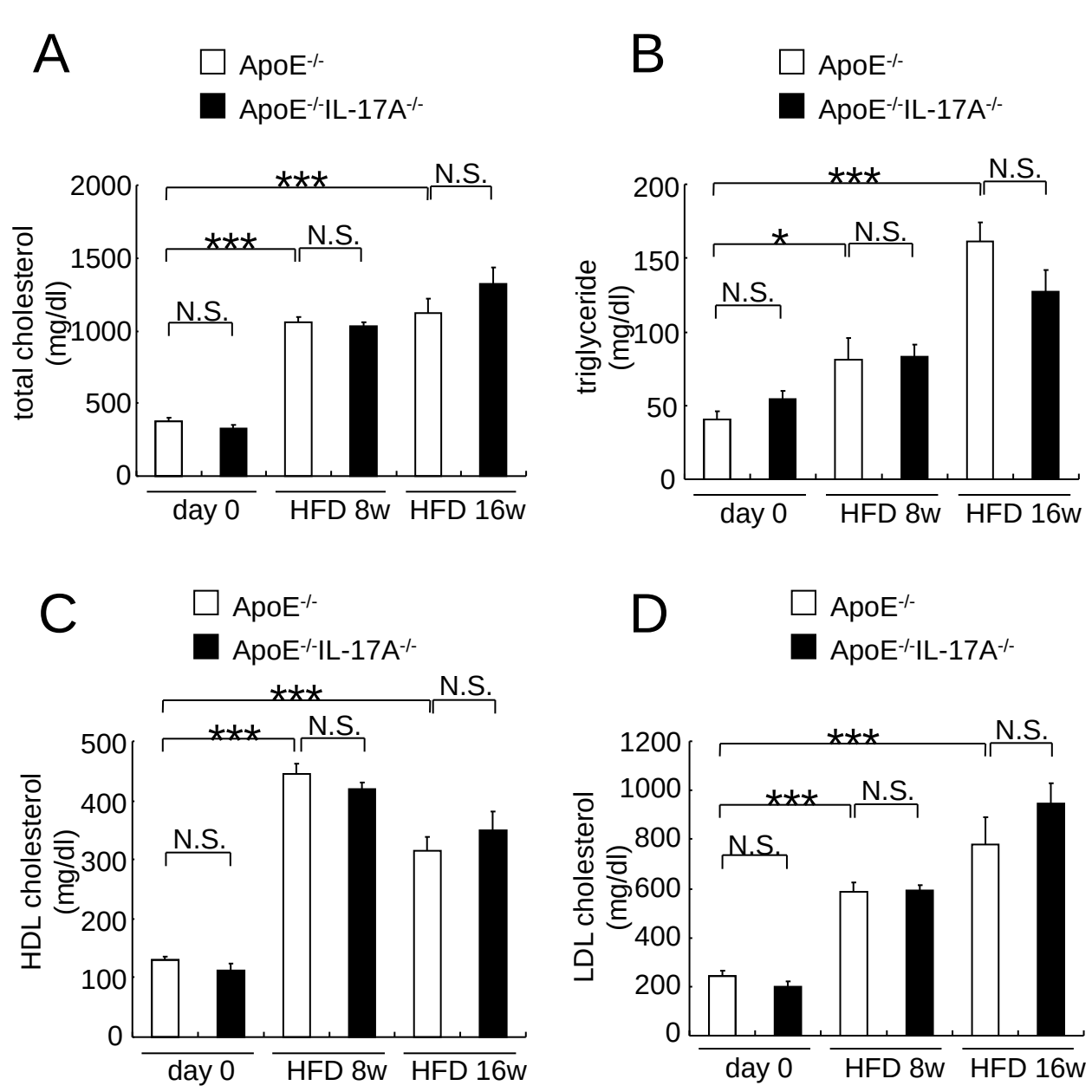
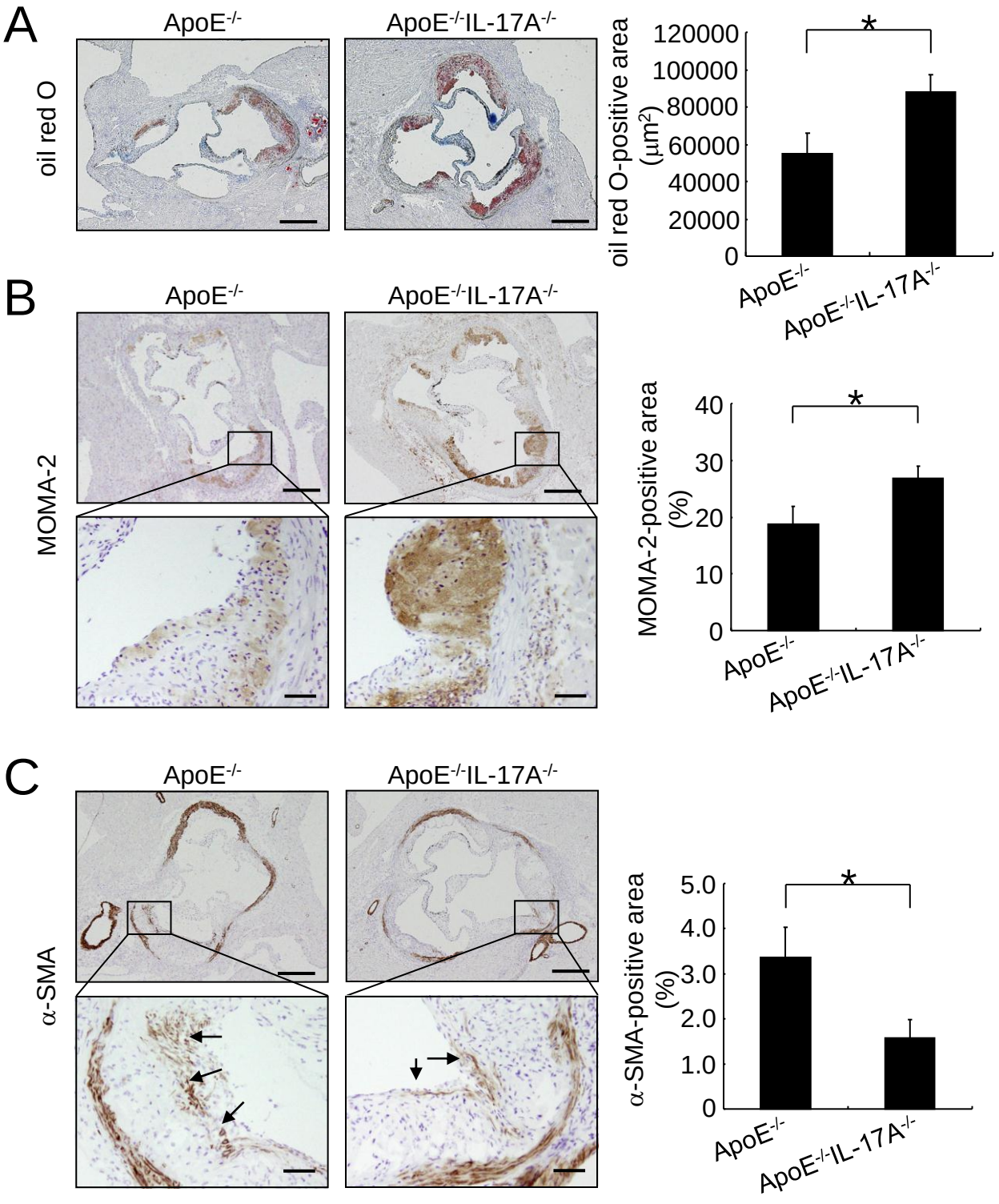

A
ApoE-/-
ApoE-/-IL-17A-/-
total cholesterol (mg/dl)
2000
1500
1000
500
0
***
N.S.
***
N.S.
N.S.
day 0
HFD 8w
HFD 16w
B
ApoE-/-
ApoE-/-IL-17A-/-
triglyceride (mg/dl)
200
150
100
50
0
***
N.S.
*
N.S.
N.S.
day 0
HFD 8w
HFD 16w
C
ApoE-/-
ApoE-/-IL-17A-/-
HDL cholesterol (mg/dl)
500
400
300
200
100
0
***
N.S.
***
N.S.
N.S.
day 0
HFD 8w
HFD 16w
D
ApoE-/-
ApoE-/-IL-17A-/-
LDL cholesterol (mg/dl)
1200
1000
800
600
400
200
0
***
N.S.
***
N.S.
N.S.
day 0
HFD 8w
HFD 16w

Figure 2


A
ApoE-/-
ApoE-/-IL-17A-/-
oil red O
oil red O-positive area (μm2)
120000
100000
80000
60000
40000
20000
0
*
ApoE-/-
ApoE-/-IL-17A-/-
B
ApoE-/-
ApoE-/-IL-17A-/-
MOMA-2
MOMA-2-positive area (%)
40
30
20
10
0
*
ApoE-/-
ApoE-/-IL-17A-/-
C
ApoE-/-
ApoE-/-IL-17A-/-
α-SMA
α-SMA-positive area (%)
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4.0
3.0
2.0
1.0
0
*
ApoE-/-
ApoE-/-IL-17A-/-

Figure 3

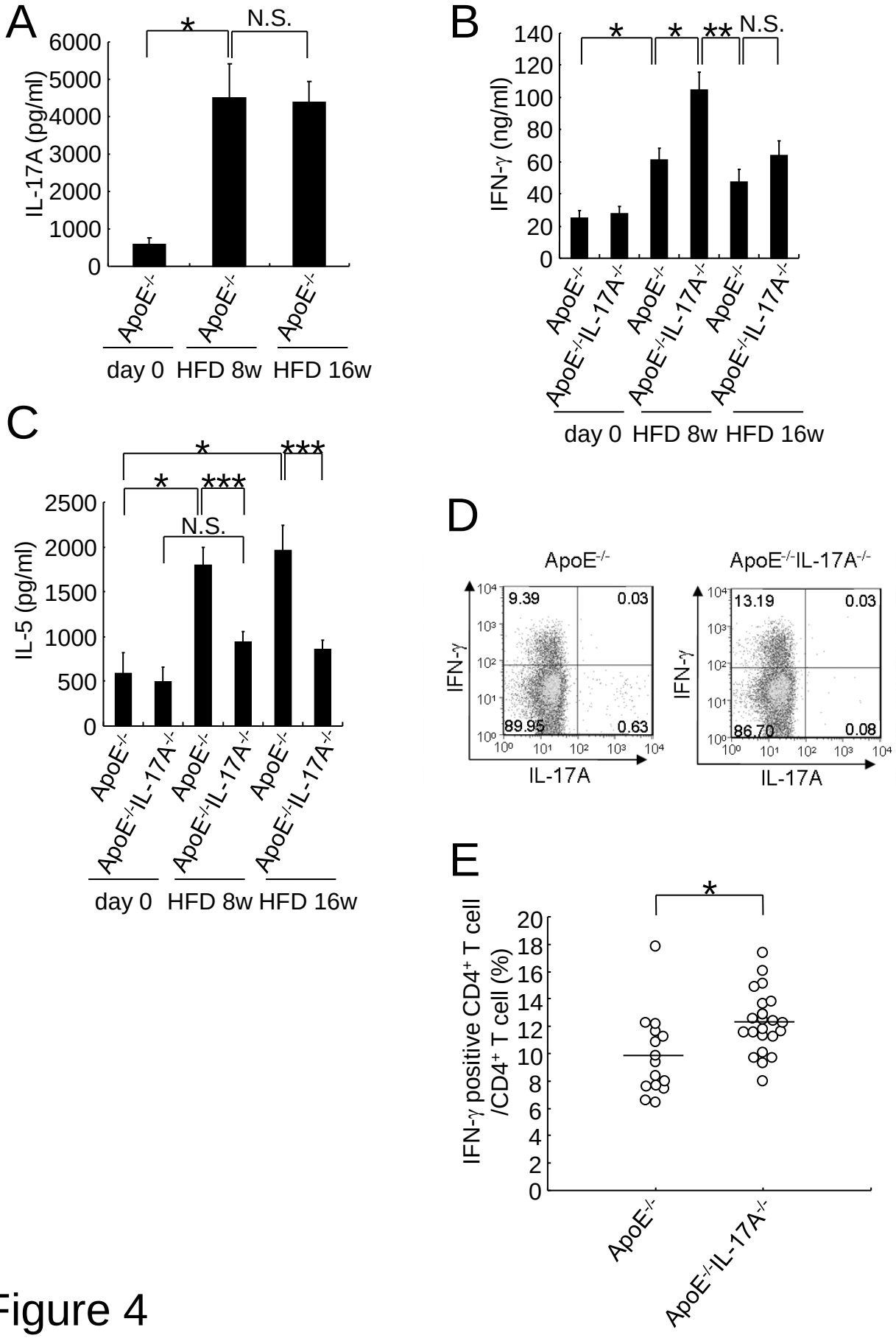
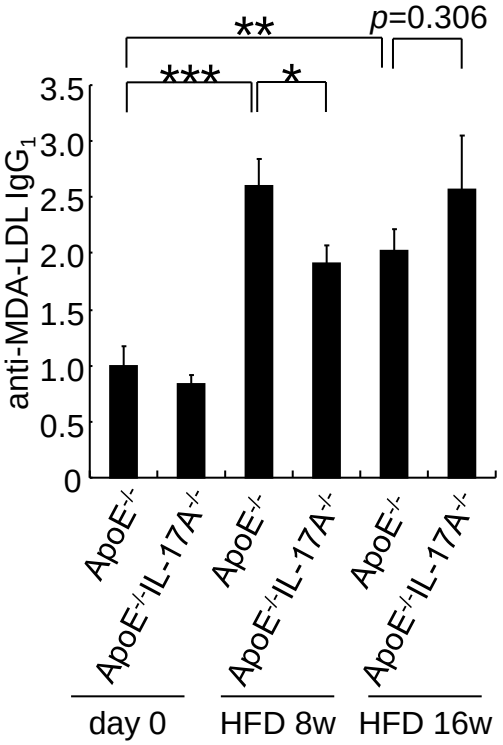

A
IL-17A (pg/ml)
6000
5000
4000
3000
2000
1000
0
*
N.S.
ApoE-/-
ApoE-/-
ApoE-/-
day 0
HFD 8w
HFD 16w
B
IFN-γ (ng/ml)
140
120
100
80
60
40
20
0
*
*
**
N.S.
ApoE-/-
ApoE-/-IL-17A-/-
ApoE-/-
ApoE-/-IL-17A-/-
ApoE-/-
ApoE-/-IL-17A-/-
day 0
HFD 8w
HFD 16w
C
IL-5 (pg/ml)
2500
2000
1500
1000
500
0
*
***
*
***
N.S.
ApoE-/-
ApoE-/-IL-17A-/-
ApoE-/-
ApoE-/-IL-17A-/-
ApoE-/-
ApoE-/-IL-17A-/-
day 0
HFD 8w
HFD 16w
D
ApoE-/-
ApoE-/-IL-17A-/-
IFN-γ
IL-17A
9.39
0.03
89.95
0.63
13.19
0.03
86.70
0.08
E
IFN-γ positive CD4+ T cell /CD4+ T cell (%)
20
18
16
14
12
10
8
6
4
2
0
*
ApoE-/-
ApoE-/-IL-17A-/-

Figure 4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1.5
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anti-MDA-LDL IgG1
ApoE-/-
ApoE-/-IL-17A-/-
ApoE-/-
ApoE-/-IL-17A-/-
ApoE-/-
ApoE-/-IL-17A-/-
day 0
HFD 8w
HFD 16w

Figure 5

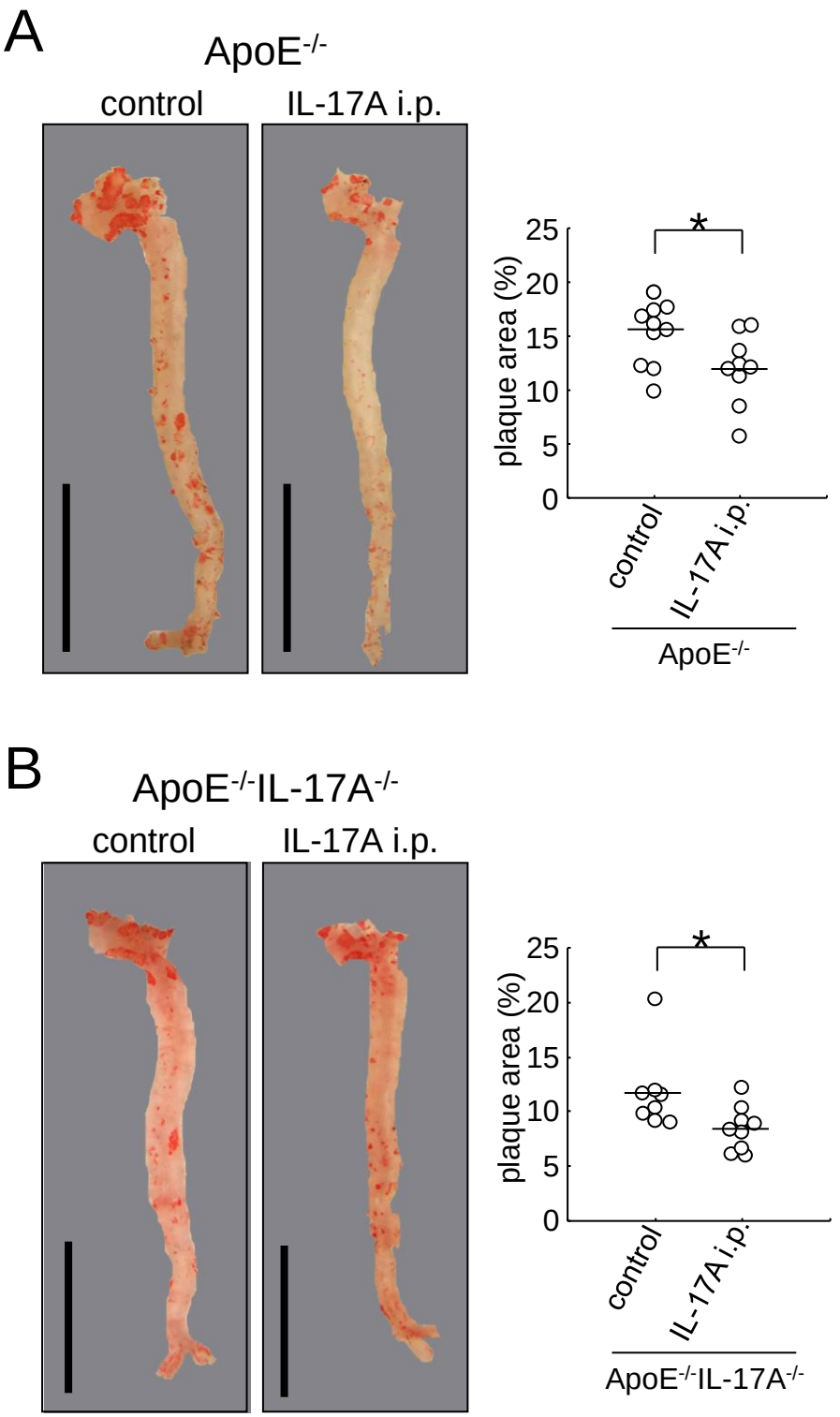
A
ApoE-/-
control
IL-17A i.p.
plaque area (%)
25
20
15
10
5
0
*
control
IL-17A i.p.
ApoE-/-
B
ApoE-/-IL-17A-/-
control
IL-17A i.p.
plaque area (%)
25
20
15
10
5
0
*
control
IL-17A i.p.
ApoE-/-IL-17A-/-

Figure 6