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

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Objective—Interleukin(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 atherosclerosis 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<sup>−/−</sup>IL-17A<sup>−/−</sup>) mice. Mice were fed with high-fat diet (HFD) for either 8 or 16 weeks, both starting at ages of 6 to 8 weeks. We found that splenic CD4<sup>+</sup> T-cells produced high amounts of IL-17A in ApoE<sup>−/−</sup> mice after HFD feeding for 8 weeks. Atherosclerosis was significantly accelerated in HFD-fed ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> mice compared with ApoE<sup>−/−</sup> mice. Splenic CD4<sup>+</sup> T-cells of ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> mice after HFD feeding for 8 weeks, but not for 16 weeks, exhibited increased interferon gamma 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<sup>−/−</sup>IL-17A<sup>−/−</sup> mice after HFD feeding for 8 weeks.

Conclusion—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 interferon gamma and IL-5 production from CD4<sup>+</sup> T-cells. (Arterioscler Thromb Vasc Biol. 2012;32:273-280.)

Key Words: atherosclerosis ■ CD4 positive T cells ■ high fat diet ■ interferon gamma ■ interleukin-17A

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. CD4<sup>+</sup> T-cells are the predominant T-cell subset in atherosclerotic lesions in apolipoprotein E-deficient (ApoE<sup>−/−</sup>) and LDL receptor-deficient (LDLR<sup>−/−</sup>) mice. On activation, CD4<sup>+</sup> T-cells differentiate into different T helper (Th) cell subsets with different cytokine profiles and distinct effector functions. Historically, CD4<sup>+</sup> T-cells have been divided into Th1 and Th2 cells on the basis of the cytokines they produce. A third subset of interleukin (IL)-17A-producing CD4<sup>+</sup> T, called Th17 cells, has been discovered. Murine IL-17A is a 21-kDa glycoprotein that is produced not only by CD4<sup>+</sup> T-cells, but also by CD8<sup>+</sup> T-cells, γδ T-cells, neutrophils, and monocytes. IL-17A induces the production of cytokines (eg, IL-6, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor), chemokines (eg, CXCL1, CXCL5, IL-8, CCL2, and CCL7), and matrix metalloproteinases (eg, 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. 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.

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 and IFN-γ-deficient mice exhibit attenuated atherosclerosis, whereas injections of recombinant IFN-γ increase...
producing T-cells within coronary plaques. 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, whereas others found contradictory experimental results, we addressed whether IL-17A plays a role in atherosclerosis by using mice that completely lack IL-17A (IL-17A−/−) and LDLR−/− mice with already low levels of IL-17A production, whereas others suggest that IL-17A suppresses the development of atherosclerosis. Because 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 that completely lack IL-17A (IL-17A−/−) mice by crossing them with ApoE-deficient mice, which is the most common mice model for human atherosclerotic disease. 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 and estrogen has antiatherogenic role in animal models. Therefore, we focused on male mice.

Materials and Methods

Expanded materials and methods are available in the Supplemental Data, 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. C57BL/6 ApoE-deficient male mice (ApoE−/−) (backcrossed 10 times; Jackson Laboratory, Bar Harbor, ME) were bred with IL-17A−/− female mice on a C57BL/6 background (backcrossed 10 times). IL-17A wild-type and IL-17A−/− mice among ApoE-deficient mice were designated as ApoE−/− and ApoE−/−IL-17A−/−, respectively. Male ApoE−/− and ApoE−/−IL-17A−/− mice were weaned at 6 to 8 weeks of age and fed an atherogenic high-fat diet (HFD) (0.15% cholesterol and 21% milk fat, 57BD; TestDiet, Richmond, IN) ad libitum for 8 or 16 weeks. Other ApoE−/− mice at ages of 6 to 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 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 to 16 or 22 to 24 weeks. Mean body weight was not significantly different between ApoE−/− and ApoE−/−IL-17A−/− mice before and after HFD feeding (body weight before diet, ApoE−/−=20.2±0.2 g. ApoE−/−IL-17A−/−=20.1±0.2 g. 8 weeks after diet; ApoE−/−=27.7±0.7 g. ApoE−/−IL-17A−/−=29.6±0.9 g. 16 weeks after diet; ApoE−/−=31.4±0.7 g. ApoE−/−IL-17A−/−=33.2±0.6 g). The development of atherosclerosis in the entire aorta was carefully analyzed. Representative macroscopic findings in ApoE−/− and ApoE−/−IL-17A−/− mice are shown in Figure 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 (Figure 1A and 1B, respectively). Individual data points are plotted by genotype in Figure 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 (Figure 1C, day 0). Importantly, however, ApoE<sup>−/−</sup> IL-17A<sup>−/−</sup> mice had significantly larger atherosclerotic lesions than ApoE<sup>−/−</sup> mice at both 8 and 16 weeks after HFD feeding, demonstrating that complete absence of IL-17A in athogenic prone mice, ApoE<sup>−/−</sup> 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<sup>−/−</sup>IL-17A<sup>−/−</sup> mice compared with ApoE<sup>−/−</sup> mice at 16 weeks after normal chow diet feeding, however, there was no statistically significant difference between 2 groups (Supplemental Figure I).

**IL-17A Deficiency Did Not Significantly Affect Lipid Metabolism in ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> Mice**

Lipid metabolism critically influences the complex processes of atherosclerosis. Total-, HDL-, and LDL-cholesterol and triglycerides levels were determined in ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> 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<sup>−/−</sup> and ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> mice, the IL-17A genotype had no significant effect on any cholesterol and triglycerides levels in ApoE<sup>−/−</sup> mice before and even after HFD feeding (Figure 2). These data indicate that the exacerbation in atherosclerotic lesions observed in ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> mice is not attributable to the alterations in serum lipid metabolism.

**Figure 2.** Interleukin (IL)-17A deficiency did not significantly affect lipid metabolism in apolipoprotein E (ApoE) double-deficient (ApoE<sup>−/−</sup>) 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 (D) in ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> mice before (day 0) and after HFD feeding (8 or 16 weeks). Day 0 and 8-week ApoE<sup>−/−</sup> (n=13), day 0 and 8-week ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> (n=16), 16-week ApoE<sup>−/−</sup> (n=16–19), 16-week ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> (n=18–22). Open bars indicate ApoE<sup>−/−</sup> mice. Closed bars indicate ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> mice. *P<0.05. **P<0.005. N.S., not significantly different.

**Figure 3.** Effect of interleukin (IL)-17A deficiency on the nature of atherosclerotic plaque in apolipoprotein E (ApoE) double-deficient (ApoE<sup>−/−</sup>) mice. A, Representative microphotographs of aortic root sections stained with oil red O in ApoE<sup>−/−</sup> (n=11) and ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> (n=16) mice after 8 weeks of high-fat diet (HFD) feeding. Red-stained areas indicate lipid-rich plaque. Scale bars indicate 300 μm. Quantitative analysis of data were shown in right panel. B, Representative microphotographs of the aortic root sections stained with MOMA-2 in ApoE<sup>−/−</sup> (n=11) and ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> (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-smooth muscle actin (SMA) in ApoE<sup>−/−</sup> (n=11) and ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> (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.

**Effect of IL-17A Deficiency on the Nature of Atherosclerotic Plaque in the Aortic Sections of ApoE<sup>−/−</sup> 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 (Figure 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<sup>−/−</sup> mice (Figure 3A). We also found that in not only aortic roots, but also abdominal aorta, atherosclerosis was prominent in ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> mice at 8 weeks after HFD feeding (Supplemental Figure II). In addition, MOMA-2–positive macrophage infiltration was greater in ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> mice than ApoE<sup>−/−</sup> mice (Figure 3B). More importantly, the α-smooth muscle actin (SMA)+ vascular smooth muscle cell (VSMC) number was significantly reduced at fibrous cap in ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> mice.
compared to ApoE−/− mice (Figure 3C). Furthermore, type I collagen-positive area was also decreased in ApoE−/− IL-17A−/− mice compared to ApoE−/− mice (Supplemental Figure 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 immunologic profile of ApoE−/− and ApoE−/− IL-17A−/− mice, splenic CD4+ T-cells were harvested from ApoE−/− and ApoE−/− IL-17A−/− mice before and 8 and 16 weeks after HFD feeding (Figure 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 (Figure 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 (Figure 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 (Figure 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 Figure IV). Therefore, these results suggest that IL-17A deficiency might modulate cytokines’ balance in the lymphoid tissue and induces a proatherogenic immunologic response, thereby leading to the augmented atherosclerosis in HFD-fed ApoE−/− IL-17A−/− mice.

**Reduced Production of MDA-LDL-Specific IgG1 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 IgG1 production, whereas Th2 cells induce IgG2a synthesis.30 There was no significant difference in the production of total IgG, IgG1, IgG2a, 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 (Figure 5 and Supplementary Figure V). ApoE−/− IL-17A−/− mice produced significantly lesser amounts of IgG1 class of anti-MDA–LDL antibody compared to ApoE−/− mice after HFD feeding for 8 weeks but not for 16 weeks (Figure 5). However, IgG2a class of anti-MDA–LDL antibody did not differ between 2 groups (Supplementary Figure 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-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<sup>−/−</sup>/H11002 mice fed with the HFD for 12 weeks starting at age 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<sup>−/−</sup> mice (Figure 6A). We also tested whether exogenous IL-17A, starting at age 5 weeks, reduces the development of atherosclerosis in ApoE<sup>−/−</sup>/IL-17A<sup>−/−</sup> mice fed with the HFD for 10 weeks and found that IL-17A significantly reduced atherosclerotic plaque area in ApoE<sup>−/−</sup>/IL-17A<sup>−/−</sup> mice (Figure 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<sup>+</sup> T-cells are predominant in both human and murine atherosclerotic lesions. Depletion of CD4<sup>+</sup> T or CD4 deficiency reduces fatty streak development in C57BL/6 mice fed with an atherogenic diet. It has been shown that Th1 cell-derived IFN-γ or IL-12 is proatherogenic. 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 and IL-17A/IFN-γ dual-producing T-cells are present within coronary plaques. In addition, IL-17A induces MMP-9 production from macrophages, which is related to vulnerable plaque. However, recently conflicting roles of IL-17A on arteriosclerosis have been reported.

Contrary to our results, numbers of papers independently supported the notion that IL-17A is proatherogenic. First, 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.
erosclerosis.13 Interestingly, plaque stability was unchanged. In their system, IL-17 signaling is lacking only in BM cells, whereas in our system IL-17 signaling is lacking in both BM cells and 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. Second, ApoE−/− mice, starting at age 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 are 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 nontreated 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 (Figure 4B, 4D, and 4E), consistent with the idea that IFN-γ is important in atherogenesis.38 Third, 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-signaling reduced atherosclerosis and accumulation of macrophages at plaque. Importantly, IFN-γ levels did not differ between control and IL-17 receptor A treated ApoE−/− mice15 and it is possible that the adenovirus-mediated blocking of IL-17 receptor A-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 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.19 Thus under our experimental model, effect of IL-17A was tested during early stage of atherosclerosis development. While they examined the effect of IL-17 in late phase of atherosclerosis development.

On the other hand, similar to what we found, there are reports that IL-17A is a protective against atherosclerosis. First, 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 atherosclerosis 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 (Figure 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. Second, 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−/− mice were reduced, whereas IFN-γ-treated ApoE−/− mice showed enhanced atherosclerotic plaque areas,11,40 suggesting that IFN-γ has proatherogenic functions. In addition, IFN-γ produced vulnerable atherosclerotic plaque by inhibiting VSMC proliferation or collagen production.11,42 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-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-17A−/− mice.

In addition, we also found that IL-5 and anti-MDA-LDL IgG1 production was decreased in HFD-fed ApoE−/−IL-17A−/− mice. Of note, anti-MDA-LDL antibody was reported to be antiatherogenic 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 antiox-LDL antibody and diminishing IFN-γ production44 and played an antiatherogenic role via production of IL-5. Therefore, it is possible that the impaired production of IL-5 led to the decreased anti-MDA-LDL IgG1 production, thus accelerating atherosclerosis in HFD-fed ApoE−/−IL-17A−/− mice.45,46 However, production of IL-4 by splenic CD4+ T-cells did not differ significantly between ApoE−/− and ApoE−/−IL-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 it 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 Figure III) and α-SMA+VSMC (Figure 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 upregulation of IFN-\(\gamma\), reduction of IL-5, and reduction of anti-MDA-LDL IgG1 increased accumulation of macrophage and reduced appearance of \(\alpha\)-SMA\(^{\*}\) VSMC at fibrous cap of plaque are evident at HFD feeding for 8 weeks, indicating the possibility that IL-17A and thus IFN-\(\gamma\) 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-\(\gamma\).

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-\(\gamma\) and decreased IL-5 productions in splenocytes and decreased IgG1 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-\(\gamma\) 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.

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Disclosures

None.

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Expanded Materials and Methods

Mice

IL-17A-deficient mice used in this study were created as described previously\(^1\).

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\(^5\).

**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-\(\mu\)m 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 10\(^5\)/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 \(\mu\)g/mL streptomycin (Wako). Supernatants were collected at 72 h and analyzed by ELISA for IFN-\(\gamma\), 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% 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-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 diet16w; 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<sup>−/−</sup>IL-17A<sup>−/−</sup> mice compared to ApoE<sup>−/−</sup> mice

A, Representative microphotographs of aortic root sections stained with type I collagen in ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> mice after 8 weeks of HFD feeding. Scale bars in upper panels indicate 300 µm and in under panels indicate 50 µm. B, Quantitative analysis of the percentage of type I collagen-positive areas in both ApoE<sup>−/−</sup> (n= 4) and ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> (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<sup>−/−</sup> 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<sup>−/−</sup> and ApoE<sup>−/−</sup>IL-17A<sup>−/−</sup> 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 <i>in vitro</i> 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
Supplemental Figure II
Supplemental Figure III
Supplemental Figure IV
Supplemental Figure V